THE CHEMISTRY AND BACTERIOLOGY OF PUBLIC HEALTH

BY

C. L. DUNN, C.I.E., D.P.H. (Lond.)

LIEUTENANT-COLONEL, INDIAN MEDICAL SERVICE

Director of Public Health, United Provinces of Agra and Oudh; Examiner in Hygiene, State Medical Faculty, United Provinces; Formerly Examiner in Hygiene, Universities of Allahabad and Lucknow and State Board of Medical Examinations, United Provinces; etc.

AND

D. D. PANDYA, D.P.H. (Camb.)

RAI BAHADUR

Assistant Director of Public Health, Provincial Hygiene Institute, United Provinces of Agra and Oudh; Professor of State Medicine, University of Lucknow; Examiner in State Medicine, University of Lucknow and in Hygiene, Universities of Patna and Punjab and the State Medical Faculty, United Provinces, in B.Hy., University of Bombay; Formerly Examiner in Hygiene, University of Allahabad and State Board of Medical Examinations, United Provinces; etc.

CALCUTTA:

BUTTERWORTH & CO. (India), LTD.,

6, HASTINGS STREET.

MADRAS BRANCH: 317, LINGA CHETTY STREET.

BOMBAY BRANCH: JEHANGIR WADIA BUILDING, ESPLANADE ROAD.

LONDON:
BUTTERWORTH & CO.
(Publishers), LTD.
BELL YARD, TEMPLE BAR.

TORONTO (CANADA):
BUTTERWORTH & CO.
(Publishers), LTD.

SYDNEY:
BUTTERWORTH & CO.
(Australia), LTD.

WELLINGTON (N. Z.):
BUTTERWORTH & CO.
(Australia), LTD.

1929

Printed by Mr. M. E. K. Majlish at Karim Bux Bros.
Government of India and General Printers,
9, Anthony Bagan, Calcutta.

DEDICATED BY PERMISSION

TO

HIS EXCELLENCY

SIR WILLIAM MALCOLM HAILEY

B.A., G.C.I.E., K.C.S.I., I.C.S.

GOVERNOR OF THE UNITED PROVINCES OF AGRA AND OUDH

WHOSE INTEREST IN THE AMELIORATION OF THE CONDITION OF THE INHABITANTS OF THE RURAL AREAS OF THE PUNJAB AND THE UNITED PROVINCES IS WELL KNOWN.

PREFACE

THIS book is meant to serve as a laboratory handbook for all students for the practical portion of examinations in Public Health and to be a companion volume to the various books on Hygiene which deal chiefly with the subject-matter for Part II of the examinations for the Diploma in Public Health. The Authors also issued a volume of this kind under the title *Indian Hygiene and Public Health* in 1925 and have had the present volume in preparation since that date. They are of opinion that this volume contains everything required by D.P.H. students for the practical portion of their work. It is also meant to serve as a practical laboratory handbook for Medical Officers of Health and their Assistants working in municipal and other laboratories. It is based on the teaching for Part I of the Diploma in Public Health of the University of Lucknow which courses have been recognised by the Conjoint Board of England as qualifying for Part I of their examination. It has been accepted as a text book for candidates appearing for the Diploma in Public Health of the University of Lucknow and the License in Public Health of the State Medical Faculty, United Provinces.

The Authors acknowledge with thanks the permission of the Authors and Publishers of the various books from which passages have been taken, and their thanks are due especially to Professor Kenwood and Messrs. H. K. Lewis & Co., Ltd., London. Some of the methods recommended by the Special Committee of the Medical Research Council, Great Britain, have been described in the text, and the thanks of the Authors are due to them also.

The Authors wish to acknowledge the great assistance they have received from the officers and staff of the Provincial Hygiene Institute, United Provinces, in the preparation of this work, especially Major D. Clyde, M.B., D.P.H., I.M.S., Assistant Director of Public Health, Malariology, United Provinces, and Lecturer on Malariology in the Provincial

Hygiene Institute and the University of Lucknow. He has prepared the subject-matter on mosquitoes and given great assistance in the preparation and revision of other portions of the work. The Authors also acknowledge the assistance given by Dr. A. N. Goyle, M.B. (Lahore), PH.D. (London), Plague Research Officer, United Provinces, for preparing the portion on fleas and other parts of the work. The thanks of the Authors are also due to Dr. H. G. D. Mathur, M.B.B.S. (Allahabad), M.R.C.P. (Edin.), DR.P.H. (U.S.A.), D.T.M. & H. (London), and Dr. B. S. Yajnik, M.B.B.S., D.P.H. (Lucknow), for revising and arranging portions of the manuscript.

Without the assistance of these officers it would have been difficult for the Authors to have produced this volume which, they hope, will meet the needs of those for whom it is intended. The volume is not designed to apply to students in India only, as it contains all the necessary information required for students for Part I of the Diploma in Public Health in England.

The Authors are also indebted to Dr. J. T. Cornelius, M.A., M.D., D.P.H., PH.D. (London), Lecturer at the Provincial Hygiene Institute, United Provinces, for going over the proofs, and to their Publishers, Messrs. Butterworth & Co. (India), Ltd., 6, Hastings Street, Calcutta, for their unfailing courtesy.

LUCKNOW: C. L. D.

August 1, 1929. D. D. P.

ERRATA

Page 12, line 31, *for* $\frac{}{10}$ *read* $\frac{N}{10}$.
„ 12, line 33, *for* $\frac{N}{}$ *read* $\frac{N}{10}$.
„ 23, line 26, *for* Hosvay's *read* Ilosvay's.
„ 32, column 3, *for* 0.6 *read* 1.6.
„ 39, line 10, *for* 20 *read* 2.0.
„ 56, line 28, *for* 11 *read* I.
„ 63, line 23, *for* $\frac{42}{}$ *read* $\frac{47}{678}$.
„ 78, line 10, *for* eutyracea *read* butyracea.
„ 87, line 37, *for* Hosvay's *read* Ilosvay's.
„ 92, line 37, *for* tranished *read* tarnished.
„ 108, line 1, *for* $\frac{N}{}$ *read* $\frac{N}{10}$.
„ 108, line 12, for $\frac{N}{01}$ *read* $\frac{N}{10}$.
„ 110, line 17, *for* bue *read* but.
„ 121, line 13, *for* is *read* it.
„ 139, line 46, *for* Law *read* Low.
„ 141, line 35, *for* shown *read* sown.
„ 150, line 20, the line is reversed.
„ 167, line 12, *for* measures *read* measure.
„ 167, line 23, in column 2, *insert* 2.
„ 173, line 44, *for* in *read* on.
„ 191, line 12, *for* agen *read* agent.
„ 200, line 27, *for* tract *read* track.
„ 203, line 26, portion of the line is reversed.
„ 218, line 21, *for* injection *read* infection.
„ 221, line 2, *for* ovid *read* ovoid.
„ 225, line 17, *for* transmitted from man to man, *read* transmitted to man.
„ 258, line 33, *for* continued *read* contained.
„ 269, line 33, *for* agglutinates *read* agglutinate.
„ 273, line 16, *for* is *read* in.
„ 284, line 4, *for* terium *read* tertium.
„ 295, line 27, delete the line.
„ 297, line 1, *for* Pfeifferis, *read* Pfeiffer's.
„ 301, line 11, *for* Wel-Felix *read* Weil-Felix.
„ 317, line 22, *for* anoneme *read* axoneme.
„ 317, line 31, *for* biniary *read* binary.
„ 319, line 20, *for* neucleus *read* nucleus.
„ 330, line 13, *for* B. coli, *read* E. coli.
„ 330, line 29, *for* B. coli, *read* E. coli.
„ 335, line 15, *for* Platyhhelminthes *read* Platyhelminthes.
„ 343, line 20, *for* 65μ *read* 65μ to 75μ.
„ 352, line 10, *for* Pygiophylla *read* Pygiopsylla.

THE
CHEMISTRY AND BACTERIOLOGY
OF
PUBLIC HEALTH

PART I—CHEMISTRY

CHAPTER I

ALKALIMETRY, ACIDIMETRY, STANDARD AND NORMAL SOLUTIONS

The Preparation of Solutions.—In public health laboratory work various kinds of solutions are used, *viz.*:—

1. Dilute (usually 10 per cent.) solutions of acids, alkalies and salts.
2. Concentrated solutions of acids and saturated solutions of other reagents.
3. Standard solutions.
4. Normal solutions.

A *standard solution* is a solution containing a known weight of a substance dissolved in a known volume of water. This is usually made up so that one c.c. of water contains 1, 0·1 or 0·01 milligramme of the substance. Standard ammonium chloride solution for example contains 0·01 milligramme of ammonia per c.c. and standard lead acetate solution contains one milligramme of lead per c.c.

A *normal solution* of a substance is one which contains the equivalent weight of the substance dissolved in and made

up to a litre with distilled water. A decinormal solution contains one-tenth of this amount in a litre of water and a centinormal solution one-hundredth.

The *equivalent weight* of a substance is the weight which will combine with one gramme-molecule of hydrogen or 8 gramme-molecules of oxygen. This weight $= \frac{\text{the molecular weight}}{\text{valency}}$.

The *Valency* of an element is the number of hydrogen atoms with which it can combine or which it can displace, directly or indirectly. The valency of an acid is the number of replaceable hydrogen atoms it contains. The valency of a base is equal to the number of hydroxyl groups it contains and this number is termed the "acidity of the base"—*i.e.*, NaOH is a mono-acid base, $Al(OH)_3$ is a tri-acid base.

All normal solutions are equivalent, all decinormal solutions are equivalent, etc.: thus, such solutions of acids and alkalies exactly neutralize each other in identical quantities.

Normal solutions are expressed by the letter $\frac{N}{1}$ placed before the name or formula of the substance; decinormal and centinormal by $\frac{N}{10}$ and $\frac{N}{100}$ respectively.

Estimation of the Equivalent Weight of Substances.—

(*a*) *Base.*—Bases with one hydroxyl group such as caustic soda (NaOH) are monovalent; with two hydroxyl groups such as barium hydrate [$Ba(OH)_2$] are divalent: so, the equivalent weight of caustic soda ($NaOH = 23+16+1 = 40$, *i.e.*, the sum of the atomic weights, while the equivalent weight of barium hydrate, $Ba(OH)_2 = (137{\cdot}37+32+2) \div 2 = 171{\cdot}37 \div 2 = 85{\cdot}68$.

40 grammes of NaOH dissolved in a litre of water is a normal solution of caustic soda and four grammes per litre is a decinormal solution.

Similarly, 85·68 grammes of $Ba(OH)_2$ dissolved in a litre of water is a normal solution of barium hydrate.

(*b*) *Acids.*—(*i*) Inorganic.—The number of hydrogen atoms that can be replaced by an alkali to form a salt represents the basicity of the acid; thus hydrochloric and nitric acids are monobasic and monovalent, while sulphuric acid (H_2SO_4) is dibasic and divalent.

The equivalent weight of hydrochloric acid (HCl) is therefore $1+35{\cdot}5 = 36{\cdot}5$ and of sulphuric acid (H_2SO_4) is $2+32+64$

$=98\div2=49$; 36·5 grammes per litre is therefore a normal solution of hydrochloric acid and 49 grammes per litre is a normal solution of sulphuric acid.

(*ii*) Organic.—Acids with one carboxyl (COOH) group are monovalent; those with two or three divalent and trivalent respectively.

Examples.—(*a*) Acetic acid $=CH_3COOH=12+3+12+16+16+1=60$, and 60 grammes per litre makes a normal solution.

(*b*) Oxalic acid, $COOH.COOH.2H_2O$, contains two carboxyl groups, so is a divalent acid with an equivalent weight of $126\div2=63$, *i.e.*, requiring 63 grammes per litre to make a normal solution.

(*c*) Similarly, citric acid, $C_3H_4OH.(COOH)_3.H_2O$, is trivalent acid with an equivalent weight of $210\div3=70$.

In (*b*) and (*c*) the H_2O equals the molecules of water of crystallization which must always be taken into account in making normal solutions.

(*d*) *Salts.*—The valency of a salt is the valency of its acid, *e.g.*, sodium chloride (NaCl) is monovalent: one atom of hydrogen only is required to replace the sodium (Na) atom to form HCl. Barium sulphate ($BaSO_4$) is divalent, because two atoms of hydrogen are required to replace the barium to form H_2SO_4. Thus, a normal solution of NaCl is made by dissolving $23+35·5=58·5$ grammes in a litre, and, of $BaSO_4$ by dissolving $\frac{138+32+64}{2}=\frac{234}{2}=117$ grammes in a litre, and of Na_2CO_3 by dissolving $\frac{46+12+48}{2}=\frac{106}{2}=53$ grammes in a litre.

Note.—The total volume is made up to 1 litre in the various normal solutions, *i.e.*, the volume occupied by the acid, etc., must be allowed for.

Preparation of Various Decinormal Solutions.—In public health work *decinormal solutions* are usually used. In preparing normal, decinormal solutions, etc., certain practical points have to be considered, in view of the physical characters of the various substances and their reactions, in order to obtain accurate results.

(1) *Sodium carbonate* usually contains impurities, and the bicarbonate should be used after heating it to volatilize the water and ammonia present in combination or as impurities and to convert it to sodium carbonate. Of this 5·3 grammes accurately weighed out in a chemical balance and dissolved in a litre of water will give a decinormal solution of sodium carbonate.

(2) *Sulphuric acid* cannot be accurately weighed owing to its great affinity for water. It is therefore better to make a

stronger solution than is required and bring it to the right strength by adding the amount of water found necessary after titrating with a normal solution of an alkali.

But to make normal solutions of liquids it is necessary to know their specific gravity to obviate difficulty in weighing. The volume of the liquid is measured, and, by multiplying by the specific gravity, the weight is calculated.

The *specific gravity* of a substance is the *ratio* of the weight of a volume of the substance to the weight of the same volume of a standard substance—water in the case of liquids and solids, air and hydrogen in the case of gases—the temperature and pressure being constant. The specific gravity of sulphuric acid is about 1·84, *i.e.*, 1 c.c. weighs 1·84 grammes. A decinormal solution of H_2SO_4 would contain 4·9 grammes in a litre of water and as 1 c.c.=1·84 grammes, this means that we require $\frac{4·9}{1·84}$ =2·66 c.c. per litre. Therefore take about three c.c. of the concentrated acid and dissolve it in a litre of water. Take 20 c.c. and titrate it with the $\frac{N}{10}$ Na_2CO_3 already prepared, until neutral.

Suppose 25 c.c. of $\frac{N}{10}$ Na_2CO_3 are required to make the 20 c.c. of sulphuric acid neutral to phenolphthalein. Had 20 c.c. been used only, the acid solution would have been a $\frac{N}{10}$ solution; so, to every 20 c.c. of the acid, 5 c.c. of water must be added to make it decinormal. As 20 c.c. were removed for titration, only 980 c.c. remain and so it requires $\frac{980 \times 5}{20}$ =245 c.c. to make it decinormal. Add 200 c.c., mix well and again test 20 c.c. Find the amount of water still to be added for this second titration and having added it, the result will be a decinormal solution.

Note that if all the 245 c.c. indicated by the first titration were added, the solution would probably be too weak.

All decinormal solutions should be stocked in well stoppered bottles.

Indicators.—To find the neutral point we need an indicator which will vary in colour according to whether the solution is acid or alkaline. The indicators which are commonly used are litmus, methyl-orange and phenolphthalein. The neutral points are not all identical (*see* P_H values).

(*a*) *Litmus* is red in acids, blue in alkalies; the colour-change is not sharp. In solutions litmus is not satisfactory because of its dichoric effect but litmus paper gives satisfactory results.

(*b*) *Methyl-orange* is scarlet in acids and yellow in alkalies, is not reliable with organic acids or when nitrites are present, but is reliable in the presence of carbonic acid (CO_2) and mineral acids.

(*c*) *Rosolic acid* is a delicate indicator of a pale yellow colour which becomes rose coloured in alkalies.

(*d*) *Phenolphthalein,* as usually used in this class of work, is colourless in acids and pink in alkalies, but is not reliable in the presence of free carbonic acid.

(3) *Caustic soda.*—From the formula NaOH it is found that 4 grammes per litre gives a decinormal solution. Caustic soda always contains sodium carbonate and other impurities. Moreover it cannot be accurately weighed owing to its hygroscopic properties.

For this reason first dissolve 4·5 grammes of caustic soda in a litre of water, titrate with the $\frac{N}{10}$ H_2SO_4 already prepared and make a decinormal solution in the manner described above.

(4) *Permanganate of Potassium.*—To make a decinormal solution of this, we must find how much of it corresponds to an equivalent weight of oxygen, which is its active principle. This is got from the equation:—

$$2KMnO_4+3H_2SO_4=5O+K_2SO_4+2MnSO_4+3H_2O,$$

i.e., two molecules of potassium permanganate (molecular weight =316·06) give only 5 atoms of available oxygen, and since 1 atom of oxygen combines with 2 of hydrogen, therefore, (5×16) *i.e.*, 80 grammes of oxygen will combine with (5×2) *i.e.*, 10 grammes of hydrogen, that is to say,

$KMnO_4$ is decavalent.

∴ a normal solution contains 31·6 grammes per litre;

∴ a decinormal solution contains 3·16 grammes per litre;

∴ 1 c.c. of a decinormal solution contains 0·00316 gramme of potassium permanganate.

(5) *Normal Oxalic Acid.*—This in its solid state is fairly stable and can be obtained pure. It is useful as the starting point for making other normal and decinormal solutions, but it does not keep well in solution, and for laboratory purposes a fresh solution should always be prepared.

N.B.—Caustic soda also deteriorates in solution as also does sodium carbonate. Therefore it is necessary always to restandardise these solutions before use.

Hydrogen-Ion Concentration.—(*a*) Although all $\frac{N}{1}$ solutions are equivalent to, and equal quantities will neutralise, each other, yet the manner in which the hydrogen exists is the factor which determines the intensity of the reaction. One litre of any normal acid solution contains 1 gramme of hydrogen; but all the hydrogen in a solution may not be free or dissociated. A strong acid is one which, in dilute aqueous solutions, is largely dissociated into ions; a weak acid is one which in dilute solutions is dissociated to a small extent only. For instance $\frac{N}{100}$ HCl is 91 per cent. dissociated, whereas $\frac{N}{100}$ CH_3COOH is only 1·05 per cent. dissociated; yet 1 litre of either of these will neutralise 1 litre of $\frac{N}{100}$ alkali. The *neutralising* power depends on the total number of hydrogen atoms; the *reaction* depends on the number of hydrogen atoms free or ionised.

The P_H value is the number used to express the total quantity of free ions, *i.e.*, the active acidity or alkalinity of a fluid.

(*b*) The majority of solutions of acids are only partially ionised; as the acid is made more dilute the percentage of ion concentration (the number of ions freed) is increased. According to the theory of Electrolytic Dissociation all aqueous solutions contain free hydrogen $(H)^{(+)}$ or free hydroxyl $(OH)^{(-)}$ ions. On dissociation certain ions become negatively charged (*Kations*) and others positively charged (*Anions*). The process is reversible, some anions and kations combining; *e.g.*,

$HCl \rightleftarrows H^{(+)} + Cl^{(-)}$, and $NaOH \rightleftarrows Na^{(+)} + OH^{(-)}$.

When the $H^{(+)}$ and $OH^{(-)}$ ions are balanced in numbers, the solution is neutral on the P_H scale.

One litre of pure distilled water contains one ten-millionth of a gramme of ionised hydrogen. 1 litre of $\frac{N}{10}$ HCl contains 0·104 gramme of ionised hydrogen. These numbers are expressed in negative powers of 10 to avoid dealing with small fractions, *e.g.*,

$\frac{1}{10}$th gramme of ionised H per litre = 0·1 gramme
$= 10^{-1}$ (written as P_H1);

$\frac{1}{100}$th gramme of ionised H per litre = 0·01 gramme
$= 10^{-2}$ (written as P_H2);

$\frac{1}{1,000}$th gramme of ionised H per litre = 0·001 gramme
$= 10^{-3}$ (written as P_H3);

$\frac{1}{10000000}$th gramme of ionised H per litre=0·0000001 gramme=10^{-7} (written as P_H7);

i.e., the number of decimal places in the fraction =the power to which 10 is raised=P_H value

Thus the hydrogen-ion concentration of water (neutrality on the P_H scale)=10^{-7}, and of HCl, $10^{-1·04}$: all concentration on the P_H scale having a numeral which is the logarithm or index of the power to which 10 is raised and which represents the fraction of a gramme of hydrogen ionised per litre of solution. Since all these numerals are minus quantities the minus sign is omitted, and the sign P_H followed by the logarithm gives the P_H values, *i.e.*, $10^{-7}=P_H7$; $10^{-1·04}=P_H1·04$. An increase of P_H1 represents $\frac{1}{10}$th the concentration of H ions present in the preceding P_H, *i.e.*, P_H9 contains $\frac{1}{10}$ th the number of free H ions existing in P_H8.

In practice the hydrogen ions only are referred to, *i.e.*, the number of grammes of active or ionised hydrogen in 1 litre of the liquid. An acid solution must have a P_H value less than 7·07; an alkaline solution a value greater than $P_H7·07$.

(*c*) There is no relationship between the P_H values of two solutions and their rate of ionisation, *e.g.*,

$\frac{N}{10}$ acetic acid is dissociated to the extent of 1·05 per cent. and has a P_H value of 2·88, while $\frac{N}{100}$ acetic acid contains 4·17 per cent. of free H ions and has a P_H of 3·38.

(1) An acid with a P_H value of 5 which is 100 per cent. ionised has an actual P_H of $10^{-5}\times1·0$=—5+log. of 1=—5 +0=P_H5.

(2) An acid with a P_H value of 5 which is 1 per cent. ionised has an actual P_H of $10^{-5}\times0·01$=—5+log. of 0·01= —5—2=—7=P_H7.

$P_H7=P_{OH}7$=neutrality, ∴ the alkalinity of a fluid may be obtained by subtracting its P_H value from 14.

(*d*) Certain buffer substances such as proteins, amino-acids and the salts of weak acids or bases such as carbonates, phosphates, and citrates diminish the amount of free ions by dissociation into anions and kations which unite with the H ions of an acid and the OH ions of an alkali, respectively, when these are added to form molecules of a weakly dissociated base.

Thus the addition of 1 c.c. of $\frac{N}{100}$ HCl to 1 litre of distilled water will alter the value from $P_H 7{\cdot}07$ to $P_H 5$ whereas the same quantity of $\frac{N}{100}$ HCl added to 1 litre of broth media will leave the P_H value almost unaffected. These buffer substances prevent the sharp change in P_H value on adding an acid or an alkali. The buffer action depends on various factors such as the concentration of the constituents (a dilute solution offering less resistance to P_H change than a concentrated solution), on the nature of the buffer constituents, on the percentage dissociation of the acid or alkali used in dissociation, on the actual P_H value at which the titration is being made and on the temperature at the time of testing.

(*e*) The colorimetric method of estimating the P_H values of solutions is generally used. The method is simple and is rapidly carried out. The indicators used consist of organic colouring matter which change colour as the P_H value varies. The hydrogen-ion concentration is ascertained by comparison with uniform glass tubes of standard buffer solutions (usually made from $\frac{5}{15}$ mono-potassium phosphate and $\frac{5}{15}$ di-sodium phosphate) coloured with the indicator used, standardised and marked with the P_H value.

Each indicator has a definite P_H range and is only of use within that range. The neutral points do not coincide and what is acid by one indicator may be alkaline according to another indicator. For instance, litmus is red at $P_H 5$ or less, purple at $P_H 7$, and blue at $P_H 8$ or over: methyl-orange is red at $P_H 2{\cdot}8$ and orange at $P_H 4$: thymol blue is acid at $P_H 8$ or less, alkaline at $P_H 9{\cdot}8$ or more. Thus, a liquid, acid to thymol blue, can only be considered as having a P_H of not more than 8: it may actually be alkaline on the P_H scale. Hence all indicators do not cover absolute neutrality (7·07).

Several firms produce universal indicators with a range of about 2 to 11, a drop of which added to about 4 c.c. of the fluid under test will produce a definite colour. Reference to a table will give the approximate P_H value for the colour obtained. This is often sufficiently correct to indicate the probability or otherwise of anophelines breeding in water under test. If further accuracy is required an indicator covering this colour is chosen, a measured quantity of this added to a measured quantity of the fluid to be tested and the colour produced compared in a "comparator" case.

A "comparator case" having two rows of three holes for tubes and side holes through which the three pairs can be compared is used. Into the front three holes are placed three

standard tubes containing the water (or media in bacteriological work) to be examined, the centre one having added to it 0·5 c.c. of the indicator which is being used. Behind this centre tube is placed a tube of distilled water. Various standard buffer tubes (which are marked, giving a rise of P_H0·2 between each) are then placed in the holes behind tubes 1 and 3 until a match is produced.

Examination by hydrogen-ion concentration methods is becoming important in relation especially to malarial problems and is used in standardising media. As a rule "carriers" anopheline mosquitos mostly breed in waters which have a P_H value of from 6·2 to 8·5 though certain species are found at a P_H of 3·5. Extremes of P_H are inhibitory to anopheline growth (*see* water opinions). Alkalinity on the P_H scale is also inimical to ancylostoma larvæ.

The following gives the P_H range of selected indicators sold by the British Drug Houses:

Thymol blue	P_H 1·2 to 2·8	Bromthymol blue	P_H 6·0 to 7·6
Methyl orange	P_H 2·9 to 4·0	Phenol red	P_H 6·8 to 8·4
Bromphenol blue	P_H 2·8 to 4·6	Cresol red	P_H 7·2 to 8·8
Methyl red	P_H 4·2 to 6·8	Thymol blue	P_H 8·0 to 9·6
Bromphenol purple	P_H 5·2 to 6·8	Cresol phthalein	P_H 8·2 to 9·8

B. D. H. Universal Indicator .. P_H 3·0 to 11·0

Atomic Weights.—From the following atomic weights the various normal solutions can be prepared:—

Aluminium	27·1	Lead	207·2
Arsenic	74·96	Magnesium	24·32
Barium	137·37	Manganese	54·93
Boron	10·9	Mercury	200·6
Bromine	79·92	Nitrogen	14·008
Calcium	40·07	Oxygen	16·0
Carbon	12·0	Phosphorus	31·04
Chlorine	35·46	Potassium	39·1
Chromium	52·0	Silicon	28·3
Copper	63·57	Silver	107·88
Fluorine	19·0	Sodium	23·0
Hydrogen	1·008	Strontium	87·63
Iodine	126·92	Sulphur	32·06
Iron	55·84	Tin	118·7
Zinc	65·37		

CHAPTER II

THE PHYSICAL AND CHEMICAL EXAMINATION OF WATER

Method of Collection of Water Samples.—The main object of chemical analyses of water from the public health point of view is simply to ascertain maximum amount of pollution present in it. The sample should always be collected for analytical purposes in such a way as to avoid all sources of extraneous contamination.

The sample can be collected in any stout glass bottle, fitted with a tight glass stopper and capable of holding about half a gallon of water. If the complete mineral analysis is required, it is necessary to collect two gallons of water. "Winchester quart" bottles are very convenient for this purpose. Stone jars or vessels made of metal or of any porous material should not be used.

Before collecting the sample the bottle should be thoroughly rinsed with dilute hydrochloric acid and then with clean water till the washings are no longer acid in reaction. Finally the bottle and stopper should be separately washed, at least three times, with the water to be subjected to analysis.

If the sample is to be taken from a shallow stream, the sediment should not be disturbed and the mid-stream water should be allowed to run into the bottle held just under the surface of the water with the mouth pointed upstream. The water near the bank should be avoided, especially in the case of lakes and tanks and as little æration as possible permitted during collection. If tap water is being taken first allow some water to run to waste and then fill the bottle directly from the tap without using any funnel.

It is very necessary to keep the sample cool and unexposed to light to avoid important chemical changes. The analysis should not be delayed, if possible, more than 48 hours and all information, specially the name and address of the sender, date and time of collection, nature of subsoil, risk of pollution, etc., should be communicated to the analyst.

Physical Characters of Water.—Examine in a white glass tube two feet long holding it above a white surface in a good light. Potable water should be *clear* and *bright* but polluted

water may also have these characters. The turbidity of a water can be measured by noting the depth at which a platinum wire 0·04 inch in diameter can be seen. Since most waters are slightly green in colour and have a very small amount of vegetable matter, iron or other suspended matter in them, these tests are of relatively little importance.

Colour is given to water by vegetable, animal or mineral contamination. A yellow or brown colour means vegetable pollution, dissolved iron salts or a peaty water. A red or green colour may be due to algæ. A good sample of water should possess *lustre*, a *pleasant taste* and *no odour*. The pleasant taste and lustre are due to dissolved gases.

Smell.—An odour indicates grossly polluted water or medicinal waters. The pollution may be organic or inorganic. To test, half fill a bottle with the water, alkalise with a weak solution of KOH, stopper, and warm. On removing the stopper the odour, if present, is easily detected.

The *reaction* of the water should be noted as it gives an indication of the contents. Most waters are neutral or faintly alkaline. Peaty waters are usually acid. An alkaline water which contains free CO_2 may give an amphoteric reaction, neither red nor blue litmus-paper being changed in colour when dipped into the water. The *temperature* of spring water may give an indication as to the depth of its source.

Suspended matter including vegetable growths, fungi, spores, plankton and animal organisms such as diatoms, ciliates, euglenids, Daphnia and Cyclops, the ova of intestinal parasites, etc., may be seen macroscopically or microscopically.

Organic pollution is indicated if the residue or deposit, filtered off and burned in a platinum dish, chars, scintillates or gives off fumes or the odour of burnt feathers.

Chemical Analysis of Water

Total Solids.—The term " total solids " usually indicates only that solid matter which is present *in solution*, in contradistinction to the suspended solids indicating solids which are not dissolved in water. Take 50 c.c. of the sample water previously filtered or the supernatant fluid after centrifugalising it, and put it in a previously weighed platinum capsule. Evaporate to dryness over a water-bath. Then dry thoroughly in a hot-air oven for half an hour at 105°C. Cool in a desiccator, weigh and note the increased weight of the capsule which represents the weight of

total solids present in that amount of water. Heat to dull redness over a Bunsen flame. Cool in a desiccator. Weigh and note the decreased weight of the capsule representing the loss due to volatilisation of volatile solids.

Example.—Capsule weighs 41·25 grammes.

Weight after evaporation = 41·275 grammes.

Weight after incineration = 41.270 grammes.

Therefore the weight of the total solids in 50 c.c. = 41·275—41·25 = 0·025 gramme, or the weight of the total solids in 100 c.c. = 0.05 gramme.

But as 0·05 gramme = 50 milligrammes and as there are 100,000 milligrammes in 100 c.c., therefore the total solids are 50 parts per 100,000.

After incineration the capsule weighs 0·005 gramme less or 0·01 gramme less for 100 c.c. of sample water, *i.e.*, in other words, 10 milligrammes per 100,000 milligrammes of water. Therefore the volatile solids constitute 10 parts per 100,000 and the remaining 40 parts represent the non-volatile solids.

Sediment in Water.—Wynter Blyth's tube is a convenient instrument for collecting water sediments for examination. The sediment can also be collected by the use of the centrifuge.

To estimate the amount of *sediment* in water, a sample, say 50 c.c., of the water are filtered or allowed to stand, the filtrate or supernatant clear fluid taken and the total solids in solution estimated as above described. The same procedure is again followed with unfiltered water and the difference between these two results gives the total solids in suspension.

Estimation of Acidity and Alkalinity.—The acidity and alkalinity of a water may be found by titrating 100 c.c. of the sample to which a few drops of an alcoholic solution of methyl orange have been added by $\frac{N}{10}$ alkali (or $\frac{N}{10}$ acid if the water is alkaline). As all $\frac{N}{10}$ solutions are equivalent and 1 c.c. of ^{N} sodium carbonate = 5·3 milligrammes of Na_2CO_3, the number of c.c. of $\frac{N}{10}$ solution used × 5·3 will give the alkalinity in terms of sodium carbonate in the water in milligrammes per 100 c.c., *i.e.*, parts per 100,000. Acidity is expressed in terms of HCl.

The Poisonous and other Metals.—

QUALITATIVE TESTS.—1. *Fe, Cu, Pb and Zn.*—(*i*) Evaporate a litre of the sample down to 200 c.c. after adding a few drops of hydrochloric acid (unless lead is suspected, when no HCl should be added). Cool and to about 100 c.c. add ammonium or sodium sulphide drop by drop. A brown or black discoloration indicates the presence of iron, copper or lead; zinc gives no discoloration.

(*a*) To half this discoloured sample add dilute hydrochloric acid: the dark colour disappears if iron is present.

(*b*) To the other half add some potassium cyanide: copper sulphide dissolves, lead sulphide is unaffected.

To confirm the presence of lead, to some of the concentrated clear water in a test-tube add a few drops of potassium chromate; a yellow precipitate forms. The precipitate may be very faint, so it should be compared with a control of distilled water plus reagent.

To confirm the presence of zinc, if no discoloration results on adding ammonium sulphide, add to some of the sample a few drops of ammonia and ammonium chloride solution, boil and filter; gently add some ammonium sulphide; a white precipitate results with zinc.

(*ii*) Ferrocyanide test to confirm the presence of Fe, Cu and Zn.—To some of the concentrated water add a few drops of hydrochloric acid and potassium ferrocyanide. A blue colour results with iron, a brown colour with copper and a white precipitate with zinc.

2. *Arsenic.*—Marsh's test. (*See* food analysis.)

3. *Calcium salts.*—Add ammonia and ammonium chloride to the concentrated water; filter and add ammonium oxalate; a white precipitate indicates the presence of calcium. Uncombined lime will give a brown precipitate of silver oxide when a few drops of silver nitrate solution are added. The uncombined lime test is given if excess is added during Clark's softening process.

4. *Magnesium salts.*—Precipitate the calcium present in water by means of a solution of ammonium oxalate, ammonium chloride and ammonia; filter until the filtrate is perfectly clear and free from lime, as shown by ammonium oxalate furnishing no opacity. Acidify the filtrate with hydrochloric acid; concentrate by boiling and add a few drops of a saturated solution of sodium phosphate with sufficient ammonia to produce strong

alkalinity. Stir; then set aside for several hours, when a crystalline precipitate of triple phosphate of sodium, magnesium and ammonia forms. Examine for the characteristic appearance under a microscope.

5. *Silica.*—Evaporate 500 c.c. of water, to which is added a drop or two of hydrochloric acid, to dryness. Treat the residue with strong hydrochloric acid; wash well with boiling distilled water; filter. Dry, ignite and weigh the residue which will be silica.

6. *Sulphates.*—Add dilute hydrochloric acid and barium chloride to a concentrated sample: a white precipitate indicates the presence of sulphates.

7. *Phosphates.*—To 500 c.c. of water add nitric acid, evaporate to dryness; dissolve the residue in 3 c.c. of dilute nitric acid and filter. Add 3 c.c. of ammonium molybdate and warm. Greenish yellow turbidity appears if phosphates are present in minute quantities.

QUANTITATIVE ESTIMATION.—*Lead.*—Take 100 c.c. of sample concentrated to one-fifth of its volume and pour into a Nessler glass.

Take a similar amount of distilled water in a number of Nessler glasses to each of which add different amounts of a standard solution of lead acetate (1 c.c.=1 mgm. of lead) varying from 0·1 to 1 c.c. Add one drop of ammonium sulphide to all the glasses and compare the colour with that produced by the sample.

Example.—Say, 0·4 c.c. of standard solution matches the sample colour.

But 1 c.c. of standard solution=1 mgm. of lead.

Therefore 0·4 c.c.=0·4 mgm. of lead.

Therefore there is 0·4 mgm. of lead in 100 c.c. of concentrated water.

The water was concentrated to one-fifth of its original bulk.

Therefore 0·08 mgm. of lead is in 100 c.c. of original water or 0·08 part per 100,000.

The amount of lead in a water should not exceed $\frac{1}{20}$ grain per gallon.

Copper.—Take 50 c.c. of the sample and 50 c.c. of distilled water in separate Nessler glasses. To each add 2 drops of potassium ferrocyanide solution (1 in 25) and 2 c.c. ammonium nitrate solution. To the distilled water add drop by drop a standard solution of copper sulphate, 1 c.c. of which=0·0001 gramme of copper, *i.e.*, 0·1 mgm. of copper, till the bronze colour produced matches that in the sample glass, the liquid being well stirred between each addition.

Example.—0·8 c.c. of the standard solution produces a colour which matches the colour in sample.

∴ there are 0·08 mgm. of copper in 50 c.c. of the sample;

∴ there are 0·16 mgm. of copper in 100 c.c. of the sample,

or 0·16 part per 100,000.

The amount of copper in a potable water should not exceed $\frac{1}{15}$th grain per gallon or 0·09 part per 100,000.

Iron.—(1) Evaporate 100 c.c. of the sample to dryness, ignite and dissolve in strong hydrochloric acid. Make up to 100 c.c. with distilled water. Add 1 c.c. of 50 per cent. nitric acid and 1 c.c. of potassium ferrocyanide solution (1-20). Put 1 c.c. of a standard solution of ferric chloride of which 1 c.c.=0·1 mgm. of iron in a Nessler glass and make up to 100 c.c. with distilled water. Add 1 c.c. nitric acid and 1 c.c. potassium ferrocyanide. If the colours do not match continue adding standard solution of ferric chloride until they do, or make up different solutions of various amounts of standard solution and treat each with 1 c.c. of nitric acid and 1 c.c. of potassium ferrocyanide, and then match the colour.

Example.—1·5 c.c. of the standard solution is required to make the colours match.

But 1 c.c. of standard solution=0·1 mgm. of iron.

∴ 1·5 c.c. of standard solution=0·15 mgm. of iron,

so that there is 0·15 mgm. of iron in 100 c.c. of the sample or 0·15 part per 100,000.

(2) An alternative quantitative test for iron present in large quantities is as follows:—

To 100 c.c. of water in a bowl add 10 drops of dilute sulphuric acid. Run in an $\frac{N}{10}$ solution of potassium permanganate until the crimson colour is permanent.

Calculation:—Say 2·5 c.c. $\frac{N}{10}$ solution were required:

Now, 1 c.c. of $\frac{N}{10}$ solution = 0·0056 gramme Fe,

∴ 2·5 c.c. of solution = 0·0140 gramme Fe,

∴ there are 0·014 gramme Fe in 100 c.c. of water,

or 14 parts per 100,000.

Only $\frac{1}{4}$ grain of iron per gallon should be permitted in potable waters.

Zinc, calcium, magnesium and sulphates can be estimated gravimetrically and the phosphates colorimetrically.

Zinc should not exceed $\frac{1}{10}$th grain per gallon.

Chlorine as Chlorides.—

QUALITATIVE TEST.—Add a few drops of silver nitrate and dilute nitric acid to the water in a test-tube: a white haze or turbidity will appear in the presence of a chloride.

QUANTITATIVE ESTIMATION.—

Reagents.—

1. A standard solution of silver nitrate, 4·79 grammes to the litre: 1 c.c. of this = 1 milligramme of chlorine.

2. A cold saturated solution of yellow chromate of potassium.

Measure 100 c.c. of sample water (neutralised if acid in reaction) in a porcelain dish, and add a few drops of yellow chromate of potassium. Put the standard silver nitrate solution in a burette and add drop by drop to the sample water until the colour permanently changes from yellow to reddish brown. When the change has occurred, read off the number of c.c. of the standard solution so used.

Say, 4 c.c. are used. 1 c.c. of the standard solution = 1 mgm. of chlorine. Therefore 4 c.c. of the standard solution = 4 milligrammes of chlorine. Therefore there are 4 milligrammes of chlorine in 100 c.c. of the water. But 100 c.c. of water = 100,000 milligrammes. Therefore there are 4 milligrammes of chlorine in 100,000 milligrammes of water or 4 parts per 100,000.

If the chlorine is in large quantities, it will probably be overestimated by this method. So take 50 c.c., 25 c.c. or 10 c.c. of water and make up to 100 c.c. with distilled water and multiply the results by 2, 4 or 10 respectively.

If it is desired to express the result in terms of sodium chloride, the result is multiplied by 1·65,

i.e., NaCl: Cl:: 58·46:35·46 (Na=23, Cl=35·46)

$\therefore \frac{58{\cdot}46}{35{\cdot}46} = 1{\cdot}65.$

Free Chlorine.—The sterilisation of water supplies by chlorine is so much in vogue at present that a qualitative test for the presence of free chlorine in water is often called for. Treatment with chlorine or hypochlorites converts the free and saline as well as albuminoid ammonias into chloramine or chloromido derivatives; therefore the amount of the ammonias as tested by Wanklyn's process is not reduced.

(1) One c.c. of normal sulphuric acid and a drop or two of potassium iodide solution (free from iodate) added to 50 c.c. of sample water in a Nessler's glass will produce a brown coloration if the sample contains free chlorine or hypochlorites.

(2) *The orthotolidine test.*—One gramme of orthotolidine, recrystallised from alcohol, is dissolved in 1 litre of 10 per cent. HCl. To 100 c.c. of the water under examination add 1 c.c. of this solution. After 10 minutes a yellow colour indicates small quantities of free chlorine; larger quantities will give an orange colour. By comparing the colour obtained with a set of standards made from solutions of copper sulphate and potassium bichromate a quantitative estimate can be made.

(3) *Winkler's process.*— Add a drop of a very dilute solution (1 in 5,000) of methyl orange to the water and acidify with 2 or 3 c.c. of 10 per cent. hydrochloric acid. If the sample of water is chlorinated or contains any hypochlorites such as bleaching powder, it is almost immediately decolorised. Nitrites, if present in abnormal quantity, also give this test, but it takes more than an hour to complete the change.

Hardness.—

Total Hardness:—

Reagents.—

A standard solution of potassic soap of a strength that 1 c.c. will precipitate 1 mgm. of calcium carbonate or its equivalent is required. 14 grammes of the soap are dissolved in a litre of 50 per cent. solution of methylated spirit and warm distilled water, filtered and standardised with a solution of calcium chloride. This solution of calcium chloride is prepared by dissolving 0·2 gramme of calcium carbonate in dilute hydrochloric acid, evaporating to dryness on a water-bath and washing the reside with distilled water. The solution is made up to 1 litre with distilled water and now contains calcium chloride equivalent to 0·2 gramme of calcium carbonate per litre.

1 c.c. of standard soap requires to equal 1 mgm. of calcium carbonate.

∴ 20 c.c. of standard soap requires to equal 100 c.c. of the solution containing 0·2 gramme per litre.

Test 100 c.c. of the calcium chloride solution with the soap solution; say 18 c.c. of the soap solution gives a permanent lather. The soap solution is too strong and must be weakened. If the total filtered soap solution is 980 c.c., it must be made up to $\frac{20}{18}$ of 980 c.c.=1,088 c.c. with extra water and methylated spirit (3 volumes of distilled water to 5 of spirit). In short, 20 c.c. of the standard soap solution should give a permanent lather with 100 c.c. of calcium carbonate solution containing 0·2 gramme per litre.

Process.—To 100 c.c. of the water sample in a bottle add soap solution in quantities of 1 c.c. at a time (shaking after each addition) until a permanent lather is formed.

The number of c.c. used *minus* 1 equal the parts of total hardness per 100,000 (1 part per 100,000 is allowed for the hardness of distilled water).

On Clark's scale 1 degree of hardness equals 1 grain per gallon which is 1 part per 70,000. Therefore, to convert parts per 100,000 into degrees in Clark's scale, multiply by 0·7.

Temporary and Permanent Hardness.—Boil another 100 c.c. of sample for 15 minutes. Cool. Make up to 100 c.c. with distilled water and test similarly with soap solution. The number of c.c. used *minus* 1 c.c.=the permanent hardness, and the difference between this and total hardness is the temporary hardness.

Note.—If more than 25 c.c. of soap solution are required, the water must be diluted with an equal quantity of distilled water and the test repeated.

The presence of magnesium in excess will produce a scum which must not be mistaken for a permanent lather.

Organic Matter in Water (Wanklyn's process).

"Free and Saline Ammonia" and "Albuminoid Ammonia."—

Special Reagents.—

1. Standard solution of ammonium chloride.—Dissolve 3·14 grammes of pure ammonium chloride in a litre of distilled ammonia-free water. Take 10 c.c. of this solution and dilute it to 1 litre. 1 c.c. of this solution=0·01 mgm. of ammonia.

2. Nessler's reagent, *i.e.*, a saturated solution of periodide of mercury in distilled ammonia-free water made strongly alkaline with caustic potash. Dissolve 13 grammes of mercuric chloride in 250 c.c. of water and 35 grammes of potassium iodide in another 250 c.c. of water. Boil both and mix the two hot solutions. Add cold saturated solution of mercuric chloride till a precipitate of red periodide just begins to remain permanently. Boil, and the precipitate will possibly be redissolved. Cool

and decant the supernatant fluid from any precipitate. Dissolve 120 grammes of caustic potash in 400 c.c. of water by boiling, and cool. Mix the two cold solutions and make up to 1 litre. The water used must be ammonia-free distilled water.

3. A strongly alkaline solution of potassium permanganate.—Mix together caustic potash 200 grammes and potassium permanganate 8 grammes; add ammonia-free distilled water up to 1 litre.

I.—The Process for Free and Saline Ammonia,—

1. 500 c.c. of the sample are placed in a boiling flask.

2. If the water is acid or neutral, add a little pure sodium carbonate to ensure alkalinity so that any "fixed" ammonia will be liberated.

3. Turn on the cold water so that it runs continuously through the condenser.

4. Connect the boiling flask to a condenser and rapidly boil. The condensing apparatus must have been cleaned by passing ammonia-free water through it.

5. A Nessler glass is placed so as to catch the distillate and when this reaches the 50 c.c. mark, a second glass is substituted and then a third.

6. When three Nessler glasses are filled to the 50 c.c. mark a fourth is placed to catch the distillate and 2 c.c. of Nessler's reagent are added to each. These will show a yellow colour, the degree of which varies with the amount of ammonia.

7. There will usually be no colour in the fourth but if there is, a fifth must be taken distilled over. If there is no colour, shut off the bunsen flame.

8. Pool the whole distillate, say from four glasses, all of which gave a yellow colour, and pour one-fourth of the mixture into a Nessler glass.

9. Take a set of Nessler glasses; add to them varying amounts of standard ammonium chloride solution and fill up to the 50 c.c. mark with ammonia-free distilled water. Now add 2 c.c. of Nessler's reagent to each and match the colours.

Note.—Note that the ammonium chloride solution must always be put in before the Nessler reagent, or a turbidity will occur.

Example.—Say 0·9 c.c. of standard solution of ammonium chloride were required to match one-fourth of the distillate;

Therefore the whole distillate=3·6 c.c. of ammonium chloride solution=the amount in 500 c.c. of the sample of water.

But 1 c.c. of standard solution of ammonium chloride=0·01 mgm. NH_3

∴ 3·6 c.c. of standard solution of ammonium chloride=0·036 mgm. NH_3

∴ in 100 c.c sample water there is 0·0072 mgm. NH_3

∴ Free and saline ammonia=0·0072 part per 100,000 parts of sample.

II.—Albuminoid Ammonia Process.—Add to the remains of the water in the boiling flask 50 c.c. of a recently boiled alkaline solution of potassium permanganate. Boil slowly and collect the distillate as before in Nessler glasses. It may be necessary to make four glasses before the distillate is free from colour on adding the reagent. In very foul waters it may not be free even then. Add Nessler's reagent and match the colours as before with the standard solution. The ammonia comes over very unevenly, and there may be more in the second and third glasses than there was in the first.

Example.—It was necessary to distil 200 c.c. before the distillate became colourless.

On matching, the following amounts of standard solution were found necessary to match the colours in the four glasses:—1·2+3·2+0·5+0·4=5·3 c.c. of the standard solution. Pool the distillates and match again to confirm results.

Therefore 5·3 c.c. of the standard solution contain the same amount of albuminoid ammonia as 500 c.c. of the sample.

Therefore there is 0·053 milligramme of ammonia in 500 c.c. of water,

i.e., 0·0106 milligramme per 100 c.c. or 0·0106 part per 100,000.

Oxidizable Organic Matter (*Tidy's process*).—

Special Reagents.—

1. A standard solution of potassium permanganate made by dissolving 0·395 gramme of potassium permanganate and making up to 1 litre with distilled water. 10 c.c. of this=1 mgm. of available oxygen.

2. A freshly prepared solution of potassium iodide made by dissolving 1 part of potassium iodide in 10 parts of distilled water.

3. Dilute sulphuric acid (1 in 3).

4. Sodium thiosulphate solution 1 gramme to a litre.

5. Starch solution 0·5 gramme to 200 c.c. of cold distilled water. Boil and decant the clear supernatant fluid.

Process.—1. To 100 c.c. of the sample water in a stoppered bottle add 10 c.c. of the standard solution of potassium permanganate and 10 c.c. of the dilute sulphuric acid.

2. To 100 c.c. of cold recently boiled distilled water in another bottle add 10 c.c. of the standard solution of potassium permanganate and 10 c.c. of dilute sulphuric acid.

3. Place both bottles in an air oven at 37°C. for 3 hours. In the presence of organic matter the potassium permanganate is able to part with five-eighths of its oxygen.

4. After 3 hours proceed to estimate the undecomposed permanganate in the bottles.

Naturally, in the distilled water bottle, owing to the complete absence of organic matter, no permanganate will be decomposed. Add sufficient potassium iodide to render both solutions distinctly yellow, due to the liberation of free iodine. The potassium iodide is broken up by the permanganate and free iodine is liberated. The free iodine is proportionate in amount to the undecomposed permanganate.

5. To estimate the amount of free iodine add sodium thiosulphate from a burette and note how many c.c. are necessary to discharge the yellow colour, *i.e.*, to take up all the free iodine. When the yellow colour is nearly gone add a few drops of starch solution and if any free iodine is left the water will turn blue. Go on adding thiosulphate till the blue colour is just gone and read off the number of c.c. used in each bottle. More will, of course, be required in the distilled water bottle as no permanganate was decomposed .

Example.—Say, 28 c.c. of thiosulphate is used in the distilled water bottle.

Therefore 28 c.c. of thiosulphate may be considered=10 c.c. of permanganate=1 mgm. of oxygen.

Say, 25 c.c. of thiosulphate is used in the sample bottle.

Therefore 25 c.c. of thiosulphate=the undecomposed permanganate.

Therefore 28—25 or 3 c.c. of thiosulphate=the oxygen taken up by organic matter.

Therefore if 28 c.c.=1 mgm. oxygen, 3 c.c.=0·107 mgm. of oxygen.

Therefore 0·107 mgm. of oxygen are taken up by 100 c.c. of water;

or 0·107 mgm. of oxygen are taken up by 100,000 milligrammes of water.

Therefore the organic matter in 100,000 parts of water required 0·107 part of oxygen to oxidise it in 3 hours at 37°C.

Nitrates and Nitrites.—

QUALITATIVE TESTS.—1. *Brucine test.*—Take half a test-tube full of the sample water. Add about 3 c.c. of a saturated solution of brucine. Mix, hold on the slant and run in some pure sulphuric acid till the acid forms a layer at the bottom of the tube. If nitrites are present a pink colour forms at once. If nitrates, it forms after some time, or if nitrates are present in large amount, the pink colour rapidly becomes yellow.

If 50 per cent. acid is used, the pink colour is produced with nitrates only and not by nitrites in the absence of nitrates.

2. *Diphenylamine test.*—Take a solution of the salt in sulphuric acid and 5 per cent. hydrochloric acid. Add 3 or 4 drops to 1 c.c. of the sample water to be tested and 2 c.c. of pure sulphuric acid. Mix well. If nitrites are present an immediate blue colour develops while in the prevalence of nitrates free from nitrites this takes some time.

3. *Starch test.*—Add a little starch solution and a drop or two of potassium iodide to the sample in test-tube, then add dilute sulphuric acid. If nitrites are present an immediate blue colour develops. But if nitrates are present in the absence of nitrites, the sample slowly turns blue.

4. *Griess' Meta-phenylene-diamine test.*—Dissolve 0.5 gramme of meta-phenylene-diamine in 100 c.c. of distilled water slightly acidified with dilute sulphuric acid. Prior to use it must be decolorised by animal charcoal. If 1 c.c. of this be added to 50 c.c. of the water in a Nessler glass, a pale yellow tint indicates nitrites and the colour varies in depth (to orange) with the amount. This test is usually performed thus:—

To a Nessler glass add 50 c.c. sample water and to another add 50 c.c. distilled water.

To each add 1 c.c. of a solution made by adding a few drops of H_2SO_4 and a pinch of meta-phenylene-diamine to a test-tube full of water. In 15 minutes the sample glass will develop a yellow colour if nitrites are present.

A more delicate test is to acidify a large bulk of water with acetic acid, distil and test the distillate. If there is sulphuretted hydrogen in the water, it must be got rid of by the addition of carbonate of lead.

Quantitative Estimations.

Nitrites.—

I.—*Griess' test.*

Reagents.—

A standard solution of potassium nitrite is made by dissolving 1·1 grammes of pure silver nitrite in hot distilled water and adding excess of potassium chloride. Cool, make up to 1 litre. The silver chloride is allowed to settle, then 100 c.c. of the clear supernatant liquid is made up to 1 litre.

1 c.c. of this=0.01 mgm. of nitrogen as nitrites. Take several cylinders containing from say 0·02 to 0·1 mgm. of nitrogen as nitrites, prepared by adding 2, 4, 6, 8 and 10 c.c. of the standard solution and make up to 100 c.c. with distilled water. Add 1 c.c. of meta-phenylene-diamine solution to each and also to 100 c.c. of the sample water. Stand 15 minutes and match the colours.

Example.—Say the colour in the sample matches that in the cylinder in which 6 c.c. of the standard solution were added.

6 c.c. of standard solution=0·06 mgm. of nitrogen.

Therefore there is 0.06 part of nitrogen as nitrite per 100,000 parts in the water.

II.—*Hosvay's test for nitrites.*—Dissolve 0·5 gramme of sulphanilic acid in 150 c.c. of dilute acetic acid (specific gravity 1·04); in another flask dissolve 0·1 gramme of naphthylamine in 20 c.c. of distilled water and then add 150 c.c. of dilute acetic acid.

Take 100 c.c. of the sample in a Nessler glass. Put varying amounts of standard potassium nitrite solution in a number of glasses and make the volume of each up to 100 c.c. with distilled water. To each add 2 c.c. of the sulphanilic acid solution and 2 c.c. of the naphthylamine solution. A pink colour is produced if nitrites are present. If there is no pink colour in 15 minutes they are absent. Match the colours to make the estimate.

Nitrites and Nitrates.—

Copper-Zinc Couple process.—In this process all the oxidized nitrites and nitrates are reduce to ammonia.

(*a*) A wet copper-zinc couple is prepared as follows:—

Take a piece of zinc foil about 9 square inches in area and clean it with dilute sulphuric acid. Put it in a saturated solution of copper sulphate for three minutes. Wash in distilled ammonia-free water, then put it in a wide-mouthed stoppered bottle, add 110 c.c. of the sample water and leave overnight at 20°C.

(*b*) Take 10 c.c. of the water and test for nitrites by Griess' or Ilosvay's test; absence of colour shows that the process is complete.

(*c*) Distil the water as in Wanklyn's test and deduct the amount of free ammonia already found in the water; the remainder is the ammonia due to nitrates and nitrites from which the amount of nitrogen may be calculated by multiplying the ammonia value by $\frac{14}{17}$. Allowance must be made for the 10 c.c. of water taken out for the preliminary test in (*b*).

Nitrates.—

The Phenol-Sulphonic Acid method (Picric Acid process).—

Special Reagents.—

1. Phenol-sulphonic acid (Sulpho-carbolic acid). Mix 6 grammes of pure phenol with 3 c.c. distilled water and 37 c.c. of pure sulphuric acid. Digest for several hours at 82°C. Preserve in a tightly stoppered bottle.

2. A standard solution of potassium nitrate, 0·721 gramme to the litre. Dilute tenfold, so that 1 c.c.=0·01 mgm. of nitrogen.

Process.—10 c.c. of the sample and 10 c.c. of the standard solution are each placed in separate platinum dishes and both evaporated to dryness on a water-bath. 3 c.c. of phenol-sulphonic acid are added to each and the dishes kept on a water-bath for a further five minutes. The contents of the two dishes are poured into two Nessler glasses and the dishes washed out with 25 per cent. ammonia solution and the washings added to the respective Nessler glasses. Then more ammonia solution is added cautiously to each Nessler glass till no further deepening of the yellow colour occurs. The contents of the glasses are then filtered, if necessary, and made up to 50 c.c. with distilled water. The glass containing the standard solution assumes a distinct deep yellow colour owing to the formation of potassium nitrophenol sulphonate and the contents of the other glass are also coloured more or less in proportion to the amount of nitrate present. By transferring measured quantities of the deeper coloured liquid into other glasses, which are again filled up to the 50 c.c. mark with distilled water, a match is obtained with the original lighter yellow sample. This reaction depends on the liberation of picric acid

from sulpho-carbolic acid by nitric acid, and the subsequent production of ammonium picrate by ammonia.

Example 1.—Say, 5 c.c. of the 50 c.c. of the darker standard liquid are required when diluted to 50 c.c. to match the sample.

But this 5 c.c. is $\frac{1}{10}$th of the whole volume of 50 c.c.

$\therefore$ it represents $\frac{1}{10}$th of the original 10 c.c. of the standard potassium nitrate solution.

$\therefore$ the nitrates in 5 c.c.$= \frac{1}{10}$ of the original 10 c.c. solution
$= 1$ c.c. of standard solution.

But 1 c.c. = 0.01 mgm. of nitrogen as nitrate,

$\therefore$ there is 0.01 mgm. of nitrogen in 10 c.c. of the sample.

$\therefore$ there is 0.01 mgm. of nitrogen in 10,000 milligrammes of the sample, or 0.1 mgm. of nitrogen in 100,000 milligrammes of the sample or 0·1 part per 100,000.

If the sample is darker than the standard solution, then measured quantities of the sample must be removed and made up to 50 c.c. with distilled water till a match is obtained.

Example 2.—Suppose 20 c.c. suffice for the match, then the sample contains $\frac{50}{20}$, *i.e.*, 2·5 times more oxidized nitrogen than the 50 c.c. of the standard solution. These calculations can be simplified thus:—

(1) Where the control glass is the deeper yellow:—

$\frac{x}{50}$ = parts of nitrogen per 100,000;

(2) Where the sample glass is the deeper yellow:—

$\frac{50}{x}$ = parts of nitrogen per 100,000 parts of water,
where x = amount required to match.

The Gases in Water

Free Carbonic Acid.—

The Lunge-Trillich method.—To 100 c.c. of the sample add a few drops of neutral phenolphthalein solution in alcohol.

Titrate with $\frac{N}{20}$ solution of sodium carbonate till the pink colour remains permanently.

A normal solution of sodium carbonate contains 53 grammes to the litre. Therefore 1 c.c. of the solution = 0·053 gramme of sodium carbonate.

$\therefore$ 1 c.c. of a $\frac{N}{20}$ solution = 0·053 ÷ 20 gramme or 0·00265 gramme of sodium carbonate.

But 106 parts of sodium carbonate neutralize 44 parts of carbonic acid.

$\therefore$ 1 c.c. of $\frac{N}{20}$ sodium carbonate = $\frac{44}{106}$ of 0·00265 or 0·0011 gramme of carbonic acid.

Say, 2·5 c.c. is used, then there is 2·5 × 0·0011 = 0·00275 gramme of carbonic acid in 100 c.c. of sample or 2·75 parts per 100,000.

Carbonic Acid as Carbonate and Bicarbonate.—

Thorpe's method.—To 100 c.c. of the sample add a drop or two of phenolphthalein.

Add standard oxalic acid solution (2·863 grammes to the litre, *i.e.*, 1 c.c. = 1 mgm. of carbonic acid) from a burette till the colour disappears.

Note the amount used, which represents the carbonic acid present as carbonate. Boil for ten minutes; the colour is re-developed as CO_2 is driven off. Add a further amount of standard oxalic acid to decolorise and note the amount used. When the water is boiled and the carbonic acid gas is driven off, the *converted bicarbonate* and the *original bicarbonate* are reduced to carbonates. The second titration will give the amount of carbonic acid remaining after boiling. Twice this quantity will represent the total carbonic acid as bicarbonates prior to boiling; subtract from this the original carbonic acid in the carbonates, to obtain the amount of carbonic acid as bicarbonates originally in the water.

Example.—100 c.c. of water required 3 c.c. of oxalic acid, *i.e.*, = 3 mgms. of carbonic acid as carbonates, or 3 parts per 100,000.

After boiling, 3·5 c.c. of oxalic acid are used.

Therefore the total carbonic acid as bicarbonates is 3·5 × 2 = 7 milligrammes.

Deduct 3 milligrammes of carbonic acid as carbonates and the remainder is the amount of bicarbonates in the original water. *i.e.*, 7—3 = 4 parts per 100,000.

Free Chlorine.—*q.v.*

Sulphuretted Hydrogen.—

Reagents required.—

1. Starch solution.

2. $\frac{N}{100}$ iodine solution. This is prepared by dissolving 1·3 grammes of iodine in a solution of 2 grammes of potassium iodide in 50 c.c. of water and diluting to 1 litre. Further dilute till 10 c.c. of the solution coloured blue with starch solution are decolorized by exactly 10 c.c. of $\frac{N}{100}$ sodium thiosulphate solution (2·464 grammes to the litre). Keep the solution in the dark.

Process.—Titrate 10 c.c. of this centinormal iodine solution in a flask with the water to be tested till the colour of free iodine just disappears. Add 5 c.c. starch solution and run in more iodine till just blue. The slight excess of iodine required to produce this colour is insignificant but it may be deducted from the total amount used.

Each c.c. of the $\frac{N}{100}$ iodine solution=0·17 mgm. of sulphuretted hydrogen.

Therefore the 10 c.c. of iodine used=0·17 × 10 or 1·7 milligrammes of sulphuretted hydrogen in the amount of water required for titration.

Oxygen Dissolved in Water.—The various processes for estimating the oxygen dissolved in water are seldom used in water analysis, though they are of importance in sewage analysis (*q.v.*). It is to be noted that the oxygen dissolved in water is not the same as the oxygen absorbed by oxidisable organic matter determined by Tidy's process.

I.—*Winkler's process.*—

Reagents required.—

1. Manganous chloride 40 grammes to 100 c.c. of distilled water.

2. Potassium hydrate (33 per cent. solution) containing 10 per cent. potassium iodide.

3. Hydrochloric acid (concentrated solution).

4. Sodium thiosulphate ($\frac{N}{10}$ solution). 1 c.c.=0·8 milligramme of oxygen.

5. Fresh starch solution.

Take a glass-stoppered bottle of about 250 c.c. capacity and fill it completely and carefully with the sample water avoiding splashing, lest the water take up oxygen from the air. Add 1 c.c. manganous chloride solution and then 2 c.c. of the potassium hydrate and iodide solution by means of a pipette passed down into the water. Insert the stopper and shake by inverting the bottle several times. Allow to stand for 15 minutes in a dark place. Manganous hydrate is formed which takes up the dissolved oxygen and is converted to the higher hydroxide, according to

the proportion of dissolved oxygen present in the sample of water. Pour the contents of the bottle into a flask containing 3 c.c. strong HCl. The precipitate will dissolve and a yellow colour of free iodine result. Titrate with $\frac{N}{10}$ sodium thiosulphate from a burette until the yellow colour is almost gone. Add a little of the fresh starch solution and again titrate till the blue colour has just disappeared.

When nitrites exceed faint traces the results are too high as no end point is obtainable owing to the reaction between nitrous and hydriodic acids.

This difficulty can be avoided by carrying out the process in the usual way, after dissolving the precipitate by adding hydrochloric acid and introducing with a long pipette 2 c.c. of potassium acetate solution (1000 grammes per litre).

The dissolved oxygen liberates free iodine in equivalent amount which is then neutralized by the thiosulphate. The number of c.c. of thiosulphate used, multiplied by 0·8, gives the amount of oxygen in the sample in milligrammes, from which the parts by weight in parts per 100,000 can be calculated.

Each c.c. of $\frac{N}{10}$ thiosulphate = 0·8 milligramme of oxygen.

Example.—Suppose the capacity of the bottle is 265 c.c. and the amount of reagent used is 3 c.c.

∴ the amount of water tested is 262 c.c.

The $\frac{N}{10}$ thiosulphate solution required to neutralise the liberated iodine is 3 c.c.

∴ the amount of oxygen in 262 c.c. is 3 × 0·8 milligramme

= 2·4 milligrammes.

= 0·91 milligramme per 100 c.c. or 0·91 part per 100,000.

II.—*Thresh's Process* for the estimation of dissolved oxygen requires special apparatus and the use of coal gas and is not now largely used.

Report on Water Analysis.—

(1) *Source.*—It is often possible to give a general opinion of the source of a water from its chemical examination. Rain-water, water from upland surfaces and peaty waters give low hardness and low inorganic solids figures. River water varies according to the season, the Gangetic waters, for instance, being alkaline from 4 to 6 months in the winter season and acid in the 4 to 6 months before, during and after the monsoon, and not only the reaction

but the hardness and ammonia figures show great variations according to the land through which the river flows, its length, the introduction of water from smaller streams, etc. Even a knowledge of the geological formations of the area does not permit of accurate statements being made as regards the source of a water, though after percolating strata such as sandstone, chalk and greensand, a high figure for hardness and for inorganic solids will be met with. A water which presents unusual results should be carefully considered and unless the results can be attributed definitely to strata a cautious opinion should be given. The chlorine and nitrite figures may be raised by chalk and greensand strata.

(2) *Suitability of water for drinking purposes.*—The complete series of figures must be considered before a report is made. The usual results of analyses of contaminated waters are given below:—

[+=figure raised; ±=figure may be raised or low;—=low figure].

	Free and saline ammonia.	Albuminoid ammonia.	Oxygen absorbed.	Volatile solids.	Nitrates.	Nitrites.	Chlorine.
Organic vegetable contamination.	—	+	+	±	—	—	—
Doubtful organic animal pollution (Condemn unless due to strata).	—	±	+	±	±	±	±
Organic animal pollution—							
A.—Old	—	—	±	±	+	—	+
B.—Recent	+	+	+	±	+	+	+

In considering the results certain limits are generally accepted and must be given due weight if any other figures except the albuminoid ammonia, oxygen absorbed and possibly also the volatile solids are raised.

Analysis Limits.—A moorland surface water must not have a free and saline ammonia figure above 0·001 part per 100,000. When the albuminoid ammonia reaches 0·008 part per 100,000 the

free and saline ammonia must not exceed 0·005 part. Nitrates must never exceed 0·1 part per 100,000 unless definitely due to strata. Nitrites, if present at all, should at once condemn a water unless the ammonias and oxygen absorbed are very low, *i.e.*, nitrites due to the reduction of nitrates by iron salts, etc. The chlorine figure means nothing by itself as near the sea or in chalk strata it may be raised, but should be about 1 part per 100,000. Oxygen absorbed must not exceed 0·1 part per 100,000 unless in vegetable contamination, where no other figure must be raised except the albuminoid ammonia and volatile solids. Iron must not exceed $\frac{1}{4}$ grain, copper $\frac{1}{15}$ grain, lead $\frac{1}{20}$ grain and zinc $\frac{1}{10}$ grain per gallon; the presence of other poisonous metals condemns a water at once. Total hardness should not exceed 30 parts per 100,000 and permanent hardness not more than 10 parts. The non-volatile solids should not exceed 100 parts per 100,000.

(3) *Suitability for washing, cooking, industrial and other purposes.*—The only factor which is of great importance is the hardness and a hard water should not be condemned unless it is not feasible to treat the water with lime, etc., or to instal a "Permutit" or other system for softening the water. The amount of temporary as against permanent hardness is, in this case, most important. A *soft water* is one which contains 10 or less parts per 100,000: a water which contains 10 to 20 parts is a *hard water* and one which contains between 20 to 30 parts a *very hard water.*

(4) *Suitability of waters for mosquito-breeding.*—The importance of water analysis in relation to the investigations regarding the breeding places of mosquitoes was noted by Waddell and more recently by Williamson, Hackett and Senior-White, the last named investigator having raised this method of investigation to an important level. From their experiments and some recent work carried out in the United Provinces it is obvious that this offers a line of research the importance of which cannot be gauged.

Hydrogen-ion concentration is only important as indicating the likelihood or not of anopheline mosquitoes breeding in a water, extremes of P_H being inimical to larvæ. According to Senior-White, the P_H itself means nothing in the presence of CO_2 while acidity due to other than CO_2 inhibits anopheline growth. Low absorbed oxygen also inhibits larval growth but is not apparently of great importance. The rôle of phosphates and carbonates is doubtful. The important estimations are that of albuminoid ammonia: a figure higher than 0·15 part per 100,000 definitely kills larvæ and the free and saline ammonias, 0·1 part per 100,000 being inimical to all larvæ of 'carrier' mosquitos.

Water Standards in the United Provinces.—The following chemical standards have been worked out at the Provincial Hygiene Institute, Lucknow, to judge the potability of water supplied by various water works in these Provinces. It will be seen that if reasonable care is exercised such standards can be easily maintained. The figures indicate parts per 100,000:—

Free and saline ammonia ..	0·002 to 0·005
Albuminoid ammonia ..	0·005 to 0·01
Chlorine	1·0
Nitrates } as nitrogen, must Nitrites } not exceed ..	0·1
Total hardness	20
Permanent hardness should not exceed	10
Total solids	20 (in well waters 20 to 60 parts per 100,000).
Oxygen absorbed from permanganate in 3 hours at 37° C.	0·1

Reaction should be faintly alkaline.

In case the albuminoid ammonia is high, the free and saline ammonia should be less than 0·005 part per 100,000.

It will be appreciated that sound judgment on a water analysis cannot be made on absolute analytical figures and in the tropics it is especially imperative for the medical officer to acquaint himself with other useful information such as (*i*) the geological nature of the district, especially the saline constituents of the strata, (*ii*) the agricultural condition of the gathering ground together with method of storage and distribution, (*iii*) the surface and subsoil drainage of the area, (*iv*) the amount of the rainfall before and after the collection of the sample, etc.

The following typical analyses of water from different sources available in these Provinces are given here to show the wide differences to be met with both in the inorganic and organic constituents of various waters. A careful study of these will undoubtedly be of considerable help in interpreting results.

TYPICAL ANALYSES OF WATER FROM DIFFERENT SOURCES AVAILABLE IN THE UNITED PROVINCES.

	1	2	3	4	5	6	7	8	9	10
Physical characters	Slightly turbid	Slightly turbid	Muddy	Transparent	Clear	Slightly brown	Clear	Transparent	Slightly hazy	Clear
Reaction	Slightly alkaline	Slightly alkaline	Slightly alkaline	Slightly alkaline	Neutral	Faintly acid	Slightly alkaline	Slightly alkaline	Alkaline	Slightly alkaline
Free and saline ammonia	0.0017	0.011	0.014	0.003	0.011	0.0005	0.015	0.004	0.004	0.0032
Albuminoid ammonia	0.0055	0.041	0.044	0.009	0.009	0.018	0.006	0.007	0.006	0.009
Oxygen absorbed from potassium permanganate in 3 hours at 37° C.	0.06	0.085	0.508	0.12	0.13	0.21	0.086	0.034	0.08	0.06
Chlorine	0.8	0.5	0.6	1.1	2.5	0.4	3.8	0.5	64	1.8
Nitrites	0.002	0.008	Traces	Faint traces	Traces	Nil	Nil	Nil	Traces	Traces
Nitrates	Faint traces	0.006	Nil	Nil	1.4	0.07	Nil	Nil	Traces	Traces
Total solids	14	16	20	20	31	13	30	40	212	32.6
Fixed solids	10	10	10	10	13	4	20	24	117	18.8
Volatile solids	4	6	10	10	18	9	10	16	95	13.8
Appearance on ignition	No charring	Brown charring	Brown charring	No charring	No charring	Brown charring	Faint charring	No charring	No charring	No charring
Total hardness	8	9	8	18	9	4	19	28	92	31.8
Temporary hardness	2	5	4	13	3	0	12	12	34	10
Permanent hardness	6	4	4	5	6	4	7	16	58	21.8

Column 1 gives the analysis of a sample taken from the Ganges water at Hardwar in the month of March. Excepting for slight turbidity the water is quite potable and this shows that the river water as it comes down from the mountains is free from organic pollution. The subsequent organic contamination, due generally to habitation and cultivation, is evidenced by analysis of the sample shown in column 2 taken down the same river on the same date, showing comparatively recent animal contamination.

Column 3 deals with a sample of raw water from the river Gomti in Lucknow taken in the last week of June before the approach of the regular monsoon. It shows organic contamination as evidenced by high ammonia, chlorine and absorbed oxygen figures in addition to the brown discoloration of the solids. The same water after sand filtration and chlorination becomes potable as is shown by the analysis of the sample in column 4 where the various figures of analysis compare fairly well with the standards.

The figures in columns 5 and 6 very clearly bring out the characteristic difference between animal and vegetable pollution. In column 5 is a sample of shallow well water contaminated with sewage as indicated by high free and saline ammonia (associated with low albuminoid ammonia), chlorine and nitrogen figures. The sample in column 6 is a typical peaty water contaminated with products of vegetable decomposition as shown by the brown colour, acid reaction, brown charring, high albuminoid ammonia and oxygen absorbed.

Column 7 deals with a water sample from a school well with sound masonry construction. The evidence of animal contamination, probably due to the well being uncovered, is furnished by the high free and saline ammonia and chlorine figures.

Column 8 represents a sample from a hill spring free from organic pollution but showing the usual hardness and fairly large amount of total solids derived from the strata.

Column 9 gives the analysis of a sample from a tube well sunk in a saline bed. It contains a large amount of soluble mineral matter derived from the underlying strata. It is free from organic contamination but the large amount of total solids consisting mainly of chlorides, sulphates and bicarbonates producing a high degree of hardness makes it unfit for drinking and other domestic purposes.

Column 10 deals with a sample from a stand post of a tube well water-supply. It is free from organic pollution but is fairly hard owing to the presence of soluble mineral matter derived

from deeper strata. A comparison with the sample represented in column 9 will show how the composition of subterranean water is influenced by the nature of the underlying strata.

ANALYTICAL SCHEME (after Kenwood)

Time.—Three hours.

1. Start Tidy's process for the oxidizable organic matter.
2. Start evaporation for the total solids.
3. Start concentration for poisonous metals.
4. Start Wanklyn's process for free and saline ammonia.
5. Start boiling for hardness tests.
6. Start the boiling of alkaline permanganate for the estimation of albuminoid ammonia.
7. Apply qualitative tests for nitrates, nitrites, sulphates and phosphates.
8. Start estimation of the nitrites and nitrates.
9. Make estimation of the chlorine.
10. Estimate the hardness.
11. Add the alkaline permanganate to the boiling flask and proceed with the second stage of Wanklyn's process.
12. Estimate the free ammonia.
13. Finish the nitrites and nitrates.
14. Examine the deposit.
15. Estimate the albuminoid ammonia.
16. Complete the Tidy's process.
17. Test for poisonous metals.
18. Estimate the total solids, both volatile and non-volatile.

Sea Water.—Sea water contains approximately from 3,300 to 3,900 parts per 100,000 of total solids and from 1,700 to 2,000 parts of chlorine. The free and saline ammonia is the best figure from which to deduce animal contamination and should not be above 0·002 part per 100,000. The free and saline ammonia figure is not only raised immediately after contamination but the high figure persists for several weeks. The albuminoid ammonia figure is also raised after contamination but owing to variations is not a reliable indicator of contamination. The oxygen absorbed only indicates gross pollution, the normal figure in sea water being 0·6 part. A negative result for nitrites means nothing, while a positive result may often be found long after contamination. The presence of phosphates is indicative of contamination since phosphates are absent in pure sea water.

CHAPTER III

THE ANALYSIS OF SEWAGE AND SEWAGE EFFLUENTS

Most of the processes are the same as in water analysis except that sewage diluted with distilled water is used instead of the crude sewage or effluent. The physical characters such as colour, smell (direct and after 48 hours' incubation at 37° C.), turbidity and amount of deposit should be noted.

The dilution of the sewage or effluent required may vary within wide limits. Generally not more than 20 c.c. of effluent or 10 c.c. of sewage should be used for Tidy's, Kjeldahl's or Wanklyn's processes.

Kjeldahl's process.—This method of estimating the total organic nitrogen is used in sewage analysis as being more complete than Wanklyn's process for the estimation of ammonia.

1. Put 10 c.c. of sewage or 20 c.c. of the effluent in a small flask, add 1 c.c. of strong sulphuric acid, mix well and then evaporate slowly to 5 c.c. over a water-bath or a small flame, guarded with wire-gauze.

2. Add 20 c.c. of pure sulphuric acid and boil slowly till the solution is pale yellow in colour.

3. Cool, transfer to a distilling flask, adding washings of the small flask as well and make up to 500 c.c. with ammonia-free distilled water and neutralise with excess potash till distinctly alkaline.

4. Put a piece of recently ignited pumice-stone in the flask to prevent "bumping."

5. Distil over about 400 c.c., receiving the first portion of the distillate into 20 c.c. of ammonia-free water slightly acidulated with 2 drops of dilute sulphuric acid.

6. "Nesslerise" the ammonia. The result multiplied by $\frac{14}{17}$ will give the amount of nitrogen.

7. Deduct the amount of free and saline ammonia (found by Wanklyn's test) in the same amount of the sewage or effluent and the difference is the *organic nitrogen.*

8. Make a blank experiment to determine the ammonia present in the reagents and water employed and deduct this.

Dissolved Oxygen in Sewage.—I.—*Winkler's process is* usually carried out. But the process as used in the case of water is unsuitable owing to the presence of a large amount of organic matter and so *Ridcal and Stewart's* modification is employed. This measures the *dissolved oxygen absorbed in five days* at 65° F.

Reagents required.—

1. Concentrated sulphuric acid.
2. Concentrated hydrochloric acid (free from traces of chlorine).
3. $\frac{N}{8}$ permanganate (3·94 grammes $KMnO_4$ per litre).
4. Potassium oxalate (2 per cent.).
5. Manganous chloride (33 per cent.).
6. A mixed solution of caustic potash and iodide of potassium, containing 70 grammes KOH and 10 grammes KI per 100 cc.
7. $\frac{N}{20}$ sodium thiosulphate solution, containing 12·4 grammes of the salt per litre (to be kept in dark bottle).

The Process.—Shake the effluent. Measure out 300 c.c. and dilute it five times by mixing it with four times its volume of tap water.

Measure the capacity of four small similar bottles of about 350 c.c. capacity and fill each up to the mouth with the mixture. Let them stand for five minutes and then stopper. Keep two bottles in an incubator at 18° C. for five days. Estimate the dissolved oxygen in the water of the other two bottles, as given below:—

1. Add 1 c.c. of concentrated sulphuric acid and then a sufficient quantity of the permanganate to retain a pink colour after 20 minutes. 1 or 2 c.c. of $\frac{N}{8}$ permanganate are usually sufficient for this purpose. Mix the contents and allow to stand for 20 minutes to oxidise any nitrites to nitrates.

2. Add 1 c.c. of the oxalate solution to remove the excess colour of the permanganate; stir well.

3. When the liquid has become colourless introduce to the bottom of the bottle by pipette 1 c.c. of the manganous chloride solution and then 4 c.c. of the potassium hydrate and potassium iodide solution. If a precipitate does not result add more of the hydrate and iodide solution.

4. Invert the bottle several times; then set it aside for 10 minutes.

5. Pour the contents of the bottle into a flask and add 5 c.c. of the hydrochloric acid; insert the stopper and place aside for ten minutes, occasionally shaking the flask.

6. Discard 20 c.c. of the liquid and titrate the remainder with thiosulphate till colourless, using clear starch solution as indicator.

7. Repeat the process with the second bottle and take the mean of the two results.

8. After five days estimate the dissolved oxygen in the two bottles incubated. Take the mean of the two results, subtract it from the first mean, and calculate the amount of dissolved oxygen absorbed per 100,000 parts in five days.

Example.—Capacity of each bottle = 332 c.c.

So, after discarding 20 c.c. the volume of the sewage (diluted five times) is 332—20 or 312 c.c. If the thiosulphate solution required for titrating the first set of bottles equals 6·82 and 6·98 c.c. respectively, the mean of the two readings=6·9 c.c.

1 c.c. of $\frac{N}{20}$ thiosulphate solution=0·4 milligramme of oxygen.

Therefore, 6·9 c.c.=6·9×0·4 milligramme of oxygen in 312 c.c. or 0·884 part per 100,000.

Similarly with the other set of bottles if the amount of dissolved oxygen represented by the mean is 3·11 c.c., the total oxygen per 100,000 parts is $\frac{3.11\times0.4\times100}{312}=0.4$.

∴ the difference 0·884—0·4=0·484 is the amount of oxygen absorbed from the mixture.

But the mixture was diluted five times by water;

So it should be multiplied by 5 to get the amount in the original sewage, *i.e.*, =2·42 parts per 100,000.

II.—*Lett's and Blake's method.*—

Reagents:—

1. Ferrous sulphate 12½ grammes with 2 or 3 c.c. of strong sulphuric acid, made up to 250 c.c.

2. A standard solution of permanganate, 5·638 grammes to the litre; or a standard solution of bichromate, 8·79 grammes to the litre;

3. Sulphuric acid, 50 per cent. solution in distilled water.

Process.—1. Measure the capacity of a separating flask of about 300 c.c. capacity and fill it completely with the sample, and then remove 7 c.c. by means of a pipette. Add 5 c.c. ferrous sulphate solution and then 2 c.c. ammonia by means of a pipette passing down to the bottom. Put in the stopper and mix for 10 minutes.

2. Invert the separator and fill the tube at the bottom with 50 per cent. sulphuric acid and open the tap. Allow the acid to mix with the alkaline sample.

3. Place in a procelain dish and titrate with standard permanganate solution if analysing ordinary water or standard bichromate solution if testing sewage or sea water.

If bichromate is used potassium ferricyanide is used as an external indicator. A small crystal is cleaned, washed and dissolved in 10 c.c. of distilled water and a series of drops placed on a porcelain slab. As long as oxidation is incomplete a blue colour will be produced on dropping a little of the liquid during the process of titration on each drop of ferricyanide solution.

A blank experiment is now made with water in the flask. About 10 c.c. of sulphuric acid must be added first and then the 5 c.c. of ferrous sulphate so that the ferrous sulphate will not absorb oxygen. In this blank experiment ammonia solution is not added. Titrate as before and the difference between the two titrations gives the amount of dissolved oxygen in the volume of sample tested. Calculate in parts per 100,000.

Example.—Say, the capacity of the bulb=333 c.c.

Subtract 7 c.c. for the reagents; so the volume of water tested is 326 c.c.

Bichromate (or permanganate) solution used for the blank experiment=4·4 c.c.

Bichromate (or permanganate) solution used for the actual experiment=3·2 c.c.

Therefore 4·4—3·2=1·2 c.c. of the bichromate (or permanganate) solution represents dissolved oxygen in the sample.

But 1 c.c. of bichromate (or permanganate) solution=1 c.c. of dissolved oxygen at normal temperature and pressure.

Therefore 1·2 c.c. of bichromate (or permanganate) solution=1·2 c.c. of oxygen in 326 c.c. of the water, or $\frac{1{\cdot}2 \times 1000}{326}$ =3·68 c.c. per litre.

But 7 c.c. of oxygen per litre=1 part per 100,000 at N. T. P.

Therefore 3·68 c.c. of oxygen per litre= $\frac{3{\cdot}68}{7}$ part per 100,000 at N. T. P.=0·52 part per 100,000.

Standards for effluents and sewage vary greatly according to the size of the river which ultimately receives them. (*See* "Indian Hygiene and Public Health" by the Authors).

Average analysis standards of effluents after treatment.—

Free and saline ammonia	.. 1·5 parts per 100,000.
Albuminoid ammonia	.. 0·1 part per 100,000.
Oxygen absorbed in 2 hours at 80° F. ..	.. 1·0 part per 100,000.
Chlorine	.. 10·0 parts per 100,000.
Nitrates and Nitrites ..	20 or more parts per 100,000.

CHAPTER IV

ANALYSIS OF SOILS

The Classification of Soils.—

A.—*Surface soil* is the superficial surface of the earth's crust—"the humus"—in which organic matter occurs. The organic matter may exist at a great depth or only in the first few feet of soil, this depending mostly on the amount of rain and subsoil water, the type of soil and the amount of surface organic decomposition.

The *classification* of surface soil is based on the amount of sand, chalk, humus, etc., it contains.

Peaty soil contains from 60 to 80 per cent. by weight of organic matter.

Sandy soil is soil containing less than 10 per cent. of clay, less than 5 per cent. of chalk and is not rich in peaty matter.

Sandy loam is soil containing between 10 and 40 per cent. clay.

Loamy soil contains between 40 and 70 per cent. of clay.

Clay loam contains 70 to 85 per cent. of clay.

A strong clay soil is a soil where clay forms from 85 to 90 per cent. of the total soil and if this is free from sand this clay is termed "pure agricultural clay."

The *composition* is extremely complex and is affected by various factors such as the addition of manures to the surface, the putrefaction, fermentation and breaking down of organic matter, the presence of matter foreign to the area which has been carried down by rivers, etc. Acids (such as sulphuric, phosphoric, carbonic, hydrochloric, humic and silicic), oxides (such as iron oxide), alkalies (soda, ammonia and potash), earths (silica, alumina, lime, magnesia), gases and moisture are present.

B.—*The Subsoil* is that layer of earth resting on the natural geological formation in which organic matter is not present. Subsoil is classified as (*i*) *sedimentary,* (*ii*) *igneous* and (*iii*) *metamorphic.*

Sedimentary subsoils are usually stratified and consist of chalk, clay, gravel, sand, coal, gypsum (calcium sulphate), sandstones and limestones (carbonates or sulphates of calcium), dolomite (calcium and magnesium carbonate), oolite (granular limestone).

Igneous subsoil is formed by the action of heat. It mainly consists of the double silicate of alumina and potash (felspar) and may contain iron oxides, lime and phosphates. Granite is an example of igneous subsoil.

Metamorphic subsoil consists of the silicate, lime, iron magnesium and alumina containing rocks which have been altered by pressure or chemical changes, *e.g.*, marble, mica, quartz and slate.

Temperature.—The temperature of the soil varies with that of external air but at a depth of 12 feet or so it is more or less constant. Independent of latitude, the temperature of the soil at 100 feet deep is 52°F. and a rise of 1°F. for every 66 feet of further descent occurs.

The **Specific heat** of soil ranges from 0·2 to 0·5, being highest in peaty soils. Heat absorption depends upon the composition of the soil. As a relative indication, if sand absorbs 100 units then clay absorbs 70, chalk 60 and humus 50. A moist soil is cold owing to the specific heat of the water being high.

Porosity or Pore Volume.—This is the sum total of the interstitial spaces between the soil particles which may be filled with air or water, and does not depend merely on the size of the particles, but on their uniformity of size and arrangement. Porosity is determined by dividing the apparent specific gravity of the soil by its real specific gravity.

(*a*) The apparent specific gravity is found by filling a previously weighed cylinder of 1000 c.c. capacity with the soil, introducing a little at a time, and packing it lightly by tapping the cylinder from time to time. The cylinder when full of soil is reweighed and, deducting the weight of the empty cylinder, the weight of soil occupying 1000 c.c. volume is found out and the apparent specific gravity is calculated by dividing the weight by 1000.

(*b*) The real specific gravity is found by a specific gravity bottle. First the bottle is weighed dry and then full of water at 15°C. After these two weighings, 10 grammes of soil, dried at 100°C., are put in the bottle which is then filled with water and reweighed. The real specific gravity is then calculated by finding out the weight of water displaced by 10 grammes of soil.

All porous soils are permeable and absorptive. Sand is highly permeable but it is not very absorptive. Sandstones are both permeable and absorptive. The more permeable the soil is, the drier it is. As a general rule permeable soils retain little water while absorptive soils are not very permeable.

Moisture.—Moisture present in soil exists mainly as capillary water held up in capillary spaces.

The amount of moisture present in soil is determined by finding out the loss in weight by drying a known weight of soil, say 50 grammes, on a water-bath and then in a hot-air oven at 95°C. until the weight is constant.

The capacity for absorbing moisture is found out by soaking a known weight of dried soil in water for several hours. The extra amount of water is drained off through muslin and the soil reweighed; the increase in weight denotes the amount of water absorbed. Another simpler method is to connect two burettes by long rubber tubing in the form of a U-tube. One is filled with a known amount of dried soil and the other with water. The water burette is gently raised till the water level in the soil burette rises above the soil level without disturbing the soil particles. After a few hours when the soil has absorbed its maximum amount of water, the soil burette is raised to drain off all the water. The loss of water indicates the amount of water absorbed by that amount of soil.

The water which rests on the first impermeable stratum is called the ground or subsoil water. The level of this subsoil water forms a curve; its flowing velocity seldom exceeds 50 ft. in 24 hours. The depth of the subsoil water is found out by trial holes and its fluctuations by an arrangement whereby a cord is made to move round a pulley. A float is attached to the end of the cord which rests on the subsoil water through a hole in the ground. The float is counterpoised by a weighted pointer moving along a vertical scale and the fluctuations of the subsoil water are noted by the movement of this pointer along the scale.

Size of Soil Particles.—The determination of the size of the particles of soil may be made by sifting through Knopp's set of sieves. The soil is first dried and then weighed. The large pebbles, roots, etc., are collected and also weighed. The set of sieves is arranged in order of diminishing diameters, one over the other, keeping the coarsest sieve topmost. The whole set is thoroughly shaken till the particles are retained by the various sieves. The contents of the several sieves are collected and

separately weighed; the percentage weight is calculated and the results are expressed as follows:—

(*a*) Coarse mass removed by hand;

(*b*) Particles on the first sieve more than 7 mm. diameter as *coarse gravel.*

(*c*) Particles on the second sieve between 7 and 4 mm. diameter as *medium gravel.*

(*d*) Particles on the third sieve between 4 and 2 mm. diameter as *fine gravel.*

(*e*) Particles on the fourth sieve between 2 and 1 mm. diameter as *coarse sand.*

(*f*) Particles on the fifth sieve between 1 and 0·3 mm. diameter as *medium sand.*

(*g*) Particles on the sixth sieve finer than 0·3 mm. as *fine sand.*

The portion retained by the last sieve is further classified by washing in a Knopp's soil-washing cylinder. This is a glass cylinder 55 centimetres long, fitted with 4 taps at intervals of 10 centimetres. The soil from the last sieve is weighed and put in the cylinder which is filled with water to 10 centimetres above the highest tap. The whole is well shaken and then allowed to settle. The top tube is first opened and the water is collected in a weighed dish, evaporated and the total solids weighed. The water from other taps and the bottom of the cylinder is similarly removed, in separate dishes, the solids calculated and the clayey matter in the fine soil classified as a percentage weight. The process is facilitated by first treating the soil by a weak acid to dissolve carbonates and decompose humates with the liberation of humic acid and then washing with ammonia to dissolve out this humic acid.

The acid employed is generally very weak hydrochloric acid so that only carbonates may be dissolved while other mineral materials may not be effected.

Estimation of Sand and Clay.—Ten grammes of soil are thoroughly shaken up with 25 c.c. of water and the whole is added to 100 c.c. of 0·1 per cent. of ammonium chloride solution and left for a few minutes. The supernatant turbid fluid is decanted and collected. The process is repeated till the fluid is clear. The residue is washed with dilute hydrochloric acid and then with water, dried and weighed and the percentage calculated and recorded as *sand.* The turbid samples of water are all

mixed and acidified with hydrochloric acid and filtered after 3 or 4 hours. The precipitate is washed, dried and weighed as *clay*.

Sand makes a soil friable and, owing to its low specific heat, is easily heated and easily cooled. It is very permeable but less absorptive. Clay, on the other hand, is less permeable but more absorptive and forms a retentive soil.

Determination of percentage of sand in clay.—Heat a weighed quantity of the soil with sulphuric acid, then boil with water, filter, dry and weigh the insoluble matter. Boil part of this insoluble matter with some sodium carbonate. The insoluble residue is sand, and, after drying and weighing it, the percentage in the original quantity of insoluble matter left after treating with sulphuric acid is calculated.

Lime may be estimated by treating the earth in a litre shaking flask for half an hour with 500 c.c. of $\frac{N}{10}$ hydrochloric acid and titrating a portion with the same normality of soda, using phenolphthalein as an indicator. The difference between the amounts of hydrochloric acid and soda solutions used indicates the quantity of lime present which can be calculated easily.

Phosphates, nitrates, sulphates, etc., can easily be estimated by macerating a weighed quantity of soil in water or other suitable solvent and proceeding as in water analysis, but this is more the work of an agricultural chemist.

Estimation of water soluble constituents of soil.—Water dissolves much of the soluble constituents of the soil, especially when it is saturated with carbon dioxide. Plants derive their food from these soluble constituents of soil and so a rough and ready method of examining the nature of mineral constituents of a soil is to analyse the ash of plants growing over that soil.

Schulze's method for the estimation of minerals in soil.—Several funnels are fitted with strong filter-paper and air-dried soil (unpulverised) is placed on them. Distilled water is poured over the soil. The filtrate, if turbid, is again poured over the soil. The filtrate from the various filters is mixed and then divided into two portions, one large and the other small. The larger portion is concentrated by evaporation and tested for chlorine and organic matter as in water analysis. The other portion is evaporated to dryness and ignited completely to burn all organic matter. The ash is then dissolved in hydrochloric acid (carbonates being indicated by effervescence), and again

evaporated to dryness. The dry ash is moistened with a drop of hydrochloric acid, dissolved in warm water and then filtered. The filtrate may be tested for sulphuric acid, phosphoric acid, iron, magnesia, potash, soda and other mineral constituents and the residue on the filter-paper for silicic acid, carbon and clay.

Humus may be estimated by extracting the soil with dilute hydrochloric acid to set free the humic acid from the humates of lime and magnesia. The residue is extracted with ammonia which is then evaporated, leaving a black lustrous humus with some ash. This is weighed, then slowly ignited and converted into ash. The weight of the ash when deducted from the previous weight of the black mass gives the amount of humus.

Total nitrogen is estimated by Kjeldahl's method. Ten to fifteen grammes of fine air-dried soil are placed in a flask and 25 c.c. of pure sulphuric acid poured over it. When frothing has subsided 10 grammes of potassium sulphate are added to raise the boiling point and ½ gramme of anhydrous cupric sulphate as an oxidising agent. Heat till the mixture is of a yellow colour. Add 50 per cent. caustic potash solution (recently boiled to expel the ammonia) till the liquid is alkaline (indicated by copper being precipitated as blue hydroxide). The rest of the process is done as described under Sewage Analysis.

CHAPTER V

THE ANALYSIS OF AIR

The composition of air is nitrogen 78·07 per cent., oxygen 20·95 per cent., argon 0·94 per cent., carbonic acid 0·04 per cent. *plus* aqueous vapour, traces of rare gases and suspended matter. In towns ammonia, sulphur dioxide, sulphuretted hydrogen and sulphuric acid are frequently present.

Estimation of Oxygen.—

I.—*By Eudiometry.*—This is done in *Hempel's double absorption pipette.* The absorbent is a solution of pyrogallic acid and caustic potash: 15 grammes of pyrogallic acid and 50 grammes of caustic potash to a litre of distilled water. The first bulb contains the absorbent and the third water.

The Process.—100 c.c. of the air to be examined are drawn into the eudiometer burette previously filled with water. This is driven into the absorbent in the pipette, left in contact with it for three minutes and then brought back into the eudiometer. The process is repeated several times till the final difference in volume is constant. The volume of unabsorbed air is noted and the difference gives the amount of oxygen and carbonic acid gas which was in the air. The latter when estimated by Pettenkofer's process described below, should be deducted to get the volume of oxygen in the original 100 c.c. of air.

II.—*Duma's process.*—A known quantity of air is passed over red-hot spongy copper in a combustion tube after having been freed of its carbonic acid by aspirating through caustic potash solution. The oxygen is estimated as copper oxide by finding the increase in the weight of the copper.

Estimation of Carbonic Acid in Air.—

I.—*Pettenkofer's Alkalimetric Process.*—

Reagents:—

1. Baryta water (4·5 grammes of barium hydrate and 0·5 gramme of barium chloride to a litre of water). The baryta water must be protected from carbon dioxide by drawing the incoming air through a tube containing soda lime.

2. Oxalic acid, 2·819 grammes to the litre. Of this, 1 c.c.=0·5 c.c. of carbonic acid at standard temperature and pressure.

3. Solution of phenolphthalein (1—250 of alcohol).

The Process.—1. Collect the air sample in a 4-litre air-jar by filling it with water and emptying out the water in the place being examined. Breathing in the direction of the jar must be avoided. Stopper the jar. Note the possible sources of vitiation in the room, *i.e.*, gas burners, lamps, number of occupants, etc. Note the temperature and pressure in the place being examined.

2. Add 50 c.c. of barium hydrate solution quickly and roll the jar round occasionally for 30 to 45 minutes. This 50 c.c. will displace 50 c.c. of air.

3. Test the alkalinity of another 50 c.c. of barium hydrate solution with standard oxalic acid and phenolphthalein.

4. Test the alkalinity of the 50 c.c. in the air-jar in the same way.

5. The difference in the number of c.c. of acid used will be due to the carbonic acid present in the volume of air used.

6. The volume of air used will be the capacity of the air-jar *minus* 50 c.c. space taken up by the barium hydrate.

7. The result must be returned as the volume of carbonic acid at standard temperature and pressure, *i.e.*, at 0°C. and 760 millimeters of mercury.

By *Boyle's law* the volume of air varies inversely as the pressure.

By *Charles' law* air contracts on cooling $\frac{1}{273}$ or 0·00366 of its bulk for every degree centigrade down to 0°C.

Therefore V volumes of air at current temperature and pressure will represent $\frac{V \times P}{760 \times (1 + 0{\cdot}00366t)}$ at standard temperature and pressure, where V=the volume of air, P=the current barometric pressure and t=the current temperature.

Example.—The difference in the amounts of acid used was 3·2 c.c.

But 1 c.c.=0·5 c.c. carbonic acid.

Therefore, 3·2 c.c.=1·6 c.c. carbonic acid.

The capacity of the jar was 4000 c.c.

Therefore the air sample was 4000—50 or 3950 c.c. at, say, 750 millimeters and 15°C.—the current pressure and temperature.

Therefore, the volume of air at standard temperature and pressure will be $\frac{3950 \times 750}{760 \times (1 + 0{\cdot}00366 \times 15)} = \frac{2962500}{760 \times 1{\cdot}0549} = 3{,}695$ c.c.

Therefore there were 1·6 c.c. carbonic acid in 3,695 c.c. or 0·043 per cent. at standard temperature and pressure.

The amount of carbonic acid in the outside air is similarly examined and the difference in the amount of carbonic acid will represent the *added carbonic acid impurity* of the inside air. 1 c.c. of carbonic acid at standard temperature and pressure weighs 1·96633 milligrammes.

Therefore the relation between the volume and the weight is 1 : 1·96633; so, if the amount is to be calculated in weight in milligrammes, multiply the volume in c.c. by 1·96633.

II.—*Lunge and Zeckendorf* have originated a process of pumping air through a decinormal solution of sodium carbonate to which a drop of phenolphthalein has been added until the colour is discharged.

The number of pressures of the rubber ball being known, the amount of carbonic acid is found by a reference to Lunge's table or by a simple calculation if the capacity of the ball is determined.

III.—*Haldane* has made an apparatus for estimating the carbonic acid by eudiometry, caustic potash being the absorbent. The apparatus is complicated and costly and the technique has to be very exact to exclude error.

The Organic Matter in Air is estimated by passing a large measured volume of air through ammonia-free distilled water which will retain all the soluble organic matter. The water can then be tested by Wanklyn's process for its ammoniacal organic matter or by Tidy's process for its oxidizable organic matter, bearing in mind that in the latter case nitrous acid, sulphurous acid, sulphuretted hydrogen or tarry matters, if present, will also reduce permanganate.

The *results* obtained from air analyses are expressed as milligrammes per cubic meter (1000 litres). 100 liters of air are usually examined by aspirating this quantity through 500 c.c. of water in two 250 c.c. flasks. The 500 c.c. are then tested for the free and saline and albuminoid ammonia and the results calculated as per cubic meter. Outside air usually contains *albuminoid ammonia* up to 0·1 mgm. per cubic meter, *i.e.*, 1000 litres. *Free and saline* ammonia in towns averages 0·06 mgm. per cubic meter.

Carbon Monoxide.—This is often found in air near fires and where coal gas is used, near cast-iron stoves, coke ovens,

brick-kilns and cement works. It is also markedly present in tobacco smoke.

QUALITATIVE TESTS.—I.—*Vogel's test.*—Put 100 c.c. of distilled water in a wash bottle and add a little defibrinated blood. 10 litres of air are then drawn through the water by an aspirator. Roll the bottle round, let it stand for half an hour and then examine some of the contents with a spectroscope.

The *spectroscope* helps us to examine the spectra of various chemical substances. In all its essentials it consists of a glass prism mounted on a circular table, a collimator and a telescope. The collimator is a long cylindrical tube having an adjustable slit on the further end and a lens nearer the prism, meant for condensing the rays and making them parallel before they fall on the refracting prism. A compound light, such as sun light, after falling on the prism, is dispersed and decomposed into component rays of different colours. These unequally refracted rays, characteristic of the source of light, form a band of colours called a spectrum. The spectrum is observed through a low power revolving telescope fitted along a circular table provided with a scale and vernier. Compound light (sun light) examined in a spectroscope gives a characteristic spectrum showing bands of red, orange, yellow, green, blue, indigo and violet (from right to left). In between these colours there are dark lines called *Fraunhofer's lines* (or absorption bands) indicating the absence of certain rays owing to absorption by metals in a state of vapour in the sun's atmosphere. These lines have definite positions and help in localising accurately the parts of the spectrum.

Spectrum analysis mainly consists in the examination of the colour, number and position of lines and such an examination affords an easy qualitative test for many metals and colouring matters. When metals are heated to a temperature of incandescence the light emitted by them has characteristic spectra. Metals or metallic salts may be examined by dissolving them in the purest hydrochloric acid, taking up a little on a clean platinum wire and holding this in the Bunsen flame. In the case of hæmoglobin, chlorophyll and other similar colouring matters, light is allowed to pass through a very dilute solution of these when examined spectroscopically. In some instruments there is a small prism placed near the slit of the collimator by which two spectra are seen —one of ordinary light and one of the material under examination— adjacently placed, one over the other, in the telescope, to help comparison.

For the examination of carboxyhæmoglobin and for other public health work a *direct vision spectroscope* is very useful. It consists of an eyepiece fitted to a draw tube. There is slit at the other end of the draw tube the opening of which can be regulated by a milled head. The spectroscope is held against a bright sky or white cloud and the eyepiece is pulled out or pushed in till the Fraunhofer's lines of the spectrum are clearly focussed. The slit is narrowed by moving the milled head until these lines are as sharp as possible, specially the lines *D* in the orange, *E* and *b* in the green, and *F* in the blue band. The instrument is held in such a position as to keep the red end of the spectrum towards the left. Some defibrinated blood is put in a tube, diluted till it is very faintly coloured and then examined for the characteristic spectrum. *Oxidized hæmoglobin* shows two well-marked bands between Fraunhofer's lines *D* and *E*

(in the yellow and green bands). *Carbon monoxide hæmoglobin* does also show this but the left-hand band at the blue end lies nearer the right and the edges are not so sharply defined. The blood also becomes pink or cherry red in carbon monoxide hæmoglobin. Add two drops of colourless ammonium sulphide to the bottle and shake; if no marked change occurs on spectroscopic examination, carbon monoxide hæmoglobin is present. If it is not present the characteristic single broad band of *reduced hæmoglobin* replaces the two bands of oxyhæmoglobin.

II.—*Welzel's test for Carbon monoxide.*—To 10 c.c. of a solution of blood (1 of normal blood to 100 of water) and 15 c.c. of a 20 per cent. solution of potassium ferrocyanide and 2 c.c. of acetic acid (33 per cent.). The precipitate becomes reddish brown if carbon monoxide is present, while oxyhæmoglobin gives a greyish-brown precipitate.

Sulphurous Acid or Sulphur Dioxide.—

QUALITATIVE TESTS.—Aspirate a quantity of the air to be tested through distilled water.

(1) A starch and potassium iodide paper will turn blue in the presence of sulphurous acid.

(2) Add potassium permanganate to the water containing sample air; if it is decolorized the presence of sulphur dioxide is indicated.

(3) Add a drop of ferric chloride and potassium ferricyanide; a blue precipitate results if sulphur dioxide is present.

QUANTITATIVE ESTIMATION.—Aspirate a known quantity of air through a dilute solution of bromine in water. Precipitate the sulphuric acid thus formed by the oxidation of the sulphurous acid, with barium chloride solution. Weigh the barium sulphate formed from whence the amount of SO_2 may be calculated.

Sulphuretted Hydrogen.—

QUALITATIVE TESTS.—(1) Lead acetate paper is blackened.

(2) A copper sulphate solution gives a black precipitate.

QUANTITATIVE ESTIMATION.—Pass a measured quantity of the air to be examined through a solution of $\frac{N}{10}$ iodine in iodide of potassium containing a little starch paste till the colour is discharged.

Each c.c. of the $\frac{N}{10}$ iodine solution = 1·7 milligrammes of H_2S.

Ozone is tested by soaking a piece of filter-paper in potassium iodide and starch solution, drying and exposing. In the presence of ozone the iodine is liberated and gives blue colour.

Hydrogen Peroxide.—Fill a bottle with distilled water and empty it, leaving a little of the water in the bottle. Shake the bottle so that the water will take up H_2O_2 if present. Add a few drops of 1 per cent. solution of potassium chromate, then a few drops of sulphuric acid and ether. On shaking, the ether assumes the blue colour of perchromic acid.

Suspended Matter.—(1) Aspirate large quantities of air through small quantities of distilled water which will retain the suspended matter. This can be weighed after evaporating the water and drying the residue. Hesse's tube with 50 c.c. of glycerin may be used to catch the suspended matter for microscopic examination.

(2) Pouchet's æroscope and sugar filters can also be used for this purpose.

Qualitative Tests for Gases

1. *Smell.*—The peculiar odours of gases are only given if the gases are present in very large quantities. Free chlorine has a peculiar irritating odour, while hydrochloric acid gives a faint odour resembling chlorine. Carbonic acid, nitrous acid and nitric acid cannot be recognised by smell.

Ammonia, ammonium sulphide and sulphuretted hydrogen have characteristic odours.

The odour of carbon disulphide resembles garlic and sulphurous acid has a pungent characteristic smell.

Tests.—Test the reaction using moistened red and blue litmus paper caught in the stopper of the bottle. If the reaction is acid or alkaline, run 10 c.c. of ammonia-free distilled water into the flask and shake vigorously.

Pour half of this water into a test-tube and test for the gas; the remaining half can be used for confirmatory tests.

A.—*If acid,* add a drop or two of silver nitrate.

(*a*) A white precipitate may be—

(*i*) Carbonic acid—a faint precipitate, insoluble in nitric acid. Baryta water becomes turbid when added to the original solution and the turbidity increases on adding ammonia.

(*ii*) Hydrochloric acid—a marked precipitate, insoluble in nitric acid but soluble in ammonia.

(*iii*) Sulphurous acid—a marked precipitate, soluble in nitric acid. On being heated the precipitate clears up with darkening of the solution.

(*b*) No precipitate.—

(*i*) Nitric acid.—Add brucine and sulphuric acid which gives a pink zone.

(*ii*) Nitrous acid.—Add starch solution and potassium iodide and then sulphuric acid: a blue colour. The metaphenylene-diamine or Ilosvay's tests may also be applied.

B.—*If alkaline.*—

(*i*) Ammonia.—Add Nessler's reagent. A yellow colour.

(*ii*) Ammonium sulphide.—Add Nessler's reagent. A black colour. Sodium nitroprusside gives a violet colour. Odour characteristic of rotten eggs *and ammonia.*

C.—*If litmus not affected.*—

(*i*) Sulphuretted hydrogen. Lead acetate paper is darkened. Odour characteristic of rotten eggs only.

(*ii*) Carbon disulphide. Odourless. Inflammable, burns with blue flame, leaving sulphur behind.

D.—*If litmus is first reddened, then bleached.*—Chlorine. Potassium iodide paper is first darkened, then bleached.

Note.—Ammonium sulphide and sulphuretted hydrogen are very similar. The reaction and nitroprusside tests distinguish them.

CHAPTER VI

MILK ANALYSIS

Fresh Milk

The following table shows the average composition of cow's and buffalo's milk in England and certain parts of India:—

	Water.	Solids					
		Total solids	Solids non-fat	Fat	Protein	Lactose	Mineral salts.
Cow's Milk—							
1. England ..	87·4	12·6	8·95	3·65	3·48	4·75	0·72
2. Bengal* ..	87·07	12·93	8·55	4·38	3·33	4·54	0·68
Buffalo Milk—							
Bengal* ..	81·12	18·88	10·86	8·02	4·72	5·38	0·76
Mixed Herd Milk—							
United Provinces.	85·0	15	9·3	5·7	4·15	4·40	0·75
Goat's Milk—							
England ..	86·04	13·96	9·33	4.63	4·35	4·22	0·76
Legal limits—							
1. England ..	..	..	8·5	3·0	..	..	..
2. Calcutta Corporation.	..	..	8·5	3·5	..	..	..
Buffalo Milk, Calcutta Corporation.	..	..	9·0	5·0	..	..	..
Mixed Herd—							
Standards for the United Provinces.	..	..	8·5	4	..	..	..

It will be seen that buffalo's milk chiefly differs from cow's milk in the larger amount of solids, especially fat. The solids are found to be uniformly higher in the cold weather than in summer.

Note.—The standards shown against the Calcutta Corporation have been adopted by them as a result of extensive investigations carried out by Drs. Datta and Ghosh (Stevenson and Simpson).

* Extracted with permission without alteration from "Principles of Hygiene" by Simpson—Stevenson and Simpson's results for cow's milk during the rains and buffalo milk in the cold season being given.

The *Sediment* in a sample of milk should not be more than 1 milligramme per 100 c.c.

Reaction.—Fresh milk is faintly alkaline or amphoteric, owing to the presence of two salts with opposite reactions, the acid reaction being due to primary sodium phosphate (NaH_2PO_4) and alkaline due to the secondary phosphate (Na_2HPO_4). As sold it is usually acid in reaction. Marked acidity indicates lactic acid fermentation.

Cream is estimated by standing the milk 24 hours in a cream tube and reading off the amount on the graduations on the tube. Cream contains 50 per cent. fat and about 45 per cent. of water, the rest being casein, milk-sugar and ash. The average amount of cream is 8 per cent. (English standard).

Specific Gravity can be taken by *lactometers* which are, however, not very accurate. The Westphal's balance is a convenient and quick method of getting the accurate specific gravity. It is based on the principle of Archimedes, which purports that when a body is immersed in a liquid it loses a part of its weight which is equal to the weight of the displaced liquid, or in other words, the weight of an equal volume of the liquid. So, if a substance is first weighed in water and then in milk, the respective weights are proportional to the specific gravities of water and milk.

Westphal's balance.—The apparatus consists of a graduated arm swinging on a knife-edge. The whole is supported on a vertical stand and can be adjusted to any height. At one end of the swinging arm is a hook by which a glass plummet thermometer is suspended by means of fine wire. At the other end is a sharp metal pointer which should rest against another similar metal point on the frame when the balance is adjusted. There are three riders or weights of different sizes; the largest indicates hundreds, the next tens and the smallest units. For example, if the largest rider is placed at a notch marked 6 it means that it measures 600 and the medium sized rider at the same notch measures 60 and the smallest 6. The hook by which the plummet is suspended represents the tenth notch and so the largest rider on the hook measures 1000. The specific gravity of water is 1000 and so the balance is adjusted to measure 1000 by immersing the plummet in a cylinder of water and putting the largest rider measuring hundreds on the hook. This adjustment is easily made by turning a screw on the vertical support of the frame till the pointer of the swinging arm is adjacent to the pointer on the frame. After this the water in the cylinder is replaced by milk and additional riders put in at suitable places on the swinging arm till the pointer is again adjusted. The weight registered by the different riders is noted and represents the spcific gravity of milk.

Suppose the riders are at the following places:—

Largest at the hook or 10th notch	= 1000
Medium at the 3rd notch	= 30
Smallest at the 2nd notch	= 2

$\therefore$ the specific gravity of milk is 1032.

Temperature corrections are necessary for accurate observation. Usually all these observations are made at 15·5°C. (60°F.). Temperature alters the volume, so the higher the temperature the lesser the specific gravity. Suppose the specific gravity of milk is measured at 20°C. it means that owing to increase in volume the observed specific gravity is lower than the actual. For ordinary ranges of temperature in a laboratory it is sufficient to add 1 to the specific gravity for every 3° of temperature on the centigrade scale above 15·5°C. and to subtract 1° for every 3° below 15·5°C.

The specific gravity can also be taken in a *specific gravity bottle* which is more exact. The bottle is thoroughly dried and weighed. It is then filled with distilled water at 15·5°C. (60°F.) and again weighed. It is now filled with the liquid to be examined and reweighed. The difference between the second and first weighings = the weight of the water the bottle holds.

The difference between the third and first weighings = the weight of the liquid the bottle holds.

Divide the weight of the liquid by the weight of the water and multiply by 1000. The result is the specific gravity of the liquid as compared to water as 1000.

Removing the cream gives a high specific gravity while adding water makes it low. Therefore neither the specific gravity nor the cream test alone gives any indication of adulteration, but both together are valuable.

The *specific gravity* is normally between 1028 and 1034.

Estimation of the Total Solids.—Pipette 5 c.c. of milk into a weighed platinum dish, curdle by adding acetic acid (1 part to 9 of methylated spirit). Dry it to a constant weight on a water-bath and then in an oven at not above 105°C.

Multiply this weight of solids by 20 to bring the result to 100 parts by volume. Convert 100 c.c. of milk to weight by deduction from the specific gravity; calculate the percentage of solids by weight.

Example.—Solids and dish weigh 11·908 grammes.

Dish weighs = 11·258 grammes.

Therefore solids = 0·65 gramme in 5 c.c.

i.e., $\frac{0{\cdot}65 \times 100}{5}$ or $0{\cdot}65 \times 20 = 13$ grammes in 100 c.c.

If the specific gravity of milk = 1032, 100 c.c. of milk weigh 103·2 grammes.

Therefore there are 13 grammes of solids in 103·2 grammes of milk, or $\frac{13 \times 100}{103{\cdot}2}$ or 12·5 grammes in 100 grammes.

N. B.—*Richmond's formula for total solids* is T=0·25G+1·2F+0·14, where T=total solids, F=fat percentage and G=the last two figures of the specific gravity.

Richmond's slide scale is useful for finding the amount of fat, total solids or specific gravity if any two of these are known and is useful for checking analytical results. The middle part of the scale, showing specific gravity figures, slides. The upper part of the scale indicates percentages of fat and the lower, percentages of total solids. There is a pointer on the sliding part pointing to fat figures. If the pointer is placed opposite the percentage of fat, the specific gravity reading will be opposite the calculated amount of total solids. Conversely, if the specific gravity be placed opposite the estimated amount of total solids, the pointer will point to the amount of fats. The other end of the sliding scale enables the user to estimate the corrections for degrees of temperature below or above 60°F.

The *ash* is estimated by igniting the total solids and weighing the residue.

The Fat in Milk.—

Note.—Of the various processes Werner-Schmidt's process is fairly accurate and is rapidly carried out; Adam's process is very accurate and is the official method used; Gerber's modification of Leffmann Beam process is recommended for rapid sampling and Gottlieb's process is advised for sweetened brands of condensed milk, sour milk, cream, butter and cheese.

II.—*The Werner-Schmidt Process.*—1. In a graduated Stokes' tube place 10 c.c. of milk and 10 c.c. of strong hydrochloric acid.

2. Boil and shake till the lactose is changed to maltose and caramel and it turns brown.

3. Stand a few minutes, then cool in a stream of water.

4. Fill to the 50 c.c. mark with ether, cork, invert several times and allow the ether to settle out.

5. Pipette off 20 c.c. of the clear supernatant fluid into a weighed dish and evaporate off the ether in hot water-bath. Dry at 100°C. and weigh.

6. Note how many c.c. of ether remain in the tube—also counting as ether three-quarters of the flocculent layer of casein lying below the ether layer.

Example.—10 c.c. of milk produced in 20 c.c. of etherial solution 0·314 gramme of fat. In the tube there remain 7 c.c. of solution counted as ether.

Therefore, the total etherial solution=27 c.c. ether, and $\frac{0{\cdot}314 \times 27}{20}$ or 0·424 gramme of fat exists in 10 c.c. milk, *i.e.*, 4·24 grammes in 100 c.c. milk.

But the specific gravity of the milk was 1032,

i.e., 100 c.c. of milk weighs 103·2 grammes;

therefore there are 4·24 grammes of fat in 103·2 grammes or 4·1 per cent.

Note.—The hydrochloric acid and milk should not be boiled for more than two minutes.

II.—*Adam's Process.*—1. After shaking the milk place exactly 5 c.c. in a small beaker and weigh.

2. Soak up as much of the milk as possible with a coil of fat-free filter-paper. The paper is rendered fat-free by extracting the paper with acid alcohol containing 10 per cent. acetic acid for at least three hours and then drying.

3. Remove the coil and reweigh the beaker to find out the amount of milk soaked in the coil of paper.

4. Dry the coil in an air-oven for two hours and then extract the fat by anhydrous ether in a *Soxhlet,* 12 syphonings at least being necessary.

Soxhlet's apparatus for the extraction of fat from milk consists mainly of a flask, a condenser and an extraction tube. The extraction tube is connected to the flask by the lower end and to the condenser by the upper end. The extraction tube contains a side tube curved to form a siphonic arrangement, having its outlet in the flask, so that when a certain amount of liquid gets collected in the extraction tube it is sucked into the flask by this siphonic arrangement. The flask is first thoroughly dried and weighed, then about half filled with ether and connected with the extraction tube and the condenser. Milk is soaked in a fat-free filter-paper and placed in the tube, taking care that no part of the paper is above the level of the siphonic bend. The whole is placed over a water-bath and the ether gradually evaporated. Ether condenses and falls over the paper containing fat. The condensed ether dissolves the fat and its amount gradually rises in the rising arm of the syphon tube till it just reaches the bend, when it overflows and, producing a partial vacuum, creates suction. Thus the whole of the condensed ether gets back into the flask. This is repeated a number of times till all the fat is extracted by ether. Immediately after the last siphon discharge, when all the ether is in the flask, the apparatus is disconnected, the ether finally evaporated and the fat dried and weighed.

5. Receive the fat in a previously weighed small light flask, drive off any ether, dry, cool and weigh.

To obviate two weighings suspend strips of fat-free filter-paper over a lighted gas ring, as near as the hand can bear. On this put 5 c.c. milk, dry, roll in coil and place in the Soxhlet and proceed as above.

III.—*Leffmann-Beam Process.*—Into a specially graduated bottle run 15 c.c. milk. Add 3 c.c. of a mixture of equal parts of hydrochloric acid and amyl alcohol. Mix well and add 9 c.c. of strong sulphuric acid, 2 c.c. at a time, shaking well between each addition. The mixture will now be of a violet colour and *very hot.* Fill up to the 0 mark on the neck of the bottle with freshly prepared sulphuric acid (1 in 3). Centrifuge after placing a similarly weighted tube in another limb to balance it. The fat will separate and rise to the top. The direct reading on the graduated neck gives the percentage of fat present in milk. The process is not very accurate, usually underestimating the amount of fat.

IV.—*Gerber's modification of the Leffmann-Beam Process.*—

Reagents, etc., Required.—

1. Sulphuric acid—specific gravity 1·825 at 15·5°C.
2. Amyl alcohol—specific gravity 0·816 at 15·5°C.
3. A small flask (Gerber's) with a thin long graduated neck, each graduation representing 0·1 per cent. by weight of fat.

Place 10 c.c. of the sulphuric acid, 1 c.c. of the amyl alcohol and 11 c.c. milk in the small flask. The alcohol and milk are allowed to flow down the sides of the bottle so that the three liquids form distinct layers; then shake till the curd is dissolved, turning the bottle upside down several times. Place the flask in a centrifuge (and a balancing weight on the opposite side if necessary) and centrifuge for 3 minutes. On removal, the flask is put in warm water (temperature about 70°C.) to keep the fat liquid. The percentage weight of fat can now be read off. Each fine division=0·1 per cent. by weight of fat.

V.—*Gottlieb's process* for the estimation of fat is reliable. 5 c.c. of milk are placed in a graduated tube, 1·0 c.c. of 30 per cent. ammonia added, and, after shaking well, 5 c.c. of alcohol are added. The tube is well shaken and placed in a hot water-bath till the mixture is uniform and free from lumps. 12·5 c.c. of ether are run in, the tube shaken and an equal quantity (*i.e.*, 12·5 c.c.) of petroleum ether added. After shaking well the tube is set aside. When the ether separates out 10 c.c. or a known volume are pipetted off and the fat estimated as in the Werner-Schmidt process.

Richmond and Hehner's formula for estimating the fat is F=0·859T—0·2186G, where F=Fat, T=Total solids and G=the last two figures of the specific gravity.

The *solids non-fat* may be calculated by subtracting the fat from the total solids.

Lactose.—

Determination by Fehling's solution.—Take 25 grammes of milk, add 0·5 c.c. of 30 per cent. acetic acid. Shake, stand, add 100 c.c. boiling water, shake. Add ammonia to alkalinise. Filter and make the filtrate up to 250 c.c. Take 10 c.c. Fehling's solution (5 c.c. of each of numbers 1 and 2), dilute to 100 c.c., boil. Run some of the clear whey filtrate into the boiling Fehling's solution till the blue colour is discharged. As an "external indicator" a solution of potassium ferrocyanide slightly acidulated with acetic acid may be used to denote the end point.

As 0·067 gramme of lactose reduces 10 c.c. of Fehling, therefore the number of c.c. of whey used must contain 0·067 gramme of lactose.

Say, 18 c.c. of the whey were used.

Therefore 0·067 gramme of lactose must be in that amount of whey.

But 250 c.c. of whey=25 grammes of milk.

Therefore 1 c.c. whey=0·1 gramme of milk.

Therefore 18 c.c. whey=1·8 grammes of milk.

Therefore 1·8 grammes of milk contains 0·067 gramme of lactose=3·72 per cent. of lactose.

The following *Fehling equivalents* are useful:—

10 c.c. Fehling=0·0475 gramme cane-sugar after inversion.

10 c.c. Fehling=0·0676 gramme lactose.

10 c.c. Fehling=0·0475 gramme glucose.

10 c.c. Fehling=0·0778 gramme maltose.

10 c.c. Fehling=0·045 gramme starch after inversion.

Proteins.—

QUALITATIVE TEST.—A few drops of copper sulphate solution are added to milk made alkaline with KOH. A violet colour indicates protein.

QUANTITATIVE ESTIMATION.—I.—*Kjeldahl's process* is described under sewage.

II.—The *Aldehyde test.*—(*i*) Titrate 10 c.c. of milk with $\frac{N}{10}$ strontium hydroxide, using phenolphthalein as an indicator.

(*ii*) Titrate 2 c.c. of formalin with the same reagent until alkaline. Mix (*i*) and (*ii*) solutions.

(*iii*) Again titrate the mixed solutions till a permanent pink colour again appears and the number of c.c. used in the final titration multiplied by 0·17 gives the total proteins as a percentage.

Vieth's ratio of proteids, sugars and ash in cow's milk is 9::13::2.

Sour Milk

When milk undergoes lactic acid fermentation, about half the milk sugar is changed into lactic acid and volatile acids; there is practically no change in fat. The total solids average from 0·2 to 0·5 per cent. less than in fresh milk.

To Estimate Fat.—1. Adam's process (*see* page 57) may be carried out by adding some ammonia to the milk before absorption by the coil.

2. Weigh 10 grammes of the sample in a platinum basin. Add 2 drops of phenolphthalein and titrate with $\frac{N}{10}$ solution of strontia.

Evaporate on a water-bath. Add 20 c.c. of dry ether and thoroughly mix with a glass rod. Decant through a dry filter paper of known weight. Repeat this several times. Distil off the ether. Dry and weigh the residue, which is fat.

The Non-Fat Solids.—Transfer the solids left on the filter-paper and the paper, to a weighed flask. Dry for one hour at 90°C. or until there is practically no difference in weight. Deduct the weight of the filter-paper and the weight of the added strontia (1 c.c. of $\frac{N}{10}$ strontia = 0·00428 gramme) and the difference is non-fat solids in the quantity of sour milk tested.

Corrections should be made for alcohol formed from lactose and for volatile acids.

To Estimate the Alcohol.—1. Distil 100 grammes of milk.

2. Neutralize the distillate with $\frac{N}{10}$ sodium hydrate solution, using litmus-paper as an indicator.

3. Re-distil and make up to original bulk and determine the specific gravity; the quantity of alcohol corresponding to this specific gravity is deduced from a table.

The percentage by weight of alcohol multiplied by $\frac{90}{46}$ gives the percentage amount of lactose which has disappeared in producing the alcohol.

To Estimate the Volatile Acids.—1. Estimate the acidity of 10 grammes of the sample by titration with $\frac{N}{10}$ sodium hydrate solution, using phenolphthalein as an indicator and thus find out the number of c.c. of soda required.

2. Neutralize another portion (10 grammes of the sample) to the extent of half the total acidity with $\frac{N}{10}$ sodium hydrate.

3. Evaporate this half neutralized sample to dryness in a platinum capsule with frequent stirring and treat with 20 c.c. of boiling distilled water; add further sodium hydrate till the neutral point is reached.

The difference between the original acidity of the milk and that of the evaporated portion multiplied by 0·006 is regarded as volatile acids in terms of acetic acid.

Example.—Original acidity of 10 grammes of sour milk in terms of $\frac{N}{10}$ soda solution is 2 c.c. Soda solution required for another portion is 1 c.c. before evaporation and 0·4 c.c. after it. So the differenec in acidity is 2—(1+0·4)—0·6 c.c. of $\frac{N}{10}$ soda representing the loss due to volatile acids.

As 1 c.c. of $\frac{N}{10}$ soda=0·006 gramme of acetic acid,

$\therefore$ 0·6 c.c.=0·0036 gramme present in 10 grammes of milk, or 0·036 per cent.

The production of each molecule of this acid (60 parts) denotes the loss of one molecule of carbonic acid and one of water, that is, a loss of 62 parts of original lactose.

Boiled Milk

Proceed as in the test for hydrogen peroxide given under "Preservatives in Milk," but in this case add hydrogen peroxide in addition to the aqueous solution of ortol. The strawberry colour means that the enzyme of the milk has not been destroyed by boiling, while absence of this colour indicates that the milk has been heated above 72°C. (181·6°F.). Pasteurised milk heated to a lower temperature will give the colour test.

Skimmed, Separated and Designated Milk

Hand-skimmed Milk.—

Specific gravity	.. about 1032.
Fat	.. 0·5 to 1·5 per cent.
Solids non-fat (English legal limit)	.. 8·7 per cent minimum.

If condensed milk is added to thicken milk, *the ash and non-fatty solids will be in excess.*

Separated Milk.—

Fat (average) .. 0·3 per cent.
Solids non-fat (English legal limit) .. 8·7 per cent. minimum.

Designated Milk.—*See* Part II, under Bacteriological Analysis of milk.

THE SOPHISTICATION OF MILK

1. The Addition of Water.—The amount is estimated by deduction from the amount of solids non-fat. If 8·5 per cent. = 100 per cent. pure milk then a sample of milk giving 8 per cent. solids non-fat = $\frac{8 \times 100}{8 \cdot 5}$ or 94·1 per cent. pure milk. Therefore added water = 100—94·1 or 5·9 per cent.

2. Cream Abstraction.—This is deduced from the amount of fat. If 3 per cent. of fat = 100 per cent. fat in pure milk, then 2·8 per cent. fat present in a sample = 93·3 per cent. fat. Therefore 6·7 per cent. of the fat has been removed.

3. Starch or Gelatin.—*Starch* is added to milk to thicken it. It may be tested for with iodine and potassium iodide paper, when a blue colour is produced.

QUANTITATIVE ESTIMATION OF STARCH.—Invert the starch by boiling milk with hydrochloric acid for 15 minutes. Cool. Neutralize with caustic soda. Estimate the dextrose in the sample by Fehling's method. To express dextrose as starch multiply the factor by 0·9.

Gelatin is sometimes added in European countries to thicken cream.

Test for Gelatin.—To 10 c.c. of the sample add 20 c.c. of cold water and 10 c.c. of a solution of acid nitrate of mercury. Shake, stand for 5 minutes and filter. If much gelatin is present, the filtrate is not clear. To a portion of the filtrate add an equal quantity of saturated aqueous solution of picric acid. A yellow precipitate is formed if gelatin is present.

4. Cane-Sugar Added to Milk.—

QUALITATIVE TESTS.—I.—To 10 c.c. milk add a few drops of hydrochloric acid and about 0·1 gramme of resorcinol and boil. A rose red colour is produced if cane sugar is present.

II.—*Cotton's test.*—Take 2 tubes. In one place 10 c.c. of a six per cent. solution of lactose and in the other 10 c.c. of the

sample. To each add 5 grammes of ammonium molybdate and 10 c.c. of hydrochloric acid (1 in 10). Put both these tubes in the water-bath and gradually raise the temperature but not above 80°C. If the sample contains cane-sugar it will give a blue colour. Even 0·1 per cent. can be detected in this way.

A more simple way to perform this test is to add 2 c.c. of a saturated solution of ammonium molybdate to 10 c.c. milk and 10 c.c. of dilute HCl. Heat for 5 minutes but not above 80°C. A blue colour appears if cane-sugar is present.

QUANTITATIVE ESTIMATION.—Take 100 c.c. of the whey made for the determination of the lactose, as before. Invert the sugar with 1 c.c. of hydrochloric acid (or 5 c.c. of 2 per cent. citric acid) by boiling; cool, filter, washing the filter-paper once or twice afterwards with distilled water. Neutralize with anhydrous Na_2CO_3 and make up to three times its original bulk with water.

Estimate the amount of reducing sugar as for lactose and subtract the amount of lactose previously found.

10 c.c. Fehling's solution=0·0475 gramme dextrose.

The reducing power of cane-sugar as compared to lactose is as 0·047 to 0·0678.

Therefore, percentage of cane-sugar equals percentage of lactose $\times \frac{47}{}$.

Tyrotoxicon in Milk.—Milk, butter, cheese, etc., may all contain tyrotoxicon. This is a diazobenzene butyrate and is produced by improper storage which conduces to fermentative changes resulting in powerful poison, giving symptoms resembling atropine poisoning.

Test.—Filter the milk and make the filtrate alkaline with sodium carbonate. Add an equal bulk of pure ether, shake, stand and then decant off the ether. Shake and dry in the air. Dissolve the residue in a little water and filter it to free the filtrate from fat. Add an equal bulk of pure ether to the filtrate, shake, decant off the ether and dry in air. Dissolve the residue in a few drops of a mixture of equal parts of pure carbolic and pure sulphuric acids. If tyrotoxicon is present a reddish colour appears.

PRESERVATIVES, ETC., IN MILK

Chemical preservatives and colouring matter are now prohibited in any type of milk in Britain under the Acts of 1912 and 1922 respectively, and are not added to milk to any extent in the East.

1. Boric Acid and Borates.—Qualitative test.—I.—Take 100 c.c. of milk. Evaporate to dryness after making alkaline with caustic soda (0·5 gramme); incinerate and extract the ash with dilute hydrochloric acid. Dip turmeric paper in the liquid and dry high over flame. If boric acid is present the paper turns reddish and becomes dark green on adding sodium carbonate or ammonia.

Boric acid and borates are the only preservatives which turn the colour of turmeric in an *acid solution.*

If the reaction is faint or doubtful, re-test, using turmeric paper which has been dipped in a *weak solution* of oxalic acid and then dried.

II.—Take two test-tubes; place 10 c.c. milk in one and 5 c.c. glycerin in the other. To each add a few drops of phenolphthalein and sufficient $\frac{N}{10}$ alkali to produce a light pink colour. Mix the solution. If boric acid is present the solution becomes colourless.

Quantitative Estimation.—*Richmond and Miller's process.*—Weigh 10 c.c. of the sample; add 5 c.c. of a half per cent. phenolphthalein solution; run in normal NaOH till pink colour appears; boil and titrate back with normal HCl till white, and again with $\frac{N}{10}$ NaOH, till a faintly pink colour appears. Add 30 per cent. of glycerol and continue the titration with $\frac{N}{10}$ NaOH.

The number of c.c. $\frac{N}{10}$ NaOH used for the final titration, multiplied by 0·0062, gives the quantity of boric acid contained in the quantity of milk used.

2. Sodium Carbonate.—*Test.*—To equal quantities of milk and alcohol add a few drops of a solution of rosolic acid. A rose-red colour indicates sodium carbonate or borax. A yellow-brown colour is given with pure milk.

3. Salicylic Acid.—Coagulate 10 c.c. of milk with acetic acid. Filter and to the filtrate add 10 c.c. of ether. Mix well. Allow the ether to separate and pour the clear layer into a capsule. Evaporate on water-bath. Dissolve the residue in rectified spirit. Add a drop of ferric chloride. Heat just to boiling, turning out the light as the spirit boils or it will catch fire. If salicylates are present, a violet colour, not discharged by acetic acid, is produced. If it is in excess, this colour may occur without heating but disappears on applying heat.

This test may be performed simply by adding a solution of ferric chloride to milk in a test-tube. A purple colour results.

The quantity may also be estimated by this method by comparing with the colours produced in a standard solution containing 0·05 per cent. salicylic acid in 50 per cent. alcohol. One per cent. iron-alum may be used instead of ferric chloride.

4. Formaldehyde (CH_2O).—Formalin is a 40 per cent. commercial solution of formaldehyde in water.

I.—*Hehner's Test.*—Dilute some milk with at least an equal bulk of water. Take 10 c.c. in a test-tube and pour 90 per cent. commercial sulphuric acid containing some iron down the side of the tube. If formaldehyde is present a violet ring is formed; if not, a green ring. This test is not given with pure formalin in the absence of milk nor if the formalin is in excess of 0·5 per cent. in the milk; hence the necessity of diluting the milk before the test is made.

II.—Acidify 100 c.c. of milk with a little dilute H_2SO_4 and filter. To the filtrate add a drop of Schiff's reagent. A pink colour appears.

This reagent is a solution of fuchsin, the colour of which has been discharged by sulphurous acid.

III.—Boil 10 c.c. of milk in a test-tube. Add a few drops of 1 in 4 of sulphuric acid. Filter. Add 5 c.c. of 0·1 per cent. solution of phloroglucinol and 5 c.c. of sodium hydrate solution to the filtrate. A pink colour appears.

QUANTITATIVE ESTIMATION.—*Romijn's process.*—Distil 100 c.c. of milk. To the distillate add 30 c.c. of $\frac{N}{1}$ NaOH in a stoppered bottle. Then add a known amount of $\frac{N}{10}$ iodine till the fluid remains bright yellow. Shake vigorously. Add 40 c.c. $\frac{N}{1}$ H_2SO_4 and titrate the excess of iodine with freshly prepared $\frac{N}{10}$ thiosulphate to get the amount of iodine absorbed.

1 c.c. of $\frac{N}{10}$ thiosulphate = 0·0015 gramme formaldehyde.

5. Hydrogen Peroxide.—To 10 c.c. of milk add 1 c.c. of a freshly prepared 1 per cent. aqueous solution of ortol. If hydrogen peroxide is present a crushed strawberry colour is produced. If the milk has been heated above 70°C. this will not take place, as the test depends on the presence of enzymes. In this case a little fresh milk (known to be free from peroxide) should be added before testing.

Buddeisation is the treatment of milk by hydrogen peroxide.

6. Mystin.—This is a proprietory substance consisting of formaldehyde and sodium nitrite in water and is added in some of the European countries as a preservative. The sodium nitrite prevents the colouration for formaldehyde being obtained in the Hehner test above described; so it should be removed by adding a few c.c. of a 2 per cent. solution of urea. Hehner's test is then performed. The nitrites may be detected by the meta-phenylene-diamine test.

Condensed and Dried Milk

Condensed Milk.—Whole or skimmed milk is evaporated to about one-third of its bulk and cane-sugar frequently added. To analyse, take 20 grammes and make up to 100 c.c. with water for the stock solution.

Under the Public Health (Condensed Milk) Regulations, 1923, full cream condensed milk in England must (unless it is to be exported), contain 9 per cent. of milk fat. "Full cream" condensed milk must contain 31 per cent. of total milk solids and "skimmed" condensed milk if sweetened 26 per cent. and if unsweetened 20 per cent.

(*i*) *Total solids.*—Evaporate 5 c.c. and proceed as usual. Average figures are from 68 to 84 per cent. (sweetened) and 29 to 35 in unsweetened condensed milk (both full cream).

(*ii*) *Ash* averages from 0·9 to 2 per cent. and may reach 3·5 per cent. in full cream sweetened milk.

(*iii*) *Fat* averages 10 to 11 per cent. but may reach 13 per cent. in full cream condensed milk or be as low as 0·2 in machine-skimmed sweetened condensed milk. Estimate by Adam's or Gottlieb's process.

(*iv*) *Proteids.*—Estimate by Kjeldahl's process, taking 5 grammes of milk. Multiply the nitrogen by 6·38 to convert into milk proteids. The proteids average 8·5 to 9 per cent.

(*v*) *Degree of concentration.*—In unsweetened milk divide the percentage of solids by 12·6; in the case of sweetened milk divide the percentage of fat by 3·7. Another method is to divide the percentage ash figure by 0·7 which is the percentage ash figure of normal milk.

(*vi*) *Total mixed sugars.*—I.—*Ash Method.*—To 10 c.c. of stock solution of condensed milk add 40 c.c. methylated spirit and a few drops of acetic acid; shake and filter. Evaporate 20 c.c. of the filtrate and weigh the residue which represents total solids. Incinerate and subtract the ash from the former weight

and thus find the amount of sugars present in the filtrate. Multiply the amount of sugars by 22·5 and then by 5 to give the percentage of mixed sugars.

II.—*Inversion method.—Saccharose.*—Boil 5 c.c. of the stock solution, diluted with water, with hydrochloric or citric acid to invert the sugar; filter, cool and neutralize the filtrate with caustic potash. Make up to 100 c.c. total bulk with distilled water and titrate with Fehling's solution. Subtract lactose figure from the result to obtain saccharose figure. Average varies from 44 per cent. in sweetened condensed milk to *nil* in unsweetened condensed milk.

(*vii*) *Lactose.*—Add a few drops of citric acid or mercuric nitrate solution to 5 c.c. of the stock solution. Make up to 100 c.c. with water; filter. Add ammonia to alkalinise and estimate by Fehling's solution. Average 13 to 15 per cent. (varies from 11 to 17·6 per cent.).

Dried Milk.—"Dried Milk," according to the Public Health (Dried Milk) Regulations, 1923, includes in England pure milk, skimmed milk or partly skimmed milk reduced to powder form by evaporation of the water. The minimum standards for the milk fat and milk solids are:—

Moisture should not exceed 5 per cent.

Milk fat varies according to the type of milk from which the powder is made, and varies from 8 per cent. in dried "quarter cream" milk to not less than 26·0 per cent. in dried full cream milk.

Total solids vary from 9·9 to 12·4 in the various types of "dried milk." (*See Infants' Foods*).

Polarimetry

Polarimetry forms one of the readiest methods of determining the presence of any one sugar and if the sugar is identified its percentage amount present in a given solution can also be known.

The Polarimeter.—This instrument is one by which the action of various substances on the plane of polarised light can be observed and measured. Ordinary light consists of undulations in ether occurring in all directions at right angles to the path of the propagation of the wave. By certain means it is possible to restrict the vibrations to any one direction and the beam is then said to be *plane polarised.* This plane polarisation of a beam is carried out by interposing a *Nicol prism* in its path, all the waves having vibrations in various directions being then intercepted excepting those which are at right angles to the principal sections of the prism. Such a prism which polarises

the light is called a *polariser* and certain chemical substances which rotate the plane of polarisation of a beam of such a "*plane polarised light*" when placed in its path are called "*optically active substances.*"

The principle of the instrument can be easily grasped by supposing a man shooting at a fence made up of narrow vertical palings with thin, broad arrows resembling lathes of a venetian blind. If the arrows are shot vertically they will pass easily through the narrow vertical gaps between the palings but if they are shot horizontally the flat arrows will not pass through the vertical gaps. A Nicol prism may be supposed to consist of narrow slits through which only those waves can pass which are parallel to the slit while others will be obstructed.

In a polarimeter, there is at one end of the instrument a fixed Nicol prism (*the polariser*) to polarise light which is made to pass through it and at the other end there is a similar Nicol prism (called *the analyser*) which is capable of being rotated about its longitudinal axis. Between these two prisms is a glass tube which can be filled with the solution to be tested. Beyond the analyser is an eyepiece. The rotation of the polarised light is measured on a circular dial divided into angular degrees and provided with a vernier capable of being read up to 0·05°.

It follows that if the principal sections of the polariser and the analyser are both parallel to each other the light polarised will also pass through the analyser and will be perceived by the observer through the eyepiece but if they are at right angles no light will be observed. At intermediate angles there will be intermediate degrees of illumination. Between the prisms is placed an empty tube or one filled with water. On looking through the eyepiece, while revolving the analyser, a position will be found at which light no longer passes through. On continuing the rotation of the analyser through half a complete revolution another field of maximum darkness will be found. For purposes of observation the points of greatest darkness are to be preferred for reference, since at these points the least movement of the Nicol produces a perceptible change in the appearance of the field of vision. If now the tube be filled with a solution of some substance capable of rotating polarised light, such as cane sugar, the analyser having been previously set to darkness, it will be found that the field of vision appears bright, and to obtain the maximum of darkness again the analyser must be turned through a certain angle, the *angle of rotation.* It follows that the introduction of the tube containing the sugar solution has rotated the rays from their previous plane of polarisation.

Optically active substances are distinguished as being *dextro-rotatory or lævo-rotatory,* according as they rotate the plane of polarised light in a right or left-handed direction, as viewed by the observer looking towards the incident light. Substances which do not possess this power are said to be *optically inactive.*

Numerous forms of the polarimeter are in use. Those constructed on what is called the "half shadow" system give sufficiently satisfactory results. A half shadow polarimeter consists, like other polarimeters, of two Nicol prisms—one fixed (polariser) and another capable of rotation (analyser). In addition there is a small Nicol occupying only half the field, thereby rotating the plane of polarisation in only half of the field, leaving the other half quite unaffected. In the zero-position of the analyser both halves of the field are equally dark.

The polarimeter is extensively used in analytical chemistry for quantitative determinations. In this connection the term *specific rotation,* introduced by Biot, is generally employed. By this term is meant the angle of rotation produced when a ray of polarised light passes through a solution containing one gramme of an optically active substance in one c.c. in a tube one decimeter long. This angle will vary with the wave-length and the temperature and is designated by $(a)D^{20}$ which indicates the specific rotation of a substance at 20°C. using the yellow (D) lines of sodium as a light source. Let a be the observed angle of rotation at 20°C., l the length of the observation tube in *decimeters* and w the weight in grammes of the substance per c.c. of solution, then the specific rotation $(a)D^{20} = \pm \frac{a}{wl}$. In this equation a and l are known by direct measurement and $(a)D^{20}$ has been determined once for all for most of the important active substances; w is therefore easily calculated to obtain a quantitative idea of the strength of the solution of an optically active substance.

The following table gives the specific rotatory power of important optically active sugars:—

Name.	Formula.	Source.	Specific rotation power $[(a)D^{20}]$.
Dextrin	$(C_6H_{10}O_5)_n$	Starch	+198·9
Saccharose	$(C_{12}H_{22}O_{11})$	Cane and beet	+ 66·5
Maltose	$(C_{12}H_{22}O_{11})$	Starch	+138
Lactose anhydrous	$(C_{12}H_{22}O_{11})$	Milk	+ 55·4
Lactose crystalline	$(C_{12}H_{22}O_{11})$ H_2O	Milk	+ 52·6
Dextrose (glucose)	$(C_6H_{12}O_6)$	Sweet fruits	+ 51·3
Lævulose	$(C_6H_{12}O_6)$	Sweet fruits	— 95·4
Invert sugar	$(C_6H_{12}O_6)_2$	Cane-sugar	— 19·6
Galactose	$(C_6H_{12}O_6)$	Lactose	+
Raffinose	$(C_{18}H_{32}O_{16})$	Molasses	+
Arbinose	$(C_5H_{10}O_5)$	Gum arabic	+
Starch	$(C_6H_{10}O_5)_n$	Plant cells	+
Inulin	$(C_6H_{10}O_5)_n$	Chicory, etc.	—
Mannite	$C_6H_8(OH)_6$	Manna	+
Dulcite	$C_6H_8(OH)_6$	Manna	+
Sorbite	$C_6H_8(OH)_6$	Mountain ash	+
Asparagine	$C_4H_8N_2O_3$	Asparagus	+
Insonite	$C_6H_{12}O_6.2H_2O$	Muscle	—

In addition to the identification of sugars or their strength in solution the polarimeter can be used to find out the adulteration of butter with other fats. With pure butter an equally distributed light can be obtained, but with butter containing fat which has been melted (margarine) this is impossible, since such fats rotate the plane of polarisation. Glucose in honey and sugar added to milk may also be detected by this means.

It is absolutely necessary that the solution in the tube should be perfectly clear and colourless, otherwise illumination of the field will be very defective and measurement of the angle of rotation impossible. To effect clarification of dark coloured liquids basic lead acetate is used. If the solution is cloudy a few c.c. of alumina cream is added and finally one or two drops of basic lead acetate and the whole shaken and filtered. Yellow coloration requires more lead acetate; a brown black solution may require 2 c.c. of 10 per cent. sodium sulphite and subsequently some lead acetate to furnish a transparently clear solution.

CHAPTER VII

BUTTER, GHEE AND CHEESE

Butter

Average composition of Butter.—

Water	13	per cent. (varies from 8 to 15 per cent.)
Fat	83·5	„
Casein	1·0	„
Lactose	1·0	„
Ash	1·5	„

Butter fat consists of the glycerides of oleic, stearic, palmitic, and myristic fatty acids and of certain volatile fatty acids, chiefly butyric. The butyric volatile fatty acid is soluble in hot water.

Salt (up to 6 per cent.) is often added to improve the taste and as a preservative. The addition of any preservatives in England is prohibited from January, 1928.

English Legal Limits.—

	Percentage	
	Water not more than—	Butter-fat not more than—
Butter	16	..
Margarine	16	10
Milk blended butter	24	..

Butter is made by churning fresh milk or cream. "Starters" (cultures of the organisms produced in cream on standing) may be added to facilitate the process especially if pasteurised milk is used. The butter which rises to the top is washed with fresh water to remove the milk and curd and so increase its keeping qualities.

Margarine is made occasionally from animal fats but more generally from vegetable fats and therefore contains a relatively small quantity of volatile fatty acids. Margarine is almost as

valuable a food as butter except that vitamins are deficient. Lard, beef and mutton fats, paraffin wax and paraffin have been used to make, or are incorporated in, margarine—the last two being rare.

Margarines in the East are sold mostly under the name of "Vegetable Ghee." "Vegetable ghee" is not a trade name but a class name which represents one of a group of margarines possessing the outward appearance and consistency of butter fat, which may contain (1) pure vegetable oil or fats such as purified cocoanut oil, (2) pure vegetable oil (or oil mixtures) which have been hydrogenated, and (3) mixtures of (1) and (2). Imported vegetable ghees consist generally of cocoanut, sesame or cotton-seed oil solidified by hydrogenation. These margarines are totally free from fat and oil of animal origin so as to make them suitable for sale in India and are of great importance owing to the recent general practice of adulterating expensive pure ghee with these cheaper substitutes.

Preservatives.—The preservatives are the same as in milk (*see* tests under Milk). To perform the tests add a little water to the butter, melt and shake or centrifuge. The water may then be tested for preservatives.

Colouring Matter can be dissolved out with warm distilled water. If the solution loses its colour when treated with sodium hypochlorite the colouring matter is generally harmless.

Annatto, saffron and *turmeric* are used as colouring matters.

Tests.—Dissolve in alcohol, evaporate to dryness and add 1 drop of concentrated H_2SO_4. Annatto gives a dark blue colour changing to green. If saffron is present this colour turns reddish brown; if turmeric, the colour is violet red, becoming brown with alkalies.

ADULTERATION WITH FOREIGN FATS, MARGARINES, ETC.

As the types of margarine now being sold in the East vary not only in the original oils from which they are made but also in the methods of preparation it is almost impossible to determine the nature and proportions of the ingredients. For instance hydrogenation so alters the physical and chemical composition of vegetable oils that by incorporating various proportions of partially or wholly hydrogenated oils a margarine may be prepared which almost defies accurate analysis.

The chief differences between butter-fat and foreign fats are as follows:—

Butter-fat.	Other fats.
1. Specific gravity at 38°C.—0·910 to 0·913. (Minimum—0·910).	1. In the case of vegetable fats specific gravity is above 0·913. Specific gravity of animal fats is below 0·904.
2. Soluble volatile fatty acid figure never below 4·5 (usually 6 to 7 per cent.).	2. Never above 0·75 per cent.
3. Insoluble fatty acids approximately 88 per cent.	3. Approximately 95 per cent.
4. The Reichert-Meissl figure averages 20 to 36.	4. Averages 1 to 2 except in respect of cocoanut oil, when it is from 7 to 8.
5. By the Valenta test the fat clears at 30° to 40°C.	5. No animal fats clear below 94°C. and no vegetable oil used as a substitute clears below 80°C.
6. Jean's test.—Glacial acetic acid absorbed exceeds 60 per cent.	6. Rarely more than 30 per cent. absorbed.
7. Polariscope.—When a thin layer of sample is examined by the micro-polariscope, at the moment the Nicol prisms are crossed, the whole field appears dark.	7. No dark field is obtained.

Admixture with vegetable fat or a mixture of fats cannot be determined by the specific gravity figures alone.

Preparation of Fat for Analysis.—The sample is heated at a temperature of 45 to 48°C. in an air oven for 20 minutes. The first layer of butter-fat floats on the top, and contains a few particles of curd and a few drops of water. Gently pour out the fat on a perfectly dry filter-paper kept warm by a special type of hot water funnel. The filtrate is pure fat.

Fat is transparent in appearance in a genuine sample, turbid in a mixed one. Adulteration is detected by estimating the soluble and insoluble volatile fatty acids and for this purpose the Reichert-Meissl-Polenske method should be used.

Estimation of the Amount of Soluble Volatile Fatty Acids.—

I.—The Reichert-Wollny Process.—

1. Take a weighed small-necked glass flask (200 c.c. capacity). Add 5 grammes (5·8 c.c.) of butter-fat at 38°C.

2. Take 2 c.c. of 50 per cent. caustic soda solution and 10 c.c. of 92 per cent. alcohol and add to flask. Fit a reflux condenser. Heat for 15 minutes on the water-bath at 100°C. The fatty acids combine with the alkali to form soap. Evaporate off the alcohol until the soap is dry.

3. 100 c.c. of hot freshly boiled distilled water are next added Heat till the soap is dissolved.

4. 40 c.c. dilute sulphuric acid (1 in 40) are then added. The soap is thus decomposed and the fatty acids are set free.

5. Add a small piece of pumice-stone to prevent "bumping" and distil over, using a special condenser. The fatty acids are melted and butyric acid is distilled over unchanged along with some insoluble volatile fatty acids. These are removed by running through a dry filter-paper.

6. Collect 110 c.c. of the filtered distillate in a graduated flask.

The acidity is estimated by means of $\frac{N}{10}$ alkali (baryta or soda), using phenolphthalein as an indicator.

The result (usually 0·3 c.c.) obtained by a blank experiment, using the reagents alone but no fat, should be deducted from this.

The result is known as the Reichert-Wollny figure.

In case of pure butter the Reichert-Wollny figure should not be less than 24, *i.e.*, 24 c.c. of $\frac{N}{10}$ alkali are required for 5 grammes of butter-fat.

1 c.c. of $\frac{N}{10}$ soda solution=8·8 milligrammes of butyric acid and so the percentage amount of volatile soluble fatty acids in terms of butyric acid can be calculated.

Example.—To find the percentage of pure butter-fat.

Suppose 16 c.c. $\frac{N}{10}$ alkali used;

then as in absence of cocoanut oil 2 is the highest figure for other fats and 24 the lowest for butter-fats, so a difference of 22 represents 100 per cent. genuine butter. The percentage in the sample with figure 16 would be represented by 16—2=14.

Therefore if 22=100 per cent., 14=63·63 per cent. Therefore 36·37 per cent. of foreign fat is present.

In Europe the highest figure for margarine is taken as 4 c.c.

Therefore, if the number of c.c. used=20, then 24—4=100 per cent. butter.

$\therefore$ 20—4=$\frac{100 \times 16}{20}$=80 per cent. butter or 20 per cent. adulteration.

Drs. Dutta and Ghosh found in Calcutta that the Reichert-Wollny minimum number for buffalo ghee is 30·5 and that for cow ghee 24.

II.—Leffmann and Beam's Modification of the Reichert-Meissl Process.—This process is useful as it needs no special apparatus.

Weigh out 5 grammes of clear butter-fat (or take 5·8 c.c.) in a narrow-necked Jena flask. Add 20 c.c. glycerol soda. Heat gently till the water evaporates and the mixture becomes clear. Add 135 c.c. of distilled water, a little at a time, to dissolve the soap formed. Put in a few pieces of recently ignited pumice-stone. Add 20 c.c. H_2SO_4 (10 per cent.) and distil over 110 c.c. Take 100 c.c. and titrate with $\frac{N}{10}$ NaOH. Note the number of c.c. used and add one-tenth of the number to this figure. The result should not be below 24 for pure butter-fat.

This process can be done in the apparatus used in Wanklyn's process for the estimation of ammonia, no reflux arrangement being required.

To make Glycerol Soda Solution.—Take a 50 per cent. solution of NaOH. Stand for 24 hours till clear. 20 c.c. of this and 180 c.c. of pure glycerin, well mixed, is glycerol soda.

The Reichert-Wollny (Reichert-Meissl) figures for various substances are given below:—

Butter-fat ..	Fresh	Buffalo milk	34
		Cow milk ..	24
Lard			0·4 to 0·6
Rape oil			0·6
Olive oil			0·6
Palm oil			1·0
Kidney fat			0·5
Cocoanut oil			7 to 8
Margarines			0·8 to 3
Butter-fat+10 per cent. cocoanut oil			26·8
Butter-fat+50 per cent. cocoanut oil			18·0
Butter-fat 50 per cent.+cocoanut oil 22·5 per cent.+margarines 27·5 per cent.			17·4

The Reichert-Wollny or the Reichert-Meissl figures indicate the amount of soluble volatile fatty acids, *i.e.*, the figure 24 for cow butter means that 24 c.c. of $\frac{N}{10}$ alkali is required to neutralise the soluble fatty acids liberated on saponification of 5 grammes of butter. The figures are practically equal whichever of the two processes is used for estimating any sample.

From the above figures it will be seen that 10 per cent. adulteration with cocoanut oil cannot be detected by these processes but the detection and estimation of cocoanut oil in a sample can be carried out by an extension of the Reichert-Meissl process for the determination of Polenske number.

This number represents the insoluble volatile fatty acids and is of great use in detecting cocoanut oil in butter. This number may be determined with the Reichert-Meissl figure by adopting the following process which is common to both.

Soluble and Insoluble Fatty Acids.—

Reichert-Meissl-Polenske Process.—Saponify exactly 5 grammes of prepared fat with 20 grammes of glycerol and 2 c.c. of a 50 per cent. NaOH solution, by heating over a flame with constant rotation until frothing ceases. This requires about five minutes, and is complete when the liquid is quite clear. While still hot add 90 c.c. of boiled water, at first drop by drop, to prevent frothing, and shake till the soap is dissolved. Cool to about 20°C. and add 50 c.c. dilute H_2SO_4 (25 c.c. to a litre) and ½ gramme of granulated pumice grains (1 millimetre in diameter). Connect with distilling apparatus with an upright Liebig condenser with a bulb and distil over 110 c.c. in twenty minutes. When 110 c.c. is distilled the flame is removed and a 25 c.c. cylinder is placed under the condenser to catch the drops. Cool the flask by immersion in water at 15°C. Stopper it, and invert four or five times. Filter through a dry filter-paper fitted close to the funnel. 100 c.c. of the filtrate is titrated with $\frac{N}{10}$ alkali (baryta best) using phenolphthalein as an indicator. The number of c.c. used, less the number of c.c. used for the blank, multiplied by 1·1 is the Reichert-Meissl number. Wash the material on the filter-paper with three 15 c.c. portions of cold water, each of which has washed out the flask, the condenser and cylinder. Dissolve the fats on the filter with three 15 c.c. portions of neutral 90 per cent. alcohol. Titrate the united alcoholic washing with $\frac{N}{10}$ barium hydrate (or soda), using phenolphthalein as indicator. The number of c.c. used less the number of c.c. used for the blank experiment, using all reagents except the fat, is the Polenske number or "*New butter value.*"

Samples of butter possessing Reichert-Meissl figures 20 to 30 will give Polenske numbers of 1·3 to 3. Samples of cocoanut oil of Reichert-Meissl values 6 and 7 will give Polenske figures 16 to 17. Lard and tallow give Reichert and Polenske figures of about 0·5 each.

This figure bears a constant relation to the Reichert-Meissl figure as is shown in the following table:—

R. M. Value	32	31	30	29	28	27	26	25	24	23
New butter number (Polenske).	3·5	3·2	3·0	2·9	2·7	2·4	2·0	1·8	1·7	1.6

A new butter value exceeding 0·5 c.c. of the corresponding Reichert-Meissl figure indicates the presence of cocoanut oil or palm kernel oil.

This new butter number varies from 1·5 to 3·0 for pure butter and for pure cocoanut oil from 16·8 to 17·8 so that adulteration is easily detected.

Valenta Test.—I.—The test depends upon intermiscibility of butter-fat with strong acetic acid at a low temperature (26°C.), while other fats become clear at a high temperature. Mix 3 c.c. of melted fat with 3 c.c. of glacial acetic acid (specific gravity 1056·2) in a narrow graduated tube and insert a thermometer. Warm the mixture and shake. Butter clears at 36°C., other animal fats clear at 94°C., vegetable oils at 80°C. or above.

II.—*Jean's modification of the Valenta test* is also helpful. It really measures the amount of acetic acid absorbed. Butter fat absorbs over 60 per cent. of glacial acetic acid but margarine rarely absorbs more than 30 per cent. After noting the temperature at which butter clears in the original Valenta test, the flame is removed and the temperature at which it becomes turbid is noted. The mean of the two temperatures is taken as the clearing temperature for the Valenta test. When the unabsorbed acetic acid has separated into a clear layer the volume of acid separated is read off and deducted from the actual amount added to determine the volume absorbed. Suppose the volume of separated acetic acid is 1·08 c.c.; then the amount absorbed by 3 c.c. of fat is 3—1·08=1·92, *i.e.*, 64 per cent.

Adulteration with cotton-seed oil may be detected by the following tests:—

1. A saturated solution of lead acetate and ammonia produces an orange colour.

2. To the sample add an equal bulk of nitric acid (specific gravity 1·375). Warm and stand for 24 hours when a brown colour results.

Ghee

Ghee is clarified butter and is made by curdling milk usually by adding a little old curd and churning the result,

ing mass of curd. The butter is strained or decanted off, melted over a slow fire and the water evaporated off. This is *pucca* ghee; if the water is not completely evaporated off, *kutcha* ghee results.

Ghee is examined in the same way as butter. The usual adulterants of ghee used in India are:—

(*i*) Mowa oil;

(*ii*) Cotton-seed oil;

(*iii*) Ground-nut oil, oils derived from the seeds of *Bassia cutyracea* and *Bassia latfolia* (Mohwa);

(*iv*) Animal fats;

(*v*) The above fats and oils partially or wholly hydrogenated (*See* Margarines and Vegetable Ghee);

(*vi*) Starches (potato, plantain, etc.).

The Detection of Adulterants.—

I.—*By Jean's Oleo-refractometer.*—This instrument makes use of the fact that different oils refract a ray of light passing through them to the right or left at a constant angle. The following is a description of the instrument.

The centre of the instrument is formed by a circular metallic reservoir, closed by two opposite lenses, from which extend two tubes, the one ending in a collimator and the other in a short-visioned telescope. In the middle of this reservoir is placed an inner metallic reservoir having glass sides placed at a suitable angle, so as to form a prism. In front of the field-glass of the telescope is a photographic scale and there is also a moveable vertical shutter, so placed as to divide the luminous field of the instrument into two parts, one being quite bright and the other in shadow. It is the position of the edge of this shadow on the scale that determines the reading with the apparatus. There are two reservoirs, one inside the other. If the same liquid be placed in both reservoirs, the shadow will come on the zero of the scale on adjustment, but if a different liquid be placed in the inner prismatic reservoir, the shadow will come at some point either to the right or to the left of the zero, acording to the nature of the refractive power of the liquid. The illumination is obtained by an ordinary gas jet placed opposite the collimator end of the instrument. Both reservoir are furnished with draw-off cocks, and are enclosed in an outer case in which water is put for the purpose of regulating the temperature and which is heated by a little lamp placed underneath so as to maintain its contents at any desired degree. M. Jean has invented a typical oil that gives no refraction, and with which the outer reservoir is always filled, and the temperature is brought to 45°C. If, now, some of this oil be also brought to 45°C. and placed in the prismatic reservoir, the shadow will mark zero, or if it does not do so it is adjusted to that point by moving the shutter. The typical oil is then run out of the prism and the oil to be tested, having been brought to 45°C., is placed therein and the reading is taken. All the vegetable oils, and some marine animal oils, deviate the shadow to the right of the zero, while the terrestrial animal oils act in the

contrary way. It takes about half an hour to regulate the instrument and get equalisation of the temperature in the reservoirs, but once this is attained one sample after another can be done at intervals of five minutes, methylated spirit being used to rinse out the inner prismatic reservoir before a new sample is observed.

The following table will give some idea of the differences that can be got between the various vegetable oils:—

Dextro-rotary oils (+)	Reading.
Olive	+ 1·5 to 2
Colza	+ 16·5 to 17·5
Ground-nut	+ 4·5
Sesame	+ 17
Cotton	+ 20
Castor	+ 40
Linseed	+ 53
Hempseed	+ 33
Poppy	+ 30 to 34
Almond	+ 6
Japonica	+ 50

Of the terrestrial animal oils and fats which go to the left (—) we have:—

Neats-foot oil	— 3
Horse-foot oil	— 12
Lard	— 12.5
Beef-tallow	— 16
Mutton-tallow	— 20
Butter-fat	— 35
Margarine (average)	— 15
Oleic acid	— 34
Cocoanut oil	— 59

Before observing any oil if it is rancid it should be shaken up in a separator with hot alcohol to remove the free fatty acids, and dried at 110°C. This is especially necessary in the examination of the lower qualities of olive oil, and indeed, with this particular oil, it is always desirable to proceed in this manner, if a preliminary examination of the oil itself does not indicate the proper refraction.

Adulteration with cocoanut oil cannot be detected by this method but if its presence is suspected the Reichert-Meissl and Polenske's tests, mentioned above, can be made.

The instrument is devised more for commercial use than as an exact scientific instrument. It is of great use, however, to a medical officer of health in his own laboratory to examine suspected ghee, butter, etc., sent to him for analysis.

II.—*Zeiss butyro-refractometer* can also be used for preliminary testing, the principle being somewhat similar to the above instrument. If these instruments are not to hand, the Reichert-Meissl or Reichert-Wollny figures will indicate the adulteration approximately, but not the adulterating oil used.

III.—*Estimation of Iodine value of Ghee.*—This value gives the amount of iodine absorbed by unsaturated acids of ghee expressed as percentage by weight. Saturated acids and their glycerides do not absorb iodine; so the iodine value is a measure of unsaturated acids present. Free iodine is absorbed by oils and fats in definite quantities and so such an estimation is also useful for testing the genuineness of ghee. For estimating this hypoiodous acid is used which is better absorbed than free iodine. This is prepared by the action of water upon iodine chloride.

Reagents Required.—

1. Wij's iodine solution.—Dissolve 13 grammes of pure iodine in a litre of glacial acetic acid (99 per cent.). Titrate the iodine solution with a standard solution of sodium thiosulphate. Then pass a slow stream of chlorine gas free from hydrochloric acid in the acetic acid solution of iodine till the amount of sodium thiosulphate required for titration is doubled. This occurs when the original dark colour of the solution changes to light yellow.

2. 10 per cent. solution of potassium iodide in water.

3. $\frac{N}{10}$ sodium thiosulphate solution (24·8 grammes to a litre of water). 1 c.c. of this solution is equal to 0·0127 gramme of iodine.

4. Dilute starch solution.

Process.—Weigh out exactly one gramme of ghee, dissolve it in chloroform and make up to 100 c.c. Measure out 20 c.c. ($\frac{1}{5}$th of the solution containing 0·2 gramme of fat) of this chloroform solution of the fat in a wide-mouthed bottle, add 20 c.c. of Wij's iodine solution and set aside for 20 minutes. Then add 25 c.c. of the potassium iodide solution and dilute the whole with about 300 c.c. of water. Titrate with $\frac{N}{10}$ sodium thiosulphate solution with vigorous agitation of the bottle until the colour is almost discharged. Add some starch solution and more thiosulphate till the blue colour of iodide of starch is discharged. Perform a blank experiment with the same quantities (20 c.c.) of chloroform and iodine solution, etc., to obtain correction for any impurities in the reagents and to ascertain the true

strength of the iodine solution. The difference between the volume of thiosulphate used in the blank and actual experiment represents the equivalent of iodine absorbed by that amount of ghee. Calculate for 100 grammes of ghee.

1 gramme of fat was dissolved in 100 c.c. of chloroform and of this only 20 c.c. were used for experiment. So the amount of fat used for experiment is 0·2 gramme. The volume of thiosulphate solution used for the blank experiment was 35·5 and in actual experiment with ghee 30·4, the difference being 5·1.

1 c.c. of thiosulphate = 0·0127 gramme of iodine.

∴ 5·1 c.c. of thiosulphate = $5{\cdot}1 \times 0{\cdot}0127$ gramme of iodine.

This much iodine is absorbed by 0·2 gramme of ghee, or $\frac{5{\cdot}1 \times 0{\cdot}0127 \times 100}{0{\cdot}2}$ by 100 grammes of ghee.

∴ Wij's iodine value = 32·38.

IV.—*Hehner's value.*—This is the percentage of fatty acids insoluble in water produced on saponification of fat. Two grammes of ghee are saponified with alcoholic potash over a water-bath. The saponified mass is washed with hot water into a beaker on a steam bath and acidified with dilute sulphuric acid. The contents are filtered through a weighed filter-paper. The beaker is thoroughly washed with small quantities of hot water and the washings poured over the filter until the filtrate coming through ceases to be acid. Allow solidification of the insoluble fatty acids on the filter-paper to occur. Dry and weigh. Deduct the weight of filter-paper and thus get the weight of insoluble fatty acids and calculate the percentage. The fatty acids can be extracted with ether in a Soxhlet and weighed after evaporation of the ether. The Hehner value of butter is between 86 and 88 and that of lard and most oils about 95.

V.—*Wellman's colour test* for the presence of vegetable oil in ghee is also useful. About a gramme of melted ghee is dissolved in 5 c.c. of chloroform, to which are added 2 c.c. of phospho-molybdic acid or of sodium phospho-molybdate, and a few drops of nitric acid. The mixture is stirred and agitated. After standing for some time a green colouration in the upper layer occurs if vegetable oils are present, but no colouration if ghee alone is present. If the ghee is rancid the test is not reliable, but if the free acids are first separated with alcohol, and the Wellman's test is then applied, the reaction will be given if any vegetable oil is present.

The following are the standards for Milk, Ghee, Butter and Oils worked out by Mr. P. S. MacMahon, M.Sc., Public Analyst to the Government of the United Provinces, and accepted by the United Provinces Government as a working guide for Public Analysts:—

Milk.—The average amount in mixed herd milk should be about 6 per cent. fat and 9 per cent. solids-non-fat.

Milk containing less than 4 per cent. fat and 8·5 per cent. solids-non-fat must be regarded as adulterated by the addition of water.

Ghee.—The constants usually relied upon as affording the necessary evidence of adulteration or otherwise, have been determined as follows:—

	Cow's ghee.	Buffalo ghee.
Sp. Gr. at 38·8°C. ..	0·9110	0·9119
Sp. Gr. at 100°C. ..	0·8649	0·8665
Iodine value (Wij's) ..	31·88	29·59
Reichert-Meissl value ..	29·91	36·53
Butyro-refractometer at 25°C.	50·35	49·85

These constants agree well with those adopted in Calcutta under the local Food and Drugs Act.

Pure ghee from cow and buffalo mixed herd should have a specific gravity at 38·8°C. of 0·911 to 0·913, a Reichert-Wollny number of not more than 40 c.c. $\frac{N}{10}$-alkali (average about 34 c.c.), and a Butyro-refractometer reading of 49 to 51 at 25°C. Ghee, being melted butter with the water driven off, should not contain more than a trace of the latter.

Butter.—The values for butter-fat are the same as for ghee. Butter should contain not more than 16 per cent. water and 4 per cent. salt.

Oils.—The following constants have been determined:

	Sp. Gr. at 30°C.	Iodine value (Wij's)	Saponification No.	Butyro-refracto-meter at 25°C.	Hehner value	Acid value
Peanut oil ..	0·909	98·32	196·0	66·4	93·2	2·32
Cotton-seed ..	0·922	100·84	186·0	70·79	94·6	..
Castor ..	0·954	87·82	179·0	77·83	95·1	7·85
Linseed ..	0·921	171·68	180·7	80·26	95·5	4·34
Mustard ..	0·909	119·72	174·8	71·3	95·4	3·44

Vegetable Substitutes for Butter-Fats.—The following average analysis figures for butter-fat and various brands of margarine (vegetable ghee) usually sold in the East and frequently mixed with ghee, have been furnished by Dr. B. M. Gupta, M.Sc., Ph.D., Assistant Public Analyst to the Government of the United Provinces. It will be seen that even with tests beyond the scope of the average public health official it is extremely difficult to give any detailed opinion on mixtures of ghee and margarine consisting of hydrogenated oils and fats.

	Butter-fat.	Pure cocoanut oil.	Cocogem.	Lily brand Vanaspati.	Cow brand vegetable product from Holland.	Phulwa butyracea fat.	Mahua latifolia fat.	Palm kernel oil.
Specific gravity at 37·8°C. (Water 37·8°C. =1).	0·910 to 0·913	0·910 to 0·9167	0·9087	.....	.....	.....	.....	.....
Solidifying point ..	19°C. to 24°C.	14°C. to 23°C.	17·5°C.	.....	35·9°C.	.....	.....	25·5°C.
Melting point ..	28°-34°C.	23°-27°C.	.....	37°C.	39°C.	.....	.....	.....
Butyro-Refractometer Index at 40°C. (Zeiss scale).	39·5 to 44·0	33·5 to 35·5	33·6	.....	.....	47·8	47·7	36·7
Saponification value (Mgms. of KOH).	221 to 233	225 to 260	255	192	.....	188·2	192·2	246·6
Iodine value ..	26% to 50%	8% to 10%	8·3%	19·5%	.....	42·6%	59·4%	14·7%
Reichert-Meissl value ..	20·5 to 36·0	6·5 to 8·0	6·5	1·9	1·4	1·31	.....	5·3
Polenske value ..	1·5 to 3·7	15 to 18	15·7	0·93	1·1	0·65	.....	9·9

Cheese

Composition.—

	Water.	Fat.	Casein.	Ash.
English "whole milk cheese"	33 to 38 %	28 to 32 %	25 to 28 %	4·6 to 4·0 %

No legal standards are laid down as the composition of cheeses varies according to the type of milk used.

Dutch cheese being usually made from skimmed milk has a lower fat figure than English cheese, the other figures being higher. Cheeses made from milk and cream (Stilton) have a much higher fat figure as a rule, while the so-called "cream" cheeses may vary enormously in their composition, the fat figure being frequently extremely low. In addition, cheese with a high water figure is particularly subject to bacterial and fungal contamination.

Certain cheeses (*e.g., Gorgonzola*) are frequently covered with an external coating of preservative. Arsenious acid, lead salts, copper and barium sulphate are frequently used for the purpose.

Adulteration.—This is rare. In cheese made from vegetable or animal fat, cheese maggots (larval stage of the fly *Piophila casei*) and the cheese mite (*Acarus domesticus*) may be found. Blue and green mould are due to Aspergilli; red mould to *Sporendonema casei. B. subtilis, Oodium lactis* and Penicillium are involved in the ripening of cheese (the round holes seen in the interior of cheese being due to gas formation). Tubercle bacilli and *B. typhosus* rapidly die off in cheese. For Tyrotoxicon *see* page 63.

CHAPTER VIII

THE EXAMINATION OF FOOD GRAINS, BREAD, BEVERAGES, ETC.

Cereals

Parasites.—The following are the commoner parasites of cereals:—Animal.—The corn weevil (*Calandra granaria*), ear cockle (*Vibrio tritici*), wheat mite (*Acarus farinæ*); Vegetable.—Mucor, aspergillus, penicillium, rust (*Puccinia graminis*), smut (*Uredo segetum*), bunt (*Uredo fœtida*), ergot (*Claviceps purpurea*) (q. v.).

Test for Ergot.—Make a paste of flour with weak caustic potash and add excess of dilute nitric acid. Neutralize with more caustic potash. A violet red colour is produced.

Starches

Microscopic examination of different starch granules.—On a clean dry slide place a small quantity of the starch to be examined. Remove the starch that does not adhere to the slide. Put a drop of dilute Gram's iodine or water on the starch and cover with a cover-slip. Examine under the low power objective.

The microscopic granules of starch are more or less characteristic of the plant from which they are derived.

1. Large round or oval granules, more or less flattened, showing no marked concentric striæ, together with other granules extremely small and ill-defined, may be *wheat, barley,* or *rye.*

Wheat.—Relatively few intermediate sizes.

Barley.—Similar but the large granules are more irregular in size and shape and somewhat smaller; intermediate sizes are more numerous.

Rye.—May show a rayed hilum and cracked edges.

2. Large pyriform or oval granules with marked concentric striæ and a circular or short hilum may be *potato* or *arrowroot.*

Potato.—Circular or stellate hilum at the smaller extremity and striæ well marked.

Arrowroot.—Hilum generally at broader extremity, granules smaller and striæ less visible.

3. Oval or reniform granules with faint concentric striæ, central linear hilum—may be *pea* or *bean.*

The granules of the pea have a central longitudinal hilum while those of the bean are larger and broader than those of pea and the hilum has a marked puckered appearance.

4. Very small angular and faceted granules without striæ —*rice, oatmeal* or *maize.*

Rice.—Minute granules which tend to collect in angular masses.

Oats.—Granules are larger than rice and tend to collect in rounded masses.

Maize.—Granules are much larger and irregular; possess visible, generally stellate, hilum. The hilum of maize turns black if examined after mounting in a drop of clove oil.

5. Irregular in size, rounded or angular with rounded edges, generally with a central hilum, ill-defined striæ—*sago* and *tapioca.*

Sago granules are large and irregular; the hilum is either stellate or linear. Tapioca granules are smaller and the hilum is generally placed towards the rounded extremity.

Flour

Flour.—The composition of flour varies (1) according to the variety of wheat from which it is made and (2) according to whether *whole meal, standard, household, white* or *patent* flour is being examined. The variations however are slight, ranging approximately to 1·5 per cent. in protein, 6·5 per cent. in carbohydrate and 1 per cent. in fat.

The analysis figures are, approximately,—

Carbohydrates	..	71 to 79 per cent.
Proteins	..	11 to 12·7 per cent.
Fat ..	..	1·2 to 2·5 per cent.
Ash ..	..	0·3 to 1·5 per cent. (should not exceed 1 per cent.)
P_2O_5	..	0·1 to 0·7 per cent.
Water	..	8·5 to 14·5 per cent. (should not exceed 15 per cent.)
Gluten (average)	..	8 to 12 per cent. (should not be less than 8 per cent.)

Physical characters.—Natural flour is of a faint yellow colour, due to carrotene, for which reason it is commonly bleached. It should be tasteless and have no musty smell. It should feel smooth and be free from animal or vegetable parasites. The dough should pull out into a string without easily breaking.

Estimation of Gluten.—Weigh out 50 grammes of flour. Mix with some water at 16°C. to form a paste. Weigh a piece of dry linen cloth, put the dough into this and tie the bag so made. Work the bag of dough with the fingers in a gentle stream of water. Ultimately all the starch, etc., will be washed away, and the water will flow clear. Dry the bag of dough in a hot-air oven. Nothing remains but crude gluten containing usually 1 per cent. of salts and fats.

The absence of starch is tested for by adding a drop of a solution of iodine in potassium iodide and water on a watch-glass.

The **organic matter** can be estimated by Kjeldahl's method using 0·5 gramme of flour and 20 c.c. nitrate-free concentrated H_2SO_4 (*see* Sewage Analysis).

Adulteration.—Mineral matter is not generally added to any great extent but more often it gets mixed with flour owing to bad storage or transport.

Test.—Shake the flour with chloroform. Flour floats while added mineral matter usually sinks. Added minerals can be detected in the ash. Ash above 3 per cent. is definitely due to added minerals, and the figure should not be above 1 per cent. The use of "improvers" (acid potassium and magnesium phosphates and potassium persulphate) will increase the ash percentage figure and can be tested for in the ash.

Flour is often *bleached.* Bleaching may be demonstrated as follows:—

To half an ounce of flour add two ounces of petrol. Unbleached flour turns yellow while there is no change in bleached flour. Nitrogen peroxide, if used to bleach flour, will increase the nitrites normally present and may be detected by mixing flour with water and testing the water for nitrites with metaphenylene-diamine. The Griess' or Hosvay's test should not show above two parts of nitrite per million in unbleached flour.

If the flour is acid it is usually old, the acidity being due to partial fermentation of the starch.

A simple test for added maize is to treat the flour with clove oil and examine characteristic starch grains under the low power of the microscope. The hilum in maize grain turns *black.*

The presence of rice starch can usually be detected by washing out the starches with water and allowing the separated starches and washing to stand for 12 hours, when *rice starch* usually separates out in the middle layer.

Self-raising flour is flour to which a definite amount of baking powder is added. Baking powders consist of tartaric acid or acid calcium phosphate and sodium bicarbonate with or without starch as a diluent. The acid calcium phosphate should not contain more than 10 per cent. of calcium sulphate as an impurity. Arsenic in acid calcium phosphate and tartaric acid must not exceed $\frac{1}{100}$ grain per lb.

Bread

"White bread" contains approximately 40 per cent. of water. The composition varies but the following is the approximate composition of dried (moisture free) bread:—

Protein	..	..	..	10·5 per cent.
Starches	..	..	..	83 per cent.
Maltose	..	..	..	4·2 per cent.
Fat	..	..	..	0·8 per cent.
Salts	..	..	..	1·5 per cent.

The moisture should not exceed 40 per cent., the ash 3 per cent., and the acidity 0·115 per cent.

Acidity in Bread.—Soak 10 grammes of bread in 50 c.c. of water for an hour, stirring occasionally. Filter and titrate the filtrate with $\frac{N}{1}$ soda, using phenolphthalein as an indicator. Calculate the amount of soda required for 100 grammes of bread. Express the result as a percentage of acetic acid (1 c.c. $\frac{N}{10}$ soda = 0·006 gramme acetic acid).

Alum is added to bread to whiten it and improve the taste. Opinions differ as to whether small quantities of alum are injurious to health, 2 grains per lb. being permissible.

Test.—Add 5 c.c. of tincture of logwood and 5 c.c. of a saturated solution of ammonium carbonate to 50 c.c. of water. Soak in this crumbs of bread. A permanent lavender colour is produced if bread is adulterated with alum. If the colour is not distinct, dry in a hot-air oven.

Infants' Foods

The majority of these consist merely of dried milk and by mixing with 7 parts of water a product resembling ordinary cow's milk is made. Others consist of flour starches mixed with dried milk or mixed with malt or ferments. The percentage composition of the various infants' foods is as follows:—

Water	.. 3·5 to 8·5 per cent.
Protein	.. 7·5 to 22 per cent.
Fat	.. Traces to 27 per cent.
Carbohydrate	.. 40 to 82 per cent.
Ash	.. 0·8 to 4 per cent.
Moisture	should not exceed 5 per cent.

The proteins, fat and sugars are estimated as under condensed milk.

Beverages

Alcoholic beverages are not drunk to a great extent by Indians with the exception of the common country spirits. A certain amount of imported spirits are, however, consumed and a short reference to the standards in use in Englind may be useful.

Foreign colouring matter is frequently added to beverages and can be detected as follows:—

Take 50 c.c. of the beverage and add 4 c.c. of concentrated hydrochloric acid, and 1 c.c. of formalin (40 per cent. formaldehyde). Heat for a few minutes over a water-bath until a precipitate begins to form. Add a slight excess of ammonia and continue heating until all ammonia is volatilised. Filter and notice the colour of the filtrate. Genuine wines give a colourless filtrate whilst those which have been coloured artificially retain the colour of dyes.

Spirits.—A beverage is not regarded as an alcoholic fluid in the eyes of the law unless it has over 2 per cent. of proof-spirit in it.

Estimation of the alcohol.—300 c.c. of the liquid is boiled until about 200 c.c. have distilled over into a flask. Make distillate up to its original bulk with distilled water. Take the specific gravity in a pycnometer at 15·5°C. If this is 1000 the fluid is free from alcohol. Absolute alcohol has a specific gravity of 0·7936.

When using a specific gravity bottle, the specific gravity= weight of bottle full of distillate minus weight of dry empty bottle, divided by the difference between the weight of the bottle full of distilled water and the weight of the dry empty bottle.

To find the percentage of alcohol in the distillate from the specific gravity, consult the "alcohol tables." In estimating the alcohol in a spirituous fluid, it should be first diluted with an equal volume of distilled water. The percentage of alcohol is determined by the specific gravity method as above described and doubled.

Proof-spirit is a mixture of 57·10 per cent. by volume or 49·28 per cent. by weight of pure absolute alcohol in water with a specific gravity of 0·91976 at 15·5°C. Solutions stronger or weaker than this are "over" or "under" proof.

The percentage of alcohol by weight in a fluid is obtained by multiplying the percentage by volume by 0·7936 and dividing the product by the specific gravity as compared to water as 1. The percentage of proof-spirit may be obtained by multiplying the percentage of alcohol by volume by 1·7535. The percentage of proof-spirit is always expressed by volume.

In England whisky, brandy, gin and rum may not be diluted with water to reduce the spirit more than 35 degrees under proof (Intoxicating Liquors Licenses Act, 1921).

Example.—Whisky is found to contain 55 per cent. of proof-spirit, how much water has been added?

The sample contains $\frac{55}{65}$ of its legal requirements,

i.e., 84·6 per cent. of whisky is present.

$\therefore$ 15·4 per cent. of excess water has been added.

The *acidity* of alcoholic beverages is estimated by titrating with $\frac{N}{10}$ alkali, using phenolphthalein as an indicator. The acidity of whisky is about 0·1 per cent., of rum 0·5 per cent., of brandy 0·05 to 0·1 per cent. The acidity of wines should not exceed 1·2 per cent. and of beer 3 per cent. of $\frac{N}{1}$ alkali.

Black beer must first be decolorised with neutral lead acetate solution. The red colour of red wines acts itself as an indicator, becoming green on neutralisation.

Note.—The approximate percentages of proof-spirit in port is 35, sherry 29·5, red wines 17 to 24, white wines 20 and beers 2 to 11.

The chemical preservatives found in wines, cordials, and beer are formalin, salicylic, boric and sulphurous acids, sulphites

and common salt. Picric acid and nux vomica may be found in beer and are harmful.

Furfural and Fusel Oil are toxic constituents of spirits and can be detected as follows:—

Furfural.—Ten drops of pure colourless aniline oil and 1 c.c. of acetic acid are added to 10 c.c. of distillate which is diluted with distilled water down to 50 per cent. of alcohol. A red colour develops. The amount present can be estimated by setting up control cylinders in which the reagents and varying amounts of furfural solution have been added.

Fusel oil (amylic alcohol) should not exceed 0·2 per cent. and is detected by decolorising the alcohol with animal charcoal and adding a few drops of hydrochloric acid and aniline oil; a rose colour results.

Quantitative estimation for fusel oil is carried out in Rose's graduated tube by placing 20 c.c. of chloroform by a long pippette into the graduated bottom of the tube, and adding to this 100 c.c. of the spirit which has been previously diluted to contain 50 per cent. of absolute alcohol by volume. The tube is stoppered and completely immersed in cold water for an hour after which the volume is read off. In the case of pure spirit the volume should be 37·1 c.c. Fusel oil will give an increase to this of 1 c.c. for each half per cent. present by volume of amylic alcohol.

Vinegar.—The specific gravity of a "strong" vinegar is about 1020, of an artificial vinegar about 1006. Arsenic should not exceed 0·14 mgm. per litre.

Estimation of the acetic acid.—Take 10 c.c. of vinegar and dilute to 100 c.c. Take 10 c.c. of this diluted vinegar (containing 1 c.c. of the original vinegar) and test with $\frac{N}{10}$ soda with phenolphthalein as an indicator.

The number of c.c. used will indicate the quantity of acetic acid in 10 c.c. diluted vinegar or 1 c.c. of the original; 1 c.c. $\frac{N}{10}$ soda = 0·006 gramme of acetic acid.

$\therefore$ number of c.c. of soda solution $\times 0{\cdot}006 \times 100$ = percentage of acetic acid in vinegar.

QUALITATIVE TEST FOR ACETIC ACID.—Boil some vinegar with sodium carbonate solution and filter. Add ferric chloride and a red colour will be produced. Pure vinegar contains 4 to 5 per cent. of glacial acetic acid.

Tests for mineral acids.—A *green colour* is produced with a dilute (1 in 10,000) aqueous solution of methyl violet if any

mineral acid is used as an adulterant to vinegar. An alcoholic solution of methyl orange gives a red colour if mineral acid, as against organic acid, is present.

Lime-Juice and Lemon-Juice.—The specific gravity should be 1030 and the acidity equal to 30 grains of citric acid per ounce.

Estimation of the citric acid.—This is done by titrating with $\frac{N}{10}$ soda, using phenolphthalein as the indicator. (1 c.c. $\frac{N}{10}$ soda = 0·007 gramme of citric acid).

Mineral acids may be detected by the methyl violet test described above.

Tartaric acid is detected by adding soda to neutralise, then shaking with calcium chloride solution to which some ammonium chloride has been added. A white precipitate indicates the presence of tartaric acid.

Note.—Free citric acid normally present in lime-juice gives a similar precipitate *after boiling.*

The following acidity equivalents are useful:—

1 c.c. $\frac{N}{10}$ alkali = 0·009 gramme lactic acid.

1 c.c. $\frac{N}{10}$ alkali = 0·006 gramme acetic acid.

1 c.c. $\frac{N}{10}$ alkali = 0·007 gramme citric acid.

1 c.c. $\frac{N}{10}$ alkali = 0·0067 gramme malic acid.

1 c.c. $\frac{N}{10}$ alkali = 0·0062 gramme boric acid.

1 c.c. $\frac{N}{10}$ alkali = 0·0075 gramme tartaric acid.

Preservatives.—Salicylic acid, sulphites and brandy (up to 4·5 per cent. strength) may be found.

Estimation of Arsenic in Food or Beverages.—Traces may gain admission from utensils in cooking or from the addition of impure chemicals as preservatives, etc. $\frac{1}{100}$ of a grain per gallon in the case of liquids (or per pound in case of solids) is the legal limit allowed in England. The presence of arsenic is tested by *Reinsch's* or *Marsh's test.*

I.—*Reinsch's test for arsenic.*—30 c.c. pure HCl are boiled for 15 minutes with 250 c.c. of the sample (beer, etc.). A piece of clean copper foil is then placed in the dish and the boiling continued for 45 minutes. Water lost by evaporation should be replaced. If arsenic is present the copper becomes tranished. If a deposit is found, wash the foil in water, then in alcohol and ether and dry at 90°C. Then heat in a glass reduction

tube. If there is any sublimate, examine under the low power of the microscope for the octahedral or tetrahedral crystals of oxide of arsenic (As_2O_3). Antimony gives a similar test but the sublimate is amorphous and not crystalline as in the case of arsenic.

II.—*Marsh's Process.*—The apparatus required consists of—

(*a*) a flask to produce hydrogen,

(*b*) a drying-tube connected to the above and filled with dry lead acetate paper and calcium chloride and plugged with cotton-wool,

(*c*) a hard glass combustion tube.

A preliminary test is made to prove that the chemicals, etc., are arsenic-free:—

The hydrogen is evolved by placing washed granulated zinc in the flask and adding hydrochloric acid. The hydrogen evolved passes through the lead acetate paper and the granulated calcium chloride and through the thick wad of cotton-wool at the end of the drying-tube, then through the combustion tube and is lighted. When the hydrogen burns with a round tip, it indicates that all the air has been removed from the flask. A Bunsen burner is then placed under the combustion tube. If there is no arsenic in any of the materials no mirror will be formed. A cold porcelain dish held in the hydrogen flame will become darkened if arsenic is present. Care should be taken not to introduce air bubbles into the flask as, if there is arsenic, the mirror will become black instead of brown.

After doing this blank experiment for testing the purity of reagents, add, through the funnel, 10 c.c. of the liquid to be tested and 10 c.c. of hydrochloric acid. If a mirror is not obtained, add more liquid and acid. Continue for 20 minutes, adding the liquid and acid gradually. The amount of arsenic present is estimated by comparing the mirror obtained with standard mirrors made by adding known quantities of arsenious oxide in hydrochloric acid to zinc in similar flasks.

To prove that the mirror is caused by the presence of arsenic seal off the portion of the tube containing the mirror after replacing the hydrogen by air; heat in a Bunsen burner flame till the mirror has disappeared. On cooling, minute crystals of arsenious oxide deposit.

The reagents and the apparatus employed should be arsenic-free. To free hydrochloric acid from the usual traces of arsenic, add bromine and excess sulphurous acid, distil, reject the first fifth of the distillate; the remainder will be arsenic-free.

Pepper

Black pepper should not leave behind more than 6·5 per cent. ash, of which 2 per cent. is insoluble in HCl.

White pepper should not leave behind more than 3·5 per cent. ash, of which 1 per cent. is insoluble in HCl.

Pepper may be adulterated with ground rice or other starches, ground olive stones, dust, sand or other mineral matter.

The starches are detected by the iodine test: the mineral matter by shaking up with chloroform.

Mustard

Adulterants:—

A.—*Colouring matters.*—Usually turmeric. To test—(1) Add ammonia and a brownish-red colour develops.

(2) A reversal of the test for boric acid in milk is performed by adding an alcoholic solution of the mustard to a solution of boric acid plus a few drops of HCl in a bowl. Evaporate to dryness: a rose colour indicates the presence of turmeric, which becomes greenish blue if touched with ammonia.

B.—*Starches* are detected by the iodine test after boiling.

C.—*Mineral matter* sinks rapidly on agitation with chloroform.

Tea

Tea consists of the leaves and leaf-buds of *Camellia thia* prepared by fermenting, or drying and firing. The two varieties of tea, black and green, differ solely in the method of manufacture; in the former, leaves are first partially dried in the sun and then rolled and allowed to ferment, while in the case of green tea leaves are artificially dried immediately they are picked, without allowing any fermentation.

The constituents of tea are tannin 6 to 12 per cent., an alkaloid, thein, 2 to 3 per cent. (occasionally 6·5 per cent.), cellulose, vegetable, albumin, alcohol soluble extractives, chlorophyll, pectin and pectic acids and dextrin, etc. Green tea contains more tannic acid and etherial oils and less thein than black tea and so is more astringent and less stimulating.

Examination of Tea.—The complete analysis of tea is difficult owing to the complexity of its composition; however, a

partial analysis gives sufficient data for finding out any adulteration.

(*a*) *Moisture* is estimated by drying a weighed quantity of leaves. It averages 6 per cent. but may be double this figure.

(*b*) *Estimation of extractable matter.*—Boil 2 grammes of the leaves with 100 c.c. or more of distilled water, using a reflux condenser for an hour. Filter while hot and repeat till no more colour is imparted to the water. Then collect the exhausted leaves and dry them in an oven and weigh. The loss of weight is determined and the percentage calculated for *dried* tea. This should not be less than 30 per cent. and adulteration with exhausted leaves is indicated by a lower extract percentage figure.

(*c*) *Total ash.*—Incinerate a weighed amount of dried tea leaves (2 grammes) in a platinum dish at a low temperature. Cool in a desiccator and find out the loss in weight. Total ash varies from 4·7 to 6·2 per cent. A figure higher than 8 per cent. of the dried leaf indicates the addition of extraneous matter and less than 4 per cent. indicates the addition of exhausted leaves.

(*d*) *Insoluble ash.*—Place the total ash (after weighing it) on a filter-paper. Wash the platinum dish with distilled water and add the washings to the filter-paper. Extract all the soluble portions of the ash with about 100 c.c. of hot boiling water. Dry the filter-paper and then ignite it and weigh the combined ashes (insoluble ash from tea and that belonging to filter-paper). Deduct the ash weight of the filter-paper; the result is insoluble ash and varies from 2 to 4 per cent. of the dried leaf. Large amounts of insoluble ash suggest admixture with extraneous mineral matter.

(*e*) *Soluble ash.*—Deduct the insoluble ash from the total ash and the remainder is soluble ash. It varies from 2·8 to 4 per cent. Generally it is at least half the total ash and legally it must be 40 per cent. of it. A lower percentage is due to adulteration with exhausted leaves.

(*f*) *Alkalinity of total ash.*—This is found out by titrating the filtrate left in the estimation of insoluble ash with $\frac{N}{10}$ HCl or H_2SO_4, using methyl orange as the indicator and returning the result as a percentage of K_2O (1 c.c. of $\frac{N}{10}$ acid=0·0047 gramme of K_2O).

(*g*) *Estimation of thein.*—Five grammes of dried leaves are extracted by successive boilings with 300 c.c. of water in a reflux condenser. At least three extractions should be done, each occupying two hours. Add neutral lead acetate solution and boil for ten minutes; filter. Excess of lead is precipitated by

sulphuretted hydrogen and the filtrate then evaporated to dryness on a water-bath with some freshly ignited magnesia and coarse sand. The dry residue is extracted 4 or 5 times with chloroform, preferably in a Soxhlet's apparatus. The chloroform is evaporated and the residue is further boiled with water and refiltered and finally the filtrate is evaporated to dryness at a temperature not above 100°C. and weighed as thein.

(*h*) *Tannin.*—Boil 2 grammes of dried tea leaves with successive changes of water till no more colour is imparted to the water. Pool the infusions and make up to 1 litre with water. Take 100 c.c., add excess of copper acetate solution to precipitate the tannin. Filter, wash the precipitate on the filter-paper with hot water till the filtrate is free from copper (*see* page 13). Dry, ignite and weigh the precipitate. Deduct the ash weight of the filter-paper. The result gives the copper oxide formed from copper tannate and, multiplied by 1·305, equals tannin.

Liquid Tea.—The infusion of tea used as a stimulant drink dissolves out about 25 per cent. of the weight of the leaves and the dextrin, glucose, tannin, thein, volatile oil and small quantity of the albuminous material present. Liquid tea also contains some acids which react on utensils made of such material as iron, copper, brass and commercial aluminium, especially when these have been allowed to rust or have undergone superficial oxidation.

Adulteration.—Adulteration of tea has practically ceased to exist in England but is still prevalent in India. Admixture with foreign leaves can be found out by soaking them in hot water or a strong potassium hydrate solution and examining them under a microscope. The genuine leaves are lanceolate in form, having spine mounted serrations on the margin but not quite to the point of attachment of the stalk; the primary veins run out from the midrib and near the margin turn back in the form of loops. The apex is slightly notched and the under surface shows a large number of oval stomata and unicellular hairs; a section of the leaf near the midrib shows characteristic long branched cells called "idioblasts." The leaves of elder, willow, sloe, etc., used as adulterants differ materially from tea leaves.

Used exhausted leaves may be coloured or "faced" to resemble fresh leaves and this adulteration is difficult to detect. The colours used are aniline dyes, Prussian blue, turmeric, sulphate of lime and black lead. Sand and gum are used to give stiffness. Tea dust mixed with other leaves, starch, gum, clay and sand is made up into small masses known as *Lie-tea*. Estimations of total ash, soluble ash and alkalinity indicate the adulteration.

COFFEE

Coffee is prepared from the seed or berry of *Coffea arabica* or *Coffea liberica,* the ripe fruit being dried and freed from husk and the beans roasted at a temperature not above 200°C. The roasted beans are afterwards ground or otherwise prepared in a form suitable for infusion. Roasted coffee contains fat, albuminous matter, cellulose, caffeic acids, dextrin, extractives, mineral matters, etc. A good sample of coffee should contain at least 10 per cent. of fat and ash up to 3 per cent.

Analyses of coffee for moisture, ash and alkaloid are done on the same lines as that of tea. Fat is estimated by extraction with petroleum ether; and the sugar by Fehling's method after inverting it by hydrochloric acid.

Adulteration.—The most important adulterant is *chicory* prepared from the root of the wild endive (*Chicorium intybus*). This adulteration increases the gum and sugar contents and decreases the percentage amounts of fat and tannin. The constituents of ash are also modified. Chicory is employed to blacken and thicken the infusion and give a slightly bitter flavour but is devoid of caffein. Chicory is detected as follows:—

1. Smell.

2. Roasted pure coffee floats for some time on water, then sinks slowly to the bottom and slightly colours the water, while roasted chicory sinks immediately to the bottom and colours the liquid. The sediment of chicory is soft and pulpy, while that of coffee is hard and gritty.

3. Microscopically—(*a*) *Coffee.*—(*i*) The skin or testa shows long lanceolate-shaped cells overlapping and parallel to each other and other ladder-shaped structures;

(*ii*) compact thick irregular mesh of tissue cells.

(*b*) *Chicory.*—Coarse oval meshes with large dotted ducts and blunt-ended testa cells.

4. Total solids.—Pure coffee—19·4 per cent. maximum.

Pure chicory—up to 66 per cent.

This is estimated by macerating the dried powdered material in proof-spirit.

5. If the caffein is extracted and estimated (as thein in tea) the percentage figure will be lower in adulterated coffee.

QUANTITATIVE ESTIMATION OF ADULTERATION WITH CHICORY

I.—*Extraction method.*—A weighed quantity of the dried sample is boiled with distilled water for 20 minutes. The extract is then strained through copper gauze or muslin. The residue is further boiled with 50 c.c. of water and strained as before and the two filtrates pooled. A portion of the filtrate is evaporated to dryness on a weighed platinum dish and the total solids determined. The total solids of—

(*i*) Pure coffee=24 per cent. of original dry coffee.

(*ii*) Chicory=70 per cent. of original dry chicory.

Therefore 70—24=46 is 100 per cent. chicory.

A sample gives 35·5 per cent. of extract.

Then 35·5—24=11·5. As 46 indicates 100 per cent. chicory, therefore 11·5=25 per cent. of chicory as an adulterant.

II.—*Specific gravity method.*—The specific gravity of a 10 per cent. decoction of pure chicory is approximately 1024·5 while pure coffee never exceeds 1009·5. Ten grammes of the sample are boiled with 80 c.c. of distilled water. The decoction is filtered and the residue washed with a minimum quantity of hot water. The filtrate is made up to 100 c.c. and cooled to 15·5°C. (60°F.). The specific gravity of this 10 per cent. decoction is determined with a Westphal balance or specific gravity bottle. As a difference of 15 (1024·5—1009·5) represents 100 per cent. chicory the amount of adulteration can be calculated.

The following table gives the average analysis of coffee and chicory:—

	Coffee	Chicory.
Moisture ..	Less than 6 per cent.	10 per cent.
Fat ..	11 to 14 per cent.	1-2 per cent.
Sugar ..	Under 1 per cent.	10-18 per cent.
Caffein ..	1·1 to 1·3 per cent.	None.
Total ash ..	3·5 to 5 per cent.	Above 5 per cent.
Soluble ash ..	Four-fifths of total ash.	One-third of total ash.
Specific gravity of 10 per cent. decoction.	1009·5.	1024·5.
Total solids in decoction.	24 per cent.	70 per cent.

Other Adulterants.—In addition to chicory there are other substances used as adulterants. Ochre, Prussian blue, etc., are used to improve the appearance and are detected by an increased amount of ash beyond 5 per cent. Artificial coffee beans made from various starches, coloured and flavoured with a little coffee and chicory, or exhausted coffee beans are sold. A ten per cent. infusion made as above, decolorised by animal charcoal or potassium permanganate, and tested by the addition of a drop of iodine solution, will detect starch. Sugar syrup is used to glaze exhausted beans and can be estimated after inversion. A figure above one per cent. indicates such adulteration. Roasted beans, acorns and other starches can also be identified by the characteristic microscopic appearance of starch granules in addition to the iodine test above described.

VITAMINS

The following tests have been recently described in current literature for vitamin A in fats and oils:—

I.—*Drummond and Rosenheim's test.*—Add 1 c.c. pure arsenic trichloride (Kahlenberg's reagent) to 1 drop of the oil to be tested; shake the test-tube. A deep blue colour results, becomes purple, then fades.

II.—*Antimony trichloride* (Carr and Price's reagent) gives a more delicate reaction than arsenic trichloride and the blue colour persists longer.

III.—Other reagents used are:—(*i*) H_2SO_4 (con.), (*ii*) phosphorous pentoxide (both these being relatively insensitive compared to arsenic trichloride), and (*iii*) Fearon's trichloracetic and pyrogallol reagent. This is not a specific test for vitamin A.

The pigments involved in these colour reactions are chlorophyll, carotin and xanthophyll. Willimott and Wokes have proved that carotin and xanthophyll give a permanent deep blue colour which interferes with the transient blue of the vitamin A test, and recommend their removal when testing natural foodstuffs by shaking a petroleum ether extract of the substance under test with vegetable charcoal and filtering.

Any reaction such as oxidation, exposure to sunlight, etc., which will destroy the vitamin destroys the substance which gives the colour reaction and this destruction, once commenced, goes on even if the material under test is kept in the dark.

No chemical tests exist for detecting the presence of other vitamins.

Preservatives and Colouring Matter in Food

Preservatives.—The test for the usual preservatives which may be added to foodstuffs have been mentioned under the various chapters. It is noteworthy that the recent English regulations permit the addition of benzoic acid or sulphur dioxide to various foods such as sausages, fresh or dried fruit, coffee extracts, pickles, sweetened mineral waters, jam, fruit juices. sugar, gelatin, beer, alcoholic and non-alcoholic wines and cordials. Other preservatives which are allowed in certain cases are saltpetre, common salt, sugar, acetic acid, alcohol, glycerin, essential oils, spices and vegetable extracts.

Colouring matter in Food.—Every year colouring matter is being increasingly added to food in the East, especially to sweetmeats and ærated mineral waters. Arsenic (q. v.) is frequently present in the aniline dyes used, being derived either from the arsenical compounds or impure chemicals used in the manufacturing processes. In England, metallic colouring matters containing compounds of antimony, arsenic, cadmium. chromium, copper, lead, mercury and zinc are prohibited; also certain coal-tar colours, *e.g.,* picric acid, and the vegetable dye, gamboge.

Harmful colours are mostly mineral in nature while harmless colours are of an organic nature and are derived from plants or animals. Mineral colours of an objectionable nature are not much in use excepting copper sulphate which is used to colour preserved vegetables, specially peas.

To distinguish natural harmless colours of an organic nature from objectionable artificial dyes various tests are employed.

Arata's wool-test.—While wool is thoroughly cleaned by boiling with sodium hydroxide solution and then washing well with water to remove all traces of alkali. An extract of the solution of the colour is made by macerating the coloured foodstuff in water, adding a few c.c. of 1 per cent. potassium bisulphate. The mixture is boiled. The clean washed wool is soaked for a few minutes in this extract of colouring matter and is then washed well with boiling water and dried. The natural colours of wines, fruits, cane-sugar, ether, etc., do not stain the wool or, if so, the colour is changed by ammonia and not restored by washing with water. Many artificial dyes colour the wool and the colour either is not changed by ammonia or, if changed, is restored by washing.

Most of the harmless colours are soluble in alcohol or chloroform and the ash furnished by incinerating them is not of a mineral nature. Organic colours are soluble in water and the

coloured solution or extract made by maceration in water is bleached by sodium hypochlorite solution, while mineral colours are generally insoluble in water and are not decolorised by sodium hypochlorite. The ash is to be examined for zinc, copper, arsenic, cadmium, lead, etc.

The common **harmful colours** used more or less for improving the appearance of foodstuffs are briefly described below:—

1. *Gamboge.*—A yellow-coloured resin insoluble in water but soluble in alcohol; gives with water a white precipitate which dissolves, producing intense red colour when ammonia is added.

2. *Picric acid.*—Colour yellow; soluble in alcohol or ether; gives a blood-red colour when an alkaline solution of potassium cyanide is added to an extract dried over a water-bath.

3. *Aniline colours.*—These are largely used owing to their cheapness, solubility and fastness. Some contain arsenic while others which are free from arsenic are themselves poisonous. The use of Victoria yellow, Martius' yellow, metanil yellow, naphthol green, Bismarck brown, methylene-blue and gentian-violet is more or less objectionable.

A.—The Iso-nitril test to detect aniline colours consists in adding an equal amount of potash-lve to the alcoholic extract, followed by 2 drops of chloroform. The whole is gently warmed to expel the alcohol and then boiled. A peculiar disagreeable odour of iso-nitril develops if any of the aniline colours is present. Iso-nitril is very poisonous, so the test should be carefully performed in a fume cupboard.

B.—Another simpler test is to add excess of sodium hypochlorite solution to the aqueous extract when a violet colour is produced in the presence of aniline dyes.

4. *Mineral colours* are not now greatly used. Copper sulphate used for colouring preserved vegetables, specially peas, forms a green coloured compound with phyllocyanin. Lead is used for yellow, red and white colours; cobalt and manganese for pink and blue; mercury for red; antimony for orange; arsenic for yellow, orange and green; iron for shades of blue and green; chromium for yellow and green; zinc, cadmium, tin and barium for white. These colours are identified by testing the ash produced by evaporating an extract of the dye.

Harmless colours are very numerous and are used with impunity where laws do not prevent it. The important tints most commonly used are as follows:—

(*a*) Red, pink and shades of these colours are produced by the use of cochineal, fuchsin (aniline dye), magenta (aniline dye), logwood and safflower. Cochineal is turned violet with alkalies and yellowish red with acids. Safflower is turned light brown and is bleached by strong sulphuric acid. Logwood gives a lavender colour when alum and ammonium carbonate are added to an alcoholic extract.

(*b*) Yellow, amber or orange colours are given by annatto, saffron, turmeric, aniline orange, marigold and chrysophanic acid. Annatto, saffron and turmeric are soluble in alcohol and give characteristic colours when an alcoholic solution is evaporated and the residue touched with concentrated sulphuric acid, annatto turning blue changing to green; saffron turning blue changing to reddish brown; marigold turning olive green; and turmeric turning violet red and brown with alkalies. Chrysophanic acid turns purple with caustic potash.

(*c*) Blue or violet colours are imparted by methylene-blue, methyl violet, indigo and litmus. If hydrochloric acid is added to the extract, methylene-blue gives a green precipitate while methyl violet turns green changing to yellow. Litmus is red with acids and blue with alkalies. Indigo gives dense violet fumes when heated and is bleached by potassium permanganate and ammonia.

(*d*) A brown colour is given by caramel produced by the incomplete burning of sugar. This is harmless.

(*e*) A green colour given by chlorophyll is identified by the characteristic spectrum.

CHAPTER IX

THE EXAMINATION OF DISINFECTANTS

Qualitative Tests

Phenols.—(1) Add ferric chloride. A purple colour results in the presence of phenols.

(2) Bromine water gives a white precipitate of tribromophenol, soluble in excess.

Formalin.—Distil; add a drop of 1: 20 carbolic acid and strong sulphuric acid; a crimson zone indicates the presence of formalin.

Perchloride of mercury.—Iodine and potassium iodide solution gives a yellow precipitate, soluble in excess of potassium iodide.

Hydrogen peroxide.—Add a few drops of potassium bichromate followed by sulphuric acid to acidify; shake with ether. A blue colour results.

Sulphurous acid.—Addition of silver nitrate produces a white precipitate soluble in nitric acid.

Quantitative Estimation

Estimation of the Phenols in carbolic disinfection.—

Reagents Required.—

1. $\frac{N}{10}$ solution of sodium thiosulphate (2·48 grammes to 100 c.c. of water).

2. $\frac{N}{10}$ solution of potassium bromide-bromate solution (2·76 grammes of potassium bromate and 15 grammes of potassium bromide per litre). This must be standardised by titrating with $\frac{N}{10}$ sodium thiosulphate after liberating the equivalent iodine from 20 per cent. potassium iodide.

3. Potassium iodide solution (20 per cent.)

Process.—In a 500 c.c. stoppered bottle put 50 c.c. of water and 5 c.c. of pure hydrochloric acid (Sp. gr. 1·2). Add 5 c.c.

of the disinfectant, diluting if necessary to approximately decinormal strength. Add slowly measured amount of $\frac{N}{10}$ bromide-bromate solution to furnish a slight but permanent yellow colour. Shake for two minutes and add 0·5 c.c. of 20 per cent. potassium iodide solution to liberate iodine from the unused bromine. Titrate this liberated iodine with $\frac{N}{10}$ sodium thiosulphate, using starch to determine the end point. The difference between the amount of thiosulphate used and the known amount of bromide-bromate solution added represents the amount of phenol present.

1 c.c. of $\frac{N}{10}$ bromide-bromate solution = 0·0015675 gramme phenol.

Example.—If the bromide-bromate solution used for 5 c.c. of phenol sample (which was diluted 100 times) is 37·5 c.c. and the thiosulphate used is 7·5 c.c.,

$\therefore$ 37·5—7·5 or 30 c.c. of $\frac{N}{10}$ bromide-bromate solution represents the phenol. 1 c.c. = 0·0015675 gramme phenol.

$\therefore$ 30 c.c. = 0·0015675 × 30 grammes of phenol contained in 5 c.c. of the diluted sample.

i.e., 94·05 per cent. exists in the original sample.

Estimation of the amount of tar oils in crude carbolic acid.—To 10 c.c. of the sample in a graduated cylinder add 40 c.c. of a 10 per cent. solution of caustic soda. Shake well, and place aside for 20 minutes to allow the oils to separate out. The amount of oil can be read off on the graduation of the tube, a light oil separating out at the top and a heavy oil at the bottom. Calculate the percentage.

Suppose 2·5 c.c. of oil separated from the original 10 c.c. of the sample.

$\therefore$ the percentage of tar oils is 25 per cent.

Estimation of the percentage of phenols and cresols in Carbolic powders.—Owing to the formation of carbolate of lime, powders which have lime for their base are of smaller disinfectant value. The tests for the estimation of powders are vitiated to the extent of the value of any of the disinfectant which is in chemical combination and thus not "free."

Process.—(*a*) (*When lime is not the base*).—50 grammes of the powder are thoroughly shaken up in ether to extract all the available tar acids. This extraction can also be done conveniently in a Soxhlet apparatus. Add 50 c.c. of 10 per cent.

caustic potash and evaporate the ether on a gentle flame over a water-bath. When all the ether is evaporated increase the flame and boil the alkaline extract of phenol. Transfer to a graduated cylinder and then add 50 per cent. sulphuric acid to render it slightly acid. Cool, and the tar acids will separate out into a layer. Read off the volume which equals the quantity of phenol in the original 50 grammes of the powder. Multiply by 2 to get percentage by volume. Since the specific gravity of phenol is 1·05, multiply the volumetric percentage by this figure to get the percentage of phenol by weight.

(*b*) (*When lime is used as the base*):—Convert all lime to calcium sulphate to free the phenols by stirring 50 grammes of the powder with sufficient sulphuric acid (50 per cent.) in a mortar until every minute particle of the powder is acid to litmus. The phenols are now extracted with ether and estimated as above described.

Phenols should not be less than 15 per cent. in carbolised powders.

Estimation of the amount of potassium permanganate in an unknown solution.—Place a known volume, say 10 c.c. of oxalic acid (6·3 grammes per litre) in a bowl and add sulphuric acid (about 3 c.c.) till it is strongly acid.

Heat to 70°C. From a burette run in the sample of permanganate solution to be tested till a permanent pink colour remains.

1 c.c. of $\frac{N}{10}$ oxalic acid = 1 c.c. $\frac{N}{10}$ potassium permanganate = 0·00316 gramme of potassium permanganate.

$\therefore$ 10 c.c. of $\frac{N}{10}$ oxalic acid = 0·0316 gramme of potassium permanganate.

If 15·5 c.c. of permanganate solution were used for 10 c.c. of $\frac{N}{10}$ oxalic acid, then 15·5 c.c. of permanganate solution contain as much permanganate as 10 c.c. of $\frac{N}{10}$ potassium permanganate or 0·0316 gramme.

$\therefore$ the percentage of permanganate in the solution is $\frac{0{\cdot}0316 \times 100}{15{\cdot}5}$ = 0·204 per cent.

The amount of available oxygen from the permanganate solution can be calculated, if required, by remembering that 2 molecules (316 parts) of permanganate is equal to 5 atoms (80 parts) of oxygen.

Estimation of the Available Chlorine in Bleaching Powder.—

I.—*Arsenite method.*—Mix carefully 5 grammes of the powder with water in a mortar, and transfer the solution gradually to a flask. Make up to 500 c.c. Take 10 c.c. of this (containing 0·1 gramme of the sample) and titrate it with $\frac{N}{10}$ sodium arsenite (4·95 grammes of arsenious acid and 20 grammes of sodium bicarbonate to a litre) until starch and potassium iodide paper no longer becomes blue when touched with a drop of the mixture taken on a glass rod. Note the reading. Now add some freshly boiled clear starch solution to the titrated liquid and estimate the excess of arsenite which has been added by titrating with decinormal iodine till a permanent blue colour appears. Deduct the amount of $\frac{N}{10}$ iodine from the amount of arsenite solution, which thus gives the amount required to neutralise the available chlorine.

Example.—To 10 c.c. of the diluted solution 6·2 c.c. arsenite was added till the starch and iodide paper was no longer blue. The excess required 0·8 c.c. of $\frac{N}{10}$ iodine solution. So 6·2—0·8 or 5·4 c.c. of $\frac{N}{10}$ solution represents the available chlorine in diluted sample.

1 c.c. of $\frac{N}{10}$ solution = 0·00354 gramme of chlorine;

$\therefore$ 5·4 c.c. = 0·00354 × 5·4. This much amount is present in 10 c.c. of the diluted sample or in 0·1 gramme; so in 100 grammes it is $\frac{5{\cdot}4 \times 0{\cdot}00354 \times 100}{0{\cdot}1}$ = 19·116 per cent.

II.—*Thiosulphate method.*—This is the method commonly used in India.

Two grammes of bleaching powder are mixed with 100 c.c. water in a mortar. 10 c.c. of the mixture with 0·5 c.c. sulphuric acid are placed in a bowl and an excess of potassium iodide added till the yellow colour is permanent. Titrate with $\frac{N}{10}$ sodium thiosulphate and estimate the free chlorine. 1 c.c. of $\frac{N}{10}$ solution = 0·00354 gramme of chlorine.

A good sample of bleaching powder should contain 35·5 per cent. of available chlorine, though 10 per cent. is a figure commonly met with in the Tropics due to the action of moist air in liberating chlorine which escapes—

$$CaOCl_2 + H_2CO_3 = CaCO_3 + Cl_2 + H_2O.$$

Estimation of the dose of bleaching powder or any other chlorine disinfectant for the purification of water by Morrison's modification of Sims Woodhead's process.—

1. Make a 1 in 1000 (7 grains in 16 oz.) solution of bleaching powder or the chlorine compound.

2. Place 500 c.c. (17 oz.) of the water to be treated in each of six large mugs, flasks or beakers.

3. To this series of vessels add 0·6, 0·8, 1, 1·2, 1·4 and 1·6 c.c. (or if no pipette is available, 10, 13, 16, 19, 22 and 25 drops) of the bleaching powder solution, stirring the contents of each thoroughly with a glass rod or vulcanite pen-holder.

4. Allow the vessels to stand for 20 minutes, then test for the presence of chlorine by dissolving in each two crystals of potassium iodide and adding a few drops of freshly prepared starch solution. If none of the vessels show a blue colour, the water is foul and the dose of bleaching powder required to render it safe will give the water an objectionable taste. For drinking, such water should be boiled. When some of the vessels give the blue colour then the lowest dose that shows a distinct blue reaction after an interval of 15 minutes indicates the correct dose to use.

5. The number of pounds of bleaching powder required for 100,000 gallons of water is obtained by multiplying the number of c.c. used for this sample by 2 or by dividing the number of drops by 8. To find the dose in grains per gallon multiply this figure by 0·07.

Thus when 0·6, 0·8, 1 c.c. give no colour and 1·2, 1·4 and 1·6 c.c. give a distinct blue colour then the correct dose per 100,000 gallons is $1{\cdot}2\times2=2{\cdot}4$ lbs. and the dose per gallon is $2{\cdot}4\times0{\cdot}07=0{\cdot}168$ grain.

Lime in water.—The strength of lime in water may be estimated by titrating with $\frac{N}{10}$ H_2SO_4 and phenolphthalein.

1 c.c. $\frac{N}{10}$ H_2SO_4 = 1 c.c. $\frac{N}{10}$ $Ca(OH)_2$

1 c.c. $\frac{N}{10}$ $Ca(OH)_2$ = 0·0037 gramme $Ca(OH)_2$.

The Estimation of Formalin.—To 5 c.c. of the formalin solution in 100 c.c. of water add 30 c.c. $\frac{N}{10}$ iodine. Then add drop by drop caustic soda solution until the colour becomes a clear yellow. If this does not occur when the solution is just

alkaline add a further noted quantity of $\frac{N}{10}$ iodine. This will be necessary if the formalin solution is concentrated.

Acidify with dilute HCl, add starch solution as an indicator and titrate with $\frac{N}{10}$ thiosulphate.

Deduct the amount of thiosulphate used from the total amount of $\frac{N}{10}$ iodine used and estimate the amount of formaldehyde this is equal to. One c.c. $\frac{N}{10}$ iodine solution = 0·0015 gramme formaldehyde.

Hydrogen peroxide.—5 c.c. of the peroxide solution and 50 c.c. of water are placed in a large glass and H_2SO_4 added until the solution is markedly acid. From a burette run in $\frac{N}{01}$ potassium permanganate until the colour becomes permanent. 1 c.c. $\frac{N}{10}$ permanganate = 1 c.c $\frac{N}{10}$ H_2O_2.

The result in volumes of H_2O_2 =

$$\frac{\text{number of c.c. permanganate} \times 0{\cdot}56}{\text{number of c.c. of original solution used in estimation.}}$$

PART II—BACTERIOLOGY

CHAPTER I

THE GENERAL MORPHOLOGY AND BIOLOGY OF BACTERIA

Medical bacteriology embraces not only the bacteria (Schizomycetes) pathogenic and non-pathogenic to man and animals but also the higher vegetable forms, namely the Hyphomycetes (moulds) and Blastomycetes (yeasts) and the pathogenic species of the lowest members of the animal kingdom, the Protozoa.

The Classification of Bacteria.—Bacteria (Schizomycetes) are unicellular organisms, devoid of chlorophyll, of minute size and of various forms. They multiply and reproduce by transverse fission with great rapidity and show no morphological nuclei.

Several classifications have been suggested from time to time. The classification advocated by the society of American Bacteriologists is probably the best on account of its elasticity and its orderly grouping of the biologically allied bacteria. Another classification is that of Zopf and Migula, the latter dividing the bacteria into five families, the Coccaceæ, Bacteriaceæ, Spirillaceæ, Clamydo-Bacteriaceæ and Beggiatoaceæ.

Bacteriological Nomenclature.—The nomenclature based upon the Report of the Committee appointed by the American Society of Bacteriologists (*Journal of Bacteriology*, 1920, Vol. V, p. 191) is recommended for adoption. The following is adopted from the *Bulletin of Hygiene*, 1928, Vol. III, pages 89-91 :—

"Where present knowledge does not permit the definite placing of a particular organism within one of the 'genera described by the Committee, the current name of the organism will be used, but without capital or italics.

"With each 'genus' are given the names of the most commonly mentioned organisms included therein, the older names being given in brackets in Roman type. It may be added that well

recognised common names will be freely used as well as the scientific names, but without capitals or italics. For example, the organism of tuberculosis will be *Mycobacterium tuberculosis* (*Myco. tuberculosis*), but will be frequently referred to as the tubercle bacillus. In the nomenclature employed, two names fill a dual role, their meaning being defined by the use of appropriate type; *Bacillus* (B) means an organism with the characters of that genus; *bacterium* means any organism whatever within the class Schizomycetes."

Actinomyces.—Organisms growing in the form of a much-branched mycelium which may break up into segments that function as conidia—sometimes parasitic, with clubbed ends of radiating threads conspicuous in lesions in the animal body. Some species are micro-ærophilic or anærobic. Non-motile. Type "species": *Actinomyces bovis* (Streptothrix actinomyces).

Mycobacterium.—Slender rods which are stained with difficulty, bue when once stained are acid-fast. Cells sometimes show swollen, clavate or cuneate forms, and occasionally even-branched cells. Non-motile. Gram-positive. No endospores. Growth on media slow. Aerobic. Several species pathogenic to animals. Type "species": *Mycobacterium tuberculosis.*

Mycobacterium tuberculosis (bacillus tuberculosis).

Myco. leprœ (bacillus lepræ).

Corynebacterium.—Slender, often slightly curved rods with tendency to club and pointed forms, branching cells reported in old cultures. Barred, uneven staining. Not acid-fast. Gram-positive. Non-motile. Aerobic. No endospores. Some pathogenic species produce a powerful exotoxin. Characteristic snapping motion is exhibited when cells divide. Type "species": *Corynebacterium diphtheriæ.*

Corynebacterium diphtheriæ (bacillus diphtheriæ).

Pfeifferella.—Non-motile rods, slender, Gram-negative, staining poorly; sometimes forming threads and showing a tendency towards branching. Gelatin may be slowly liquefied. Most "species" do not ferment carbohydrates. Growth on potato characteristically honey-like. Type "species": *Pfeifferella mallei.*

Pfeifferella mallei (bacillus mallei).

Pf. whitmori (bacillus whitmori). Motile and produces acid from certain carbohydrates, but not gas.

Vibrio.—Cells short bent rods, rigid, single or united into spirals. Motile by means of a single (rarely two or three) polar flagellum, which is usually relatively short. Many "species" liquefy gelatin and are active ammonifiers. Aerobic and anærobic. No endospores. Usually Gram-negative. Water forms, a few parasites. Type "species": *Vibrio choleræ.*

Vibrio choleræ (bacillus choleræ).

Neisseria.—Strict parasites, failing to grow or growing very poorly on artificial media. Cells normally in pairs. Gram-negative. Growth fairly abundant on serum media. Type "species": *Neisseria gonorrhœæ.*

Neisseria gonorrhœæ (gonococcus).

N. catarrhalis (micrococcus catarrhalis).

N. meningitidis (diplococcus intracellularis meningitidis).

Streptococcus.—Chiefly parasites. Cells normally in short or long chains, sometimes in pairs and small groups, never in large packets. Generally stain by gram. Capsules sometimes present, no zoögleal masses. On agar streak, effused translucent growth, often with isolated colonies. In stab cultures little surface growth. Many sugars fermented with formation of large amount of acid, but inulin is rarely attacked. Generally fail to liquefy gelatin or reduce nitrates. Type "species": *Streptococcus pyogenes.*

Streptococcus hæmolyticus.
Str. mucosus.
Str. viridans.

Str.pneumoniæ (diplococcus pneumoniæ; pneumococcus).

Staphylococcus.—Cells in groups and short chains, very rarely in packets. Generally stain by gram. On agar streak good growth of white or orange colour; glucose, maltose, sucrose, and often lactose fermented with formation of moderate amount of acid. Gelatin often liquefied very actively. Type "species": *Staphylococcus aureus.*

Proteus.—Highly pleomorphic rods, filaments and curved cells being common as involution forms. Gram-negative. Actively motile. Characteristic amœboid colonies on moist media liquefy gelatin rapidly and produce vigorous decomposition of proteins. Ferment glucose and sucrose (but not usually lactose) with formation of acid and gas. Type "species": *Proteus vulgaris.*

Proteus vulgaris (bacillus proteus).

Prot. vulgaris X_{19} (bacillus proteus X_{19}).

Bacterium.—Gram-negative, evenly staining rods. Often motile, with peritrichic flagella. Easily cultivable, forming grape-vine leaf or convex whitish surface colonies. Liquefy gelatin rarely. All forms (except the alkaligenes and abortus group, see *Brucella*) attack the hexoses and most species ferment a large series of carbohydrates. Acid formed by all, gas (CO_2 and H_2) only by one series. Typically intestinal parasites of man and the higher animals although several species may occur on plants and one (*Bact. ærogenes*) is widely distributed in nature. Many species pathogenic. Type "species": *Bacterium coli.*

Bacterium coli.

Bact. dysenteriæ (Flexner)

Bact. dysenteriæ (Shiga)

Bact. paratyphosus A, B and C

Bact. typhosus

Bact. ærtrycke

Bact. suipestifer

Bact. enteritidis (Gärtner)

Lactobacillus.—Rods, often long and slender. Gram-positive, non-motile, without endospores. Usually produce acid from carbohydrates, as a rule lactic. When gas is formed, it is CO_2 without H_2. The organisms are usually somewhat thermophilic. As a rule micro-ærophilic; surface growth on media poor. Type "species." *Lactobacillus caucasicus.*

Lactobacillus acidophilus (bacillus acidophilus).

Lacto. bulgaricus (bacillus bulgaricus).

Pasteurella.—Aerobic and facultative. Powers of carbohydrate fermentation slight; no gas produced. Gelatin not liquefied. Parasitic, frequently pathogenic, producing plague in man and hæmorrhagic septicæmia in the lower animals. Gram-negative. Type "species": *Pasteurella cholerœ-gallinarum* (*i.e., Past. aviseptica*).

Past. pestis (bacillus pestis).

Past. lepiseptica (bacillus lepisepticus).

Past. boviseptica (bacillus bovisepticus).

Hæmophilus.—Minute rod-shaped cells, sometimes thread-forming and pleomorphic, non-motile, without spores, strict parasites, growing best (or only) in the presence of hæmoglobin and in general requiring blood serum or ascitic fluid. Gram-negative. Type "species": *Hæmophilus influenzæ.*

Hæmophilus influenzæ (bacillus influenzæ; Pfeiffer's bacillus).

Hæm. conjunctivitidis (bacillus of Koch-Weeks).

Hæm. pertussis (bacillus pertussis of Bordet-Gengou).

Brucella.—Small, Gram-negative rods. Coccal forms frequent in some species. Non-sporing. Some "species" motile by peritrichic flagella, others non-motile. Some "species" sensitive to changes in O_2 or CO_2 tension. All species fail to produce acid or gas in carbohydrate media, produce an alkaline reaction in litmus-milk, and tend to produce a brownish pigment on potato. The known species are parasitic on man and animals, producing characteristic infections. Type "species": *Brucella melitensis.*

Br. melitensis (micrococcus or bacillus melitensis).

Br. abortus (bacillus abortus of Bang).

Br. bronchiseptica (bacillus bronchisepticus).

Bacillus.—Aerobic forms. Mostly saprophytes. Liquefy gelatin. Often occur in long threads and form rhizoid colonies. Form of rod usually not greatly changed at sporulation. Type "species": *Bacillus subtilis.*

Bacillus anthracis

B. mycoides

B. subtilis

B. mesentericus

Clostridium.—Anærobes or micro-ærophiles. Often parasitic. Rods frequently enlarged at sporulation, producing clostridium or plectridium forms. Type "species": *Clostridium butyricum.*

Cl. botulinum

Cl. chauvei

Cl. sporogenes

Cl. tetani.

Cl. welchii

The Anatomy of Bacteria

The Structure of the Bacterial Cell.—The bacterial cell consists of a sharply contoured mass of cytoplasm which has an affinity for the basic aniline dyes like the nucleus of an animal cell. It is difficult to demonstrate the nucleus in the bacteria. In ordinary preparations no cell-membrane is visible but it can be demonstrated by treating the bacteria with 2·5 per cent. saline solution when a retraction of the protoplasm takes place. Metachromatic granules at the poles or scattered throughout the cell may be seen and represent reserve food supply.

Motility.—This depends upon "flagella" which consist of delicate protoplasmic threads only seen by special methods of staining. Flagella vary in length, in number and in position. Some organisms such as *B. pyocyaneus* possess a single flagellum (monotrichic) situated at one pole, others have a tuft of terminal flagella (lophotrichic), *e.g., Spirillum rubrum,* and some organisms, *e.g., B. typhosus,* are surrounded by flagella (peritrichic).

Size.—The size of bacteria is measured in microns (0·001 millimetre) and may vary enormously from ultramicroscopic filter-passers to spirochætes of 40 μ. The average size of the bacilli is 2 $\mu \times 0{\cdot}5$ μ.

Reproduction.—Bacteria multiply by transverse fission and not by sexual processes. A constriction appears in the middle of the bacterial cell followed by a transverse line which cannot be stained, and the protoplasm finally separates in two parts. Division occurring in one plane results in chains (streptococci); division in two planes results in groups (staphylococci); while raised clusters (sarcina) are due to division in three planes.

Bacteria multiply with great rapidity in favourable surroundings but when placed in unfavourable conditions they grow and multiply with difficulty.

Spore Formation is rare and takes place in some of the species of bacteria, *e.g., B. tetani,* to enable the organism to retain its vitality under unfavourable external condition such as desiccation. Only one spore develops in each cell. The spore appears as a refractile body within the protoplasm, increases in size and may become round, oval or rod shaped. The rest of the protoplasm may retain its affinity for stains as in *B. tetani* or it may lose this property as in *B. anthracis* in which case the spores are termed "endospores" and the process of spore formation termed "endogenous." The spores vary considerably in

size and also in position (terminal, subterminal and central spores) and are not stained by ordinary methods.

Bacterial Capsules.—The cell membrane sometime becomes thickened, sharply defined and gelatinous on its outer margin, forming a distinct capsule round the organism which is then known as a "capsulated" bacterium.

The Chemical Composition of Bacteria varies considerably. The application of microchemical methods has revealed the presence of substances allied to glycogen (stained brown with iodine) and of fats (stained black with osmic acid). Bacteria contain proteins, lipoid substances and salts in varying proportions. The bacterial protein (mycoprotein) is said to differ from ordinary protein in that it is not precipitated by alcohol and does not contain sulphur. The bacterial proteins are chiefly globulins and nucleoproteins. The cell wall is made of chitin. The salts contained in bacteria are mainly of sodium, potassium and magnesium.

The Physiology and Biochemistry of Bacteria

Food Supply varies with the natural adaptation of the different species. As a general rule, in supplying artificial food for bacterial growth the principle ought to be to imitate as nearly as possible the natural environment of a given organism. In the case of pathogenic bacteria the medium should approximate to the composition of body fluids and tissues. Bacteria require protein, nitrogen and salts for their growth. The reaction (H-ion concentration) of the medium is a matter of considerable importance. Most bacteria thrive best in a slightly alkaline medium, but others, such as *V. cholerœ*, will not grow even if a trace of free acid is present in the medium.

Moisture is essential for bacterial growth. Resistance to drying varies enormously: *B. coli* and *V. cholerœ* are rapidly killed while *B. tuberculosis* may resist desiccation for several months.

Relation to Gaseous Environment.—Some species of bacteria will only grow in the presence of free oxygen and are described as "obligatory ærobes," *e.g., B. subtilis;* others will grow in the absence of free oxygen—"obligatory anærobes," *e.g., B. tetani;* but these derive their oxygen from compounds in the media. To the majority of bacteria the presence or absence of free oxygen is a matter of indifference though they prefer an atmosphere containing oxygen: these are called "facultative anærobes."

Temperature.—(*a*) *Influence on growth.*—For each species there is an optimum temperature at which it grows best. In each case, growth ceases above a certain temperature ("maximum") and also below a certain temperature ("minimum"). As a general rule, the optimum temperature of a bacterium is that of its natural habitat, *e.g.*, at 37·5°C. in the case of organisms inhabiting animal tissues and 20°-24°C. in the case of organisms taking part in the ordinary process of putrefaction. Some organisms (thermophilic bacteria) isolated from manure and the intestinal tract, etc., prefer a very high temperature (60°C. to 70°C.).

(*b*) *Influence on Viability.*—Bacteria which do not form spores are quickly killed at a temperature above 57°C. The lowest temperature at which a particular organism is killed in 10 minutes is known as the "thermal death point" Bacteria are more readily killed by moist than by dry heat. Spores may resist a dry temperature of 140°C. for 3 hours or boiling at 100°C. for 6 hours. All known organisms are destroyed by exposure to steam in an autoclave for 15 minutes at 125°C. Fractional sterilisation on several occasions does not always result in sterility owing to the subsequent growth of resistant spores.

Light.—Bacteria prefer darkness for their growth and viability and are readily killed by direct sunlight, the green, violet and ultraviolet rays of which are most fatal. The action of light is superficial and the rays are stopped by very thin glass. Röntgen rays and radium emanations have little, if any, effect except after long exposure.

Chemicals.—Various organic and inorganic chemical substances are inimical to bacterial growth.

Mutual Influences.—Some organisms grow in living animal tissues or plants (parasites), others flourish in water, decaying matter, soil. etc., (saprophytes) and many organisms may live either as parasites or saprophytes. Besides parasitism, there is a relationship of organisms known as "symbiosis" in which both the organisms (symbionts) are favourable to each other's growth, *e.g.*, the organism growing in the nodules of the roots of the *Leguminosæ* which fixes the nitrogen for these plants. Antibiosis, on the other hand, denotes a condition of life that is more or less inimical to the growth of the associated organisms.

Bacterial Enzymes break up the highly complex molecules of the organic matter of animals and plants into simpler substances. In the process of putrefaction albuminous bodies are split up into such simple substances as indol, cretinin, etc., and

various alcohols, acids, etc., are produced by the breaking up of carbohydrates by bacteria.

Chromogenic bacteria, as their name signifies, produce pigments, usually lipochromes. These are probably excretory products. Besides "chromogen" pigment being produced within the organisms themselves or for excretion, pigment may be produced by the oxidation of media.

Bacterial Toxins are elaborated by bacteria and in virtue of their poisonous effects, produce various pathological lesions. The toxins are proteins and are divided into:—

Exotoxins or extracellular toxins, soluble poisons elaborated by a limited number of organisms such as *B. diphtheria, B. tetani* and *B. botulinus, B. dysenteriæ* (Shiga). The exotoxins are characterised by extreme specificity inasmuch as a particular toxin has an affinity for particular tissues. They are prepared by cultivating the organism in a fluid medium from which they can be obtained by filtration.

Endotoxins or intracellular toxins are the toxic constituents of the bacterial cells and do not diffuse in the surrounding medium. They are less specific in their action and are produced by the great majority of the pathogenic bacteria.

A toxin having a specific effect on the constituents of blood has been termed "hæmolysin" (causing lysis of the red cells) and "leucocidin" (causing destruction of the leucocytes).

Ptomains are bodies produced during the breaking down of proteins. Proteins are changed into albumoses and peptones, then to aminoacids, etc., and the ptomains which are elaborated are similar to the vegetable alkaloids and may be non-poisonous, *e.g.,* methylamine or poisonous, *e.g.,* tyrotoxicon in cheese and mytilotoxin in mussels.

Variation and Mutation

Though the term "variation" includes mutation it more accurately means "fluctuating" variations in biological characters from the standard characteristic, while the term "mutation" is restricted to the evolution of new types which have developed characters different from those of the original strains, these new characters being permanent. Bacteria may show variations in form and function.

Anatomical Variations.—Variation in morphology is a common occurrence especially marked in cultures, *e.g.,* the short and long forms of organisms "pleomorphism"; the altered large swollen "involution" forms occurring in old cultures, and the Gram-positive organisms may become Gram-negative.

Physiological Variations.—Variation in function is extremely important and may take place in one of the following directions:—

Colony variations.—The typical appearances of colonies on the various media produced by each bacterium have long been known but within recent years this subject has attracted much attention. Weil and Felix obtained strains of *B. proteus* which instead of forming a bacterial film of growth developed discrete colonies. Arkwright, by plating old broth cultures of *B. dysenteriæ, B. paratyphosus A, B. typhosus, B. enteritidis* (Gärtner) obtained two types of colonies, one of which designated "smooth" (S) resembles more closely the normal, grows in colonies which have a smooth glistening surface with a well defined margin and is not agglutinated by 0·85 per cent. NaCl; the other is larger and more flattened, designated "rough" (R) and presents a granular surface and an indented margin. The "R" colonies differed from "S" colonies in being agglutinated by 0.8 per cent. salt solution. Mucoid colonies described by Fletcher as being large, slimy, white and mucilaginous have been obtained from cultures of *B. paratyphosus, B. dysenteriæ* and *B. enteritidis* (Gärtner), etc. In the case of *Proteus* Weil and Felix found that the "H" form represented by the normal type grew with a spreading film (*Hauch*) and the other "O" form occurred as a variant which grew in isolated colonies without any film (*Ohne hauch*).

Virulence.—Different strains of any particular organism exhibit wide variation in virulence. Another kind of variation is associated with the morphological characters of the colonies, *e.g.*, "R" forms of pneumococci, streptococci and *B. enteritidis* (Gärtner) have been shown to be much less virulent than the "S" forms of these organisms.

Serological variation.—The "R" forms have been further shown to differ from the "S" forms in their antigenic, agglutinating and absorbing properties. According to Goyle "H" forms are identical with the "N" forms and "S" forms, all of which contain two antigens; one of these is heat-labile and agglutinates in large fluffy flocculi, the other is heat-stable and agglutinates in small granular clumps. The "O" forms are rich in the heat-stable antigen of the "N" forms and are relatively poor in their heat-labile antigen content and may even be devoid of it. The "R" forms in addition to the heat-labile antigen of the other forms possess another antigen which is peculiar to these forms and which is heat-stable.

Variation in the fermentation properties of bacteria may occur either spontaneously or by cultivating an organism for

a long time on a medium containing a particular substance, *e.g.*, one of the sugars or some substance inimical to the growth of a bacterium. Twort, for example, obtained a strain of *B. typhosus* which fermented lactose by growing it in a medium containing lactose for a long period. Some strains of *B. typhosus* produce acid from dulcite though this is not usually attacked by strains of *B. typhosus.*

The Bacteriophage—The Twort-d'Herelle Phenomenon

In 1915, Twort observed peculiar changes in the growth of a micrococcus obtained on cultivation from vaccine lymph. The cultures on agar showed glassy transparent areas which could not be subcultured and showed only reddish granules with Giemsa's stain. He found that if a young colony of the micrococcus isolated from lymph vaccine were touched with a small portion of one of the glassy colonies the growth at the point became transparent and this gradually spread over the whole colony.

In 1917 d'Herelle described a similar phenomenon in connection with dysentery bacteria. The general name of "Bacteriophage" is now given to this substance causing this degeneration of bacteria.

Method of Obtaining Bacteriophage.—*D'Herelle's method.*—Four or five drops of a stool are added to broth, incubated at 37°C. for 18 hours and the culture then filtered through a Chamberland filter. If, now, a young broth culture or saline emulsion of a given organism is inoculated with the filtrate, it gradually becomes clear as a lysis goes on. A trace of this clear culture if added to another turbid broth culture of the same organism renders the latter clear and the phenomenon can be repeated indefinitely. When an agar culture is inoculated with bacteriophage, the growth at the point touched soon becomes clear and this change may spread and involve the whole growth. Occasionally colonies form on the clear surface after some time, and these consist of organisms which have resisted the action of the lytic agent. Similarly, if a broth culture is inoculated with the bacteriophage and plated, resistant colonies develop and the growth on agar slopes and agar plates presents a "nibbled" appearance owing to partial lysis. Samples of bacteriophage may be obtained from other sources in addition to the stools of dysentery, cholera, typhoid, etc., *viz.*, from the alimentary tract of man and animals, from the exudate produced by an intraperitoneal inoculation into a guinea-pig, from ordinary cultures in the laboratory and from sea water, sewage, earth and rivers. D'Herelle believes that

there is only a single strain of bacteriophage, whilst others hold that there are several. There is, however, no doubt, that a bacteriophage of a particular organism is restricted in its action on other organisms.

Properties of the Bacteriophage.—Only living bacteria are lysed and the propagation of the bacteriophage occurs only in the presence of living bacterial cells. Dead bacteria are unaffected and are incapable of reproducing the lytic agent.

The bacteriophage is filterable and is a very active substance, being active in a dilution of 1 in 1,000. It can withstand drying for a considerable period. It is destroyed by heating to 60°-70°C. It is very resistant to the action of some chemical agents such as alcohol, ether, acetone, etc. D'Herelle found that its activity was not lost after being kept in 1 : 200 corrosive sublimate or in 1 : 100 carbolic acid for three days. It retains its vitality for a long period in strong solutions of glycerin. It can be precipitated with acetone, alcohol and other reagents. Immunisation of an animal with the bacteriophage yields an antiserum which inhibits the action of the bacteriophage, *i.e.*, if a mixture of the antiserum and the bacteriophage is added to a culture of the organism no dissolution takes place and the organism continues to multiply.

Nature of the Bacteriophage.—Various hypotheses have been advanced with regard to the nature of this phenomenon but precise knowledge is still lacking. That the lytic property is due to the presence of a living organism was first conceived by Twort who described it as "an acute infectious disease of micrococci." He, however, did not stress this view which has been vigorously defended by d'Herelle. D'Herelle has attempted to distinguish Twort's phenomenon from his own and has given the name of bacterioclysis to the former since Twort found his transparent areas to consist of granules, *i.e.*, a fragmention of the cocci. D'Herelle calls his lytic agent a "bacteriophage" and believes that it is a living microscopic virus, *Bacteriophagum intestinale*. It is generally held that these two phenomena are identical and that the actual process of dissolution of the bacteria depends upon an enzyme. Opinion is, however, divided as to the sources of this enzyme, *i.e.*, whether it is a product of the bacteria themselves or of a definite virus parasitic on these bacteria. Against its being a definite living virus it has been urged that it is not visible by the ordinary microscopic methods and has not been observed directly, that it has not yet been successfully cultivated on any medium apart from the living bacterial cells, and lastly that it is resistant to the action of various agents. All these arguments, with the exception, perhaps, of the last, could be used to refute the general

belief that the filter-passing viruses of smallpox, hydrophobia, etc., are living organisms. With regard to the enzyme being a non-living ferment, one has to note that the property of multiplication or reproduction distinguishes it from other enzymes. Bordet believes that the bacteria for some reason or other are so modified that an autolytic process is started and is transmitted to the descendents of the bacteria. The autolysin so produced acts on other bacteria in the medium and more and more of the lytic agent is produced. Fleming has described a lytic agent as occurring in tears and other fluids of the body, in egg white and in some plant tissues. The lytic agent in tears is capable of lysing bacteria in a dilution of 1 in 240,000. He calls is *lysozyme* which, however, differs from the bacteriophage in that it is not capable of multiplication.

CHAPTER II

THE MICROSCOPIC EXAMINATION OF BACTERIA AND THE STAINING OF SPECIMENS

In the microscopic examination of bacteria micro-organisms may be examined (*i*) alive, (*ii*) in film preparations and (*iii*) in sections.

Hanging-drop Preparation.—Living microbes are generally examined as hanging-drop preparations to ascertain the motility and the method and rate of multiplication. A hanging-drop preparation must be made from a young culture 6 to 8 hours old. preferably grown in a fluid medium. A clean hollow ground glass slide and a cover-glass are warmed in the flame, and the depression of the slide is ringed with vaseline by dipping into vaseline the end of a rubber stopper that has been hollowed out and applying it to the slide. A droplet of the bacterial suspension is transferred to the centre of the cover-glass. In the case of a culture on a solid medium, a loopful of sterile broth or salt solution is placed in the centre of the cover slip and inoculated with a very small amount of the culture. The glass slide is now inverted over the cover slip, taking care that the droplet of bacterial emulsion is situated in the middle of the hollow and does not touch the slide at any point. The slide is turned over quickly so that the fluid has no time to run. To seal the chamber and prevent drying, the edges of the cover-glass are pressed gently and uniformly all round into the vaseline. When no hollow ground glass slides are available, a raised ring of vaseline is made in the centre of an ordinary slide and the cover-glass with a drop of the emulsion in its centre is inverted carefully over the vaseline.

In examining the preparation the edge of the drop is focussed with a low power so that the edge crosses the centre of the field and then $\frac{1}{6}$-inch lens is used with a small diaphragm. On examining with the oil-immersion lens true motion is to be distinguished from Brownian movement, which is an oscillatory movement around one point, and from the movement of organisms due to currents in the fluid, in which case all the particles move in the same direction. To diagnose motility the movements of organisms must be observed in all directions, especially the movement of individual organisms close together in opposite directions.

Film Preparations.—The slides and cover-glasses are cleaned by boiling them for some time in a mixture of sulphuric acid 6 parts, potassium bichromate 6 parts and water 100 parts, then washing them thoroughly in water and storing in 50 per cent. alcohol. Before use, the slide is dried with a soft cloth.

Films may be made on cover-glasses as "cover-glass specimens."

Dry Method.—Some of the material is transferred to a clean glass slide that has been passed through a flame and cooled. In the case of growth on a solid medium a drop of saline is added to a small amount of the growth on the slide and emulsified. A uniform, thin film is spread and allowed to dry. After drying in air the film is fixed by passing it slowly 3 or 4 times through the flame and for this purpose the slides may be held in a Cornet's forceps. Chemical fixing agents such as a mixture of formalin and absolute alcohol or that of alcohol, ether and alcoholic solution of bichloride of mercury may be used. The slides after treatment with chemicals should be washed in water.

Impression Films.—These are used in studying the characteristic appearance of colonies under the microscope. A clean cover-glass is placed with its edge on the surface of the medium a little to one side of the selected colony, is lowered until horizontal and then gently pressed downwards at its centre with the points of the forceps. The opposite edge is raised slowly by the point of a platinum needle until vertical and the cover-glass grasped with forceps and removed from the plate.

Wet Method.—Films are made as before; when still wet they are placed in a solution of formalin 1 part and absolute alcohol 9 parts for three minutes, and then washed thoroughly in methylated spirit and stained.

Smear Preparations are particularly applicable to sputum and certain blood examinations. The material may be pressed between two cover-glasses which are then slid apart. Smears are made from *post-mortem* and other specimens by touching a freshly cut surface of the organ with a cover-glass. The smears are dried in air and stained.

Staining

In staining for bacteria it is to be noted that (*i*) stains and reagents which have been used once must not be used again; (*ii*) the film sides of the slides must not come in contact with each other; (*iii*) the stains should be filtered before use; (*iv*) the stain is to be poured on the film sides of the slides which must be placed upwards, and (*v*) the slides must be marked with some appropriate identification mark.

General Principles.—*The staining solution.*—The protoplasmic matter of bacteria has almost the same refractive index as media, so structural peculiarities are not observed under the microscope unless stained or examined by special methods of illumination. Staining not only helps in the examination of bacteria, but owing to the selective action of the protoplasm of certain bacteria to different dyes their differentiation is also possible. Bacterial protoplasm reacts to basic dyes like the nuclear material of tissue cells. Some organisms are easily stained while others require special methods. Bacteria are best stained by aniline dyes, the time and intensity of staining depending on the protoplasmic affinity for the dye, concentration and nature of the dye, presence or absence of chemical substances called "mordants," temperature, etc. Aniline ($C_6H_5NH_2$) is a coal-tar product and the dyes derived from it are either acid salts of basic dyes or alkali salts of acid dyes. They are called basic or acid dyes although their reaction is neutral or amphoteric. The important basic dyes used as bacterial stains having a special affinity for the nuclear chromatin are: fuchsin, methylene-blue, bismarck-brown, gentain-violet and methyl-violet. Acid aniline dyes such as eosin and acid fuchsin, having an affinity for the cytoplasm, are not suitable for bacteria but are used as counterstains to bring out tissue elements.

Vegetative bacteria of almost all species can be stained with aqueous or alcoholic solutions of aniline dyes.

The Staining of Films.—Film preparations are dried and fixed. A few drops of the filtered stain are placed on the film and allowed to remain on it for the requisite period which depends upon the nature of the staining solution, its strength and the organism under investigation. The stain is poured off and the preparation washed in tap water or distilled water, the excess of water being drawn off with a filter-paper. The film is either allowed to dry in the air with the film side downwards or high on a flame, taking care that it is not overheated. A drop of cedar-wood oil is then placed on the film which is examined under 1|12 oil-immersion lens. To preserve the slide the cedar-wood oil is removed with xylol.

Staining Solutions and Methods.—(1) *Löffler's Methylene-blue* is mostly used and consists of a mixture of 30 c.c. of a saturated solution of methylene-blue in alcohol and 100 c.c. of a solution of caustic potash in distilled water (1 in 10,000). Bacterial films are stained for 3 to 5 minutes and sections for one quarter of an hour.

(2) *Carbol-Thionin-blue* is a good substitute for methylene-blue for all purposes and consists of one per cent. solution of thionin-blue in 1 in 40 carbolic acid, diluted four times when used. Sections may be stained up to half an hour and if necessary decolorised with weak acetic acid. The counterstaining with 1 per cent. watery solution of eosin should be carried out before staining with the thionin-blue.

(3) *Carbol-fuchsin* (Ziehl-Neelsen's solution) diluted with three to six parts of water may be used for staining ordinary preparations.

(4) *Gram's method.*—Certain bacteria, when stained with one of the pararosaniline dyes, *e.g.*, methyl-violet, crystal-violet or gentain-violet and then treated with iodine, form an iodine-pararosaniline compound which is insoluble in alcohol or aniline oil so that on subsequent treatment with alcohol some organisms resist decolorisation while others are decolorised and can be stained with a counterstain. Organisms which retain the stain are known as Gram-positive while those which become decolorised are called Gram-negative. In order to render the decolorised organisms evident and to distinguish them from those retaining the colour a red counterstain is applied after decolorisation.

Solutions Required:—

(1) *Carbol-gentian-violet.*—Saturated alcoholic solution of gentian-violet 1 part, 5 per cent. solution of carbolic acid in distilled water 10 parts. This mixture should as far as possible be used fresh, as it tends to precipitate. Any precipitate must be filtered off before use.

(2) *Gram's iodine.*—Iodine 1 gramme, potassium iodide 2 grammes, distilled water 300 c.c.

(3) *Acetone-alcohol.*—Acetone 10 c.c., alcohol 100 c.c. Methylated spirit can be substituted for acetone-alcohol.

(4) *Dilute Carbol-fuchsin.*—Ziehl-Neelsen's carbol-fuchsin 1 part, distilled water 9 parts.

After the film is dried and fixed in the usual manner it is stained with carbol-gentian-violet for about five minutes. The stain is poured off and replaced with Gram's iodine solution which is allowed to act for one minute. Without washing in water it is treated with acetone-alcohol solution or methylated spirit, using several changes, until the film is completely decolorised and then washed.

The counterstain is dilute carbol-fuchsin which is allowed to act for ten to twenty-five seconds, then washed off with water and the slide dried.

Jensen's Modification of Gram's Method.—The film is dried and fixed in the usual manner. When cool it is stained with 0·5 per cent. aqueous solution of methyl-violet for one quarter to half a minute. The stain is poured off and the slide washed with a few drops of strong Lugol's solution (iodine 1 gramme, potassium iodide 2 grammes, distilled water 100 c.c.). Water should not be used to wash the film. A fresh quantity of strong Lugol's solution is poured on and allowed to act for half to one minute. The iodine is now washed off with absolute alcohol, (*i.e.*, 98 per cent. or over), fresh absolute alcohol added and the slide rocked from side to side for more than two minutes. The film is again washed with a few drops of fresh absolute alcohol and immediately, without any intervening washing in water, neutral-red solution is poured on and allowed to act for ½ to 1 minute. The solution consists of neutral-red 1 gramme, distilled water 1,000 c.c., 1 per cent. glacial acetic acid 2 c.c. The film is then washed in distilled water, dried with blotting-paper and mounted in the usual manner.

If Gram's method is properly carried out, Gram-positive organisms are stained dark violet in colour. Gram-negative organisms, the nuclei and protoplasm of pus cells and tissue cells are stained pink with the counterstain.

To obviate errors from over-decolorising, a film of a known Gram-positive organism, (*e.g.*, pure culture of Staphylococcus aureus) may be made at one side of the film. This "control spot" is stained along with the film. For the recognition of Gram-negative organisms such as gonococci or meningococci, the "control spot" must retain the violet stain while the Gram-negative organisms are stained only with the counterstain.

For staining sections the Gram-Weigert's modification is recommended. After the preliminary treatment the sections are stained lightly with alum-hæmatoxylin and washed in running water, then stained in one per cent. aqueous solution of eosin for 1-5 minutes. The section is washed in water and then stained with aniline methyl-violet for half to one hour. After washing Lugol's solution is added for 1-2 minutes and again washed with water. After blotting and dehydrating the section is cleared with several changes of a mixture of equal parts of aniline and xylol (not alcohol) and mounted in balsam.

Reaction of Some Organisms to Gram's Stain.—

GRAM-POSITIVE.	GRAM-NEGATIVE.
Staphylococcus.	Gonococcus.
Streptococcus.	Meningococcus.
Pneumococcus.	Micrococcus catarrhalis.
	Cocco-bacillus melitensis.
Micrococcus tetragenus.	Pneumobacillus of Friedlander.
B. diphtheriæ.	Koch-Weeks bacillus.
Hofmann's bacillus. (Pseudo-diphtheria bacillus).	Diplo-bacillus of Morax.
B. xerosis.	B. proteus.
Tubercle bacillus. (The organism is not stained by the ordinary Gram's method).	B. typhosus.
	B. coli communis.
	B. pyocyaneus.
Smegma bacillus.	B. dysenteriæ.
B. lepræ.	B. enteritidis Gärtner.
Streptothrices.	B. mallei.
B. anthracis.	V. choleræ.
B. tetani.	B. pestis.
B. welchii.	B. influenzæ (Pfeiffer).
B. sporogenes.	B. pertussis.
	B. fusiformis.
Vibrion septique.	Spirochætes.
B. botulinus.	Bacterium pneumosintes.

Staining for Tubercle and Other Acid-Fast Bacilli.— *Ziehl-Neelsen Method.*—These organisms do not stain with the ordinary aniline dyes on account of a resistant covering of a fatty nature which prevents penetration of the stain. The stain used consists of basic fuchsin with phenol as the mordant. As the organism is resistant to ordinary stains it also tends to resist decolorisation. Any strong acid can be used as the decolorising agent, but sulphuric acid in a 20 per cent. solution is usually employed. In order to show what has been decolorised and to form a contrast with the red-stained bacilli, the preparation is counter-stained with methylene-blue.

Ziehl-Neelsen's (strong) Carbol-fuchsin.—

Basic fuchsin	..	..	1 part.
Absolute alcohol	..	..	10 parts.
Solution of carbolic acid (1 : 20)	..	..	100 parts.

The dye is dissolved in the alcohol and the solution added to the carbolic acid.

Films are made, dried and fixed in the usual manner. The slide is flooded with filtered carbol-fuchsin and heated until steam rises. The steaming dye is allowed to act for 5 minutes. After washing in water the slide is immersed in 20 per cent. sulphuric acid, and after about a minute in the acid, is washed with water and placed in the acid again. This process should be repeated several times until decolorisation is complete, *i.e.*, till, after washing, the smear is a very faint pink. The slide is then well washed in water, counterstained with Löffler's methylene-blue for half to one minute and washed, dried and mounted.

Sections are stained as above and dehydrated, cleared and mounted. The bacilli stain bright red, while the tissue cells and other organisms are stained blue.

Besides the tubercle bacilli there are other acid-fast organisms such as the smegma, leprosy, Timothy grass, mist and "butter" bacilli. The first is frequently found in samples of urine, and is decolorised by alcohol, whereas the tubercle bacillus is not. In other words, the tubercle bacillus is "acid-fast," and "alcohol-fast" but the smegma bacillus and similar organisms are "acid-fast" but not "alcohol-fast." The decolorisation with spirit is particularly important when examining urine for the presence of the tubercle bacillus. Leprosy bacilli are also acid-fast, but not to the same extent. They are stained in smears or sections in the same way as the tubercle

bacilli, except that three per cent. sulphuric acid is used for decolorisation.

Stains for the Diphtheria Bacillus.—The diphtheria bacillus gives its characteristic staining reactions only in young cultures (twelve to eighteen hours) on Löffler's blood-serum medium.

Neisser's Stain.—

Solutions Required:—

1. *Neisser's methylene-blue.*—Methylene-blue 1 gramme, absolute alcohol 50 c.c., glacial acetic acid 50 c.c., distilled water 1000 c.c.

2. Crystal violet (Höchst) 1 gramme, absolute alcohol 10 c.c., distilled water 300 c.c.

3. Chrysoidin 1 gramme, distilled water 300 c.c. (Dissolve by gentle heat and filter).

The films are dried and fixed by heat, then (*i*) stained for 5 to 10 minutes in a mixture of 2 parts of No. 1 solution and one part of No. 2 solution, (*ii*) washed in water, (*iii*) stained in chrysoidin solution No. 3 for three seconds, and (*iv*) washed with water, blotted and dried.

The protoplasm of the bacillus is brown, while the granules are blue black.

This stain is frequently used as follows:

The film is fixed and stained in No. 1 solution (above) for 30 seconds, then washed in water and counter-stained for one minute with Bismarck-brown. The counterstain is made by dissolving two grammes of Bismarck-brown in 1 litre of boiling distilled water and filtering.

Pugh's stain.—Mix toluidin blue 2 grammes, absolute alcohol 20 c.c., glacial acetic acid 50 c.c. and add distilled water up to a litre. Films are stained for 5 minutes and washed in water. Granules are stained reddish-purple and the protoplasm light-blue.

The Staining of Spores.—Spores possess resistant outer envelopes which prevent the ordinary stains from penetrating the protoplasm, hence appear as clear areas. All clear areas are not spores; once the spores are stained they retain the dye inspite of decolorising agents.

Films, which must be thin, are made, dried and fixed in the usual manner.

In the heat method films are stained with steaming Ziehl-Neelsen's carbol-fuchsin for five minutes, washed with water

and decolorised with (*a*) 1 per cent. sulphuric acid, or (*b*) methylated spirit. After washing well with water the film is counter-stained with Löffler's methylene-blue for two minutes, washed in water, blotted and dried. The spores are stained bright-red and the protoplasm of the bacilli blue.

The spores only may be stained by first heating the film for about 10 minutes in the flame then staining with Ziehl-Neelsen and washing in water. The heat destroys the power of the bacilli to take up the stain.

Möller's Method is more satisfactory than the above. The fixed film is placed in chloroform for two minutes and washed in water. A 5 per cent. solution of chromic acid is then allowed to act on the film for 2 minutes. After washing in water the film is treated with steaming Ziehl-Neelsen for 10 minutes, decolorised carefully in 1 per cent. sulphuric acid, washed and counter-stained with Löffler's methylene-blue.

Neisser's method consists of staining with warm carbol-fuchsin for 10 to 20 minutes, washing in water, decolorising in 2 to 3 per cent. alcoholic solution of hydrochloric acid and staining, after washing, with Löffler's methylene-blue.

The Staining of Capsules.—*Hiss's Method.*—

Solutions Required:—

1. Saturated alcoholic solution of basic fuchsin— 1 part,
   Distilled water .. .. .. 19 parts.

2. Copper sulphate solution—20 per cent. in distilled water.

Films, which should be thin and uniform, are fixed by heat in the usual manner, then treated for 30 seconds in steaming fuchsin solution and the stain washed off with 20 per cent. copper sulphate solution. Without washing in water the film is dried between blotting-papers and mounted, if necessary, in balsam. Films of solid cultures should be made without any diluent. If necessary, a drop of fluid serum may be used. In order to avoid an excessive deposit of copper sulphate crystals on the film successive amounts of copper sulphate solution should be poured on the film until the slide is quite clear.

The bacteria are stained deep reddish brown, while the capsules are pale reddish brown in colour.

Richard Muir's "modified" method of staining capsules consists of treating a thin film with steaming carbol-fuchsin for 30 seconds, washing slightly in alcohol, then in water, and then with a

mordant consisting of 2 parts of a saturated solution of mercuric chloride, 2 parts of a 20 per cent. solution of tannic acid and 5 parts of a saturated solution of potash alum. The film is then washed well in water, decolorised till pale pink in methylated spirit and then rewashed and counter-stained with methylene-blue. It is then finally washed and dried, or if desired, dehydrated with alcohol, cleared in xylol and mounted.

The Staining of Flagella.—Staining of flagella depends on rigorous cleanliness of cover-slip and a suitable culture. The slides should be heated in a smokeless flame to remove the grease before use. Cover-slips are boiled in bichromate-sulphuric acid and washed in distilled water. To make the bacterial emulsion a little of the surface growth of a 10 to 20 hours' agar culture is transferred to 2-3 c.c. of sterile tap water in a test-tube by means of a platinum loop which is drawn to and fro in the water and not rubbed on the sides. One or two loopfuls are placed on a clean cover-glass and the suspension allowed to flow over the surface by inclining the cover-glass. The film must be thin and dried in air and fixed high on a gentle flame.

Richard Muir's Modification of Pitfield's Method.—

Solutions Required:—

1. *The mordant.*—

Tannic acid, 10 per cent. aqueous solution, filtered ..	10 c.c.
Corrosive sublimate, saturated aqueous solution ..	5 c.c.
Alum „ „ „ „ ..	5 c.c.
Ziehl-Neelsen's carbol-fuchsin	5 c.c.

Mix thoroughly. A precipitate is formed which is deposited by centrifuging or by allowing the fluid to stand. In either case the clear supernatant fluid is removed to a clean bottle. The mordant keeps well for one or two weeks.

2. *The stain.*—

Alum, saturated aqueous solution	10 c.c.
Gentian-violet, saturated alcoholic solution	2 c.c.

This stain will not keep for more than two days.

The mordant is poured on the film and heated for one minute. After washing well in running water for two minutes and drying carefully over the flame the stain is added and steamed for one minute. The slide is then washed well in water, dried and mounted in Canada balsam.

The Romanowsky Stains.—The original Romanowsky stain was made by dissolving the compound formed by the interaction of watery solution of eosin and methylene-blue in methyl alcohol. The original stain has now been replaced by various modifications which are easier to use and give better

results. The peculiar property of Romanowsky stains is that they impart a reddish-purple colour to the chromatin of malaria and other parasites.

Leishman's Stain.—This stain is made by dissolving 0·15 gramme of Leishman's powder in 100 c.c. of acetone-free methyl alcohol. Impure methyl alcohol must not be used. The powder is ground in a mortar with a little of the methyl alcohol, the residue of undissolved stain allowed to settle and the fluid decanted into a bottle. The residue in the mortar is treated with more methyl alcohol and the process is repeated until all the stain goes into solution. The remainder of the methyl alcohol is now added. The stain is improved by keeping overnight in an incubator at 37°C. before use and is used after 2 weeks.

The film is dried in air. The stain is first used undiluted so as to allow the methyl alcohol to fix the film, and is then diluted with distilled water, and the staining proper carried out. The water must be added before the methyl alcohol in the stain has time to evaporate.

The undiluted stain is poured on the unfixed film until it is completely covered and after 30 seconds double the volume of distilled water is added and the fluids mixed by alternately sucking them up in a pipette and expelling them or drawing the edge of a clean slide over the surface of the stain. After 5 to 7 minutes the stain is flushed off with distilled water. This flushing is important as it prevents the deposit of the stain settling on the film. The preparation is now allowed to differentiate in the distilled water for half a minute. The film is then washed in water and allowed to dry in air by placing the slide slantwise with the film down; it should not be dried by shaking or by blotting.

Giemsa's Stain.—The formula of the stock solution of the stain is as follows:—

Azur II.-eosin	3 grammes.
Azur II.-eosin	0.8 gramme.
Glycerin (Merck, chemically pure) ..	250 grammes.
Methyl alcohol (Kahlbaum)	250 grammes.

It is convenient to obtain the stain ready prepared. Grubler's stain is very reliable.

The film is dried in air and fixed in absolute alcohol for 15-20 minutes, then flooded with dilute stain.

The dilute stain is made by adding 1 drop of the stock stain to 1 c.c. distilled water. If intense staining is desired,

the distilled water before mixing with the stains is rendered alkaline by the addition of one drop of one per cent. potassium carbonate to 10 c.c. of water. The stain is allowed to act for 10 to 15 minutes, the slide then washed in a stream of distilled water and dried in air. The slide is examined by the oil-immersion lens after placing a drop of cedar-wood oil directly on the film, or the preparation may be mounted in Canada balsam.

The above procedure is adopted in examining a specimen of blood for malaria parasites. In staining for spirochætes, the preparation is fixed for 15-20 minutes and the distilled water is made alkaline by the addition of potassium carbonate.

For the preparation of blood slide and other methods of staining these, see under "Malaria."

The Staining of Bacteria in Sections

Fixation and Hardening of Tissues.—The tissue is cut into blocks of not more than 1½ to 2 cm. square by 1 cm. in thickness and treated by one of the following methods:—

(*a*) *Absolute alcohol.*—The blocks of tissue are placed in alcohol for 24 hours and if not cut immediately are preserved in 80 per cent. alcohol.

(*b*) *Formalin solution.*—The tissue is soaked in 10 per cent. commercial formalin in saline for 24-48 hours, then washed in water for a few hours and placed for 24 hours in each of the following strengths of methylated spirit: 30 per cent., 60 per cent., 90 per cent. After the last bath the tissue is placed in absolute alcohol for 24 hours before cutting.

(*c*) *Zenker's fluid.*—This consists of bichromate of potassium 2·5 grammes, corrosive sublimate 5 to 8 grammes, water 100 c.c., glacial acetic acid 5 c.c. The acetic acid is not added to the stock solution but only immediately added before use. The tissues are steeped in the fluid for 18-24 hours, washed in a stream of running water for 24 hours and passed through increasing strengths of alcohol as in (*b*).

The Cutting of Sections.—(*a*) *Freezing method.*—The tissues are freed from alcohol by washing in a stream of running water for 12-24 hours, after which they are placed in a solution of gum acacia 5 grammes, cane sugar 0·5 gramme and water 100 c.c. (To prevent decomposition a piece of thymol or a little carbolic acid may be added).

The tissues are then cut on the freezing microtome and the cut sections washed with several changes of warm water to remove the gum and stained at once or stored in 70 per cent. alcohol.

(*b*) *Paraffin method.—Embedding.*—The preserved pieces of tissues are dehydrated by soaking first in 95 per cent. alcohol and then in absolute alcohol for 24 hours, then transferred in sequence to (*i*) a mixture of equal parts of absolute alcohol and chloroform for 24 hours, (*ii*) pure chloroform for 24 hours or longer, (*iii*) a saturated solution of paraffin in chloroform for 24 hours and (*iv*) passed through two separate baths of melted paraffin to remove the chloroform (2-4 hours). The temperature of the bath should be just above the melting point of paraffin. A little melted paraffin is poured into the cavity formed by two L-shaped pieces of metal or into a watch-glass or paper box. Pieces of tissue are placed in this paraffin by means of warmed forceps, their position adjusted and more paraffin poured in to fill the cavity. When the paraffin has begun to set, cold water is run over the trough to hasten the hardening process.

Whatever microtome is used, the sections should be thin and in no case should exceed 8 μ in thickness. The sections when cut are transferred with a needle or camel hair brush to a beaker containing water at a temperature of 50°C. in which the sections float, and, at the melting of the paraffin, spread out perfectly flat.

Fixation on Slides.—(*a*) *Gulland's method.*—A clean slide is introduced obliquely under the water below the sections, an edge of the section fixed on the slide in the required position with a needle and the slide withdrawn. The slide is dried in the incubator at 37°C. for 24 hours.

(*b*) *Mayer's method.*—Mayer's glycerin-albumin mixture is composed of equal parts of egg-white and glycerin, mixed thoroughly and filtered. To every 100 c.c. of the mixture 1 gramme of sodium salicylate is added to prevent decomposition. A thin coating of the mixture is made on the slides, the section transferred to it and dried in the incubator.

Staining and Mounting.—*Frozen sections* are stained in the same manner as paraffin sections except that the latter require preliminary treatment before staining.

Paraffin section.—The slide containing the section is placed in xylol or a series of drops of xylol dropped over it and the excess removed with a clean cloth. The xylol is now completely removed with absolute alcohol which is in turn drained off and removed by washing in distilled water. The section is now ready for staining after which the excess stain is removed by

rinsing in water, the section dehydrated with absolute alcohol, cleared with xylol and mounted in Canada balsam.

Note.—*At no stage should the preparation be allowed to dry.*

For staining the bacteria in sections carbol-thionin-blue, Gram's method and Ziehl-Neelsen's methods may be employed.

The Staining of Spirochætes.—(*a*) *Levaditi's Method for Spirochætes in Sections.*—Pieces of tissue about 1 mm. thick are (*i*) fixed in 10 per cent. formalin solution for 24 hours, (*ii*) rinsed in water for one hour, (*iii*) placed in 95 per cent. alcohol for 24 hours, (*iv*) placed in distilled water until the tissue sinks to the bottom of the container, (*v*) transferred to 1·5 per cent. solution of nitrate of silver contained in a dark bottle which is then kept in the incubator for three days at 37°C., (*vi*) washed in water for 20 minutes and (*vii*) placed in the following mixture in a dark bottle for 48 hours at room temperature:—

Pyrogallic acid	.. ..	4 grammes.
Formalin	.. ..	5 c.c.
Distilled water, up to	.. ..	100 c.c.

After 48 hours the tissue is rinsed in distilled water, dehydrated in increasing strengths of alcohol and embedded in paraffin in the manner above described. The sections are cut and fixed to the slides with the egg-albumin mixture, the paraffin removed with xylol and the section mounted in balsam.

The spirochætes are stained black and the tissue yellow to brown but may be counter-stained by weak carbol-fuchsin.

(*b*) *Staining for Spirochætes in Films.*—

1. *Fontana's Method.*—

Solutions Required:—

1. Acetic acid 1 c.c., formalin 20 c.c. and water 100 c.c.
2. Tannic acid 5 grammes, carbolic acid 1 gramme and water 100 c.c.
3. 0·25 per cent. solution of silver nitrate in water.

For use a small quantity of solution No. 3 is placed in a test-tube and dilute ammonium hydrate solution is added drop by drop until a faint permanent turbidity appears. An excess of ammonia clears up the turbidity and must be avoided.

The film is dried in air without heating, washed in several changes of solution (1) for a minute and then washed well in

water. Solution (2) is dropped on the film which is heated until steam rises, then allowed to remain covered with solution (2) for about 30 seconds. After washing again in water solution (3) is poured on, heated until steam comes off and allowed to cool for half a minute. The film is then washed, dried and mounted, the spirochætes being stained from brown to black.

2. *Becker's Method.*—The film is dried in air without heating and washed with two changes of a mixture consisting of acetic acid 1 c.c., formalin 2 c.c. and distilled water 100 c.c. After washing in distilled water the film is covered with a 1 per cent. aqueous carbolic acid solution in which 10 per cent. tannic acid is dissolved and heated gently until steam rises. After 30 seconds the slide is washed in water, stained with warm Ziehl-Neelsen's carbol-fuchsin solution for 30-45 seconds, washed, dried and mounted.

Note.—See also under *S. pallida.*

CHAPTER III

THE PREPARATION OF APPARATUS AND MEDIA AND THE CULTIVATION OF BACTERIA

In order to study the characters of micro-organisms, it is absolutely necessary to obtain *pure cultures* on artificial media free from other living bacteria which are always present in media, containers and apparatus. Sterilisation of media and apparatus may be accomplished in a variety of ways each of which has its particular use and limitations. The usual methods of sterilisation are by heat, chemicals and filtration.

Sterilisation

1.—Sterilisation by Heat.—

1. *Dry Heat.*—This is the most suitable and satisfactory method. Dry heat requires a much higher temperature or longer time of exposure than moist heat to effect the same degree of sterilisation. Useless articles, contaminated papers, carcasses, etc., of infected animals are best destroyed by burning. In laboratory work dry heat is employed in the following ways:—

(*a*) *Using the bright red heat of the flame.*—This is most suitable for platinum needles or loops. Other metal articles such as the points of forceps, searing spatulas, etc., may also be sterilised by this method, red heat from an ordinary Bunsen burner being applied.

(*b*) *Using the dull red heat of the flame.*—This method of sterilisation is achieved by passing the articles through the Bunsen flame without allowing them to become red hot. It is very convenient for knives, needles, mouths of culture tubes, glass rods, cotton-wool stoppers, glass slides, cover-slips, capillary pipettes, etc. The time of flaming should not be so prolonged as to fuse the glass. The change of flame to a yellow colour is a certain indication of sterilisation of the apparatus. Small metal articles may be sterilised by dipping into methylated spirit and then burning this off.

(*c*) *Hot air.*—Larger articles of glass such as test-tubes, pipettes, flasks, Petri dishes, cotton-wool, quill tubes, etc., cannot be sterilised by flaming. For such articles the *hot air steriliser* is used. This consists of a double walled metal chamber

heated from below by a number of Bunsen burners or electricity. Hot air rises between the inner and the outer walls and after heating the inner chamber escapes finally through holes on the top of the outer case. A thermometer passes down the inner chamber to register the temperature. Modern hot air ovens, heated by electricity, have an automatic arrangement for keeping a constant temperature at the required degree. Temperature and time are both important factors. A temperature of 160°C. for one hour, or, in emergency, a temperature of 180°C. for half an hour, is required for proper sterilisation but as cotton-wool, paper and other organic matter may be more or less charred at such a temperature and may stick to the glass tubes, a temperature of 140°C. for three hours is preferable. Articles sterilised by hot air are dry when removed from the oven and this is very advantageous in routine bacteriological practice.

The following precautions must be observed when glassware is being sterilised, to avoid cracking due to uneven expansion:—

(*i*) Articles should be thoroughly dry and made of thin glass and of even thickness.

(*ii*) The articles must never be put in a warm oven but put in when it is cold and then heated to the required temperature and kept for the requisite time.

(*iii*) The articles must be removed only when the temperature has fallen to 60°C. or below.

2. *Moist Heat* is a much more efficient agent for destroying bacteria than dry heat. It is employed in the following forms:—

(*a*) *Boiling.*—Ordinary non-sporing germs are destroyed by boiling for five minutes. Boiling is very useful for sterilising distilled or tap water required in various bacteriological manipulations. Syringes, surgical instruments and other metal articles are sterilised by this method but to avoid rusting, sharp instruments are placed in boiling water containing some soda. Fluid articles are also sterilised by boiling for an hour and a half.

(*b*) *Steam at 100°C.* (ordinary pressure).—This is one of the most important methods of sterilising ordinary culture media, specially those containing sugar or gelatin. Steam at 100°C. (saturated) kills all organisms in an hour and a half, but prolonged steaming destroys the physical property of the solidification of gelatin on cooling and may decompose carbohydrates

(sugars). To avoid this a method of intermittent or fractional sterilisation is employed by which instead of one full steaming for 1½ hours, three exposures of twenty minutes each on three successive days are employed. Steaming is done in a Koch's steamer, which, in its simplest form, consists of a tall metal vessel provided with a movable lid; water is placed in the bottom and there is a perforated tray placed inside the vessel above the water space to serve as a stand for the containers of media. The outside is covered with felt or asbestos to avoid loss of heat. A thermometer is also provided in the lid to ensure proper steaming. Water is boiled and the rising steam fills the whole vessel and thus effects sterilisation of media placed on the perforated tray. By application of this method both the media and containers are effectively sterilised and there is no loss of media due to evaporation. The cotton-wool plugs of containers should be wrapped in papers to prevent them from becoming wet.

(*c*) *Steam Under Pressure.*—This is the most rapid and effective method of destroying bacteria. It can be used for those media which are not decomposed or injured at a temperature above 100°C. The process depends on the principle that the boiling point of a liquid is raised by increasing the pressure surrounding the liquid. The apparatus used for this purpose in bacteriological practice is an *autoclave* which consists of a gun-metal cylinder supported in a sheet-iron case having a strong lid fastened by screws and nuts and made air-tight by an asbestos washer. A steam tap, pressure-gauge and a safety valve capable of being set to blow off at any desired temperature are also fitted and within the autoclave is a perforated shelf on which to keep the vessels containing media. In using the apparatus sufficient water is placed in the autoclave, materials are put on the shelf, the lid placed in position and tightened while the steam tap is kept open to expel all air, the presence of which considerably interferes with the correct pressure reading. The safety valve is adjusted to the required pressure (15 lbs.). When after heating for sometime the steam escapes and all air is expelled the steam tap is closed. When the required pressure is reached the safety valve automatically opens to allow the extra steam to escape. The time is noted and after the necessary interval the gas is shut off and the autoclave allowed to cool. The steam tap is not to be opened until the pressure in the autoclave has fallen to that of external air otherwise the fluid media will boil vigorously and expel the plugs from the tubes and flasks. When the pressure has fallen the steam tap is opened and the lid unscrewed.

(*d*) *Sterilisation at Law Temperature.*—The proteins of serum or other body fluids are coagulated by heat so that these

cannot be sterilised above their coagulation temperature. Such materials are sterilised on Tyndall's principle of intermittent sterilisation at the low temperature of 57°C. in a water-bath for one hour on eight consecutive days. The temperature is not allowed to rise above 59°C. to avoid inspissation of serum. Higher temperatures destroy bacterial toxins, therefore vaccines should also be sterilised at low temperature (60°C.) to preserve their immunising power.

II.—Sterilisation by Chemical Means.—

Chemical disinfectants are not much used in bacteriological work. Biniodide or perchloride of mercury (1 in 1000) should be available on every laboratory bench for sterilising the hands, contaminated articles and discarded cultures. Antiseptics of the phenol group, lysol and cresol (5 per cent.) are also used for similar purposes. Carbolic acid (0·5 per cent.) or tricresol (0·3 per cent.) is used as a preservative for sera and vaccine. Chloroform (2 per cent.) is often used to sterilise serum but this is later removed by heating at 57°C.

Great care must be exercised in handling cultures and other materials to avoid their contamination and to prevent the infection of the worker. A large dish containing 1: 1000 solution of mercuric chloride in water should be kept on the laboratory table and all the tubes, plates and hanging-drop preparations put into it as soon as done with. The worker should wash his hands with antiseptic lotion and soap before leaving the laboratory.

III.—Sterilisation by Filtration.—

Filtration through filter candles is performed—(*i*) to obtain the soluble toxic products of bacteria, (*ii*) to remove extraneous organisms from liquids such as serum, etc, which are likely to be injured during sterilisation by heat, etc., (*iii*) in investigating the "ultramicroscopic" or "filterable" viruses and (*iv*) to remove the organisms in water for domestic use. Filters of different grades of porosity are available in the market and are made of earth or porcelain.

Coarse Filters, made of Kieselguhr, a diatomaceous earth, have relatively large pores owing to the large size of the particles of the clay from which they are made. The Berkefeld filter is the commonest type and is made in three different grades of porosity; the coarsest is "V" (Viel); the finest is "W" (Wenig) and the intermediate is "N" (Normal).

Fine Filters, made of unglazed porcelain, such as Chamberland (French), and Pukall (German) allow the passage of

only certain ultramicroscopic viruses, *e.g.*, the viruses of foot-and-mouth disease, of hog cholera and of chicken plague. These filters are commonly used for obtaining the bacterial toxins from cultures such as diphtheria and tetanus. Still finer filters are obtained by the use of collodion membrane; these are so fine that even the virus of hog cholera cannot pass through them.

Method of Filtration.—For ordinary bacteriological work the most convenient form of filtering apparatus is the following:—A filter candle such as the Berkefeld is fitted tightly into a cylindrical glass mantle by means of screws and washers, and the metal tube of the candle inserted into the rubber stopper of a conical flask having a lateral opening. The openings are plugged with cotton-wool, the whole wrapped in paper and sterilised by steaming or autoclaving. In assembling the parts of the apparatus, the paper is removed and the rubber stopper fitted into the mouth of the flask. The lateral opening is connected by means of "pressure" rubber tubing with the exhaust pump. The fluid to be filtered is poured into the glass mantle and the air from the flask exhausted. Water suction or the air pump working at a negative pressure of 200-300 mm. of mercury may be used.

In filtering small quantities of fluid as in dealing with "filterable" viruses, Berkefeld candles of small size ($2\frac{1}{2}'' \times \frac{1}{2}''$) should be used and a test-tube slightly wider and larger than the candle placed over the latter.

After use, the filter is scrubbed in distilled water with a stiff nail-brush and boiled subsequently in distilled water.

In the case of filterable viruses the filtrate should be cultivated ærobically and anærobically to determine if it is sterile. If inoculated into susceptible animals the filtrate should reproduce the characteristic symptoms of the disease.

Cultivation of Bacteria

In sowing a culture it is essential that (1) the instrument used is sterile (2) the material to be shown is collected without introducing extraneous organisms to the sterile medium and (3) is transferred uncontaminated to the medium.

The media employed vary with each individual organism to be cultivated. The methods employed for inoculation depend upon the nature of the medium to be sown and the inoculum. Certain terms commonly used in bacteriological books require explanation. A *culture* is a tube of medium which has been inoculated with an organism and on which growth has appeared.

A *pure culture* consists of only one species of organism. A *subculture* is one which has been made from an already existing culture. A culture in a medium solidified in the upright position and made by inoculating the medium by means of a straight platinum wire is known as a *puncture* or *stab-culture* and the growth on a medium which has become solidified when the tube was placed on its side is variously known as a *sloped, stroke* or *slant* culture. A *shake culture* is one which is obtained by mixing the organism with gelatin or agar when still fluid.

Apparatus Required:—

Platinum wires.—These are fixed on the ends of glass rods, 8 inches long. No. 26 S. W. G. wire is generally used. The following should be at hand:—

(*i*) A straight platinum wire, 2½ inches long, for making stab-cultures and also for picking off single colonies.

(*ii*) A platinum wire with a loop turned upon its free end. The loop must be flat and completely closed and is used for transferring material from either a solid or fluid culture.

(*iii*) A thick stout wire with a loop at its free end, used for picking up tenacious growths and thick viscid sputum.

When sterilising platinum wires the glass rod is held at the junction of the upper and middle thirds with the fingers of the right hand as in holding a pen, and the wire heated in the flame in a vertical position till the entire wire becomes red. The wire is allowed to cool and used immediately. *Platinum wires must be thoroughly sterilised in a flame before and after use.* All needles when not in use rest on a rack.

Sterile capillary pipettes are used for inoculating the Smith-Noguchi medium and bullock's heart medium and for transferring a large amount of fluid material. They are made by heating the middle of a piece of glass tubing of 5-7 mm. calibre and of 25 cm. length and drawing out quickly into a fine tube. The thin portion is divided into two in the middle by melting it in the flame. The wide ends are plugged with cotton-wool and the pipettes placed in a large copper cylinder provided with a tight-fitting lid and sterilised in a hot air steriliser. Before use, the closed capillary end is broken off and a rubber teat fitted to the other end. *Sterile graduated pipettes* (1 c.c., 5 c.c., 10 c.c.) are used in water and milk analysis, the pipettes being first wrapped in paper, placed in a copper cylinder and sterilised.

Scalpels may be required in making inoculations with the scrapings of tissues and are sterilised by dipping in alcohol and then flaming.

The Methods of Inoculating Media Tubes.—

1. *Method of inoculating one "slope" from another.*—The test-tubes are held firmly in a slanting position between the thumb and the first two fingers of the left hand, with the sloped surfaces towards the worker. The culture tube should be on the proximal and the uninoculated tube on the distal side. With

the right hand the cotton-wool plugs are rotated to see that they are not adherent to the lips of the tube. The wire is flamed and the plug of the original culture removed by the crooked little finger of the right hand. The mouth of the tube is flamed and the flamed platinum needle introduced at once into the tube without touching the mouth or the sides and a portion of the medium free from growth is touched to insure that the wire is sufficiently cool. If too hot, the wire will melt the medium, so a few seconds' delay must be allowed. The growth is then touched with the wire which is withdrawn carefully, without touching the sides, and the plug replaced. The plug of the uninoculated tube, *i.e.*, medium tube, is withdrawn in the same manner, the wire charged with growth introduced and the whole surface of agar rubbed gently with the needle without cutting into the medium. The wire is withdrawn and sterilised in the flame, the mouth of the tube flamed and the stopper flamed and inserted. With a grease pencil the date and the nature of the material is written on the tube.

Stab-culture.—The tubes are held in the same manner and the needle, charged with bacterial growth, plunged down the centre of the medium, taking care to withdraw it in the same line so as not to cause splitting of the medium.

Inoculation of a fluid medium.—The tubes being held as above, a loopful of the growth is rubbed on the wall of the tube at the level of the fluid and the tube tilted so as to wash down the growth.

Isolation of Pure Cultures of Aerobic Organisms.—If an organism occurs unmixed in a material, *e.g.*, *B. typhosus* in blood, there is no difficulty in obtaining a pure culture: all that is required is to inoculate the material in an appropriate medium. If, however, a mixture of organisms is present in a material such as fæces or urine, the isolation of the individual species may be attended with some difficulty.

I.—*By Plating.*—In such circumstances it is customary to sow the material on a solid medium in such a manner that a large number of isolated colonies are obtained and the individual colonies can be picked off and investigated. For this purpose Petri dishes or circular shallow glass capsules with overlapping glass covers are employed.

A.—Preparation of plates.—The plates are placed in a copper cylinder and sterilised in a hot air steriliser. Tubes of agar are placed in a water-bath until melted, then cooled to 45°C. when the agar tube is taken out of the bath and wiped with a towel so as to remove drops of water. The plugs are flamed and the agar poured into the sterile Petri dish, the lid

of which is at once replaced. The medium should be at least 2 to 3 mm. in thickness at the centre of the plate. When the medium has solidified, the agar-containing-half of the Petri dish is placed in an incubator with the free surface of the medium facing downwards, one edge resting on a piece of glass tubing which may also support the cover. Plates are always inverted thus to prevent moisture dropping on to the agar and washing the colonies into one another. A piece of clean paper is placed under both. When the medium has dried it can be inoculated with the material.

B.—Method of inoculating plates.—Several different methods of making inoculations are employed:—

(*i*) *Dilution method.*—"Shake cultures."—The emulsion of the material is added to agar or gelatin when liquid and gently mixed by rolling the tubes between the palms of the hands. Different dilutions of the material are used in order to secure a number of discrete colonies. The tubes of gelatin are prepared by placing them in a water-bath at 40°C. till the gelatin is liquefied. It is essential that no gelatin be allowed to touch the cotton-wool plug.

(*ii*) The method of *successive strokes* is more convenient and is carried out as follows:—

(*a*) A loopful of an emulsion of the material is placed on the surface of the plate and is spread over the surface with a sterile glass rod bent at a right angle. Without charging the rod with fresh material the surface of a second or even a third plate is smeared in succession.

(*b*) An alternative method consists in placing a drop of the material on the medium at one side of the plate and spreading this in a straight line. Other straight lines are drawn parallel to this original line, without *recharging* the platinum loop, and then diagonal lines are drawn. This method is especially useful when plates are not plentiful.

Plate cultures are made for three purposes:—

1. For isolation of a particular organism in pure culture from a mixture of organisms.

2. For the enumeration of organisms (in water and milk analysis).

3. As an aid in the identification of an organism from the appearance of the colonies.

C.—Incubation.—Agar plates are incubated in an inverted position. Gelatin plates on the other hand are incubated with

the lid uppermost and the date and the nature of the material are noted on the lid with a glass pencil.

D.—The Fishing of Colonies.—After an appropriate period of incubation, usually from 18 to 24 hours, the plates are examined and searched for the colonies of the organism which is to be isolated. The lid of the plate is removed and placed inner side down on the working bench. The medium-containing-half is then held round the sides of the thumb and middle finger with the glass surface facing the worker. Suspicious colonies are then marked on the glass with a grease pencil. After steadying the hands by resting the elbows on the table the selected colony is picked off with a sterile platinum needle—care being taken not to touch the neighbouring colonies—and an appropriate medium inoculated.

II and III.—Separation may be carried out by *single cell* culture or by *shake* culture.

IV.—Separation by Animal Inoculation.—In the case of certain organisms such as *B. tuberculosis,* pneumococcus, etc., occurring in association with other bacteria it is impossible to separate them by ordinary plate methods as they may require special media and some of them grow so slowly that they are soon outgrown by other organisms. In such cases, inoculation of a suitable animal with the mixture of the bacilli is resorted to. When the animal dies or is killed on developing the characteristic disease, suitable material is obtained from it with aseptic precautions and cultivated on special media.

Incubation

Cultures on gelatin are grown at room temperature (22°C.). Agar and serum media are employed to grow bacteria at higher temperatures and for this purpose various incubators have been devised. The aim is to secure a constant temperature and this is accomplished by a mechanical contrivance which automatically shuts off the supply of gas or electric current when the temperature begins to rise above a fixed limit. Regulators to work at different ranges of temperature are supplied and each can be adjusted to work at any point in its own range. For the cultivation of bacteria a temperature of 37°C. is generally required whereas some incubators are regulated at 56°C. for killing cultures or for inactivating the serum for the Wassermann test.

Centrifuges

These form part of the necessary equipment of a bacteriological laboratory and are used for various purposes. They are driven by hand, water or electricity. For ordinary

purposes the machine should be capable of attaining a speed of 4,000 revolutions per minute.

Preparation of Culture Media

For the successful cultivation of bacteria upon artificial media it is important that the medium should resemble, as nearly as possible, the natural surroundings in which the bacteria grow. In the case of pathogenic organisms the media should, as far as possible, approximate to the composition and reaction of the body tissues in which these organisms ordinarily multiply. With many pathogenic organisms it is usually found expedient to use serum or blood. The media commonly used for pathogenic organisms contain proteids or carbohydrates in fluid or semi-solid form, in a transparent or opaque condition.

Watery extract of meat forms the basis of most of the common media used and a great advance was made by Koch in providing a transparent solid medium by adding gelatin to it. For those organisms which have their optimum temperature higher than the melting point of gelatin, another gelatinous product, called agar (carbohydrate), a Japanese seaweed, which does not melt below 98°C. is used instead of gelatin. Culture media are almost innumerable, but broth, gelatin, agar and blood serum are all that are necessary in the vast majority of cases. Gelatin having a low melting point is not so suitable as agar in tropical countries, so in all media containing gelatin, agar is substituted with advantage. Gelatin and agar media are not quite suitable for the isolation of a pure culture, for the colonies of most of the organisms on these media resemble one another closely. To overcome this difficulty, two methods are available. In the first, *differential media method*, a sugar, *e.g.*, lactose, is added to the medium which is tinted with an indicator such as litmus, neutral-red, basic fuchsin, etc. to reveal the change produced by the organism. The colonies fermenting the particular sugar used will be of a different colour from those which do not ferment it. Well known examples of differential media are MacConkey's bile-salt media, Endo's medium, etc. In these lactose is used.

In the *second method* an attempt is made to cause a particular organism in a mixture to grow more luxuriantly than the rest by a variety of physical and chemical agencies. *V. choleræ*, for example, will multiply in media so alkaline that *B. coli* will fail to grow. In the brilliant green enrichment method advantage is taken of the fact that brilliant green, when present in a suitable concentration in a culture medium, has a greater inhibitory effect on *B. coli* generally than *B. typhosus* or *Paratyphosus A and B.*

General Technique

A.—Preparation of Glass-Ware.—The glass-ware is immersed in 1 per cent. hydrochloric acid solution in order to remove free alkali, transferred to 1 per cent. sodium hydrate solution for a few hours, then washed in running hot water. In the case of old glass-ware containing cultures, sterilisation in an autoclave and subsequent boiling in 5 per cent. washing soda is necessary. After thorough mechanical cleaning, the glass-ware is treated as above and dried. After plugging the clean flasks, test-tubes, etc. with non-absorbent cotton-wool, these are sterilised in a hot air sterilizer for one hour at 150°C.

B.—Ingredients of Media.—The various constituents are mixed in the order described under the special media.

C.—Standardisation of Media.—The reaction of the medium influences bacterial growth considerably.

(*a*) The majority of the pathogenic microbes prefer a slightly alkaline medium and to obtain the proper reaction of a media containing meat extract, which is somewhat acid when prepared, a rough and ready method is to add a 10-20 per cent. solution of caustic soda, a few drops at a time. Between each addition the medium is well shaken and tested by immersing in it a litmus test paper. At first the blue paper is changed to red, while the red remains unchanged, then the amphoteric reaction is obtained. With further neutralisation with the alkali, the red paper is turned blue whereas blue litmus remains unaffected. The reaction will now be suitable. The more delicate methods for adjusting the reaction of media are:—

(b) *Eyre's Method.* According to Eyre's scale the reaction or titre of media is expressed by indicating the number of cubic centimetres of normal alkali or acid required to render *one litre* of the medium exactly neutral to phenolphthalein. The sign + (plus) is placed before this number if the original solution reacts to acid and the sign — (minus) if it is alkaline. For example meat extract +10 indicates that the extract is acid in reaction and it requires 10 c.c. of normal alkali *per litre* to make it neutral to phenolphthalein. In the United States of America a similar scale is used but the unit volume is 100 c.c. instead of a litre and so a medium made +1 *per cent.* is the same as +10 on Eyre's scale. The optimum reaction for most bacteria is between +10 and +15 (Eyre's scale), *i.e.*, acid to phenolphthalein. Litmus and phenolphthalein have not the same neutral point: the neutral point of litmus corresponds to +25 with phenolphthalein. Therefore the optimum reaction of media in general lies between the neutral points of these two indicators, *i.e.*, acid to phenolphthalein and alkaline to litmus.

Technique.—The meat extract is generally acid in reaction due to the presence of acid phosphates of sodium and potassium and some weak organic acids. Hydrolytic changes are produced by prolonged boiling whereby its acidity is increased, but prolonged boiling for about 45 minutes is necessary to make it stable, so the reaction has to be adjusted after allowing all the chemical changes to take place in the medium by heat. To 20 c.c. of the warm medium in a procelain basin is added an equal amount of distilled water and a few drops of phenolphthalein solution (0·5 gramme of commercial phenolphthalein to 100 c.c. of 50 per cent. alcohol) as an indicator. The media is kept boiling over a gentle flame or water-bath and, gradually, decinormal solution of caustic soda is added from a burette till a faint but permanent pink colour is obtained. The amount of alkali added is noted and the titration repeated with a similar quantity of another sample. The mean of two readings is taken.

Suppose 5·3 c.c. of $\frac{N}{10}$ NaOH were required to neutralize 20 c.c. of the medium.

Then 20 c.c. of broth require 5·3 c.c. $\frac{N}{10}$ NaOH to neutralize.

$\therefore$ 100 c.c. of broth require 26·5 c.c. $\frac{N}{10}$ NaOH.

$\therefore$ 1 litre of broth requires 265 c.c. $\frac{N}{10}$ NaOH.

$\therefore$ 1 litre of broth requires 26·5 c.c. $\frac{N}{1}$ NaOH.

The reaction of the medium is then said to be +26·5 on Eyre's scale. But the final reaction required is +10. Therefore 16·5 c.c. (*i.e.*, 26·5-10) of normal soda solution per litre are added to standardise the medium.

On theoretical grounds the method is accurate but owing to the presence of buffer salts, the end point is not quite sharp and therefore the point of exact change of colour varies with the personal factor of the observer.

(c) *Estimation of Hydrogen-Ion Concentration.*—The true reaction of the medium is determined by the amount of hydrogen or hydroxyl ions which are present in it. (See Chemistry section).

Apparatus Required:—

1. A set of standard tubes which can be purchased containing solutions of known P_H value with definite concentration of an indicator such as phenol-red. These tubes are usually 8 in number, having a range of P_H value varying from 6·6 to 8·0 with a difference of P_H 0·2 between each tube and the next.

2. Cordite tubes of a uniform thickness of wall and bore and identical in shape with standard P_H tubes.

3. Colour comparator. This consists of a rack to hold two rows of three cordite tubes and has a ground glass at the back to disperse the light. Tubes 4, 5 and 6 are behind tubes 1, 2 and 3 respectively.

4. Phenol red solution (0·01 per cent. in water) made by diluting the 0·02 per cent. solution with an equal volume of distilled water.

5. A micro-burette measuring upto 0·01 c.c.

6. $\frac{N}{20}$ soda solution prepared as follows:—

$\frac{N}{10}$ NaOH.	..	..	.. 500 c.c.
Phenol red (0·01 per cent.)		..	.. 91 c.c.
Distilled water—upto		..	.. 1000 c.c.

The indicator is added to the soda solution so that the actual concentration of the dye remains constant during titration.

7. Bacteriological pipettes of 0·5 c.c. and 5 c.c. respectively.

Method.

Suppose a reaction of P_H 7·5 is desired. The standard tube of P_H. 7·4 is placed in space 1, and that of P_H. 7·6 in space 3. Pure distilled water is placed in the tube in space 2 and 5 c.c. of medium in tubes 4 and 6. In the tube number 5, five c.c. of the medium and half a c.c. of phenol red (0·01 per cent.) are placed. By this arrangement tubes 1 and 4, and 3 and 6 are superimposed when examined in the comparator box. The standard soda solution from the micro-burette is gradually added into tube 5 till the pink colour produced is between the colours of P_H. 7·4 and 7·6 while looking through the medium tubes. After the addition of each small quantity of $\frac{N}{20}$ soda the contents of the tube should be well mixed before comparison. Exact matching with a particular tube is not so convenient as producing a colour midway between two tubes having a higher and lower P_H. value than that actually required. The quantity of alkali used is noted to calculate the quantity required per litre of medium.

Example.—Suppose 1·25 c.c. of $\frac{N}{20}$ NaOH is required to bring the reaction of the 5 c.c. medium to P_H 7·5.

$\therefore$ 5 c.c. of medium require 1·25 c.c. of $\frac{N}{20}$ NaOH.

$\therefore$ 5 c.c. of medium require $\frac{1·25}{20}$ c.c. of $\frac{N}{1}$ NaOH.

and 1000 c.c. require $\frac{1·25 \times 1000}{5 \times 20}$ c.c. of $\frac{N}{1}$ NaOH.

$= 12·5$ c.c.

D.—Methods of Clearing Media.—*Clearing with eggs* is carried out in the case of media containing no coagulable protein by the addition of white of egg in the following manner: The

whites of some eggs (one egg to each litre of medium) are beaten up with a little water. The white mixture is added to the medium, which, if hot as in the case of melted agar or gelatin, must first be cooled to 55°C. The flask containing the medium is shaken vigorously so as to break up the coagulum and again steamed for 15 minutes. The medium is now filtered. The albumin coagulation precipitates the fine particles present in the suspension.

Filtration is best done through cotton. Two square pieces of cotton are placed, one above the other, in the bottom of a large glass funnel in such a way that the direction of the fibres of the one layer is at right angles to the other. Funnel should be warmed in the case of gelatin and agar.

Agar may be filtered through cheese cloth and cotton; gelatin through paper pulp; vitamin media through glass-wool and broth through filter-paper.

E.—The Tubing of Media.—The medium is placed in a large glass funnel fitted to the discharging glass tube by a short piece of rubber tubing provided with a thumb cork. The plug is removed from the sterile test-tube and the glass outlet thrust deeply into the test-tube. About 8 c.c. of the medium are allowed to flow into the test-tube, taking care to prevent the medium from touching the upper portion of the tube.

F.—Sterilisation of Media.—(*i*) *By Heat.*—Media not liable to be injured by high temperatures are sterilized in the autoclave for 15-30 minutes at 15 pounds pressure. Media sensitive to heat, such as sugars, serum or glycerin, are steamed in a Koch's steamer for twenty minutes on three consecutive days. During the intervals between sterilisation the media are kept at room temperature or in the incubator.

(*ii*) *By Filtration.*—Blood serum and ascitic fluid, etc., are filtered.

Indicators are added to the various media to reveal changes in reaction during bacterial growth.

Litmus is added to the medium before sterilisation as a strong watery solution.

Andrade's Indicator is made up as follows:—To 100 c.c. of an aqueous 0·5 per cent. solution of acid fuchsin is added 16 c.c. of $\frac{N}{10}$ NaOH. The indicator should be yellow after standing for 2-3 hours. If still red, 1 c.c. more of the caustic soda solution should be added. This indicator is added to the

medium in a concentration of 1 per cent. Such a medium is red when hot and colourless or yellow when cold. Acid formation turns this indicator red.

Neutral-Red.—The agar and bouillon media containing 0·5-1 per cent. of the sugar to be tested is tinged with watery solution of neutral-red, (0·5 per cent. of a 1 per cent. solution). The medium is yellowish-brown when alkaline and of a rose-red colour when acid.

Preparation of Individual Culture Media

A.—Bouillon Media consist of meat extract and certain other substances favourable to the growth of bacteria.

A (1) Peptone Broth or Bouillon has the following composition:—

Meat extract	1000 c.c.
Peptone	10 grammes.
Sodium chloride	5 grammes.

One pound of finely minced meat, freed from fat, is soaked in a litre of distilled water, and, if practicable, left over-night in a cool place, preferably in an ice-chest. Very young veal is the best to use but fresh lean beef is quite satisfactory and cheap. Horse flesh is cheap and contains less fat than beef but it is unsuitable for certain purposes owing to the presence of fermentable sugars. Next day the extract is filtered in fine muslin and the juice of the meat residue is expressed in a meat press and added to the filtrate. The fluid is bright red and the film of fat floating over it is skimmed off with filter-paper. It is then boiled for 3 hours or steamed in a Koch's steriliser for two hours whereby the colour is changed from bright red to dark brown with the appearance of turbidity owing to the change in the hæmoglobin and the coagulation of proteins. It is then refiltered and the volume made up to 1000 c.c. The finished extract should be clear and light yellow in colour; if not, it is reboiled and filtered. Owing to the lack of the nitrogenous matter removed as a result of its coagulation by heat, it is not yet fit for culture media. Ten grammes of uncoagulable and freely digested protein in the form of commercial peptone are therefore added and 5 grammes of sodium chloride to prevent the precipitation of the carbonates and phosphates on alkalinisation. These substances are dissolved by heat and the medium then filtered again till it is quite clear. After this the reaction is adjusted as above described, the media placed in a flask, stoppered with a cotton plug and sterilized in an autoclave for thirty minutes.

Lab-lemco is an extract of meat which is found to be a good and convenient substitute for fresh meat extract and broth can be prepared by dissolving 10-20 grammes of lemco in a litre of water and adding salt and peptone in the usual quantities. Liebig's meat extract (2 grammes per litre) is also a suitable substitute for fresh meat extract.

A (2) *Glucose Broth.*—To the other constituents of bouillon, 1-2 per cent. of glucose is added. The mode of preparation is the same as A (1).

A (3) *Glycerin Broth.*—To 1000 c.c. of ordinary filtered broth 10-60 c.c. of glycerin of sp. gr. 1·25 are added.

B.—Gelatin Media.—These are simply the above broths solidified with gelatin. (The "gold label" gelatin of Coignet et Cie, Paris, is the most suitable).

B. (1) *Nutrient Gelatin.*—To 1000 c.c. of peptone broth (see A (1) above) 100-150 grammes of gelatin are added. The gelatin is dissolved in broth by heating (½ hour) in a steam steriliser and then filtering through two thicknesses of filter paper, care being taken that the contents of the flask are not shaken. To avoid solidification of the medium during filtration, a funnel with a water jacket, which is heated, is used. The medium is cleared with egg after cooling to 50°C. and finally filtered till the filtrate is quite clear. The finished medium is sterilised in the steam steriliser for twenty minutes each day on three successive days.

The proportion of gelatin required varies with its quality and the time of the year. In very hot summer days 15 per cent. may be necessary while during cold weather 10 per cent. may be sufficient. Nutrient gelatin melts at 22-23°C. and can therefore be used for those becteria which grow best at atmospheric temperature and not at that of the animal body.

B (2) Glucose Gelatin.—To the other constituents of peptone gelatin (see B (1)) 1-2 per cent. of glucose is added before sterilisation (for anærobic organisms at the ordinary temperature). The steps in the preparation are the same as given in B (1).

C.—Agar Media.—Agar is a seaweed found in the China sea and is available in the market in the form of dried strands. For organisms growing at blood temperature (37°C.) agar is used to stiffen the medium instead of gelatin.

C (1) Nutrient Agar.—The agar is cut into small pieces with a pair of scissors and soaked in water for 24 hours. This preliminary washing is very necessary if the sample of agar

contains much sea salt. To 1000 c.c. of peptone broth [A (1)] 15 grammes of agar are added and this medium heated in a Koch's steriliser for 1-2 hours, or in an autoclave at 115°C. for 30 minutes. Phosphates are further precipitated by re-heating for 20 minutes. After clearing with the white of an egg the medium is made slightly alkaline, filtered and sterilised in the Koch's steriliser for 20 minutes or in autoclave for 15 minutes on each of three consecutive days.

C (2) *Glycerin Agar.*—To C (1) after filtration, 1½ per cent. of glycerine is added. Sterilisation is done on three consecutive days in Koch's steriliser for 20 minutes. (For tubercle bacilli).

C (*3*) *Glucose Agar.*—To 1000 c.c. of ordinary agar C (1) 100-200 c.c. of a 10 per cent. sterile solution of glucose are added. (For anærobic organisms).

Robertson's Bullock's Heart Medium (cooked meat medium or minced meat medium) is useful for the cultivation of anærobic organisms.

To 500 c.c. of tap water, 1 lb of minced bullock's heart is added and the mixture heated in the Koch for several hours so as to cook the meat thoroughly. Sufficient normal sodium hydrate is added to the medium to render it alkaline to litmus and the mixture strained through gauze and added to that squeezed out of the meat. The residue is minced again. The fluid and the minced meat are placed in separate flasks and autoclaved for 30 minutes at 115°C. When required for use, sterile tubes are filled to one-fourth with the mince and the fluid is added till half full. The autoclaving is done at 15°C. for 20 minutes on two successive days.

Peptone Water is mainly used as the basis for the sugars employed for fermentation tests. Broth and agar media not free from muscle-sugar cannot be used for carbohydrate fermentation tests. This is a very simple medium, being a solution of peptone (1 per cent.) and sodium chloride (0·5 per cent.) in warm water which is filtered and sterilised in the autoclave. Peptone water is used to test for the formation of indol and also for the isolation of the cholera vibrio. For the cultivation of the cholera vibrio the reaction should be neutral to phenolphthalein (P_H 8·0) as the vibrio prefers an alkaline medium.

Special Culture Media

Blood or Serum Media.—Those organisms which do not grow will on ordinary media require some enriched media for

their proper cultivation. This enrichment is usually accomplished by the addition of blood, serum or any other body fluid or tissue. Agar is used as the basis for solid media when it is necessary to have some uncoagulated serum proteins.

Coagulated Serum.—A sufficient quantity of blood serum is poured into sterile test-tubes which are placed in a slanting position in a tray, and heated in the Koch at 80°C. for an hour. To prevent tearing up of the medium by gas bubbles the tray is placed high up in the Koch. The tubes are sterilized by heating at 80°C. for two hours on each of the two successive days. If the tubes are to be stored a small quantity of broth is added after the serum has solidified and sterilization then performed. This prevents the medium from drying up. Before use, the broth is poured off.

Loffler's Blood Serum.—This medium is most suited for the cultivation of the diphtheria bacillus and may also be used for other organisms. Three parts of ox, sheep or horse-serum are mixed with one part of ordinary 1 per cent. glucose broth. The mixture is placed in tubes which are arranged in a sloped tray and put in a serum inspissator. The temperature is raised to 70-80°C. when all proteins are coagulated to a yellowish white mass. The tubes are further steamed at 90°C. for twenty minutes on three successive days. Over-heating should be avoided so that the expansion of air bubbles and the formation of steam may not disrupt the medium.

Douglas' Medium.—Ingredients required:—(*a*) Nutrient agar; (*b*) 1 per cent. solution of potassium tellurite. As much as possible of the salt is dissolved in distilled water and the insoluble portion allowed to settle. The clear supernatant fluid is pipetted off and 10 per cent. potassium hydrate added to the deposit. After neutralisation by hydrochloric acid, it is added to the portion of salt which is readily dissolved and the solution is made up to the requisite volume. To 100 c.c. of the melted agar, 4 c.c. of a 1 per cent. solution of potassium tellurite are added and the mixture cooled to 60°-65°C. when 15 c.c. of trypsinized serum, which is prepared by filtering a mixture of 100 c.c. of fresh horse-serum and 5-8 c.c. of liq. trypsinæ Co., (Allen and Hanbury) is mixed in thoroughly. The medium is then distributed with aseptic precautions into sterile test-tubes.

Hiss's Serum Water Media are used for testing the fermentative changes of those pathogenic organisms which require serum for their growth, *e.g.*, streptococcus, diphtheria, pneumococcus, gonococcus, meningococcus and other delicate bacteria. Acid production by fermentation is indicated by the coagulation of the media and change of colour (*vide* page 164).

Serum Agar consists of ordinary nutrient agar containing 3 per cent. agar to which 5 per cent. of *sterile* uncoagulated serum is added. Agar is first melted and then allowed to cool to about 50°C. when the requisite amount of serum is added gradually. This medium is indistinguishable from ordinary agar so the tubes or Petri dishes containing serum agar should be labelled "S." Sterile hydrocele fluid or ascitic fluid withdrawn aseptically can be used instead of serum.

Blood Agar is a very useful medium for the gonococcus and Pfeiffer's bacillus, the bacilli of soft sore and whooping cough and other delicate organisms which grow with difficulty on ordinary media. Defibrinated blood obtained by cardiac puncture from a rabbit is warmed to 45°C. and is added to 3 per cent. agar at the same temperature in the proportion of 5 to 10 per cent. Sometimes even 20 per cent. of blood may be necessary. It is necessary to incubate to see if the medium is sterile.

Blood (Pfeiffer's Medium) and Serum-Smeared Agar.—In these the surface of an agar slope is smeared with blood or serum respectively. The blood must be obtained aseptically as the media cannot be sterilised when the smear has been made. Serum-smeared agar is sterilised on 3 successive occasions as usual.

Bordet and Gengou's Medium for the Bacillus of Whooping-Cough.—An extract of potato is first prepared by adding 100 grammes of sliced potato to 200 c.c. of 4 per cent. glycerin. This is steamed in an autoclave and the fluid is decanted. To 50 c.c. of this extract, 150 c.c. of physiological saline (0·85 per cent.) and 5 c.c. of agar are added. Of this medium 2-3 c.c. are placed in sterile test-tubes and sterilised. To each tube after sterilisation, an equal volume of sterile defibrinated rabbit's or better, human blood is added.

Blood-Alkali Agar (Dieudonne's Medium) is used to isolate the cholera vibrio from other intestinal bacteria. Blood-alkali solution is prepared by mixing equal parts of normal caustic soda solution (40 grammes per litre) and defibrinated blood. This is then sterilised in Koch's steamer for about 30 minutes. By the action of the alkali, ammoniacal substances are volatalised, so the steaming should be repeated till no ammoniacal smell is given off. Three parts of this mixture are now added to seven parts of 3 per cent. ordinary peptone agar rendered neutral to litmus. The medium is poured into plates which after drying should be kept at room temperature for 24 hours before being plated. Alternative methods for rendering

the plates ready for sowing are:—(*i*) Placing them in a bell jar containing Co_2 gas for one hour; (*ii*) Keeping the blood alkali mixture in a flask plugged with cotton instead of an air-tight cork for 6-8 weeks; (*iii*) Mixing the ox blood with 11·4 per cent. of anhydrous sodium carbonate in place of normal caustic soda solution.

Egg Media.—*Egg Medium* (*Dorset's*) is useful for the cultivation of the tubercle bacillus. Four fresh eggs are washed with soap and water and then dried. The contents of the eggs are well beaten up, mixed with 25 c.c. of distilled water and filtered through sterile muslin. The medium is now run into tubes and solidified in a sloped position in the serum inspissator at 75°-80°C. The tubes are then sterilized at 90°C. at the top of a steam steriliser for twenty minutes on three consecutive days. The medium may be covered with a few drops of 0·8 per cent. sodium chloride solution to prevent drying. A sufficient amount of basic fuchsin may be added to the medium to make it pale pink so that the growth may be identified earlier. Before inoculation, two drops of sterilised water are placed on the surface of the medium and the inoculating material is well rubbed over the surface. Growth develops after some weeks and so it is advisable to seal the tube by pushing down the cotton-wool plug below the top of he tube and then pouring in some melted paraffin wax.

Glycerin Egg Medium is used for the cultivation of the human type of tubercle bacillus. One part of saline glycerin (6 per cent. glycerin in 0·85 per cent. saline solution) is added to three parts of beaten eggs. The mixture is sterilised, as described, with Dorset's egg medium.

Petroff's Medium.—

Ingredients required:—

1. *Meat juice.*—This is prepared by mixing 500 grammes of finely ground beef or veal with 50 c.c. of 15 per cent. glycerin solution. After 24 hours standing in the ice chest, the meat is squeezed through a sterile meat press and the infusion collected in a sterile beaker.

2. *Eggs.*—Place the eggs in 70 per cent. alcohol for 10 minutes. Dry and break them into a sterile beaker. Beat and filter through sterile gauze. One part of meat juice is added to two parts of eggs by volume.

3. *Gentian-violet.*—One per cent. alcoholic solution of gentian-violet is added to make a final concentration of 1: 10,000.

The three ingredients are well mixed, tubed and inspissated in the form of slants at 85°C. and then heated for an hour on two consecutive days at 75°C.

Litmus Milk is used for observing the effect of bacterial growth on milk, *e.g.,* production of acid by fermentation of the lactose present in the milk and the coagulation of soluble albumin. Fresh milk is centrifugalised to separate the cream, then steamed in the Koch for fifteen minutes and set aside to cool, preferably in an ice chest over-night to allow further separation of the cream. The fat-free milk is pipetted off and placed in sterile tubes. A little litmus is added to colour it and the final sterilisation is done in a steamer for 20 minutes on three successive days. The reaction of milk is alkaline and if necessary, the proper reaction may be adjusted by titration.

Potato Slopes.—Potatoes are used as slices in tubes. A large sized potato is well washed and peeled. Cylindrical slices are cut off by means of a large cork borer and are then cut obliquely, leaving two wedges which are placed in Ehrlich's test-tubes. These are filled with sterile water, boiled in the Koch for 30 minutes after which the water is poured off and the tubes autoclaved at 115°C. for 20 minutes. To prevent the potato from drying up, a pledget of wet cotton-wool is placed under the broad end of the wedge at the bottom of the tube or the wedge may rest on a glass rod with a little water below or Roux or Ehrlich's bottles may be used, the bulb at the lower end of these bottles being filled with water.

Glycerin Potato is used for the cultivation of the tubercle bacillus. Potato slopes in tubes are covered with 6 per cent. glycerin solution in distilled water and steamed for 30 minutes at 100°C. The glycerin is then decanted and sterilisation further continued for half an hour. Potatoes ought not to be prepared long before use.

MacConkey's Neutral-Red Bile-Salt Peptone-Water (Rebipelaqua) is often used for the bacteriological examination of water and milk. It differentiates most of the intestinal bacteria and has been extensively used for the study of *B. coli, B. dysenteriæ,* etc. Other sugars can be substituted for the lactose to study various fermentative changes.

A stock solution of bile-salt and peptone is prepared by dissolving by heat 2 grammes of Witte's peptone and 0.5 gramme of commercial sodium taurocholate in 100 c.c. of tap water. The solution is steamed for two hours, filtered while hot, allowed to stand for 24 hours and then filtered. To this stock solution, 0.25 per cent. of freshly prepared 1 per cent. neutral

red solution is added to give it a distinct reddish-brown colour. If the medium is acid, the reddish brown colour appears only on adding some alkali. Finally one per cent. lactose (or any other of the sugars required) is added and the media distributed in fermentation tubes. Sterilisation is effected by steaming for 15 minutes on two successive days, avoiding over-heating.

MacConkey's Neutral-Red Bile-Salt Peptone Lactose-Agar (Rebipelagar) is the same as the above with the addition of 1·5 or 2 per cent. agar which is dissolved in the stock solution and cleared with white of egg. Neutral red and lactose are added as in the case of the liquid medium. Organisms which produce acid from lactose form rose-pink colonies whereas the colonies of non-lactose fermenters are yellowish.

Endo's Medium is made as follows:—To 1000 c.c. of ordinary agar (2-3 per cent.) heated in the autoclave at 115°C. for 30 minutes, 6 c.c. of 10 per cent., sodium carbonate are added to make it alkaline. The mixture, when cool, is cleared with the white of an egg, then boiled in the Koch for 2 hours and filtered through Chardin's paper while still hot. To the filtrate, 10 grammes of pure lactose, 0·5 c.c. of saturated alcoholic solution of basic fuchsin and 25 c.c. of 10 per cent. sodium sulphite (crystals) solution are added. The medium is sterilised in the Koch for twenty minutes on three successive days and is kept in the dark subsequently.

N. N. N. Medium (Novy, MacNeal, Nicolle) is used for the cultivation of trypanosomes and Leishmaniæ. Meat extract is prepared in the ordinary way by using 125 grammes of rabbit or ox flesh per 1000 c.c. of water. To this are added the following ingredients:—

Peptone	20 grammes.
Sodium chloride	5 grammes.
Agar fibre	20 grammes.
Normal sodium carbonate solution ..	10 c.c.

The medium is put in tubes and autoclaved. When cooled to 50°C. double the quantity of defibrinated rabbit's blood is added to each tube. The tubes are rotated between the palms so as to thoroughly mix their contents and solidified in sloped positions, preferably on ice. A large amount of water of condensation should be present as the growth of Leishmaniæ occurs mostly in the water of condensation.

Smith-Noguchi Medium is useful for the cultivation of spirochætes (q.v.)

Anærobic Methods for Cultivating Bacteria.

For those bacteria which grow only in the complete or partial absence of free oxygen special methods of cultivation are employed to produce the required anærobic conditions.

It is always preferable to add some reducing substance to the nutrient media to favour anærobic growth. Sodium formate (0·4 per cent.), sodium sulphindigotate (0·1 per cent.), methylene-blue, glucose (2 per cent.) are all suitable substances for this purpose, the last named also serving as a suitable medium for growth. Reducing substances like methylene-blue or sodium sulphindigotate, if added, may indicate the conditions of anærobiosis by change of colour. Additions of fresh sterile animal tissue favours the growth of anærobes and this principle is utilised in Noguchi's method for spirochætes. The various methods employed have certain limitations and so their choice depends mainly on the character of the organism and method of cultivation. The principles underlying the various methods are:—

(*a*) Removal of oxygen with or without replacement with an inert gas such as hydrogen.

(*b*) The addition of reducing substances such as glucose or formate of sodium to the medium.

(*c*) The addition of a catalase in the form of portions of fresh animal or vegetable tissues which without excluding air favours the growth of anærobes.

Method of Liborius.—Many anærobes grow in the depths of solid media, (glucose agar or gelatin). The medium is first boiled to remove dissolved oxygen, then cooled rapidly to 42°C., inoculated with a deep stab and finally solidified with the help of running water or ice. The anærobic conditions in the bottom of such a stab can further be safeguarded by sealing the opening of the stab by another layer of melted agar or gelatin. Similar conditions can be maintained in the case of fluid media by covering the fluid in the tubes with a layer of melted paraffin or olive oil or vaseline. In this method (Hamilton) inoculation or the withdrawal of culture is performed with a sterile glass pipette.

In *Roux's biological* method some strong ærobe such as *Bacillus prodigiosus* is allowed to grow on the surface. To obtain pure cultures a second or third tube may be inoculated as in the usual dilution method.

To prepare a subculture from a pure growth a ready method is to prepare a Petri dish of melted agar or gelatin, inoculate it at one point with the pure culture and cover the point of

inoculation with a cover-slip pressing it down to exclude air and taking care not to include any air bubble.

Vignal's Method consists of the preparation of fluid media or melted solid media and sucking it up in a glass tube of 3-4 mm. in diameter and 1 meter long which is drawn to capillary ends. After inoculation, both ends are sealed in a flame. In order to get at the colony the tube is filed and broken at the desired point.

Wright's Method.—A deep culture in glucose agar or gelatin is made after boiling the media to get rid of dissolved oxygen. A cotton plug is flamed and then pushed down to ¾-inch below the mouth of the tube which is filled with pyrogallic acid to ¼ of an inch. Then 2 or 3 c.c. of 10 per cent. caustic soda solution are added and a rubber stopper inserted rapidly. This method is very convenient and rapid.

Zinsser's Method.—This is a fairly satisfactory method for plate cultures. Two small crystallising dishes about 1″ in depth are sterilised. Inoculated glucose agar is poured into the smaller, and dry pyrogallic acid is placed in the centre of the larger dish. The smaller one is inverted over the larger and then 5 per cent. caustic soda solution is quickly poured in the space left between the dishes. When the pyrogallic acid is being dissolved, paraffin oil is poured quickly into the space to prevent the access of air.

Buchner's Method.—This method is very convenient and gives excellent results with tubes as well as plates. The principle is to put the inoculated tubes or plates in a large vessel containing alkaline pyrogallic acid. (One gramme for every 100 c.c. of space). Rubber-stoppered wide-mouthed bottles or large test-tubes can be used to accommodate the tube cultures and an ordinary desiccator used for plates. First dry pyrogallic acid is placed in the bottle or desiccator and then the inoculated tube or plate. Ten per cent. caustic soda solution is then added and the bottles or desiccator quickly closed and the whole incubated.

Bulloch's apparatus for anærobic culture consists of a bell-jar placed on a strong glass plate, the junction being made air-tight with vaseline or plasticine. The top of the bell-jar has two openings through each of which passes right-angled glass tubing fitted with stop-cocks. One of the tubes reaches to a dish placed on the glass plate forming the bottom of the jar while the other terminates immediately below the stopper. The culture plates are put on the stand and the tubes in a beaker. Two to four grammes of dry pyrogallic acid are placed in a dish at a point well away from the long tube reaching to the bottom of the jar. The small tube is connected with a hydrogen apparatus and thus all air is expelled as indicated by a blue flame produced

by the outgoing hydrogen. (The hydrogen is usually passed from a Kipp's apparatus after removing all traces of sulphuretted hydrogen, arsenic and oxygen by passing it through three wash-bottles each containing in turn lead acetate (10 per cent.) silver nitrate (10 per cent.) and alkaline pyrogallic acid. The tube leading from the last bottle is sterilised and a small plug of cotton-wool is placed in it to filter the incoming hydrogen). When a current of hydrogen has passed through the apparatus both stoppers are closed. The short tube is then connected with an air pump and a partial vacuum created. By repeated exhaustion and hydrogenation, complete anærobiosis is obtained. Finally a slight degree of vacuum is created and the longer tube is connected by means of a rubber tube to a beaker containing strong soda solution which is then sucked in by opening the stop cock. Some boiling water is also allowed to be sucked in to prevent erosion of the glass tubing, then the stop cock is closed and the whole apparatus incubated. *Novy's jar* can also be used similarly. It is preferable to put in a tube containing a control sample of glucose broth to which is added 0·1 c.c. of 1 per cent. of methylene-blue to indicate the anærobic condition by change of colour. (Methylene-blue remains colourless as long as anærobic conditions are maintained).

McIntosh and Fildes' Anærobic Jar is an excellent apparatus which consists of a strong metal or glass jar with an air-tight lid fastened with screws and nuts. In the middle of the lid there is a tap for passing in hydrogen and below it is suspended a copper gauze cover containing an insulated spool of palladium asbestos. The spool is surrounded by a piece of resistance wire which is connected with two electric terminals on the outside of the lid. The palladiumised asbestos spool is surrounded by a wire cage to prevent the explosion of the hydrogen and oxygen mixture. Petri dishes and culture tubes are placed in the jar with some methylene-blue solution as an indicator. The lid is clamped and hydrogen from a cylinder or a Kipp's apparatus (after freeing from all impurities) is passed into the jar. Current is turned on to heat the palladiumised asbestos which helps the combination of hydrogen with oxygen present in the jar. Water is formed till in about 20 minutes there is no oxygen left to combine with hydrogen. The hydrogen supply is then disconnected, the tap is closed and the jar incubated.

Blood Culture.

1. Collection of Blood.—Blood is aspirated by means of a sterilised 10 c.c. syringe from the median basilic vein. The skin over the bend of the elbow is washed with soap and water, dried and swabbed with antiseptic solution such as

alcohol followed by ether. One of the best antiseptics for this purpose is—brilliant green 0·5 gramme, crystal violet 0·5 gramme, 50 per cent. alcohol 100 c.c. This solution renders the skin quite sterile. The middle of the arm is bandaged by several turns of the tourniquet firmly enough to make the veins prominent, but not too tightly to compress the arteries. If the patient is in bed, the fully extended and partially abducted arm is supported on a pillow. Holding the syringe in the right hand, the skin is rendered tense with the left and the needle sharply pushed through the skin in the direction of the blood current and parallel to the blood vessel. The syringe is transferred to the left hand and the piston slowly withdrawn with the right until about 10 c.c. of blood have been obtained. If no blood enters the barrel on withdrawing the piston the syringe is raised slightly and the needle pushed a little deeper. In fat persons the vein can generally be felt as an elastic band if it cannot be seen. In nervous subjects, hot fomentation may make the veins turgid, failing which an inhalation of a capsule of amyl nitrite should be given. Having obtained the blood, it is added to the various media at once at the bed side.

2. Transmission.—If blood is to be sent by post or messenger to the laboratory, the procedure is as follows:—

1. Part of the blood is distributed into sterile tubes containing sterile ox-bile, and the remainder placed in a sterile tube containing ammonium oxalate solution (ammonium oxalate 2 grammes, sodium chloride 6 grammes, distilled water 1000 c.c.), or,

2. The blood is mixed with sterile melted gelatin in a proportion of 1 to 4, or,

3. One part of Liq. Trypsinæ Co. (Allen & Hanbury) is mixed with 4 of blood.

The tubes must be sealed hermetically or closed with a sterile rubber bag.

When only Wright's capsules are available some of the oxalate or trypsin solution is run in before drawing the blood into them and some more after the blood has been added.

3. Cultivation of Blood.—The aim underlying the numerous recent improvements in the technique of blood culture is to neutralise or diminish the factors such as the bactericidal power of the serum and plasma, the phagocytic power and coagulation of the blood, which militate against the successful making of blood cultures.

The choice of culture media is adapted to each individual case. In the case of the enteric bacilli:—

1. One c.c. of blood is added to 500 c.c. of sterile broth contained in a flask or 1 c.c. of blood is mixed with 5 c.c. of broth containing 0·25 c.c. of Liq. Trypsinæ Co. (Allen & Hanbury).

2. Five c.c. of blood are added to 10 c.c. of sterilised oxbile or to 50 c.c. of a sterile 0·5 per cent. solution of sodium taurocholate.

The culture is incubated for 24 hours at 37°C. and films made from the sown media and stained. A daily examination for at least a week is required before a diagnosis of no growth is returned.

If the film shows organisms, subcultures are made on agar slopes or on an agar plate by the successive stroke method. The organisms are identified by biochemical and serological tests.

In cases of suspected typhoid, direct plating from the blood culture on MacConkey's medium is necessary. Even when no organisms can be detected in films from the primary blood culture, subcultures should always be made.

Biochemical Reactions.

1. **Fermentation Tests** are of considerable value in the differentiation of organisms. The substances commonly employed are:—

Sugars: (*a*) *Monosaccharides*: include pentoses such as xylose, arabinose, etc., and hexoses such as glucose (syn. dextrose), fructose (syn. lævulose), galactose, etc.

(*b*) *Disaccharides*: lactose, sucrose, (syn. saccharose) and maltose.

(*c*) *Trisaccharide*: raffinose.

(*d*) *Polysaccharides*: dextrin, inulin and starch.

Alcohols: glycerol, dulcitol, mannitol, etc.

Glucosides: salicin, coniferin, etc.

Milk may become acid or alkaline with or without curdling as a result of bacterial growth.

Inosite and Organic Salts of certain acids such as formates and citrates are also used.

Durham's fermentation tube, which consists of a large test-tube containing an inverted small test-tube, is very convenient for the observation of gas-formation. These tubes are filled with suitable sterile media with aseptic precautions and plugged and heated in the Koch's steriliser for 15 minutes on each of the three successive days. The medium employed for this purpose consists of 1-2 per cent. peptone water (1-2 part of peptone, 0.5 part of NaCl and 100 parts of water) and ½-1 per cent. of the fermentable substance, to which a few drops of an indicator such as litmus, Andrade's reagent or phenol red are added.

Hiss's serum water medium consists of 1 part ox serum and 3 parts distilled water. The mixture is heated at 100° C. for a short time. To 100 parts of this mixture are added 1 part of the various carbohydrates and 1 per cent. litmus solution. The mixture is again heated at 100°C. for 30 minutes on three consecutive days. It is a suitable medium for studying the fermentative properties of diphtheria bacilli, streptococci and pneumococci. The medium is inoculated with the organism and incubated. Acid fermentation is indicated by the change in reaction and the resulting coagulation and gas production by bubbles in the coagulum.

Einhorn's saccharimeter consists of a bent tube, one limb of which is much longer than the other and is graduated and closed; the other is open and forms a bulb. The graduated limb is filled with the medium upto the bend. The medium is inoculated and incubated. Any gas developed collectes in the closed limb of the tube and its amount can be read off the scale.

Formation of Indol by Bacteria.—Several different methods have been suggested. The most delicate is the Ehrlich's test, which is carried out in the following manner: Two solutions are made:—

(1)	Paradimethylamidobenzaldehyde	4 grammes.
	Absolute alcohol (96 per cent.)	380 c.c.
	Concentrated hydrochloric acid	80 c.c.
(2)	Potassium persulphate	Saturated watery solution.

The organism under investigation is grown in 5 c.c. of peptone water or, better, casein digest broth for 48—72 hours at 37°C. One or two c.c. of ether are added to the culture which is shaken for a few seconds. When the ethereal layer has separated and risen to the top, it is either pipetted off into a dwarf test-tube containing a few drops of water and 1 c.c. of solution (1) and 1 c.c. of solution (2) or 1 c.c. each of No. (1) and No. (2) are added to the culture itself and if indol has been formed,

either at once or after standing for a variable time, a rose-red ring appears between the ether and the culture. In carrying out this it is necessary to make sure that the medium in which the organism is grown (*i*) contains free tryptophane or a tryptophane polypeptide, (*ii*) does not contain glucose, (*iii*) does not already contain indol.

Cotton-wool plug test.—It consists in moistening the under surface of the cotton-wool plug of the culture tube with the above solutions (1) and (2) and heating the tubes for a few minutes gently over a water-bath. If indol is present a rose-pink colour develops on the under surface of the plug.

Reduction of Nitrates to Nitrites.—The organism under investigation is grown for 48 hours at 37°C. in a medium containing 10 grammes of peptone, one litre of ammonia-free distilled water and 2 grammes of nitrite-free potassium nitrate, which is filtered, tubed and sterilised for half an hour on three consecutive days before use.

Two solutions are required:—

1.	Sulphanilic acid	0·5 gramme.
	Dilute acetic acid (S. G. 1·04) ..	150 c.c.
2.	*a*-naphthylamine	1 gramme.
	Water	22 c.c.
	Filter and add dilute acetic acid ..	180 c.c.

In carrying out the test, 2 c.c. each of (1) and (2) are added to 10 c.c. of the culture. The reduction of nitrates to nitrites is indicated by the appearance of a pink colour.

CHAPTER IV

SERA, SERUM TESTS, VACCINES AND COLLECTION OF MATERIAL FOR EXAMINATION

The Agglutination Test.—The agglutination test is of great practical utility and is employed for determining the nature of an infectious disease on the one hand and establishing the identity of an organism on the other. In the case of many pathogenic bacteria separate types antigenically distinct from one another have been demonstrated by means of this test.

Agglutination may be observed microscopically or macroscopically.

Macroscopic test.—(*a*) *Dreyer's Method.* The directions given below are taken from the circular sent with the standard cultures from the Department of Pathology, Oxford.

1. *Apparatus and reagents*:—

Pipettes.—Use special dropping pipettes, but if none are available the ordinary graduated 1 c.c. pipettes or those made out of glass tubing may be used.

Note.—*The same pipette must be used throughout the operation.*

Agglutination tubes (Dreyer's) should be of uniform size about 5 cm. in length with internal diameter of 5-6 mm. They should be clean and devoid of any scratches. They may also easily be made out of glass tubing.

A stand to hold about 15 agglutination tubes (or a plasticine bed may be used for this purpose).

2. *The diluting fluid* is normal saline (0·85 per cent. solution of sodium chloride in distilled water). In the case of spontaneous agglutinating cultures lower concentrations of salt such as 0·42 per cent. or 0·21 per cent. are used.

3. *Bacterial emulsion* is in the form of a young broth culture or of a saline emulsion of a young agar culture. The emulsion

should be diluted to Brown's opacity tube No. 2 or it may be standardised to contain 250-300 millions of bacteria per c.c. Emulsions of various organisms should be prepared in bulk and stored in the ice-chest after the addition of commercial formalin (40 per cent. formaldehyde), in a strength of 0·1 per cent. It may be noted that cultures of dysentery bacilli prepared in this way at first undergo a considerable reduction in agglutinability but after two or three months they become constant and should then be used. Standard agglutinating cultures may be obtained from Oxford.

Dilution of the serum.—With the proper dropping pipette held vertically, measures out into the dilution tube 36 or 54 drops of normal saline by means of gentle pressure on the teat. The pipette is washed with distilled water and rinsed with successive quantities of absolute alcohol and of ether and dried by gentle warming.

Measure out 4 or 6 drops of the serum to be tested into the dilution tube already containing 36 or 54 drops of saline. Mix the fluid in the tube well to make a dilution of 1 in 10.

The Test.—Properly arrange a series of tubes in the stand. Wash out the pipette and measure out the following into the tubes:—

Tube in the first row			3	4	5
Drops of normal saline	0	5	8	9	10
Diluted serum (1 in 10)	10		2	1	0
Standard culture	15	15	15	15	15
Ultimate dilution of serum in each tube	1 in 25	1 in 50	1 in 125	1 in 250	(Control without serum)

If a mark is made corresponding to the volume of 15 drops the bacterial emulsion may be added without counting the drops.

A similar procedure is followed in rows Nos. 2, 3, 4 and 5, using a different culture each time, *e.g.*, in the second row an emulsion of *B. paratyphosus A* may be added if in the first *B. typhosus* emulsion has been added.

Shake the tubes thoroughly from each row beginning with the highest dilution. If the limit of agglutination is not reached within this series higher dilutions are made in a similar manner. For example, 3 drops of a 1 in 10 serum if added to 57 drops of normal saline will give a serum dilution of 1 in 200 and using the same quantities as before the ultimate serum dilutions will

be, 1 in 500, 1 in 1000, 1 in 2500, 1 in 5000. Similarly, still higher dilutions can be made.

Temperature and time of incubation.—Place the stand in a water-bath at 50-55°C. taking care that the level of water is only half way up the column of the fluid in the tubes. Two hours in the bath are required for *B. typhosus, B. paratyphosus A* and *B, B. enteritidis gartner, B. coli* and *Vibrio cholera.* Dysentery bacilli and meningococci require 4-6 hours. Take the readings after incubation and leave the stand at room temperature for 24 hours for final readings.

Method of reading agglutination results.—Readings are taken by comparing each tube in succession with the control tube. Complete sedimentation with a perfectly clear fluid above is recorded as +++; incomplete sedimentation with a partial clearing of the fluid as++. Distinct agglutination visible to the naked eye but without sedimentation by + and the highest dilution in which this is seen is *Standard agglutination.*

Dreyer employs the drop method for all measurements, and uses thin formalized broth cultures which are standardised, a water-bath at 55°C. and a defined degree of agglutination. Detailed instructions regarding Dreyer's technique are sent out with the emulsions obtained from the Department of Pathology, Oxford University. An excellent account of agglutination is given in the Medical Research Council Report No. 52.

Stands, dropping pipettes, agglutination tubes, etc., as used in Dreyer's method can be obtained from Messrs. Baird & Tatlock, Hatton Garden, London, E. C.

(*b*) *Garrow's Agglutinometer* is an exceedingly handy and useful apparatus. It consists of a glass slide, divided by grooves at regular intervals, which is rotated on its long axis by a mechanical contrivance. Dilutions of serum are made on the slides and mixed with a bacterial culture, the clockwork is set in motion and when it stops the slides are examined for any evidence of clumping.

(*c*) *Agglutination in Capillary Tubes.*—Agglutination can also be observed in glass capillary tubes. A mixture of the bacterial emulsion and serum of known dilution is sucked up in a pipette and the capillary end is sealed. After 18 hours' incubation the tubes are examined for any sedimentation.

Microscopic test.—Equal quantities of the diluted serum and a young culture are mixed on cover-slips which are mounted as hanging-drops. Starting with the dilutions of the serum

1 in 15, 1 in 25 and 1 in 60 the ultimate dilutions of the serum in the specimens will be double the original dilution, *i.e.*, 1 in 30, 1 in 50 and 1 in 120. The specimens are examined under high power ($\frac{1}{6}$-in.).

The Technique of the Absorption test.—1. Dilute the serum to be absorbed to 1 in 25. Complete removal of the agglutinins for the absorbing bacterial emulsion is to be aimed at.

2. Make a uniform thick emulsion of the culture. Add the diluted serum (1 in 25) and the emulsion in equal quantities so that the ultimate dilution will be 1 in 50.

3. Mix thoroughly the contents of the tubes and leave them at room temperature for 2 hours and then at 37°C. for an hour.

4. Centrifuge the tubes at once or after they have been left standing overnight at room temperature.

5. Pipette off the clear supernatant fluid and test its agglutinating power on the organism with which the serum was prepared. If the tested organism is homologous with the organism with which the serum was prepared, the supernatant fluid (absorbed or saturated serum) will no longer agglutinate it or only to a slight extent.

Discussion of Results.—

A.—*Identification of an organism.*—The organism is put up against dilutions corresponding to $\frac{1}{8}$, $\frac{1}{4}$, $\frac{1}{2}$ and full and even double titre of a known serum.

(*a*) If the tested organism is agglutinated to the full titre it is obviously homologous with the organism which was employed in producing the serum.

(*b*) If the tested organism is agglutinated to $\frac{1}{4}$ or $\frac{1}{2}$ titre only it is possible that it is identical with the organism with which the serum was prepared.

(*c*) If the tested organism shows no reaction with serum even after several subcultures it is certainly not homologous with the organism which produced the serum.

B.—*Specific nature of the Disease.*—The following table gives the dilution in which the serum in different diseases should react with the infecting organism before a diagnosis of a particular disease can be made. Variations due to previous inoculation against a disease are mentioned under the organism concerned.

B. *typhosus*	.. .. ..	1 in 25
B. *paratyphosus* A	.. ..	1 in 20
B. *paratyphosus* B	.. ..	1 in 200
B. *dysenteriæ* (Shiga)	.. ..	1 in 50
B. *dysenteriæ* (Flexner)	.. ..	1 in 100
B. *melitensis*	.. ..	1 in 500 or over.

The test as applied in the diagnosis of typhoid infection is known as *Widal's reaction.*

The Preparation of Agglutinating Sera

Inoculation.—Organisms can be identified by their agglutination reactions with specific agglutinating sera of a high titre, that is, sera which give the agglutination reaction in very high dilution, for example 1 in 500 or more. Rabbits are the most suitable animals from which to obtain agglutinating sera. A young healthy rabbit is selected. The animal is held in a towel to prevent sudden movement, the hair from the margin of one of the ears is clipped and the ear rubbed until the veins become prominent. The area is disinfected with alcohol. The ear is held by the first three fingers of the left hand below and the thumb above and the needle of the syringe containing the bacterial culture is plunged into the vein. The needle should be parallel to the vessel and point towards the heart. The bacterial culture for the inoculation is made as follows:—

To a young broth culture about 24 hours' old is added 0·1 per cent. formalin and the mixture is placed in the ice chamber for 4-5 days. Standardisation, etc., is carried out as usual. The initial dose is from 200-500 millions of bacteria. On the 5th or 6th day after the first injection another similar dose or stronger dose is given intravenously as before. About the tenth or eleventh day a small quantity of blood is removed from the marginal vein and the serum tested for agglutination against the organism which was employed for the inoculation. Such a serum will generally be found to be of an adequate titre, *i.e.*, 1: 6000; *i.e.*, one part of the animal serum diluted with 5999 parts of saline will agglutinate the organism in a standard manner. In case the titre is low (in dysentery 1: 2000 is satisfactory; in typhoid 1: 8000, etc.) a third or even a fourth injection of larger doses, or even of living bacteria, may be given, but for ordinary purposes a titre of even 1 in 2000 is accepted as sufficient. To ascertain the progress of immunization samples of blood are removed at intervals of 5 to 6 days from the marginal vein of the ear under aseptic conditions.

When, on examination, the serum is found to be of a high titre the animal is bled.

Bleeding.—1. The animal is anaesthetised with ether and after shaving, etc., the carotid artery is dissected out. Two clips are put on the artery and a cannula is introduced between them, pointing towards the heart. The cannula is secured in the artery by means of a thread tied round it. The clip below the knot is now released and the blood allowed to flow into a sterile flask. The flow of blood is interrupted every 15 to 20 seconds for 2-3 minutes. This increases the yield of the serum.

2. An alternative procedure and one which does not involve the killing of the animal is to wrap it in a large cloth to prevent movement and shave and disinfect the marginal vein area of the ear with ether or alcohol. The prepared area is smeared with sterile vaseline to prevent coagulation of the blood. The vein is pricked with a hollow large-sized needle and the blood allowed to flow into a sterile test-tube. When the flow of blood ceases the ear is scratched gently or if this fails a drop of xylol is put on the tip of the ear, away from the prepared area, as xylol is hæmolytic. In this way, about 30-40 c.c. of blood can be collected. The process can be repeated the following day. The animal should be given plenty of water to drink after bleeding.

Separation of the Clot is facilitated by collecting the blood in a sterile test-tube which has been rinsed out with saline. The tube is placed in the incubator for an hour at 37°C. to hasten separation and then kept at room temperature overnight. The blood may also be collected in a test-tube or flask, the inside of which is coated with a thin continuous layer of agar (1·5 per cent. agar in 0·85 per cent. NaCl solution).

Preservation of the Serum.—The serum is pipetted off into a sterile vessel and preserved with 0·3 per cent. phenol. To obtain a final concentration of 0·3 per cent. phenol in the serum requires the addition of 0·64 c.c. of 5 per cent. phenol to each 10 c.c. of serum. The serum is kept in sealed ampoules or glass-stoppered bottles in the dark in a cool room.

Determination of the Opsonic Index

I.—Preliminary.—(*a*) *Preparation of the Sera.*—Clear serum is obtained from the patient's blood and from healthy persons. In the case of the coli-typhoid group of organisms which are liable to undergo lysis, both the sera (*i.e.*, from the patient and a normal person) are heated at 55°C. for ½ hour to destroy the complement.

(*b*) *Preparation of Bacterial Emulsion.*—The bacterial emulsion must be perfectly uniform and free from clumps. In the case of staphylococci and streptococci some of the 18-24 hours' living culture is emulsified in normal saline and centrifuged to remove the clumps, if necessary. The emulsion should exhibit only a cloudiness to the naked eye and as a general rule a suspension containing 500,000,000 bacteria per c.c. is quite suitable. In the case of such pathogenic organisms as *B. pestis* and *B. tuberculosis* the culture is killed by mixing it with 40 per cent. formalin and centrifuging. The supernatant formalin is pipetted off, the rest washed in normal saline, centrifuged and the salt solution pipetted off. Sufficient saline is then added to make a satisfactory suspension of the organisms.

(*c*) *Preparation of the Washed Leucocytes.*—A centrifuge tube is filled with a 1·5 per cent. solution of sodium citrate in 0·85 per cent. saline. About 1 c.c. of blood obtained from a finger-prick is added drop by drop, inverting the tube frequently to prevent coagulation. After centrifuging, the supernatant fluid is drawn off, fresh saline added, mixed and centrifuged and the supernatant fluid again poured off. The greyish surface layer of the blood deposit is pipetted off by means of a fine pipette. This layer is rich in leucocytes.

II.—The Test Proper.—A mark is made with a wax pencil at a point about an inch from the end of the capillary thread of a pipette. This forms the unit volume. The pipette is fitted with a teat and corpuscles sucked in up to the mark, then an air bubble allowed to enter the capillary, an equal amount of the bacterial suspension drawn in, then an air bubble and finally one volume of the patient's serum. The contents of the capillary tube are expelled on to the surface of a glass slide and mixed. The mixture is sucked up in the capillary pipette and the tip sealed. A similar preparation is now made with the normal serum instead of the patient's serum. The mixtures are incubated at 37°C. for 15 minutes.

Each pipette is now dealt with as follows:—

The tip is marked with a file and broken off and the mixture blown on to a clean slide, smeared out into a film, dried in air and stained with Leishman. If it is desired to stain with carbol-methylene or thionin-blue the slide is fixed with a mixture of alcohol and ether. For tubercle bacilli the film is fixed in a saturated solution of mercuric chloride (1-2 minutes), stained with carbol-fuchsin in the usual manner, decolorised with 2-5 per cent. sulphuric acid and cleared with 4 per cent. acetic acid, the counterstain being a watery solution of methylene-blue.

Both the preparations, *i.e.*, patient's and normal serum, are examined under the microscope, and the number of bacteria in the protoplasm of about 100 leucocytes counted. The *opsonic index* is given by the ratio between the number of organisms contained in the leucocytes of control and those in the sample of the patient's serum. If, for example, 200 bacteria are ingested by 100 leucocytes in the presence of a patient's serum, while in the control serum there are 300 bacteria in 100 leucocytes, the index would be $\frac{200}{300} = 0{\cdot}66$.

Hæmolytic Test

The test is employed for the determination of the hæmolytic action of a blood serum or bacterial products, (*e.g.*, in cholera), and forms part of the Wassermann test. The test can be applied in two ways:—

1. Melt agar tubes in a water-bath and cool to 45°C. Add defibrinated rabbit's blood to the cooled agar. To mix the blood with the medium hold the tube vertically upright between the opposed palms and rotate it between them. Pour the contents of the tube into a sterile Petri dish. When the medium has solidified, plant the organism under investigation on it so as to obtain isolated colonies. The colonies of the hæmolytic organism will be found to be surrounded by a clear, well defined halo after 24 hours of incubation. As a substitute for blood agar, human or rabbit's blood (sterile) may be used for smearing agar plates.

2. A more reliable method is as follows:—Centrifuge the culture of the organism under investigation (grown in broth for three days at 37°C.) at high speed for a long period or place in the ice-chest for some days. Pipette off, the clear supernatant fluid to be used in the test. Arrange a series of small test-tubes ($3'' \times \frac{3}{8}''$); add 1 c.c. of the undiluted clear culture fluid to the first tube; 1 c.c. of a 1 in 2 dilution to the second tube; 1 c.c. of a 1 in 10 dilution to the third tube; 1 c.c. of a 1 in 20 dilution to the fourth tube; 1 c.c. of a 1 in 100 dilution to the fifth tube. The sixth and seventh tubes serve as controls:—in No. 6 place 1 c.c. of saline and in No. 7 place 1 c.c. broth similar to that in which the organism was cultivated. To each of these tubes add 1 c.c. of a 5 per cent. emulsion of red blood cells and agitate the contents by bubbling air through the fluid by means of a capillary pipette provided with a rubber teat working from the higher dilution up to the higher concentration. Incubate the tubes at 37°C. for four hours. Keep them in the ice-chest overnight and examine for hæmolysis (the solution of the red-blood cells) in

the following morning. In a negative reaction red cells form a buff-coloured deposit.

The Wassermann Reaction

The following technique for carrying out the Wassermann reaction is based on "Method No. 4" as described in the Medical Research Committee's Special Report No. 14. Details of three other standard methods are also given in this report.

I.—Apparatus required:—

Stoppered glass amber-coloured bottles ..	250 c.c. 100 c.c. 50 c.c.
Glass graduated cylinders	25 c.c. 50 c.c. 250 c.c. 1000 c.c.
Erlenmeyer flasks	1000 c.c. 500 c.c. 250 c.c.
Glass pipettes, graduated to tip	10 c.c. in 0·1 c.c. 5 c.c. in 0·05 c.c. 1 c.c. in 0·01 c.c.
Wright's teat pipettes	0·5 c.c. 0·25 c.c. 0·1 c.c.

(These should be standardised to deliver the exact quantity of distilled water at the normal rate of working, as weighed on a chemical balance).

Test-tubes	12″ x 1″ 6″ x ¾″ 3″ x ½″

Rubber teats.

Electrically or water-driven centrifuge with four buckets.

Refrigerator.

Dry air steriliser.

Koch's steam steriliser.

Autoclave.

Water-bath, thermo-regulated to 37°C. and fitted with removable trays, the front row of holes of each tray being numbered in series, thus:—first tray—1—12; second tray, 13—24; and so on. Each tray to take 48 tubes in four rows of 12 each.

Water-bath, thermo-regulated to 56°C., similar to above, but to hold two trays, the holes in both trays being numbered in series, thus:—No. 1 tray, 1—48; number 2 tray, 49—96.

The glass-ware used in the test should be perfectly clean. After use, empty the tubes and pipettes in tap-water and allow them to remain in water for several hours. Brush the tubes in running water and place them in a solution of washing soda (sodium carbonate) and brush again. Rinse the pipettes in water and then in washing soda solution. Wash the glass-ware with a weak solution of hydrochloric acid and rinse in tap water until free from acid. Place the glass-ware in distilled water for some hours. Drain the tubes by inverting them in wire baskets and dry in the hot-air steriliser for 30 minutes at 150°C.

II.—Chemicals required:—

1. Sodium chloride 0·85 per cent. solution in distilled water.
2. Sodium carbonate.
3. Hydrochloric acid.
4. Absolute alcohol.

III.—Special Reagents required:—

Complement.—Select well-fed guinea-pigs which should not be pregnant. On the day of performing the test, obtain the blood by cutting the animal's throat over a glass funnel, or by aspirating from the heart or from the ear if only a small quantity is required. The animal must not be bled until several hours after feeding, to avoid chylous serum. Mix the samples of blood obtained from bleeding 3 or 4 guinea-pigs thus, as such a pooled serum is not likely to be anti-complementary. Place the blood in the incubator or water-bath at 37°C. Remove the serum and centrifugalise it for 5 minutes or until free from cells. Pipette off the serum into clean tubes, cork and place in the cold room until required. A dry complement which will keep in good condition for several weeks in unopened ampoules may be obtained from Messrs. Gans, of Oberwesel. For use, 1 gramme of this is dissolved in 9 c.c. of distilled water.

"Antigen" or Extract.—Obtain a fresh human or ox heart, cut away the fat and mince the muscular portion. To every 1 gramme of the mince add 9 c.c. of absolute alcohol in a mortar with clean sand and grind. Transfer the whole to a well stoppered bottle, shake the contents at frequent intervals during the next few hours, then allow to stand overnight. Wash a filter-paper with ether and allow it to dry. The extract is filtered through this filter-paper into an amber-coloured glass bottle. The filtrate is allowed to stand for a week by which time a deposit forms. Only the clear extract is used in the test. As a rule it keeps good in the cold for six months.

Solution of Cholesterol.—Place one gramme of pure cholesterol such as Kalbaum's in a well fitting glass-stoppered bottle and add 100 c.c. of absolute alcohol. Stopper tightly. Shake and warm in the water-bath at 37°C. until the cholesterol is dissolved. The bottles containing the extract and solution of cholesterol should be kept in a dark cupboard when the extract will retain its vitality for about six months. Immediately before use, mix two parts of cholesterol solution with three parts of the extract. Then make a 1 in 15 dilution of the heart-extract cholesterol mixture by adding saline to the necessary amount of the mixture contained in a test-tube.

Suspension of Washed Sheep's Cells.—Sterilise a glass-stoppered litre bottle containing a number of large glass beads or short pieces of glass tubing in an autoclave or a Koch's steamer. Obtain blood from the jugular vein of a sheep or from a slaughter-house, in the latter case collecting the blood when the sheep has bled for some time. When half a bottleful of blood is obtained, stopper and shake vigorously for at least five minutes, then keep in the refrigerator until required for the test for immunising purposes. The blood will keep for 3-4 days. As an alternative the blood may be received into a bottle containing an equal quantity of 2 per cent. solution of citrate of soda in 0·85 per cent. salt solution. However obtained, when required, place 5 c.c. of the blood in each of two 15 c.c. centrifuge tubes and fill these up to the top with sterile 0·85 per cent. salt solution and centrifuge until corpuscles are deposited. Pipette off the clear supernatant fluid, shake up with saline and centrifuge. Repeat this process four times, using a clean pipette each time. After the last washing remove the supernatant salt solution without disturbing the corpuscles and prepare a 6 per cent. solution of the deposit in normal saline.

Anti-sheep Cell Amboceptor.—This may be obtained from a central laboratory or prepared in the following manner:—Make six intravenous injections, each of 2 c.c. of a 5 per cent. suspension of washed red cells, into a rabbit at weekly intervals. Immunize several rabbits at the same time as some may die and others may yield only a weak serum. Ten days after the last injection, bleed the animal. Defibrinate and centrifuge the blood, or allow the blood to stand for 24 hours before removing the serum. As soon as the blood clots, remove the clot from the sides of the flask containing blood. Bottle the clear serum into 1 c.c. vaccine ampoules and heat it for half an hour at 55°C. so as to destroy the complement. Kept in the ice-chest the potency will be retained for many months.

Patient's Serum.—Collection.—Collect at least 1 c.c. of the blood by pricking the thumb or puncturing a vein in the lobe of

the ear in a Wright's capsule or a small tube. Label the specimen carefully.

When received in the laboratory enter the particulars concerning the specimen in the laboratory register together with the day's serial number, thus:—

July 1, 1929.

1. Syphilitic control serum.
2. Patient's blood—Name—
3. Patient's blood—Name—

so on.....

The last number in the day's tests represents a known normal control. The tubes containing the specimens are also numbered serially.

Preparation of the Serum.—Arrange test-tubes, 3 in. by ½ in., in a rack and number these 1 to 12 depending upon the number of samples to be examined on that day. Allow the blood to clot. Remove the clear serum, centrifuging if necessary. Place about 0·5 c.c. of the clear serum in the test-tube bearing the number corresponding to that of the patient's name in the register. Place the rack in a water-bath at 55°C. for half an hour. According to Harrison ten minutes in a water-bath at 55°C. is sufficiently long to inactivate 0·5 c.c. of the patient's serum.

IV.—Standardisation of Reagents—

Anti-sheep Cell Amboceptor or Hæmolytic Immune Serum.—The object of carrying out this titration is to ascertain the minimum amount of the amboceptor which is sufficient to produce complete hæmolysis of the standard amount of the sheep corpuscles used in the test with the aid of an excess of complement.

Method:—

Dilute the amboceptor serum as follows:—

1. Arrange a number of large test-tubes in a rack and number 1-11.

2. With a Wright's capillary pipette, to tube No. 1, add 0·1 c.c. of the hæmolytic serum and 9·9 c.c. saline. Mix. Dilution=1 in 100. To tube No. 2 add 0·5 c.c. of the diluted serum in tube No. 1 and 4·5 c.c. of saline; dilution=1 in 1000.

To tube No. 3 add 0·25 c.c. of 1 in 1000 (tube No. 2) and 0·25 c.c. saline=1 in 2000;

To tube No. 4 add 0·25 c.c. of 1 in 1000 (tube No. 2) and 0·5 c.c. saline=1 in 3000;

To tube No. 5 add 0·25 c.c. of 1 in 1000 (tube No. 2) and 0·75 c.c. saline=1 in 4000;

To tube No. 6 add 0·25 c.c. of 1 in 1000 (tube No. 2) and 1·0 c.c. saline=1 in 5000;

and so on to tube No. 11 add 0·25 c.c. of 1 in 1000 (tube No. 2) and 2·25 c.c. saline=1 in 10,000.

3. Set up a number of small test-tubes, 3″×½″, in a test-tube rack. Add 0·25 c.c. of each of these dilutions in small test-tubes. Pipette into each tube 0·5 c.c. of a 1 in 4 dilution of 6 per cent. red cell suspension prepared in the manner described above. To each tube also add 0·25 c.c. of a 1 in 10 dilution of the guinea-pig serum. Each tube then contains 0·25 c.c. of 3 per cent. suspension of red cells. Shake thoroughly and incubate in a water-bath at 37°C. Take the readings after an hour and note the tube containing the highest dilution of amboceptor in which there is complete hæmolysis. This dilution of the amboceptor serum represents the minimum hæmolytic dose (M.H.D.) of the amboceptor under investigation. The hæmolytic serum must be titrated at once after preparation or as soon as received in the laboratory and at intervals of about 3 months afterwards. No amboceptor of the M.H.D. lower than 1 in 1000 should be used.

Preparation of Sensitised Sheep Cells.—Assuming that 30 c.c. of sensitised cells are required for the day's test and that the M.H.D. of the amboceptor serum is 1 in 5000, add 15 c.c. of a 1 in 500 dilution of the amboceptor (a dilution which is ten times as strong as that representing the M.H.D.) quickly to an equal volume of a 6 per cent. suspension of sheep cells, thus making 30 c.c. of a 3 per cent. suspension of cells in a 1 in 1000 dilution of amboceptor.

Complement Titration without antigen and *in the presence of Antigen* is carried out each day before the reactions are done. Measure 1 c.c. of the guinea-pig's serum into a test-tube and add 4 c.c. of saline, making thus a dilution of 1 in 5.

From this, make successive dilutions as follows:—

0·25 c.c. of a 1 in 5 dilution and 0·75 c.c. saline=1 in 20.

0·25 c.c. of a 1 in 5 dilution and 1·00 c.c. saline=1 in 25.

0·25 c.c. of a 1 in 5 dilution and 1·25 c.c. saline=1 in 30.

0·25 c.c. of a 1 in 5 dilution and 1·50 c.c. saline=1 in 35.

0·25 c.c. of a 1 in 5 dilution and 1·75 c.c. saline=1 in 40.

0·25 c.c. of a 1 in 5 dilution and 2·25 c.c. saline=1 in 50.

0·25 c.c. of a 1 in 5 dilution and 2·75 c.c. saline=1 in 60.

0·25 c.c. of a 1 in 5 dilution and 3·75 c.c. saline=1 in 80

Three rows of eight tubes are set out in a rack and are marked A, B and C respectively. Add 0·25 c.c. of the 1 in 80 dilution of complement into the first tube of each row, *i.e.*, A, B, C. Place the same quantity of 1 in 60 dilution into the second tube of each row and so on to the 1 in 20 dilution. Place 0·5 c.c. saline into each tube of row A, and 0·25 c.c. of saline into each tube of row B. Pipette into each tube of row B 0·25 c.c. of 1 in 15 dilution of heart-extract cholesterol mixture. Shake the contents of the tubes. Allow them to stand at room temperature for half an hour and at 37°C. for another half hour. After incubation, place in each tube 0·25 c.c. of the 3 per cent. suspension of sensitised cells prepared as described above. The total volume of fluid in each tube is 1 c.c. as in the test proper. Shake immediately and at every 10 minutes interval. Read the results after half an hour and note the tube in row A containing the highest dilution of the complement in which there is complete hæmolysis. This dilution represents the minimum hæmolytic dose of the complement. The antigen control (row B) serves to eliminate an anti-complementary guinea-pig serum. The tube in row B (complement and antigen) containing complement twice as strong as the last tube of row A showing hæmolysis is usually completely laked. For example, the tube containing a dilution of 1 in 60 in row A shows complete hæmolysis, the tube containing 1 in 30 of the complement in row B also shows hæmolysis. If not, the particular sample of complement should be rejected and a fresh sample is obtained.

Row C is intended for incubation with the test proper and indicates any deterioration of the complement during standing. In the test proper 3 M.H.D. are employed. If the titre of the complement is less than 1 in 30, it should be rejected.

V.—Daily Routine.—

1. Wash or complete the washing of red corpuscles.

2. Kill or bleed guinea-pigs to obtain complement.

3. Make fresh saline solution.

4. Standardise complement alone and in the presence of antigen.

5. When the standardisation of complement is proceeding or at any convenient time on the day of the test, inactivate the patients' sera.

VI.—The Test Proper.—Lay out the inactivated sera in the back row of each tray which has 4 rows of twelve holes. The front holes of each tray are numbered in series, thus 1 to 12, second tray 13 to 24 and so on. For example, the control syphilitic serum is contained in the test-tube of the hole of the back row opposite No. 1, first patient's serum in the hole opposite No. 2 and so on to the last number—the control normal serum. Place empty test-tubes (3" by ½") in the remaining holes so that three tubes stand in front of each specimen of inactivated serum.

Remove 0·25 c.c. of No. 1 serum and add 1 c.c. of saline to it in a separate test-tube (not in the rack). Mix. Pipette off 0·25 c.c. of the dilution so obtained into each of the three tubes standing in front of No. 1 serum. Repeat this procedure with No. 2 serum and so on, using a fresh pipette each time.

Make two dilutions of complement—one three times and the other five times as strong as that representing the minimum hæmolytic dose of the complement on the day in question. For example, if the M.H.D. is 1 in 60, make a dilution of (60 divided by 3) 1 in 20 and (60 divided by 5) 1 in 12.

Measure 0·25 c.c. of the diluted complement (in this example 1 in 20) into tubes of the first and third rows, using the same pipette each time. Similarly add 0·25 c.c. of 1 in 12 dilution (5 M. H. D.) of the complement to each tube of the second row.

Place 0·25 c.c. of the 1 in 15 dilution of antigen prepared in the manner described above into each tube of the first and second row. Add 0·25 c.c. of normal saline to each tube of the third row.

Also add 0·5 c.c. of saline to each tube of the fourth row of the complement titration, which was reserved for detecting any deterioration of the complement which may have occurred (*q.v.*)

Shake the tubes and allow them to stand at room temperature for half an hour. Then place the rack in water-bath, incubate at 37°C. for half an hour. After incubation, add 0·25 c.c. of sensitised cells (*q.v.*) to each tube of all the four rows. Shake at once and again at an interval of 15 minutes.

Ten minutes after hæmolysis is complete in the last tube of the first row (normal serum control) marked X in the table transfer the tubes to a water-bath containing ice. Take the

final readings after an hour or so but an experienced observer will find it easy to read results ten minutes after there is complete hæmolysis in the serum control tubes (last set of three tubes).

VII.—Reading and Recording the Results.—(*a*) Failure of the normal serum test [last tube (X) of the first row] to show complete hæmolysis or the presence of the hæmolysis in the first tube of the first row entails rejection of the whole set of tests.

(*b*) If any third row tube has lagged definitely behind the others in this row in showing complete hæmolysis, the test of the serum of which that tube is the control should be rejected and repeated with another specimen, unless the corresponding tube in the first row shows hæmolysis indicating that the serum is giving a negative reaction. Various methods have been recommended for recording the various grades of reaction. The following notation is recommended by the Medical Research Committee (*vide* Report No. 14).

(*i*) Complete inhibition of hæmolysis in the first and second row iubes, *i.e.*, no tinge of hæmoglobin in the fluid around or above the cells = + +

(*ii*) Complete inhibition of hæmolysis in the first row tube, some opacity in the second row tube, but also some hæmolysis = + ±

(*iii*) Complete inhibition of hæmolysis in the first row tube, but complete hæmolysis in the second row tube = +

(*iv*) Slight hæmolysis in the first row tube = ±

(*v*) Considerable hæmolysis in the first row tube = $\overline{\pm}$

(*vi*) Complete hæmolysis in the first row tube = —

VIII.—Interpretation of Results.—1. A reaction weaker than + in a case which has not been previously diagnosed as one of syphilis is of no diagnostic value.

2. If in such cases the reaction is ± or $\overline{\pm}$ it should be repeated, preferably after a provocative injection of one of the arsenical preparations.

3. In a previously diagnosed case of syphilis a reaction of the grade of ± or $\overline{\pm}$ is an indication that further treatment is required.

Preparation of Bacterial Vaccines

Definition.—Bacterial vaccines are "sterilised and enumerated suspensions of bacteria which furnish, when they are dissolved in the body, substances which stimulate the healthy tissues to a production of specific bacterio-tropic substances which fasten upon and directly contribute to the destruction of the corresponding bacteria" (*Wright*).

1. The Collection and Examination of Infected Material.—This is described in the various chapters dealing with bacteria. Here it is sufficient to emphasise that every precaution should be taken to avoid contamination and to obtain material that is representative of the focal secretions.

2. The Isolation of Pure Cultures has also been described. Pure cultures of the organisms are obtained by plating on suitable media and an attempt should be made to select one or more of the organisms that are considered to be the cause of the disease. The selected colonies are picked off, subcultured on agar or other suitable media and incubated at 37°C. for 24 to 48 hours.

3. Preparation of the Emulsion.—With strict aseptic precautions a few drops of sterile normal saline solution are added to the tube or tubes showing growth and the tube shaken. Emulsification is greatly facilitated by gently scraping the growth off the medium by means of a sterile platinum loop, care being taken not to cut into the medium. If the emulsion is not satisfactory it may be filtered through a piece of sterile fine linen placed in a sterile glass funnel. This arrests not only the clumps but any extraneous matter such as fragments of the medium that may be present. The bacterial suspension should be shaken up in a stout flask containing glass beads or in a mechanical shaker so that the masses of the organisms will be broken up and an even distribution of the bacteria assured.

4. Standardisation of the Bacterial Emulsion.—Standardisation is best done before sterilisation.

(*a*) *Wright's method.*—The number of red corpuscles in the blood of a human donor is estimated. A mark is made on the stem of a glass capillary pipette about an inch from the tip. The blood is sucked up to this mark by means of a teat fitted to the tube, then an air bubble and then an equal volume of the bacterial suspension which has previously been diluted according to a rough estimate. The whole contents of the pipette are mixed thoroughly by aspirating and re-expelling them several times on to a clear slide. Films are made and stained by Leishman's method. If red corpuscles and bacteria are

evenly distributed and the emulsion is free from bacterial clumps the count may be made. If either the bacteria or the red cells are in excess the procedure is repeated. The bacteria and red cells are now counted separately in each of a series of fields in different parts of the film. As the number of red cells per c. mm. is known, the number of bacteria can be calculated. For example, 500 cells and 1000 bacteria have been counted in the same number of fields. But a c. mm. of blood contains 5,500,000 red cells and equal volumes of blood and emulsion were used in this procedure; a c. mm. of the emulsion therefore contains $\frac{5500000 \times 1000}{500}$ = 11,000,000 organisms or 11,000,000,000 per c.c.

(*b*) *Hæmocytometer method.*—The following diluting and staining fluid is prepared:—

Sodium chloride	..	..	1 gramme
Formalin ..	..	..	2 c.c.
Alcohol gentian-violet	..	..	5 grammes
Distilled water	..	..	100 c.c.

The bacterial suspension is sucked into the white corpuscle pipette of a hæmocytometer up to the 0·5 mark and the diluting fluid up to mark 11 and the contents mixed thoroughly. A drop of the mixture is placed in the counting chamber and covered with a special thin cover-glass (so that an oil-immersion lens can be used). The count is made after half an hour. The number of bacteria per c.c. of the emulsion can be calculated by multiplying this figure by 4000, then by 20 and then by 1000.

(*c*) *The Opacity method* is a simple, quick and fairly accurate one and is recommended for daily use. The methods devised by Macfarland and Brown are the most satisfactory. Permanent standards for comparison are made by preparing different dilutions of barium sulphate and Brown's standard opacity tubes and tables of corresponding bacterial strengths can be obtained from Messrs. Burroughs and Wellcome. The bacterial emulsion is poured into the testing-tube which is then placed by the side of one of the standard tubes in a good light upon some clearly printed book. The opacity of the two suspensions can then be readily compared by rolling the two tubes from side to side. If the bacterial suspension has an opacity greater than that of the standard tube, then one volume is placed in the testing-tube and successive volumes of saline are added until the resulting opacity corresponds to the standard. The volume of the diluent to be added to the total amount of the bacterial suspension prepared or the number of bacteria present in 1 c.c. of the original suspension can be estimated.

5. Sterilisation of the Vaccine.—Two methods for the sterilisation of bacterial suspensions are available: (*a*) heat and (*b*) chemicals.

(*a*) *Heat.*—The bacterial emulsion is placed in a sterile test-tube, the mouth sealed and the tube heated in a water-bath at 56°-60°C. for one hour. Some bacteria are killed much more readily than others, and therefore the temperature of the water and the length of time required for complete sterilisation depend not only on the density of the bacterial suspension but also on the organism itself.

(*b*) *Chemicals.*—To the bacterial emulsion sufficient formalin is added to make the ultimate strength of this substance in the emulsion 0·1 per cent. The sterile glass bottles containing the emulsion are closed with waxed corks and placed in the incubator at 37°C. for 24 hours. Other chemicals such as 0·5 per cent. carbolic acid can be used.

Test for sterility.—After sterilisation, either by heat or chemicals, the bacterial suspension is cultured under aseptic precautions on agar or other suitable media such as Löffler's blood-serum or blood agar. The subcultures are incubated for 24 to 48 hours at 37°C. If no growth appears on these media the preparation of the vaccine may be completed. Some bacteriologists make it a practice to inject guinea-pigs and mice with the vaccine so as to exclude *B. tetani* before they are issued for use.

6. The Dilution of Vaccine and the Addition of a Preservative.—Having made the count or estimated the opacity and sterilised the vaccine, it is next diluted with sterile salt solution containing 5 per cent. phenol until each cubic centimeter contains the dose decided upon, and the percentage concentration of the phenol equals 0·25. The vaccine is placed in a sterile vaccine tube provided with a rubber cap and labelled. In using, the cap is sterilised with tincture of iodine and the needle of a sterilised syringe plunged through the cap and the required dose drawn off. Single doses of vaccines, especially autogenous vaccines, may be put up in separate sterile ampoules.

Sensitised Vaccines.—These vaccines are prepared by mixing a highly immune serum of the organism with emulsions of the living organism. The experience of bacteriologists has failed to establish the claims of Metchnikoff and Besredka that a sensitised vaccine produces little or no negative phase, or local or general reaction.

Stock Vaccines are those prepared from diseases caused by the same organisms or the organisms isolated from similar types of diseases.

Polyvalent Vaccines are made from several strains of the same organism and also include various species of allied organisms.

Autogenous Vaccines (personal vaccines) are those made from the chief organism isolated from, and supposed to be the cause of, a lesion or disease. Their success depends greatly on the ability of the bacteriologist who makes them to pick the right organism and unless polyvalent, it is often wiser to use stock polyvalent vaccines.

Detoxicated Vaccines are prepared by treating the bacteria with alkali, then precipitating with acid. The *endotoxins* remain in solution and are separated out by centrifuging. Much larger dose of these can be given.

The Negative Phase

The injection of a large amount of vaccine is generally followed by a state of depressed immunity, termed the "negative phase" by Wright. Attempts have been made to eliminate this negative phase. A fresh dose of vaccine should not be administered during this phase.

Inoculation and Examination of Laboratory Animals

Animal inoculations are performed for the purposes of (1) the isolation of pathogenic bacilli, (2) identification of an organism and experimental investigation of the disease produced and (3) preparation of agglutinating, hæmolytic, bactericidal and antitoxic sera. The animals used are guinea-pigs, mice, rats, rabbits, pigeons and dogs.

Methods of Inoculation.—Animals selected should be in good condition and gaining in weight and the age should be known. The area to be injected is prepared by clipping the hair or using a depilatory (*e.g.*, a paste of barium sulphide, corn starch and water), shaving and disinfecting with alcohol.

The methods of inoculation generally adopted are:—

1. *Intravenous.*—(In the case of a rabbit a vein of the ear is chosen).

2. *Subcutaneous.*

3. *Intramuscular.*

4. *Intraperitoneal.*—(The needle will be found to move freely when the abdominal cavity is reached).

5. *Intracutaneous Inoculation* (made either by scarification of the skin and rubbing in the inoculum, the tissue being held in

forceps or by direct injection into the skin as in the Schick reaction).

6. *Intraspinal Injection*—(between the 7th lumbar and first sacral vertebræ). Make sure that the needle is within the canal by aspirating a little of the fluid into the syringe, then inject the inoculum very slowly.

After inoculation the animals are kept in cages and any local lesions are noted carefully as well as the rectal temperature, weight and general condition of the animal.

Examination of Animals—Dead or Killed—After Inoculation.

The *objects* of autopsies on animals are to study the nature of the lesions produced by the organisms and to collect material for further investigation.

The following *general rules* must be observed in making a post-mortem examination:—

1. Soak the animal in 1 in 1000 corrosive sublimate before fastening it to a wooden board.

2. Use sterile forceps for raising the skin muscles, etc., and *never* the fingers.

3. Conduct the examination as soon as possible after death.

4. Burn the carcases and boil the instruments and the trough after the examination.

5. Always have two sets of sterile instruments ready, one for cutting the skin, the other for cutting the organs.

6. Use sterile instruments throughout.

Instruments required.—Scalpels, dissecting forceps, scissors, capillary pipettes, platinum wires, tubes of media, slides, cover-slips and a metal rod.

Examination.—Fasten the animal securely to a wooden board laid on a trough.

External examination.—Examine the surface for abscesses, etc.

Internal Examination.—Shave the skin over the abdominal wall. If peritoneal fluid is required cauterise a band, reflect the skin and draw the peritoneal fluid into a sterile capillary pipette. If no peritoneal fluid is required an incision is made

in the middle line extending from the pubis to the episternum or chin and the skin is reflected. The thorax is opened by making an incision on either side of the sternum which is divided at its upper attachment.

Heart.—Draw off the pericardial fluid, if present, by means of a sterile capillary pipette. The hot end of the pipette will sear the surface of the pericardium as it passes through it. To collect heart-blood, cauterise the surface of the ventricle with a red-hot rod and aspirate blood through this area by a sterile capillary pipette.

Lungs.—Examine for any evidence of congestion, pneumonic patches, etc. Collect material from the lung by sucking fluid in a capillary pipette through a cauterised portion of the organ.

Liver, Kidneys, Spleen, Lymphatic glands.—Examine for any gross lesions such as tubercles, etc. In collecting material for cultures, pass a sterile stout wire bent in the form of a hook through a cauterised surface of the organ; twist the wire round; withdraw it and plant the material at once in a suitable medium.

For microscopic examination obtain the smear by rubbing a portion of the organ on a clean slide.

Fix pieces of the organs for section cutting.

Bone Marrow.—Cut across one of the long bones and collect the material by means of a platinum loop or capillary pipette.

Nervous System.—Lay the animal on its ventral surface and open the skull, expose the brain and make the usual smears and sections.

The Collection of Pathological Material for Examination

Blood required for culture or for obtaining large amounts of the serum, as for the Wassermann test, should be obtained from a vein. Small quantities of blood, e.g., for the agglutination test and for making blood films, are obtained from the fingers; in the former case a straight quill tube with capillary ends is used and, when filled, the ends are sealed in a flame. A Wright's capsule can be used.

The *collection of secretion from the throat and of fæces* for examination are referred to elsewhere.

Urine.—To obtain uncontaminated samples a catheter specimen should be drawn off into a sterile vessel. For ordinary purposes in the male it is sufficient to disinfect thoroughly the

glans penis and meatus with 1 in 1000 perchloride of mercury solution and then the patient is asked to pass his urine into a series of sterile flasks, the first of which is not examined.

Cerebro-spinal Fluid is obtained by lumbar puncture. The patient lies on his right side on the edge of the bed with the back bent and the knees and shoulders approximated. The skin over the lumbar region is sterilised with alcohol and ether. The line connecting the highest points of the iliac crests intersects the vertebral column at the tip of the 4th lumbar spine and the puncture is made immediately below this line. The left index finger is placed on the lumbar spine as a guide and a sterile antitoxin needle (about 3″ long) inserted about half an inch below and half an inch to the right of this spot. The needle is directed slightly inwards and forwards and as soon as it enters the subdural space, fluid will issue from the needle. If a lamina is struck instead of the ligamentum subflavum, it is necessary to withdraw the needle slightly and try a point above or below. From 3 to 5 c.c. of the fluid are collected in a sterile test-tube, the needle withdrawn and the puncture area painted with collodion. The patient should remain in bed for some hours afterwards.

Cerebro-spinal fluid is examined for its cell count and the following organisms: Meningococcus, Sp. pallidum, Streptococcus, Pneumococcus, Tubercle bacillus and rarely for Staphylococcus, B. typhosus, B. coli and B. anthracis.

CHAPTER V

IMMUNITY

Immunity is the power which enables an animal to dispose of or at least prevent a given organism from proliferating and elaborating its poison. Before discussing immunity we must consider the characters of the infecting agent, for infection is the result of two opposed forces—the capability of an organism to multiply and function in the tissues of a particular animal and the resistance of the infected animal.

Among the innumerable bacteria present in nature a very limited group only is parasitic on higher animals. A very small proportion of this group is pathogenic, *i.e.*, capable of inflicting disease; the majority are harmless (saprophytes).

In *infection* we must consider the following factors:—

(*a*) *Virulence.*—This is the disease-inciting power of an organism and depends upon its property of resistance or self-preservation after lodgment in the tissues and its power of producing poisonous substances of the nature of toxins, endotoxins or exotoxins or aggressins. *Aggressins* are substances which are produced by an organism only as a result of its struggle for existence against the defence of the animal. They are not found in test-tube cultures and are non-toxic in themselves. Bail obtains aggressins from the exudate produced by the injection of a virulent culture into the peritoneum of a guinea-pig. The exudate is centrifugalised until free from cells and bacteria, and a small quantity of chloroform is added to the clear supernatant fluid to kill off the organisms. Bail found that the sterile exudate so obtained was capable of making a subletha| dose of that organism lethal for a fresh animal.

(*b*) *Number.*—If the number of bacteria gaining entrance is small they will be readily eliminated by the animal. If on the other hand their number is large the defensive mechanism of the invaded animal may break down and the bacteria may continue to multiply and produce toxins.

(*c*) *The Path of Infection.*—The cholera vibrios and dysentery bacilli injected subcutaneously do not reproduce disease; on the other hand if swallowed they may cause the characteristic infections. Only a local abscess is produced by the subcutaneous injection of staphylococci into rabbits whereas an intra-

venous injection may cause a fatal infection with multiple abscesses in certain organs. Conversely, a subcutaneous injection of pneumococcus produces death more readily than an intravenous injection. In the case of syphilis, infection occurs more readily by scarification than by any other means.

(*d*) *Defence of Bacteria.*—Organism such as the pneumococci and the anthrax bacilli are most virulent in the capsulated stage. Attenuation of virulence which takes place during artificial cultivation on media is associated with a loss of capsule.

(*e*) *Susceptibility of Host.*—Susceptibility is the reverse of immunity and varies according to (*i*) species, (*ii*) race and individual peculiarities and (*iii*) age. This subject is discussed in greater detail elsewhere. The pathogenic effects of bacteria are due to the production of poisonous substances which are: (*a*) Toxins, (*b*) Enzymes, (*c*) Aggressins and (*d*) Ptomains.

Immunity may be natural or acquired as the result of disease or as the result of artificial methods which may be either active or passive.

Natural Immunity is the resistance to an infection normally possessed by certain animals, for example, rats can withstand 1000 times the lethal dose of diphtheria toxin for guinea-pigs Natural immunity is of various kinds:—

(*a*) *Species immunity* is illustrated by the immunity possessed by man against certain diseases of the lower animals, such as swine plague and fowl typhoid. The lower animals are immune to certain diseases of man, *e.g.*, typhoid, measles, cholera, gonorrhœa and syphilis. Frogs and fowls which are naturally immune to anthrax can be infected by raising the temperature to 37°C. in the former case and lowering it to 37°C. in the case of the fowls.

(*b*) *Racial Immunity* is a type of natural immunity found in members of the same species. For example, negros are believed to be immune to yellow fever and Indians to scarlet fever. Algerian sheep enjoy immunity from anthrax whilst ordinary sheep are susceptible to it.

(*c*) *Individual Immunity.*—Young subjects are more liable to diphtheria and infants are extremely susceptible to tubercle and the acute suppurative affections of bones and joints.

Mechanism of Natural Immunity.—Natural immunity may depend upon phagocytosis, natural antitoxins and bacteriolysins, anti-aggressins and lack of suitable receptors for a particular toxin or a bacterium.

Acquired Immunity.—(1) *Naturally Acquired Immunity as a Result of Disease.*—In the case of certain diseases such as smallpox, scarlet fever, typhoid, etc., recovery from an attack is followed by a lasting immunity. The immunity following an attack of an infectious disease may be transient as in pneumonia, diphtheria, cholera, plague, etc.

(2) *Artificially Acquired Immunity* is the second type of acquired immunity and is of two kinds: Active and Passive.

I.—Active Artificially Acquired Immunity is the resistance to infection brought about by the activity of the tissues of an animal as a result of artificial inoculation with either an attenuated form or sublethal quantity of the infecting agen of a disease or its products. The process of conferring protection by these methods is known as " active immunisation " and canbe accomplished in various ways:—

1. By injection of the living organisms.—The introduction of living virulent organisms is attended with danger to the life of the animal. The organism in question has to be either attenuated in virulence or injected in non-lethal doses.

(*a*) *Immunisation with Living but Attenuated Cultures.*—

(*i*) *Attenuation by heat.*—Exposure of an organism to a high temperature not sufficient to kill it may reduce virulence: Touissant heated anthrax blood at 65°C. for 10 minutes. Prolonged cultivation of the bacteria at temperatures above the optimum for growth may attenuate their virulence, *e.g.*, Pasteur diminished the virulence of anthrax bacilli by growing them at 42°C. to 45°C.

(*ii*) *Attenuation by prolonged cultivation of bacteria on artificial media.*—A great many organisms such as the pneumococci and streptococci lose their virulence to a greater or lesser extent when grown outside the animal body. This is the method employed by Pasteur in the case of chicken cholera.

(*iii*) *Attenuation by passage through animals.*—The virulence of an organism for a particular animal may be diminished by passing the organism through the body of another animal, *e.g.*, rabies virus by passage through monkeys becomes markedly attenuated for rabbits. The attenuation of anthrax bacilli for the ox by passage through guinea-pigs is another instance. In the case of swine plague,

Pasteur found that by passing the organism through rabbits, its virulence was increased for rabbits but was diminished for pigs.

(*iv*) *Attenuation by drying.*—This is illustrated by Pasteur's method of prophylactic immunisation against rabies. The spinal cords of rabbits inoculated with *virus fixe* are dried in bottles containing caustic potash. With each day of drying, the virus shows a progressive loss of virulence.

(*v*) *Attenuation by the use of chemicals* such as weak antiseptics. Roux and Chamberland found that the anthrax bacilli if cultivated in a medium containing 1 : 600 carbolic acid became avirulent for sheep.

(*b*) *Immunisation with Living Cultures in Non-lethal doses.*—Protection may be conferred by the injection of virulent cultures in non-lethal doses. The dose may be increased in subsequent inoculations.

2. By injection of the dead organisms.—This method is extensively used for experimental purposes and in anti-plague and anti-typhoid vaccines. Repeated injections of a killed culture in increasing doses confers a high degree of protection. The dead cultures are less toxic than the living ones and in the laboratory immunisation of animals, it is usual to inject dead organisms once or twice before living bacilli are introduced. Dead cultures may be mixed with antiserum (sensitised vaccine) and employed for immunisation.

3. By injection of bacterial products.—The organism is grown in a fluid medium and filtered through a Chamberland or other porcelain filter. The filtrate which contains the soluble toxins of the organism can be employed to immunise animals.

II.—Passive Artificially Acquired Immunity.—In this form of immunity the tissues of the animal protected do not take any part in its defence but the immunity is acquired by the transference to the animal of protective bodies contained in the highly immunised serum. This type of immunity is of shorter duration than active immunity and is either antitoxic as in the case of diphtheria antitoxin administered to man, or antibacterial as in immunisation by anti-plague serum. Passive immunisation has achieved its greatest usefulness in the case of those diseases which are caused by bacteria producing soluble toxins: two well known examples are diphtheria and tetanus. Passive immunity is specific. This means that the serum of an animal immunised against a particular

organism will protect another animal against this organism but against no other.

Local Immunity.—Besredka has called attention to the elective localisation of the viruses of certain diseases and has shown that whether dysentery, paratyphoid or cholera virus be introduced into the peritoneal cavity, into the circulation or even into the skin, the small intestine finally attracts it and the organisms are found in the lumen of the intestine. The immunity produced is also limited to the intestine. In the case of staphylococci and anthrax bacilli the receptive cells are located in the skin. In his opinion, vaccination by mouth in the case of bowel diseases is a more certain method than subcutaneous vaccination. In staphylococcal and anthrax infections cutaneous vaccination produces better results than subcutaneous injections. Filtrates of staphylococcal cultures are also effective.

The Phenomena following upon Active Immunisation.—*Antigen and Antibodies.*—The serum of an animal immunized against a particular disease affords protection to a second animal and contains substances which neutralise the action of the bacteria or their products. These substances which are absent from the serum of normal animals are called *antibodies.* Any substance which can induce the formation of antibodies is known as an *antigen.* The blood serum of an immunised animal containing antibodies is termed an *immune serum.* The different types of interactions between antibody and antigen are called *immunity reactions, e.g.*, agglutination, Pfeiffer's, etc., and are characterised by extreme specificity. The substances which act as antigens are numerous, are probably proteins and include the various toxins, bacteria, tissue cells, red corpuscles and soluble constituents of animal and vegetable tissues. Immunity does not invariably depend upon the presence of antibodies, for example an animal may be immune after the antibodies have disappeared. In the case of naturally immune animals, no antibodies may be demonstrable *in vivo* or *in vitro.* The immunity of the rat against diphtheria, for example. cannot be attributed to natural antitoxin in the blood since rat's serum has no protective power for guinea-pigs against diphtheria toxin. In the case of artificially immunised animals it is believed that the immunity persisting after the disappearance of demonstrable antibodies depends upon certain changes effected in the cells of the body. It is to be noted that in such animals antibodies are more readily produced than in normal animals.

Immune serum may be antitoxic or antibacterial:—

Antitoxic serum is produced by the injection of bacterial toxins, or toxic proteins such as snake venom, ricin and abrin,

etc. The method of production of the antitoxins, and their standardisation as applied to diphtheria have been described on page 259; the nature of the antitoxin and toxin reaction requires consideration. With regard to the former Calmette working with snake venom found that the poison was relatively more resistant to heat than the corresponding antitoxin and that when a mixture of the poison and the antitoxin, so proportioned that it was harmless on injection, was heated to 68°C. for some time, it became toxic again, indicating that the venom was not really destroyed. Martin and Cherry subjected neutral mixtures of the toxin and antitoxin of the Australian snake to filtration through gelatin filters under pressure and found that on filtration of newly made mixtures a certain amount of the toxin will pass through while the relatively large molecule of the antitoxin is held back. If, however, the interval between the preparation of the mixture and filtration is prolonged, less and less toxin comes through. A mixture of snake toxin and antitoxin can also be dissociated by the addition of small amounts of hydrochloric acid. These experiments indicate that the harmlessness of a toxin and antitoxin mixture does not depend upon toxin destruction. It is now generally held that the behaviour of toxin and antitoxin towards each other *in vitro* is not physiological but is of the nature of a physico-chemical reaction.

Regarding the exact nature of the union between toxin and antitoxin, there is a divergence of opinion. According to Ehrlich there is a firm chemical union between the toxin which is composed of heterogenous molecules and the antitoxin. Arrhenius and Madsen believe that the behaviour of the toxin and antitoxin mixture is analogous to that of two substances in weak chemical union. Bordet on the other hand regards this combination as physical depending upon absorption. An important factor in the toxin and antitoxin union is the element of time.

With regard to the constitution of toxins, it may be noted that the difficulty of explaining the facts relating to diphtheria toxin led Ehrlich to postulate the presence of *toxoids*. Ramon found that the diphtheria toxin after the addition of 0·3 per cent. formalin was not toxic but was still antigenic and gave the flocculation reaction as before. This detoxicated toxin was termed *anatoxin* by him but it appears to be analogous with Ehrlich's toxoids.

Antibacterial serum is prepared by the methods outlined above. It is advisable to employ killed cultures, the virulence of which has been increased by passage through animals in the initial stages of immunisation. Later, the immunisation can be

continued by giving living doses. The serum so obtained is found to contain protective bodies against the particular organism and these can be transferred to another animal. The following are some of the chief properties of an antibacterial serum:—

(*a*) *Bactericidal, and lysogenic or bacteriolytic property of the blood serum.*—Pfeiffer found that the injection of *V. choleræ* into the peritoneal cavity of a guinea-pig highly immunised against the organism was followed immediately by a loss of motility by swelling and granular changes and finally by destruction and disappearance of the cholera spirillum. The bacteriolytic substances can be transferred to another animal so that when the bacteria are injected into a normal animal with a sufficient quantity of the cholera immune serum, the vibrios undergo complete solution. These changes constitute what is generally described as " Pfeiffer's Reaction or Phenomenon " or " Bacteriolysis." Metchnikoff and Bordet obtained lysis of *V. choleræ* outside the body in hanging-drop preparations. Bordet further found that the bactericidal properties of the immune serum were destroyed by heating to 50° to 60°C. The addition, however, of a fresh normal serum to the heated immune serum restores the bactericidal property. These subsequent investigations established the fact that immunisation of an animal incites the production in the blood of the animal of an antibody or of a protective substance which is generally known as the " immune body," or " amboceptor " (Ehrlich). This substance is moderately resistant to heat; a temperature of 65°C. for an hour does not destroy it. It is specific for the bacterium employed in immunisation. The immune body cannot act upon the bacteria alone but requires the addition of another substance present universally in normal serum. This second substance is variously known as " complement " (Ehrlich), " alexin " or " cytase." Unlike the immune body, the complement is not increased by the process of immunisation; it is relatively unstable, being destroyed by a temperature of 60°C. It is not yet settled if there is a multiplicity of complements, each having an affinity for a particular immune body as held by Ehrlich, or if there is a single complement, as believed by Bordet.

The function of the amboceptor is to link the complement to the bacterium. The destruction of bacteria does not depend upon the immune body but upon their sensitiveness to the action of a particular complement.

Neisser and Wechsberg found that if they mixed increasing amounts of the immune serum with constant quantities of bacteria and complement, there was an optimum amount of immune body for bacteriolysis. If the immune body was present in

excess of this amount there was inhibition of the bactericidal effect (Neisser-Wechsberg phenomenon).

(*b*) *Hæmolytic Action.*—The serum of an animal may cause dissolution of the red cells of another animal. The serum of an animal, *e.g.*, a rabbit, repeatedly immunised with the red cells of another animal such as a sheep, is strongly hæmolytic for the cells of the latter animal. Bordet found that as in the case of bacteriolysis, an immune serum, the hæmolytic activity of which was destroyed by heating, could be rendered active again by the addition of a little normal serum or peritoneal exudate. The immune body (hæmolysin) combines with the red corpuscles at low temperature, namely 0°C., whereas complement does not enter into combination at this temperature but at 37°C. It is then easy to remove the immune body from a hæmolytic serum and allow the complement to remain in it. If, for instance, a mixture of the red corpuscles, immune body and complement is kept at 0°C. no hæmolysis takes place. Yet, if centrifugalised, the immune body is found to have united with the red cells, leaving the complement free in the supernatant fluid. If the same mixture is incubated at 37°C. hæmolysis at once occurs. These observations led Ehrlich to believe that complement did not directly enter into combination with the red cells but did so through the intervention of the immune body. Bordet on the other hand found no evidence of the direct union of complement and the immune body and concluded that the immune body merely acts as a sensitising agent which enables the complement to unite with the antigen.

Antisera analogous to the hæmolytic sera may be prepared by the injection of cellular elements, *e.g.*, leucocytes, ciliated epithelium, kidney and liver tissues, etc. The production and mode of action of these sera are identical with those of the hæmolytic serum. These sera, for example, acquire the property of dissolving leucocytes, immobilising the spermatozoa, causing destruction of kidney and liver tissues, etc. They are, however, not so specific in their action as hæmolytic serum, for a serum produced against tissue cells is often markedly lytic to red cells.

It is evident that complement is fixed only when the homologous antigen and amboceptor are present. On the other hand, if antigen and amboceptor are heterologous, *i.e.*, amboceptor does not correspond with the antigen, the complement remains free. The relationship of antigen and amboceptor can thus be determined by finding out if the complement is fixed or not, to do which it is necessary to employ some reagent which will react with the free complement in some obvious manner. For this

purpose, sensitised corpuscles, *i.e.*, corpuscles treated with the specific immune serum, are used. For example, if an antibacterial serum is heated to 55°C. to destroy any complement that may be present and is added to the homologous bacteria (*i.e.* bacteria which produced the serum), it fixes the complement which is added as fresh normal serum, and thus prevents hæmolysis of sensitised red cells. If, on the other hand, the original mixture contains no antibodies for the antigen (bacteria) used, the complement present is not fixed and is free to combine with the sensitised cells and cause their hæmolysis. The Wassermann reaction or the complement fixation or deviation test is based upon these principles.

(*c*) *Opsonic Action.*—Wright demonstrated the presence in blood-serum of substances on which depends the phagocytosis of bacteria by leucocytes. These substances are called *opsonins*, on account of their action in preparing the bacteria for ingestion by the leucocytes. Leucocytes washed free of serum by saline solution are not phagocytic but become so on the addition of fresh normal serum. Serum heated to 55°C. is also without effect in inducing phagocytosis. If bacteria are added to fresh serum and incubated at 37°C. they are readily ingested by the leucocytes which have been washed free of the serum by saline solution. The opsonic content of a serum is increased during the process of immunisation. It has been further shown that the opsonins of the immune serum (bacteriotropins of Neufeld) which are thermostable, being resistant to heating at 55°C., are highly specific in contrast to the thermolabile non-specific opsonins which are present in the normal serum. It is not yet settled whether or not the thermostable opsonins of the immune serum are distinct from the opsonin of normal serum. The practical importance of the opsonic substances in immunity was emphasised by Wright who showed that the opsonic content of the serum which was diminished during illness from staphylococcal or tuberculous infections could be increased by active immunisation. The ratio of the average number of bacteria ingested by a given number of phagocytes in the presence of a patient's serum to the number of the organisms ingested by the same number of phagocytes in the presence of normal serum is known as *opsonic index.*

(*d*) *Agglutinating Action.*—When an animal such as a rabbit is injected with sublethal doses of living or dead bacterial culture, *e.g., B. typhosus*, its serum acquires the property of clumping the typhoid bacilli when mixed with a uniform suspension of that organism. The sera of persons suffering from certain infectious diseases are also capable of clumping the infecting organism. This property depends upon the presence of bodies

in the serum called agglutinins which are not destroyed by heat at 55°C. The presence of salts is necessary for agglutination. The property of being agglutinated is not confined to bacteria alone; red corpuscles may be clumped by a suitable immune serum (*hæmagglutination*). Nor is the property of clumping bacteria confined to specific serum alone for (*i*) an immune serum may agglutinate a number of different species of organism (*group agglutination*), (*ii*) various concentrations of acid may cause bacterial agglutination (*acid agglutination*), (*iii*) bacteria may be precipitated in the presence of normal saline without the aid of serum (*spontaneous agglutination*).

With regard to group agglutination, as a general rule a given bacterium, when injected into a suitable animal, stimulates the production of agglutinins for itself alone, but in a number of cases agglutinins for another species are also produced, though to a lesser extent. This phenomenon is most marked in closely related organisms, *e.g.*, amongst members of the *Salmonella group*, but Goyle has recently studied a remoter type of group agglutination in the case of *B. typhosus* and *B. enteritidis* (Gärtner). He has shown that the "R" forms of these two organisms possess a common heat-stable antigen which is absent from the "N" and "S" forms. The presence of another heat-stable antigen in the "N" strains of *B. typhosus* and *B. enteritidis* may also account for the cross-agglutination phenomenon common with these organisms.

(*e*) *Precipitating Action.*—Kraus demonstrated that the sera of animals immunised with *B. pestis, B. typhosus* and *V. choleræ* when added to a clear filtrate of broth cultures of the corresponding organisms produced cloudiness and afterwards a visible precipitate. The substances in the immune serum taking part in this phenomenon are known as *precipitins.* The reaction is analogous to the agglutination reaction in many respects.

Koch's Postulates.—An organism can only be regarded as specific to a disease if it—

(*i*) is present in the tissues, organs or body fluids of the animal suffering from the disease;

(*ii*) can be isolated and cultivated from these tissues on artificial media;

(*iii*) can, after isolation, and cultivation, reproduce the disease in a suitable animal;

(*iv*) can be recovered from this inoculated animal.

To these may be added the necessity of an organism to conform to specific immunity reactions, *e.g.*, agglutination, complement-fixation, etc.

Theories regarding the Mechanism of Acquired Immunity

1. Pasteur's Theory of Exhaustion assumes that the bacteria extract from the blood some substances necessary for their growth and that these substances require renewal before the organism can grow.

2. Theory of Retention assumes that organisms produce some substances inimical to their own growth so that they die out.

3. Metchnikoff's Theory of Phagocytosis.—This assumes that the large mono-nuclear and polymorpho-nuclear leucocytes ingest and destroy the organisms in the blood stream and are attracted by positive chemiotaxis to the site of the disease. This phagocytosis depends upon the presence of opsonins.

4. The Humoral Theory assumes that substances exist or are developed in the serum which are inimical to the organism.

5. Ehrlich's Side-Chain Theory.—This assumes that unstable side-chains are attached to the bacterial cell (the haptophore group) and to the cells of the body (the receptor group). A tissue cell when stimulated may produce numerous receptor groups. The receptor groups unite with haptophore groups. If the receptor groups are few and are not sufficient to unite with all the haptophore groups present, free haptophore groups are able to damage the tissue cells. On the other hand, if an excess of the receptor groups is present not only are all the haptophore groups rendered innocuous as they are all attached to the receptor groups but free receptor groups exist in abundance to attach themselves to any fresh toxic group introduced. The free receptor cells form the antitoxins, agglutinins, etc.

CHAPTER VI

THE NON-PATHOGENIC BACTERIA AND THE HYPHOMYCETES

Non-pathogenic Bacteria

Bacillus prodigiosus.—*B. prodigiosus* is an organism which produces a red pigment and is found in air and water. It may grow upon various food stuffs, and give rise to a suspicion of foul play. Parkes describes cases of diarrhœa which he suggests were caused by this organism. On one occasion it contaminated a water-supply and infected the bread made with it.

The organism is a short rod, almost coccoid, 1 to 2 μ long, Gram-negative, motile and non-sporing. It grows well at 37°C., produces turbidity in broth and liquefies gelatin. On agar the growth is slightly red, thick and creamy, on gelatin, red or pink and on potato red and creamy.

Bacillus subtilis.—This occurs universally, *i.e.*, in air, water, fæces, etc. Along with its spores it is especially abundant in packing material (straw and hay), hence the popular name, "the hay bacillus." It is about 2 to 3 μ in length and 1 μ in breadth, has rounded ends, is actively motile and forms ovoid spores which are very resistant to heat. The bacillus is a strict ærobe and is Gram-positive. The spores resist boiling at 100°C. for 6 hours.

Cultivation.—Gelatin stab.—A white growth is seen along the needle track, and the gelatin is rapidly liquefied with the formation of a tough pellicle on its surface. Frequently, however, from lower part of the tract of the needle horizontal branches extend into the medium, producing a fine feathery growth.

Agar streak.—The cultivation is very characteristic. It is opaque, white and moist at first, but afterwards it becomes much drier and wrinkled.

Broth.—There is uniform turbidity with a much wrinkled dry white pellicle on the surface which is characteristic of this organism.

Potato.—The growth is moist and white.

Bacillus megatherium.—This was first isolated from boiled cabbage leaves and has been found in water. It is a rod-shaped bacillus (often slightly curved) with rounded ends. It is slightly motile, contains black granules and spores and is Gram-positive. This organism is responsible for producing a "sour-beef" condition in meat insufficiently cooled after killing.

Gelatin stab.—The growth is very similar to that of *B. mycoides*, but the hair-like filaments which spread out in the medium are unequal in size. Liquefaction rapidly occurs.

Agar streak.—The growth is white and moist and from its edge fine filaments pass outwards.

Potato.—A yellowish-white growth which is not so profuse as in the case of *B. subtilis* and *B. mycoides*.

Bacillus mycoides.—This is one of the commonest organisms found in the superficial parts of cultivated soil and grows well on all media. It is a rod-shaped bacterium with rounded ends resembling the bacillus anthracis. It measures 1·5 to 2·5 μ in length and 0·8 μ in breadth and is motile and possesses oval spores of about the size of those of *B. subtilis*. It is stained by carbol-thionin and by Gram's method.

Gelatin plates.—The growth is characteristic. Under the low power of the microscope a felted mass throwing out irregular offshoots from the centre is seen. Later, the naked-eye appearance resembles that of a mould. The gelatin is liquefied.

Gelatin stab.—From the track of the needle uniform branches spread out.

Agar streak.—The growth is white and misty with a fringe-like edge.

Broth.—Turbidity rapidly occurs. A white scum forms on the surface and later on becomes of a brownish colour.

Potato.—A white slimy growth appears.

Putrefactive Organisms.—*A.—Proteus vulgaris; B.—Proteus mirabilis; C.—Proteus zenkeri.*—These are types of a class of organisms which cause putrefaction—a term used to describe a series of chemical changes allied to those of fermentation.

They were first isolated from putrid meat, and are found in putrifying substances, sewage and water. Members of this class are found in the alimentary canal, and after death rapidly spread into all the tissues of the body producing decomposition.

Under the microscope every variety of shape is met with, cocci, diplococci, short rods and longer ones (they may be slightly curved), threads and involution forms. They vary in length from 1 to 4 μ the width being, on an average, about 0·5 μ. They generally remain stained when treated by Gram's method but *Proteus vulgaris* is decolorised by this method. Spore-formation is not known.

Proteus vulgaris and *mirabilis* are motile but *Proteus zenkeri* is non-motile.

Cultivation.—Proteus vulgaris.—Gelatin stab.—Gelatin is rapidly liquefied, the upper layer being of greyish-white tint, while an opaque, white, flocculent growth is seen at the bottom of the tube.

Gelatin plates.—The colonies are very variable in outline, being, for the most part, more or less spindle-shaped, with numerous branches ramifying in various directions and terminating in bulbous processes. These branches are known as " swarmers " or " swarming islets." They may assume a craggy, irregular, appearance, owing to central liquefaction, the growth being of a greyish-white tint.

The growth on *agar* and *potato* is moist and of a dirty greyish-white tint.

There is a decidedly putrefactive odour noticeable in the culture.

Proteus mirabilis.—Gelatin stab.—A dense white layer of growth, circular in outline, forms on the surface of the medium. The liquefaction of the gelatin is not so rapid as in the case of *Proteus vulgaris.*

Gelatin plate.—The colonies look white to the naked eye but under the low power of the microscope appear brownish-white, circular, or oval and finally granular, the appearance being much the same as seen in the case of *Proteus vulgaris.*

Proteus zenkeri.—Gelatin stab.—A fine branched growth spreads from the track of the needle. It either does not liquefy gelatin or the liquefaction is very slow.

Gelatin streak.—A very characteristic, finely branching almost transparent growth is seen. The delicate branches spreading laterally from the original streak are curved, often with their concavity upwards, presenting an appearance which has been compared with that of a pine tree.

Gelatin plates.—The colonies, when seen by the naked eye, look like little pieces of fluff, which are often mistaken for moulds. Under a low power of the microscope they are seen to be of a light yellow or greenish-yellow tint, elongated and with a denser oval circular portion, from which branches pass in all directions. Many of these branches are charac teristically beaded, others are distinctly spiral, and most join up with similar branches from neighbouring colonies.

Impression preparations of the colonies show that they are made up of numerous bacilli, and their branches, which are known as "swarmers" as in the case of *Proteus vulgaris* are seen to be made up of bacilli which are concentrically arranged in the bulbous extremity. Odour is absent from the cultures.

Proteus vulgaris and *mirabilis* exhibit a peculiar phenomenon when plates are made with 5 per cent. nutrient gelatin. A few hours after incubation, before liquefaction is visible, the bacilli themselves can be seen to be in active motion. Such motility is not observable if 10 per cent. gelatin is employed.

Micrococcus ureæ hydrolyses the urea of urine to produce ammonium carbonate. The cocci vary from 0·8 μ to 1·0 μ in diameter, occur in pairs, tetrads, or chains, and sometimes assume a bacillary form. A ferment (urease) can be extracted from cultures, or from the slimy urinary deposit of cystitis, which produces the ammoniacal change in solutions of urea.

B. acidi lactici is an ærobic, non-motile and non-sporing organism. It is 1 μ to 1·7 μ long and 0·3 μ to 0·9 μ broad, generally found in pairs and in fours. It does not liquefy gelatin but acts on milk sugar, transforming it into lactic acid. In the case of pasteurised milk the souring is delayed on account of the previous destruction of these bacteria.

Hyphomycetes

The fungi are multicellular organisms consisting of tubular branched filaments (hyphæ) which become interlaced in a loosely woven or hard compact mass known as the "mycelium." The hyphæ may be continuous tubes (as in the group *Phycomycetes*) or may be divided up into segments by cross septa, forming chains of continuous cells (the group *Mycomycetes*). Both asexual and sexual reproduction occur, the reproduction in the higher fungi being chiefly asexual. The further classification of the fungi is based on the method of spore formation. Phycomycetes are divided into (*i*) those in which no structural difference in the male and female cell can be seen (*Zygomycetes*) and (*ii*) those in which the large female cell containing the

Oospheres contrast with the small indefinite male cell, the *Oomycetes*. Mycomycetes are divided into two groups—(*i*) those in which the seed capsule (sporangium) contains a definite number of spores for each type (the Ascomycetes) and (*ii*) the *Basidiomycetes* in which the terminal ærial hyphæ (the conidiophores) branch at the apex, forming the basidia on which a definite number, usually four, conidia or spores are formed. Other fungi difficult to classify are known as the *Fungi imperfecti*.

Zygomycetes.—Genus Mucor. Species *Mucor mucedo*.—This occurs on damp bread and animal excreta as a white or grey mould of fine non-septate hyphæ. From the mass of the mycelium erect hyphæ stand out, each hyphæ ending in a swollen spherical columella surrounding which is the sporangium or seed capsule. The protoplasm of the sporangium breaks up into spores which finally escape by bursting the outer wall of the sporangium.

The sexual method of reproduction resulting in the formation of a zygospore may occur. Two apparently similar ærial hyphæ come together at their free ends which both become shut off from their respective hyphæ by a transverse wall; the portions of the wall between this terminal cell dissolve and the protoplasmic contents fuse. The thick-celled capsule which results is called a *Zygospore*.

Mucor mucedo is non-pathogenic, grows well on an acid medium and frequently occurs on contaminated agar plates as a white fur. *Mucor corymbifer* gives a growth in damp bread resembling cotton-wool, has a pear-shaped sporangium, is pathogenic to rabbits and is said to cause inflammatory infection in man. *Mucor rhizopodiformis* produces an aromatic odour and *Mucor ramosus* a growth which rapidly becomes brown and both these fungi are pathogenic to rabbits.

Ascomycetes.—(1) *Aspergillus herbariorum* (*A. niger, A. albus, A. glaucus, A. flavescens* and *A. fumigatus*) grow on dead vegetable matter, bread, gelatin, etc. They consist of a mass of septate filaments, the heads of the ærial hyphæ being studded with small finger-like sterigmata at the apex of each of which is a chain of spores (conidia).

These conidia are the result of the asexual method of reproduction. In the sexual method, which rarely occurs, a mass of closely woven hyphæ (the asocarp) is formed, within this being numerous asci, each containing 8 ascospores. The contents of asocarp disintegrate, leaving the ascospores loose within the outer shell, which ultimately ruptures, freeing them.

Aspergillosis occurs chiefly among birds as a pulmonary disease and may occur in man producing otomycosis and nasal and lung infections (*A. flavesceris* and *A. fumigatus*). In addition to *streptothrix maduræ* a fungus closely allied to aspergillus is associated with Madura disease. Pinta or Caraate is due to various fungi of a similar type, in which the spores measure 8 to 12 μ and contain abundant pigment, while the white mycelial hyphæ 20 μ x 2 μ, broaden out at the end bearing the spore in a funnel-shaped manner (phialides).

(2) *Penicillium crustaceum* (*P. glaucum*).—This, the common green mould of cheese and jam, produces a musty odour and is very difficult to eradicate from a laboratory; the aerial hyphæ (conidiophores) grow upwards and branch at their ends into fork-shaped *basidia.* On each finger-like branch a thin sterigma forms and from each sterigma grows a row of oval conidia or spores.

On gelatin plates penicillium radiates out over the surface from central points and the gelatin is liquefied. All the moulds possess the power of liberating arsenic from damp arsenical wall paper and *P. brevicaule* has been used to detect minute quantities of arsenic.

Mossy foot, a painful moss-like papylomatous condition affecting the feet and legs, met with in Brazil and Honduras, is due to *P. hialophora verrucosa* which produces a dark brown growth in Sebouraud's medium in which chalamydospores (large thick-walled "resisting" spores in the middle or end of the hyphæ) and conidia (asexual spores) are seen.

(3) *Saccharomycetes or Yeasts* (Torula).—These consist of round or oval unicellular organisms 3-5 μ in diameter, which usually reproduce by budding, the bud after enlarging, separating itself from the parent cell. Spore formation may occur—occasionally subsequent to the fusion or conjugation of two yeast cells—in which process the cell contents divide into 2, 4 or 8 parts (ascospores) each of which becomes surrounded by a cell membrane.

The yeasts contain certain enzymes, maltase, zymase, invertase, etc.—which have the power of reducing maltose to glucose and glucose to alcohol, inverting saccharose, etc.—and also produce ferments capable of destroying certain of these enzymes. They are of great importance in baking, brewing, the fermentation of grape juice, etc. They occur chiefly on fruits such as grapes, cherries, plums and raisins, and can be differentiated by their action in fermenting the sugars.

Blastomycosis, as a chronic dermatitis, chronic suppuration or generalised condition, may occur in man due to the inhalation of or the infection of wounds by a group of yeasts which are all pathogenic to laboratory animals and includes *Saccharomyces hominis* (producing an acute pseudo-lupus condition). *Cryptococcus capsulatus* (a kala-azar-like disease), *Cryptococcus linguæ pilosæ* (ulceration of the mouth), *Cryptococcus gilchristi, Oodium coccoides* (an acute and severe cutaneous granuloma), etc. The spherical bodies of this group vary from 10 to 30 μ, appear singly or in twos or groups, are Gram-positive, stain irregularly, reproduce by budding and produce mycelia in cultures. They can be cultivated on Sabouraud's maltose agar medium when paste-like or dry colonies appear. Glucose and maltose are fermented and gelatin generally not liquefied.

Fungi imperfecti: *Oospora lactis* (*Oidium lactis*).—This fungus occurs commonly in sour milk, butter and bread as a white fur. It can be cultivated in gelatin or agar and is non-pathogenic to man.

Oidium albicans (*Monilia candida*) which resembles *Oospora lactis* is found as white patches of growth on the tongue, fauces and pharynx of children suffering from thrush. A large spore (5 μ × 4 μ) and small spore form exist, spores and septate mycelia being seen in both culture on ordinary media and in tissue preparations. It can be cultivated on milk, bread, gelatin (white or reddish colonies with or without liquefaction), agar and potato (whitish raised growth).

Intravenous injections are very lethal to rabbits. *Oodium albicans* is also said to cause generalised infection with abscesses in the internal organs and has been reported in sprue (*Monilia psilosis*).

Examination of Moulds.—Mucor, aspergillus and penicillium should be examined by placing a piece of the mycelium in alcohol and mounting in glycerin. The hyphæ stain with methylene-blue while the spores remain unstained. Though growth occurs in ordinary and in bread paste media, the organism should be cultivated on maltose and peptone agar, on glucose agar or media to which wort has been added.

Tinea.—Ringworm is a common disease in domestic animals, dogs, cats and horses frequently transmitting the disease to man. The fungus is a microsporon, two varieties of which, the small spored and the large spored, occur.

Microsporon audouini is a small spored fungus and its fine whitish mycelia (1 to 5 μ long) may be seen on a diseased scalp, surrounding and penetrating the involved hairs which are

also surrounded by irregular masses of spores (about 23 micron in diameter). *M. audouini* occurs chiefly in the scalp in young children (80 to 90 per cent. of ringworm cases) and is said never to be met with in the scalp in adults nor in diseases of the beard or nails. Other allied species (*M. umbonatum, M. tardum, M. velveticum*) may occur in man. In culture on Sabouraud's maltose agar large flat white colonies covered with ærial hyphæ occur. Gelatin is slowly liquefied. Numerous sub-terminal condia appear on the hyphæ.

Trichophyta.—These forms are characterised by having large spores (4 to 12 μ which are not distinctly differentiated from the mycelia but appear as rows of oval bodies. Small spored forms also occur. In the human endothrix variety (*Trichophyton megalosporon endothrix*) the fungus occurs chiefly in the medullæ of the hair while in the *ectothrix* variety, which is derived from various animal infections, the fungus grows down between the follicle and the hair before penetrating the hair sheath, and the spores lie on the outside of the hair. This ectothrix variety chiefly affects the beard and nails. "Dhobie itch" (*Tinea cruris*) has been said to be chiefly due to this variety but various species of Epidermophyton and Trichophyton, especially *Microsporon minutessimum* may also cause this condition.

Trichosporosis or Piedra is a disease common in British Guinea, caused by *Trichosporum giganteum,* wherein the hair of any part of the body may be found affected with small hard nodules scattered along the length of the hair. The spores measure 2-12 μ and adhere to the hair which is not penetrated by the fungus. Trichomycosis, a disease resembling piedra, affecting chiefly the axillary hairs, is met with in Central Africa but in this the hairs are penetrated by the fungus which is probably *Nocardia tenuis.*

Achoria.—Several varieties of this fungus occur in dogs, cats, rats, rabbits and fowls, the most common variety found in human being *Achorion schönleinii*—the cause of "favus." Great destruction of hair follicles occur, the fungus growing round each hair where it penetrates the skin as a thick yellow disc (*scutula*) in the centre of which is a depression from which the affected hair emerges. Under the microscope the affected hair shows a series of wide parallel spaces within the hair, while the yellowish crust shows irregular mycelia and spores varying in size.

Sporotricha.—Sporotrichosis occurs in man, dogs and rats as a granulomatous condition of the skin, pharynx, synovial membranes, bones, etc., resembling syphilis and tuberculosis, and also as a generalised infection. The organism—*Sporotrichon*

beurmanni occurs on vegetables, in certain animals and often in the nasal cavities of healthy man. In disease, in the pus may be seen highly refractile Gram-positive oval spores (3 μ long); mycelia generally are not found and cultivation of the parasite is necessary to prove the mycotic nature of the condition. On Sabouraud's medium and on glucose gelatin the colonies, at first white, soon present a raised brown convoluted appearance. In hanging-drop preparations the spores are generally seen surrounding the mycelium at intervals along its course and large spore-like bodies (5 μ) may occasionally also be observed.

Hemispora stellata, a common saprophyte in nature, may give rise in man to a condition resembling sporotrichosis (Hemisporosis).

Microsporon furfur—This organism occurs as a saprophytic growth in debilitated patients and gives rise, as do other allied fungi, especially *M. mansoni*, to *pityriasis versicolor*. It is common in the tropics in young adults. The growth occurs as masses of mycelia (approximately 10 μ×3 μ) with refractile irregular groups of oval spores (4 to 7 μ). Attempts at cultivation have been unsuccessful.

M. mansoni cultivated on Sabouraud's media gives rise to black circular raised colonies.

Examination of affections of the Skin and Hair.—This is usually done by placing a few hairs or a scraping of the affected part in liquor potassæ, B. P., heating the slide gently for a few seconds and examining under the low power objective, after staining, if desired, by Gram's method. The fungi can grow on any of the ordinary media but owing to the variations in the rate of growth and characteristics it is advisable to adhere to one type of culture media. Sabouraud's maltose agar medium should be used and it consists of pure tap water 1000 c.c., maltose 40 grammes, peptone 10 grammes and agar 18 grammes. The media is prepared, filtered and sterilised by gently heating in an autoclave to 120° C. Affected hairs are pulled out by the roots which are then cut off or sterilised by a hot wire, and planted in the medium. In pustular condition the hair may first be placed in absolute alcohol for a few minutes and then dried before inoculating the medium.

CHAPTER VII

THE PATHOGENIC BACTERIA

I.—AEROBIC

Staphylococcus Pyogenes Aureus

Synonyms.—*Staphylococcus aureus* Rosenbach; *Micrococcus pyogenes aureus*—the causative organism of boils, abscesses and many surgical suppurations. Found also in ulcerative endocarditis, osteomyelitis, etc.

Morphology and Staining.—The organism is a spherical coccus (0·8 μ in diameter), tends to grow in irregular grape-like clusters but may be met with singly or in pairs. It is non-motile, non-sporing and Gram-positive and stains readily with ordinary basic dyes.

Culture.—Aerobe and facultative anærobe; grows readily on the usual laboratory media at 10°-40°C., optimum temperature 37°C.

Agar.—A stroke culture occurs as a thick moist, shiny growth, yellowish in colour at first but after a day or two assuming a characteristic orange-yellow colour. Colonies on surface agar are similar in appearance and look like circular discs (2 mm. diameter).

Gelatin.—In stab cultures growth appears along the needle track on the day after inoculation. Liquefaction commences on the 2nd or 3rd day at the top leading to the formation of a funnel-shaped depression. The liquefied medium is rendered turbid by yellowish masses of growth which later fall to the bottom as a flocculent orange-yellow deposit. Finally, complete liquefaction occurs. Single colonies on gelatin plates are visible with the low power of the microscope in 24 hours as small whitish granular points. On the second day they can be seen with the naked eye as shining, pinhead-shaped whitish-yellow colonies. Liquefaction of the medium around the colonies leads to the formation of little depressions in which the colonies occur as yellowish masses. The ferment of staphylococcus responsible for liquefying gelatin known as "gelatinase" is thermostable and can be obtained by the filtration of the cultures.

Blood agar.—The colonies are surrounded by a clear zone due to hæmolysis.

Broth.—A diffuse turbidity occurs and later a deposit of a brownish-yellow tint.

Milk is coagulated.

Staphylococcus Pyogenes, Var. Albus and Var. Citreus

Both these organisms are identical in their morphological characteristics with *Staph. aureus,* except that *Staph. albus* produces a white growth and *citreus* a lemon-yellow growth on agar.

Pathogenesis.—The *pyogenes* are by far the most common organisms associated with suppurative conditions. They occur in localised abscesses, in pustules on the skin, boils and carbuncles, in acute suppurative peritonitis and in some cases of puerperal fever and infective endocarditis. *Staph. aureus* may be associated with a calculus in the bladder but *Staph. albus* is the commoner organism in such cases. Injected subcutaneously into man or animals in sufficient quantities it produces an abscess. If, however, the dose is large, the organism may invade the blood stream and cause secondary suppurative foci in the internal organs. An intravenous injection of the organism produces small abscesses in the kidneys, liver, heart wall, etc. and, in case of injury to a heart valve, infective endocarditis.

Exotoxins.—Some of the strains of staphylococci have been shown to produce an exotoxin (staphylolysin) which is destroyed by heating to 55°C. This exotoxin produces an antitoxin on intradermal inoculation only. Antistaphylolysin can readily be obtained by subcutaneous inoculation of staphylolysin into rabbits.

Hæmolysins.—*Staphylococcus aureus* and to a less extent *S. albus* produce a hæmolysin which can be readily obtained by filtering (through a Berkefeld or Chamberland filter) alkaline broth cultures after incubation at 37°C. for 8 to 14 days. This hæmolysin is thermolabile; heating to 50°C. for 20 minutes destroys it.

Leucocidin (Van de Velde) is a substance which kills leucocytes and which is produced not only *in vivo* but also *in vitro* by growing the organism in a mixture of broth and blood-serum for 48 hours. When injected into animals *leucocidin* leads to the formation of an antitoxin.

Occurrence.—*Staph. pyogenes* are met with in the diseased conditions named above. They are frequently met with as normal commensal organisms on all parts of the skin, throat and

nose. They are therefore frequently present in the air, clothing and in dust, etc. *Staph. aureus* may occur in urine in septicæmia or pyæmia due to this organism.

Resistance.—Fresh cultures are killed at 55°C. in 24 hours and at 80°C. in 15 minutes. In dried pus or other albuminous material the organism withstands heating at 100°C. for several minutes. It is destroyed by 1-1000 mercuric chloride solution in 5 to 10 minutes.

Immunity.—Sera of animals immunised with staphylococci exhibit protective properties, which depend upon their opsonic content. Agglutinins are also produced on artificial immunisation.

Vaccine.—A vaccine is prepared by heating a suspension of an agar culture of staphylococci to 65°C. for half an hour and proceeding as usual in regard to standardisation and sterilisation. In chronic infections the vaccine has been used with much success. Treatment is commenced with a dose of 2-5 millions and continued with increasing doses at weekly intervals. Besredka's method of immunisation is discussed on page 193. The production of an efficient antiserum against *Staph. pyogenes* is impossible as toxin production is very feeble.

The Streptococci

Certain pyogenic cocci multiply by division in one plane only and thus become arranged in chains and are known as streptococci. The group includes a number of organisms of varying virulence and biological characters.

Morphology and Staining.—The individual streptococcus is a spherical organism about 1 μ in diameter which by regular division, especially in a fluid medium, gives rise to a chain-like appearance. It is non-motile and non-sporing. Streptococci are easily stained by aniline dyes and are Gram-positive.

Culture.—Most of the streptococci are easily cultured on the ordinary culture media at 37°C. and usually grow both ærobically and anærobically. The addition of blood or serum to the medium renders it more favourable for their cultivation and blood agar is a suitable medium for routine culture. The organism must be subcultured every four days as it dies out in about ten days.

On *agar*, colonies are small circular semi-transparent discs which appear in 24 to 48 hours. Microscopically they are seen to possess a slightly woolly margin. On *blood agar* growth is

more luxuriant and rapid and colonies are surrounded by a clear halo of hæmolysis in the case of hæmolytic streptococci such as *Strept. pyogenes.*

Gelatin.—In stab cultures the faint delicate growth consists of a row of minute rounded colonies of whitish colour. Colonies on gelatin plates show no tendency to spread on the surface or into the surrounding medium and are similar to those on agar except that they are more opaque and more distinctly white. Gelatin is not liquefied.

Broth.—Growth which is at first granular later settles to the bottom as a sandy deposit. The appearance of the fluid is sometimes clear and sometimes turbid.

Potato.—No growth. *Milk.*—Acid without clotting.

Biochemical Reactions.—The *fermentation reactions* of the organisms of this group are extremely irregular. *Strept. pyogenes* produces acid from glucose, lactose, saccharose and salicin. Unlike pneumococci, most of the streptococci fail to ferment inulin.

Indol.—This is produced in 7 to 14 days but the reaction can only be obtained by the addition of nitrites.

Bile solubility.—Most of the streptococci are insoluble in bile.

Resistance.—Exposure to a temperature of 55° to 60°C. kills the streptococci within ten minutes. They are killed by weak solutions of disinfectants, *e.g.*, 1-500 bichloride of mercury, 1-200 lysol, 1-200 carbolic acid, 1-35 peroxide of hydrogen, in ten minutes. They retain their virulence if subcultured on serum media. The streptococci may remain alive for several weeks in sputum or animal excreta but are killed by direct sunlight in a few hours.

Classification.—Attempts have been made to classify streptococci on the basis of (*i*) morphology (longus and brevis), (*ii*) morphology and the appearance of cultures upon human blood agar (*Strept. hæmolyticus* and *Strept. viridans*, etc.), (*iii*) hæmolysis (the "alpha" type is less hæmolytic, the "beta" type is markedly hæmolytic and the "gamma" type produces neither discoloration nor hæmolysis), (*iv*) hæmolysis and the fermentation of lactose, mannite and salicin (16 principal varieties are included in the classification), (*v*) various reactions such as the clotting of milk, reduction of neutral-red and the fermentation of saccharose, lactose, raffinose, inulin, salicin,

coniferin and mannite, and (*vi*) agglutination and absorption tests. The principal characters of the chief streptococci according to Gordon are:—

	Hæmolysis	Raffinose	Mannite
Strept. pyogenes ..	+	—	—
Strept. salivarius ..	—	+	—
Strept. fæcalis ..	—	—	+

The classification of streptococci into "hæmolyticus" and "viridans" is of practical importance to the bacteriologist.

Serological Reactions.—The spontaneous agglutination of cultures renders agglutination tests difficult and unreliable.

Pathogenesis and Occurrence.—Streptococci vary considerably in their virulence and by passing the organisms through a series of susceptible animals such as rabbits the virulence is very much increased. The pathogenic effects of experimental inoculation depend upon the size of the dose given, the manner of inoculation and the virulence of the strain. Non-hæmolytic streptococci when introduced intravenously into an animal have a tendency to settle down on the endocardium, producing endocarditis with vegetations.

In man, streptococci are associated with a number of pathological processes such as spreading inflammation with or without suppuration, erysipelas, suppuration of the sinus membranes, joints and periosteum, ulcerative endocarditis, puerperal septicæmia and the peritonitis secondary to appendicitis.

Non-hæmolytic streptococci are found in lesions of a less acute nature, *e.g.*, tonsilitis, otitis, dental abscess, etc., and in subacute infective endocarditis.

Toxins.—Filtrates of streptococcal cultures if injected into human beings produce a distinct reaction of an inflammatory nature but are without any effect on animals.

Hæmolysins may be obtained by filtering young broth cultures of streptococci grown in a liquid medium containing ascitic fluid or serum. The streptolysin is destroyed by heating to 55°C.

Leucocidin is also produced by streptococci (see page 210).

Vaccines have been used with some benefit in relatively chronic conditions, *e.g.*, infective endocarditis.

Serum.—On the preparation of the serum the virulence of the organism is first increased by passage from rabbit to rabbit. The virulent cultures are grown for a fortnight on human serum or on a mixture of ass' serum and broth, and inoculated into horses. For the first few injections dead cultures are employed; later living organisms are injected. Now-a-days polyvalent sera are prepared by using a mixture of the various streptococci for inoculation into horses.

Anti-streptococcal serum has been used in streptococcal infection such as erysipelas, puerperal fever, etc. It is most useful in erysipelas.

Diagnosis of Pyogenic Infections.—

1. *Microscopic examination.*—Films from pus or other fluids are stained with (*a*) methylene-blue, (*b*) Gram's method.

2. *Culture.*—In routine examination of streptococcal infections pus or other fluids are plated on blood agar in order to distinguish the hæmolytic from the non-hæmolytic type. Individual colonies are subcultured on agar and identified.

3. *Animal inoculation.*—To ascertain the virulence of a culture rabbits may be inoculated both subcutaneously and intravenously.

Besides the staphylococci and streptococci, there are other organisms which may be associated with suppuration. Of these, in addition to *B. coli* the important ones are:

(*a*) *Micrococcus tetragenus.*—(Syn.—*Staphylococcus tetragenus,* first described by Gaffky). The organism may occur singly or in pairs or in groups of four or tetrads (tetrads generally in the tissues) which are enclosed within a capsule. They are Gram-positive and they grow readily on the ordinary media. Gelatin is not liquefied. Subcutaneous injection particularly into white mice may be followed by a local abscess but usually a general septicæmia ensues. The organism is found in large numbers in the blood, organs and pus of acute abscesses.

(*b*) *Bacillus proteus.*—The organism is greatly pleomorphic, hence the name. It is 1·5 to 2·5 μ in length. Actively motile; no spores; Gram-negative; it stains with basic dyes and grows well on ordinary media at 20° to 37°C. The organism has a tendency to form a film of growth on a solid medium. Gelatin is lique-

fied. The organism is occasionally found in abscesses, peritonitis and cystitis.

(*c*) Several varieties of proteus have been described, *e.g.*, *vulgaris, zenkeri, mirabilis, capsulatus.* Of these only two, *i.e.*, *vulgaris* and *mirabilis,* are definitely recognised; the former ferments maltose, the later does not. A strain of *vulgaris,* namely X_{19}, is agglutinated by typhus serum (see page 301).

Streptococcus Scarlatinæ.—This is found in the throat of scarlet fever cases. It is found in blood only after the rash has appeared. The organism is similar to *Strept. pyogenes.* The causal relationship of this organism to scarlet fever was established by George and Gladys Dick when they were able to reproduce scarlet fever in a volunteer by swabbing his throat with the cultures of a hæmolytic streptococcus isolated from a known case of the disease. They also showed that an intradermal injection of the filtrate of these streptococci produced an inflammatory reaction in persons susceptible to scarlatina and no reaction in non-susceptibles, *e.g.*, convalescents. The reaction associated with their names is known as Dick reaction which is analogous to the Schick reaction in diphtheria. The reliability of the test is now well established. With the aid of the Schultz-Charlton reaction the sera of convalescent patients have been found to possess neutralising substances. The test consists in the intradermal injection of 0.5 to 1 c.c. of the convalescent scarlet fever serum. This produces blanching of the rash in an active case of scarlet fever.

Pneumococcus

Synonyms.—*Streptococcus lanceolatus, Diplococcus pneumoniæ, Streptococcus pneumoniæ, Fraenkel's pneumococcus*—the causative organism of pneumonia.

Morphology and Staining.—Occurs in pairs of small oval or lanceolate organisms with the bases apposed; is about 1 μ in its long diameter and in exudate and in the animal body shows a capsule which is stained by special methods. It is non-motile, non-sporing, Gram-positive and stains well with aniline dyes.

Culture.—Aerobe, optimum temperature 37°C. It does not grow below 22° or above 42°C. It grows on ordinary media but best in the presence of blood or serum.

Agar.—Minute, transparent, almost invisible colonies which remain discrete.

Serum agar.—Same as on agar but more abundant.

Broth.—Slight uniform trubidity with a subsequent granular deposit, growth enhanced by the addition of blood serum or ascitic fluid.

Blood agar.—Colonies somewhat larger than those on plain agar and surrounded by a zone of greenish coloration due to methæmoglobin production.

Gelatin.—It does not grow at ordinary temperature but in 15 per cent. gelatin at 25°C. minute white colonies develop without liquefaction.

Milk is coagulated.

Biochemical Reactions.—The organism causes fermentation without gas formation in glucose, lævulose, saccharose, lactose and inulin—the last is not fermented by many streptococci.

Action of bile.—Add one part of sterile ox bile or of a 10 per cent. solution of sodium taurocholate in normal saline to 4 to 9 parts of a fresh broth culture of pneumococci. Rapid lysis occurs. Serum or sugars must not be present in the culture used for the test as they inhibit the reaction. The reaction is hastened by placing the tube in a water-bath at 37°C. for one-half to one hour.

Serological Reactions.—An anti-pneumococcic serum contains the various types of antibodies including agglutinins, precipitins, opsonins, and bacteriolysins.

Serological types.—Pneumococci have been divided into four chief types by agglutination and absorption tests. Types I, II and III are clearly defined and distinguished by their agglutination with specific type anti-sera. Type IV comprises all strains which are not agglutinated by either type I, II or III sera. Type III which is the most virulent has a distinctive cultural appearance; it is the *Pneumococcus mucosus* and produces raised mucoid or slimy colonies. It is the least common and is recovered in about 15 per cent. of the cases. Types I and II (epidemic strains) are often associated with epidemics of pneumonia and occur in more than 50 per cent. of all the cases of pneumonia. Type IV consists of a number of hetrogenous organisms of low virulence and possessing no immunological affinity with one another or the other three types. Pneumococci present in the mouth and throat of healthy persons belong to this type. It occurs in about 20 per cent. cases of pneumonia.

Occurrence.—The pneumococcus occurs in the exudation of lung alveoli and in pleural exudates in pneumonia and also in other types of primary or secondary infection such as empyema, endocarditis, pericarditis, meningitis, arthritis, otitis media, etc.

Pathogenesis.—The organism is pathogenic to mice, rabbits, rats, guinea-pigs and dogs in this decreasing order. Injected subcutaneously, it produces a fatal septicæmia in 24 to 48 hours. At autopsy characteristic encapsulated lanceolate-shaped organisms in pairs are found in the blood. Pigeons and fowls are immune. Experimental pneumonia has been produced in mice, monkeys and rabbits by injecting virulent cultures directly into the trachea

Virulence and viability.—Virulence is lost under cultivation, but can be regained by a series of passages through susceptible animals.

The identification of the type is a matter of considerable importance from the therapeutic aspect as an antiserum of Type I has beneficial effect on cases due to this type alone.

A smear from sputum or any other pneumococcal exudate is made. In the case of sputum the patient rinses his mouth thoroughly with salt solution before coughing and the sputum is received into a sterile dish. A piece of sputum is planted by a platinum loop into 3 or 4 Petri dishes containing sterile salt solution or broth and stirred gently about. The broth or salt solution is sucked into a syringe or if necessary ground in a sterile mortar with 1-2 c.c. of sterile salt solution or broth and 1 c.c. of this is inoculated into the peritoneum of a white mouse. In 7 to 24 hours after injection the mouse will die and the peritoneal cavity is then opened with sterile precautions and the exudate stained by Gram and examined. Cultures are also made on blood or serum plates for confirmation. If pneumococci are present in large numbers the peritoneal cavity is washed with 4 to 5 c.c. of salt solution with a pipette provided with a rubber teat and the salt solution washings are centrifuged at low speed to throw down cells and fibrin. After drawing off the supernatant fluid into another tube and throwing down the organisms by centrifuging at high speed, the deposit is emulsified in saline solution to make a moderately dense suspension. The agglutination test is carried out as follows:—

A row of five agglutination tubes is set up and the following measured out:—

Tube 1—
0·5 c.c. of serum type I (1 in 20) + 0·5 c.c. of bacterial suspension.

Tube 2—
0.5 c.c. of serum type II (undiluted) + 0·5 c.c. of bacterial suspension.

Tube 3—
0·5 c.c. of serum type II (1 in 20) + 0·5 c.c. of bacterial suspension.

Tube 4—
0·5 c.c. of serum type III (1 in 5) + 0·5 c.c. of bacterial suspension.

Tube 5—
0·1 c.c. of sterile ox bile + 0·4 c.c. of bacterial suspension.

These are incubated at 37°C. in a water-bath for an hour after which the result of the test is noted. Tube 5 serves to separate the pneumococci from streptococci and to prevent the mistake of classifying a streptococcus as type IV pneumococcus.

Vaccines.—For protective inoculation against pneumonia the first injection should contain 4000 millions of the mixed types in 0.5 c.c. and the second and third doses 8000 millions or more in 1 c.c. Opinions differ in regard to the utility of vaccine in the treatment of pneumonia. A dose of 5 millions cocci should be given as soon as the disease is recognised, 10 millions on the next day and 25 millions on the day following. Vaccine is of distinct aid in cases of delayed resolution and broncho-pneumonia.

Serum.—An hour after producing antianaphylaxis with a subcutaneous injection of 0·5 c.c. of serum, the patient is given an intravenous injection of 90-100 c.c. of the serum diluted 50 per cent. with salt solution. This dose is repeated every eight hours until the temperature falls and the general condition improves. The serum which is warmed to the body temperature is injected very slowly. The serum treatment is of distinct value in type I pneumonia and of doubtful utility in injections due to types II, III and IV.

Diagnosis.—(*a*) *Sputum* obtained by ordinary precautions is—

(*i*) stained with Gram's stain and examined,

(*ii*) washed and diluted with saline and 1 c.c. injected into the peritoneal cavity of a mouse which dies within 20 hours. Culture of the organism may be obtained from the peritoneal fluid by plating,

(*iii*) planted on blood-agar plates.

(*b*) *Lung puncture.*—The fluid is withdrawn from the pneumonic lung with a 10 c.c. sterile syringe containing 0·5 c.c. of sterile saline and is examined by staining, culture and animal inoculation.

(*c*) *Pus* and *exudate* are examined as above.

Meningococcus

Synonyms.—*Micrococcus meningitidis; Neisseria meningitidis; Micrococcus intracellularis meningitidis; Neisseria intracellularis meningitidis; Diplococcus intracellularis meningitidis*—the causative organism of cerebro-spinal fever, discovered by Weichselbaum in 1887.

Morphology and Staining.—A small coccus (1 μ in diameter); occurs in pairs with the adjacent sides flattened; found free or within the polymorpho-nuclear leucocytes in the exudation. Non-motile; no spores. Stains easily with aniline dyes; Gram-negative.

Culture.—Obligatory ærobe, optimum temperature 37°C. Does not grow readily on ordinary media but requires blood, serum or ascitic fluid for its growth. The optimum reaction is one neutral to phenolphthalein. On these media the colonies appear as moist grey circular discs (2 mm. in diameter); after a further 24 hours' growth they may attain a diameter of 4 mm.; later the centre becomes opaque while the periphery remains transparent.

In serum bouillon it causes a general turbidity with a deposit.

Biochemical Reactions are tabulated on page 224.

Serological Reactions.—In some cases the patient's serum may agglutinate the organism in a dilution of 1 in 50 on the 4th day; later the titre may rise to 1 in 1000. Agglutination and saturation tests have been employed to identify the organism and to classify it, at least four types being known.

Types I and II represent 80 per cent. of all the cases. In order to obtain a satisfactory agglutinating serum an intravenous injection is made in a young rabbit, using a dose of 1000 millions cocci grown on 25 per cent. hæmoglobin serum agar and heated at 65°C. One hour later a dose of 500 millions is given. Six days later 3000 millions cocci are given. The serum is tested on the 8th day and if satisfactory the animal is bled on the following day. Such a serum will probably have a titre of 1 in 1200.

Pathogenesis.—Intracerebral or intrathecal injections of the organism may produce in monkeys a condition analogous to that occurring in man. Injected intraperitoneally or intravenously into mice, rabbits, guinea-pigs or dogs, it causes a fatal septicæmia.

Occurrence.—The organism is most commonly found in the cerebro-spinal fluid, nasopharynx and meninges and rarely in blood, conjunctiva, secretions from broncho or lobar pneumonia and otitis media.

Carriers.—The meningococcus has been shown to be present in the naso-pharynx of a certain number of contacts and healthy non-contacts. It is suggested that such persons serve to maintain the source of infection.

Vaccine.—Immunisation with polyvalent saline vaccines of meningococci in three doses of 2000 millions, 4000 millions and 8000 millions by subcutaneous injection at weekly intervals has been reported to be of value in the prophylaxis of this form of meningitis.

Serum.—Horses are immunised by repeated injections of increasing doses of dead culture of several strains of the meningococcus followed by injections of old autolysed cultures and of living cultures. Intraspinal injection of 30 c.c. of such a serum repeated on subsequent days in an adult has a favourable influence on the course of the disease.

Diagnosis.—1. *Cases of Cerebro-spinal Fever.*—(*a*) Microscopical examination.—The cerebro-spinal fluid obtained by lumbar puncture is centrifuged at once and smears are made with the deposit. After drying and fixing some films are stained with Löffler's methylene-blue or Leishman's stain and some by Gram's method. The presence of Gram-negative diplococci either free or within the polymorpho-nuclear leucocytes is practically diagnostic of a case of cerebro-spinal fever.

(*b*) Cultures are obtained by smearing some of the lumbar fluid or deposit on blood agar or serum agar and incubating at 37°C. The colonies are examined microscopically after 24 or 48 hours. A mixture of equal volumes of the fluid and 1 per cent. glucose broth is also incubated at 37°C. In cases where no organism can be seen or isolated a provisional diagnosis may be made by the presence of a large number of polymorpho-nuclear leucocytes in the cerebro-spinal fluid.

2. *Carriers.*—The material obtained by swabbing the naso-pharynx is cultured as described above. Special post-nasal swabs (West's) are required; the medium must be inoculated at once as the organism dies rapidly and the medium must be incubated at once or kept warm till it can be placed in the incubator. Three successive negative reports are necessary before a carrier can be considered as free from infection.

Gonococcus

Synonyms.—*Neisseria gonorrhœæ; Micrococcus gonorrhœæ; Diplococcus gonorrhœæ*—the causative organism of Gonorrhœa, described by Neisser in 1879.

Morphology and Staining.—A small coccus (0·7 μ × 0·5μ ovid in shape; occurs in pairs with the opposed surfaces flattened or concave; the appearance has been compared to two kidneys or haricot beans. In gonorrhœal pus, gonococci are found sometimes free but more often situated within the pus or epithelial cells. This intracellular position is one of the important morphological characteristics of the organism. It also stains with basic dyes such as methylene-blue and is Gram negative.

Culture.—Aerobe; temperature range—22° to 39°C. Optimum—36° to 37°C. Does not grow on ordinary media and requires blood or serum for its growth. The best medium is an agar tube or plate smeared with human or rabbit's blood (Pfeiffer's medium for growing the influenza bacillus).

Colonies are semi-transparent discs about the size of pin head and tending to remain more or less separate. The margin is at first circular but later shows undulations.

Biochemical Reactions are tabulated on page 224.

Serological Reactions.—The *agglutination and absorption reactions* have been employed to classify the various gonococcus strains into a number of serological types. The *complement fixation test* has been found to be a useful diagnostic method. The test fails frequently in acute gonorrhœa and in mild and localised cases of the disease and also in "carriers." The test is inadmissible in patients who have been treated with vaccines. In women the reaction is seldom positive until the infection has reached the cervix. The test may replace examination of the genito-urinary passages which is frequently objected to by women in India, the removal of blood from one of the arms not being objected to.

Pathogenesis.—Gonorrhœa is essentially a disease of man and it is impossible to reproduce it in the lower animals though injection of pure cultures into the peritoneum of white mice and into the joints of rabbits, dogs and guinea-pigs produces a slight amount of inflammatory reaction which however soon subsides.

Occurrence.—In the male urethra the organism causes a suppurative inflammation with purulent discharge, in which the gonococci are present in very large numbers; later on the disease becomes chronic and the organisms decrease in number and are associated with other pyogenic cocci. They may infect the prostate, seminal vesicles, bladder mucosa and peri-urethral tissues. In the female the urethra, the cervix uteri, and Bartho-

lini's glands, the endometrium and Fallopian tubes and peritoneum and rarely the vaginal mucosa, may be infected. Purulent conjunctivitis and arthritis may occur as complications. The organism is occasionally met with in the blood and causes pneumonia, pericarditis, endocarditis and meningitis.

Vaccines.—These have been used extensively in the treatment of a variety of conditions due to gonococcus and the results vary greatly. They appear to have been useful in cases of chronic gonorrhœal rheumatism. The first dose should be about 10 millions, the injections being given once a week in increasing dosage.

Serum is sometimes useful in acute gonorrhœal complications such as epididymitis, prostatitis and orchitis, etc., and should be given early and intravenously in a dose of 30 to 50 c.c. repeated in about 12 hours.

Diagnosis.—The routine procedure should be as follows:—
A.—*The Collection of Material.*—In the Male.—The patient is examined about six hours after he has last passed urine.

(*a*) The meatus is swabbed clean and secretion expressed from the deeper parts of the urethra. Films and cultures are made from this.

(*b*) The first ounce of urine passed by the patient is received into a sterile tube. This is centrifuged and the deposit examined for gonococcus by microscopic and cultural methods.

(*c*) The prostate, seminal vesicles and Cowper's glands are massaged and films made of any secretion appearing at the meatus.

(*d*) In cases of infection of the kidney samples of urine are obtained by ureteral catheterisation.

In the Female:—

(*a*) The meatus is swabbed, the urethral canal massaged forward with the finger through the anterior vaginal wall and films made of any discharge that appears at the meatus; or a platinum loop is introduced into the urethra.

(*b*) The cervix is exposed with a speculum, cleaned with a swab and a platinum loop passed into the cervical canal. Films and cultures should be prepared of any material thus obtained.

(*c*) The contents of Bartholini's glands should be expressed.

In chronic cases in females positive results are more often obtained if the examination is carried out immediately after the menstrual period.

B.—*Examination of Material.*—1. Microscopic examination.—Uniform films of the material are stained with Löffler's methylene-blue or Leishman's stain, while other films are stained by Gram's method, or better, by Jensen's modification of the Gram.

A diagnosis cannot be based simply on the presence or absence of intracellular diplococci. It must be shown that the characteristic organisms are Gram-negative.

A positive diagnosis is justified by the film alone. A negative microscopical examination is not sufficient for diagnosis, and the result must be confirmed by cultural and other methods.

2. Cultural methods.—The material obtained as described above is cultured on blood-smeared agar and further tests carried out to identify the culture. For differential characters of the chief Gram-negative cocci, see the table given on the next page.

3. Complement-fixation test.—This is conducted in exactly the same manner as the syphilis reaction herein described. The antigen consists of many different strains of gonococci.

The Brucella

This group of organisms includes *B. melitensis* and *paramelitensis, B. abortus, B. bronchisepticus* and the *bacillus of guinea-pig pneumonia.*

B. melitensis

Synonyms.—*Brucella melitensis; Micrococcus melitensis; Alcaligenes melitensis*—described by Bruce as the cause of Malta fever (Mediterranean fever, Undulant fever).

Morphology and Staining.—This is a minute cocco-bacillary organism (0·4 μ in diameter) occurring singly or in pairs or short chains. It is non-motile, non-sporing, stains readily with basic dyes and is Gram-negative.

Culture.—Ærobe and facultative anærobe; optimum temperature 37°C.; grows well on ordinary media.

Agar.—The colonies which are usually not visible before the 3rd or 4th day in primary cultures are small transparent discs. The growth is more rapid and abundant when the organism has been grown for sometime on artificial media.

Gelatin.—Growth is scanty and slow. No liquefaction.

THE CHARACTERS OF THE CHIEF GRAM-NEGATIVE COCCI

(*After Gordon*)

Organism or source.	Growth on nutrose ascitic agar at 37°C.	Growth on gelatin at 20°C.	Pathogenicity.	Glucose.	Galactose.	Maltose.	Saccharose.
M. catarrhalis. Nasal and pharyngeal discharge.	Opaque, granular. ..	Positive (grows on ordinary agar at 37°C.).	Mice and guinea-pigs by intraperitoneal inoculation only.	o	o	o	o
M. intracellularis (Meningococcus.) Cerebro-spinal meningitis.	Clear, smooth or no growth.	Negative.	In some monkeys, mice and guinea-pigs by intraperitoneal inoculation only.	+	o	+	o
M. gonorrhœæ (gonococcus). Urethral discharge.	No growth unless blood added.	Negative.	*ib.* ..	+	+	o	o
M. (*B.*) *melitensis.* Malta fever (probably actually a bacillus).	Creamy and slightly yellowish.	Positive.	Monkeys; also rabbits and guinea-pigs by intracerebral inoculation.	o	o	o	o

+ = acid, o = no reaction.

Gelatin stab.—In 2 to 3 weeks growth occurs along the line of puncture and as a small flat depression on the surface.

Bouillon.—Diffuse turbidity with flocculent deposit without a pellicle on the surface occurs.

Potato.—No visible growth.

Serological Reactions.—Agglutinins appear in the blood of Malta fever patients early and the test is usually employed in diagnosis.

Pathogenesis.—Rabbits, guinea-pigs and mice are generally insusceptible to intravenous or subcutaneous inoculation. Intraperitoneal injection or even an intravenous injection of an organism, the virulence of which has been increased by intracerebral inoculation, causes illness in the various animals with, at times, a fatal result. The disease has been reproduced in monkeys by inoculation of a culture. Disease in man occurs as a septicæmia with rise of temperature and enlargement of spleen. The disease is transmitted from man to man by the infected milk of goats.

Occurrence.—The organism occurs in the blood, urine and fæces and in the milk of infected goats.

Vaccines and Sera have been employed in the treatment and prevention of undulant fever but with indifferent results.

Diagnosis.—*During Life.*—The agglutination test forms the readiest means of diagnosis. Normal blood serum may agglutinate *B. melitensis* in a dilution of 1 : 100 or in 1 : 200 but in the disease the reaction occurs with a much higher dilution, *e.g.*, 1 : 500 or even more. If the serum of the patient is heated to 55°C. before applying the test the normal agglutinating effect may be destroyed. In a small number of cases the infection is due to *B. paramelitensis* which is serologically different from *B. melitensis.* In such cases the serum may not agglutinate *B. melitensis* but will agglutinate the *B. paramelitensis.* The diagnosis of the infection in goats can also be made by the agglutination test, using the animal's serum.

Culture.—(*a*) Blood cultures are positive very early in the disease; later the organisms appear in the urine and fæces. Five c.c. of the blood, obtained by puncture, is incubated in 250 c.c. of broth with or without bile. The broth culture is plated on

agar plates and a few colonies subcultured on agar. It is best to examine the blood during the height of the fever.

(*b*) The pulp obtained by spleen puncture may be cultured and subsequently the growth subcultured.

(*c*) The organism may be recovered from the urine and fæces of patients and from milk of infected animals.

Post-mortem.—Scrapings of the internal organs such as the spleen, liver and kidneys are cultivated in broth which is plated after incubation at 37°C.

The identification of the organism is based on the following characteristics:—

1. Gram-negative, non-motile coccus.

2. Non-fermentation of sugars, non-coagulation of milk; alkalinity in litmus milk.

3. Agglutination test with a specific serum.

It is necessary to differentiate it from *B. abortus.*

Note.—Great care must be exercised in handling melitensis cultures as cases of laboratory infection with the organism are not uncommon.

B. abortus

Synonyms.—*Alcaligenes abortus; Brucella abortus*—bacillus of cattle abortion, described by Bang in 1897 as the cause of bovine infectious abortion.

Morphology and Staining.—*B. abortus* is a short, slender, pleomorphic, non-motile, non-sporing and Gram-negative bacillus.

Culture.—In primary culture it does not grow on ordinary media. It grows under conditions of partial anærobiosis, which are obtained by cultivation in a closed jar in the presence of *B. subtilis* cultures. Glycerin and serum media are favourable for making primary cultures. After continued cultivation it grows readily on ordinary media.

Agar.—Colonies appear as dew drops in 2 to 3 days.

Gelatin.—No liquefaction.

Potato.—Scanty greyish-brown growth after several days. The medium is discolored subsequently.

Bouillon.--Slight cloudiness of the medium.

Milk is rendered alkaline.

Biochemical Reactions.—*B. abortus* like *B. melitensis* does not attack any of the carbohydrate media.

Serological Reactions.—*B. abortus* can be distinguished from *B. melitensis,* to which it is closely related, by its agglutination reaction. The sera of infected cattle have an agglutinating effect on the organism.

Pathogenesis.—*B. abortus* causes epidemic abortion in cattle with little general effects. Intravenous inoculation of the culture into pregnant cattle, guinea-pigs and rabbits may also lead to abortion in these animals. Experimental infection in guinea-pigs produces characteristic lesions not unlike tubercles in about 8 to 10 weeks and it is important to remember this fact when making routine injection of milk into guinea-pigs for the detection of *B. tuberculosis.*

The injection of *B. melitensis* of human origin causes abortion in pregnant guinea-pigs and cow. The organisms, however, differ in their pathogenicity to monkeys, *B. abortus* being less virulent than *B. melitensis.* The majority of the strains obtained from cattle and pigs are of *B. abortus* though a few of the strains isolated from human cases also belong to this type. As a further evidence in support of the close relationship of these organisms it may be pointed out that cases of malta fever are recorded in which the infection could not be traced to goats and presumably belonged to the abortus type.

B. bronchisepticus

This bacillus is the cause of canine distemper described by Ferry and M'Gowan. It is a short, Gram-negative, slightly motile, bacillus.

It grows readily on glycerin agar and produces diffuse turbidity in broth. In litmus milk it produces progressive alkalinity, beginning with a deep blue ring at the surface and ending with blue black discoloration of the whole of the medium in 5 to 10 days. On potato the growth is yellowish-brown in 24 hours with a greyish darkening of the medium. The organism produces a hæmolysin for guinea-pig and rabbit red corpuscles.

The *bacillus of guinea-pig pneumonia* described by Smith is a motile, Gram-negative rod.

B. Pyocyaneus

Synonym.—*Pseudomonas æruginosa*, Migula—described by Gessard in 1882 as the causative organism of a peculiar type of purulent inflammation associated with the development of pus of a bright green colour.

Morphology and Staining.—The organism is a short rod (1·5μ to 3μ by 0·5μ); occasionally it forms short chains and pairs. It is actively motile, possessing a single flagellum at one end and does not form spores. It stains with ordinary dyes and is Gram-negative.

Culture.—Aerobe and facultative anærobe; grows well on ordinary media at room temperature. The cultures produce a green pigment which may penetrate the media.

Agar.—The growth is an abundant slimy greyish layer which afterwards turns green. There is a diffuse bright green discoloration of the medium.

Gelatin stab.—Greyish-yellow colonies appear with liquefaction. The colonies form shallow cups with flocculi of growth at the bottom and around the colonies a greenish tint appears.

Potato.—The growth, not unlike that of glanders, is an abundant reddish-brown layer.

Broth.—Diffuse cloudiness of the medium occurs with a thick pellicle and pigment formation at the surface.

Milk is coagulated and becomes greyish in colour.

The pigment is due to "pyocyanin" which can be extracted from cultures with chloroform and crystalised in the form of long, delicate, bluish-green needles.

Pathogenesis.—*B. pyocyaneus* is pathogenic to rabbits, goats, mice and guinea-pigs. Subcutaneous injection of small doses in rabbits produces a local abscess; large amounts cause a spreading hæmorrhagic œdema which may be associated with septicæmia and death.

Toxins.—A ferment-like body "pyocyanase" has been isolated by filtering old broth cultures of *B. pyocyaneus*. The pyocyanase destroys certain other bacteria by lysis and has been found to possess curative and preventive properties for anthrax and diphtheria infections. This substance is thermostable.

Old broth cultures also contain a substance "pyocyanolysin" which hæmolyses the red blood cells of sheep, dogs and rabbits.

Occurrence.—*B. pyocyaneus* is widely distributed in nature. It is found in water, dung, soil and in the effluent from filter-beds and may occur as a saprophyte upon the skin and in the upper respiratory tracts of men and animals. It is associated with suppuration of the various parts of the body and may cause diarrhœa with green stools in children. It may be cultivated from the blood in some cases of marasmus in children and in cases of general sepsis.

Bacillus tuberculosis

Synonyms.—*Tubercle bacillus; Bacillus of Koch; Mycobacterium tuberculosis*—the causative organism of tuberculosis, discovered by Koch in 1882. There are three main types, namely, the human, bovine and avian.

A.—Human Type

(*Mycobacterium tuberculosis*)

Morphology and Staining.—A thin, straight or slightly curved rod-shaped bacillus (2 to 5μ by 0·3μ). In old cultures the organisms may present a segmented or irregularly-stained appearance or may grow out into long filaments and show branching. It is non-motile, has no spores, does not stain with the ordinary stains and is Gram-positive. The most useful staining method is the Ziehl-Neelsen method. The "acid-fastness" is due to the waxy covering of the organism.

Culture.—Aerobe; temperature range 30° to 41°C; optimum 37° to 38°C. *B. tuberculosis* may be cultured directly from the tissues, *e.g.*, glands, etc., but the sputum, pus, etc., should be treated with antiformin in order to destroy the more rapidly growing contaminating organisms.

Dorset's egg medium and *Glycerin egg medium* are the best for primary cultures. The growth, which becomes visible in 3 to 7 days, is abundant, buff-coloured and has a dry wrinkled appearance.

Blood serum.—Minute dry scales with corrugated surface appear at the end of 8 to 14 days in primary culture. The growth is more luxuriant in subcultures.

Glycerin agar.—Growth is similar to that on blood serum.

Glycerin potato.—A dry wrinkled growth results. This medium is suitable for primary cultures.

Glycerin broth.—The growth consists of a dull wrinkled surface pellicle or white flocculi which later sink to the bottom, forming a powdery deposit leaving the broth clear.

Serological Reactions.—Though agglutinins appear in the blood of tuberculous patients the test is not much employed on account of the difficulty of obtaining a uniform emulsion of the culture. A complement-fixation test has been devised for diagnosis but the results vary considerably. The chief difficulty consists in obtaining a suitable antigen.

Pathogenesis.—The guinea-pig and the rabbit are susceptible to experimental infection. In about 10 days after inoculation with the organism a swelling appears at the site of inoculation consisting of hard nodules which later undergo caseation and ulcerate. The inguinal glands become involved, the animal wastes and dies in 1 to 3 months. At autopsy, tubercles and areas of caseation and necroses are seen in the internal organs, especially the spleen and liver. There is a tuberculous lymphadenitis. Bacilli can be demonstrated in smears from glands, spleen, etc.

Occurrence of Tubercle Bacilli in Human Lesions.—Both the human and bovine organisms may be recovered from human lesions, the latter being commonly found in cases of cervical adenitis, abdominal tuberculosis and generalised tuberculosis of the abdominal organs in children and rarely in adults except in young subjects who suffer from tuberculosis of the intestine as a result of the infection by milk from tuberculous cows. In pulmonary tuberculosis the human type is generally found. Tubercle bacilli are found in the sputum in pulmonary tuberculosis, in the urine in the tuberculosis of the genito-urinary system, in the spinal fluid in tuberculous meningitis, in the fæces in intestinal tuberculosis and in the discharges of lesions occurring in the skin, bones, joints and lymph glands.

B.—Bovine Type

(*Mycobacterium bovis*)

The morphological and staining characteristics of the bovine type are identical with those of the human type. The differ-

ences between the two types in their cultural characters and pathogenicity are shown below:—

	Human Type.	Bovine Type.
1. Morphology ..	Larger and thinner in size.	Shorter and thicker in size.
2. Culture ..	Growth is luxurious. (Eugonic).	Growth less luxurious. (Dysgonic)
(*a*) Dorset's egg medium ..	Abundant, dry wrinkled growth, yellowish-buff pinkish, not easily broken.	Thin, slight, moist, smooth growth which is easily broken up.
(*b*) Glycerin egg medium ..	Abundant growth.	Slight and no growth—especially in primary culture.
3. Pathogenicity ..	Less virulent to bovines and laboratory animals. In the ox it causes only a localised lesion; intravenous injection of 0·1 mgm. of the culture in 1 c.c. saline into a rabbit does not kill it.	More virulent to bovines and laboratory animals. It causes fatal tuberculosis. Intravenous injection of 0.1 mgm. of the dried culture in 1 c.c. of saline into a rabbit produces an acute generalised tuberculosis which ends fatally in about 2 months.

C.—Avian Tuberculosis

Synonym.—*Mycobacterium avium*—the causative organism of tuberculosis in birds, and corresponds with the mammalian type in morphological and staining characters.

The growth on glycerin agar and on serum is more rapid and luxuriant at 41° to 45°C. whilst the human type is unable to thrive at a temperature above 41°C. The mammalian type moreover does not cause disease in fowls. Guinea-pigs which are very susceptible to the human type are relatively immune to the avian infection.

D.—Tuberculosis in the Fish

Synonym.—*Mycobacterium piscium.*

This is identical with the human organism in many respects. It grows at low temperatures, 15° to 30°C., and is non-pathogenic for mammals.

Vaccines.—"Tuberculin" was the name given by Koch to the filtered and concentrated glycerin broth culture of tubercle bacilli which is now principally used in diagnosis and called

Koch's "old tuberculin." The injection of 0·002 c.c. into a guinea-pig with tubercle gives rise to pyrexia and laboured breathing and is very apt to light up a dormant infection. The injection of 0·1 to 0·2 c.c. into tuberculous cattle causes in about 12 hours a rise of 2°F. in the temperature.

Tuberculin-R is sold as a standardised fluid containing 2 mgm. of solid matter in 1 c.c. and is diluted to the required dosage with sterile salt solution.

The preparations most frequently employed are Koch's old tuberculin, (Tuberculin-O), Tuberculin-R, Koch's new tuberculin, Deny's tuberculin and Beraneck's tuberculin. The most favourable cases for treatment appear to be those in which regressive changes have already begun in the infected tissues and which have no pyrexia. Tuberculin must be given in doses which are so small that no general reaction follows and a common dose to begin with is 1|20000 mgm. (Tuberculin-R). This is given in very slightly increased doses at weekly intervals for 4 to 6 doses but the dosage and the intervals depend upon the condition of the patient. Calmette has reduced the virulence of the tubercle bacillus by growing it for over 230 generations in three years on a medium of 5 per cent. glycerin potato saturated with beef bile. This modified organism is called Bacillus *Calmette-Guerin* (B. C. G.) and is employed to vaccinate children as a prophylactic measure. It is supplied by the Pasteur Institute, Paris, and may be given to new born infants within the first ten days of life. Three doses are given at twenty-four hours' intervals in milk at body temperature half an hour before a meal.

Serum.—Many sera have been employed in the treatment of tuberculosis but none are of decided value.

Diagnosis.—*Microscopic Examination.—Sputum, pus and fæces.*—A film is made and stained by the Ziehl-Neelsen method. If bacilli are scanty or absent the sputum must be treated with antiformin, *i.e.*, a mixture of equal parts of liquor sodæ chlorinatæ (B. P.) and of a 15 per cent. solution of caustic soda. One part of sputum is added to 2 or 3 parts of 20 per cent. antiformin and the mixture is shaken occasionally. When the sputum has become liquid, the mixture is centrifuged for an hour. Films are made from the deposit and stained. Petroff's method consists in mixing equal parts of sputum and 3 per cent. sodium hydrate solution and incubating the mixture at 37°C. for 20 to 30 minutes to liquefy the sputum. Hydrochloric acid is then added to neutralise the alkali, the mixture centrifuged and films are made from the deposit and stained as before. *Urine, pleural, peritoneal*

and cerebro-spinal fluids are centrifugalised, films made from the deposit and stained with Ziehl-Neelsen. To avoid risk of contamination with smegma bacilli, clear the meatus of the urethra with a swab, reject the first part of the urine and treat the film with alcohol (2 minutes) after decolorisation with acid (Ziehl-Neelsen method) or draw off the urine with a sterile catheter and stain the film in the ordinary way. If sputum has to be sent by post or messenger a little 1 in 20 carbolic acid should be added to it.

Culture.—Material from glands or other tissues which are not likely to be contaminated are grown directly on Dorset's egg medium. For the isolation of tubercle bacilli from sputum and other contaminated materials Petroff's sodium hydroxide method mentioned above may be used and then hydrochloric acid added to make the mixture neutral to litmus and the centrifuged deposit is inoculated into Petroff's medium which consists of meat-juice, egg and gentian-violet. The best method, however, of obtaining pure cultures is to inoculate the tubercular material into a guinea-pig and then to inoculate suitable media with portions of a resulting tubercular focus, *e.g.*, spleen.

Animal Inoculation.—If the material to be tested is contaminated, it should be treated with antiformin before the guinea-pig is inoculated or inoculation should be made subcutaneously. If the material is not contaminated or has been treated with antiformin it should be injected into the peritoneum. Inject ½ to 1 c.c. of the material under the skin on the inner side of the right leg.

The Tuberculin Reaction.—This is carried out in various ways:—

(*a*) *Subcutaneous method* (Koch).—Koch's old tuberculin is employed, being injected subcutaneously in a dose of 0.0001 c.c. and the temperature taken at four-hourly intervals. In tuberculous patients a local inflammatory reaction with a temperature of 2° to 3°F. results and a subsequent focal reaction. This method is not safe.

(*b*) *Cutaneous test* (von Pirquet).—After sterilisation of the patient's fore-arm, two drops of a 25 per cent. solution of old tuberculin are placed upon the skin about 4 inches apart. Scarification is done through these drops and also in the skin between them so as to serve as a control. Within 24 to 48 hours in tuberculous individuals a reaction occurs, consisting of a distinct inflammatory papule or even vesicles. About 70 per cent. of adults show a reaction and this diminishes the value of the test. Moro has modified the method by applying tuberculin to the skin in the form of 50 per cent. ointment in lanolin.

(*c*) *The ophthalmic tuberculin reaction.*—Calmette instils into the eye a drop of 1 in 100 solution made from the deposit obtained by adding alcohol to tuberculin. In tuberculous patients this causes conjunctivitis in 6 to 16 hours.

B. tuberculosis has to be distinguished from other acid-fast organisms such as:

1. *The Smegma bacillus.*—(*Mycobacterium smegmatis: Bacillus smegmatis*).—This occurs as a saprophyte in the region of the external genitals and resembles the tubercle bacillus very closely in size and appearance. It is not pathogenic to animals. For primary culture it requires human serum or hydrocele fluid. The organism is decolorised after treatment with alcohol unlike *B. tuberculosis* which is alcohol-fast as well as acid-fast.

2. *Bacillus butyricus*—found in milk and butter. The organisms grow readily on agar and on gelatin without liquefaction and are slightly pathogenic to guinea-pig on massive inoculation but not to man.

3. *Bacillus of Timothy.*—(*Mœller's Grass bacillus, Mycobacterium phlœi*) isolated by Mœller from infusions of Timothy grass hence called "Timothy-grass bacillus" and also from other grasses. They grow readily on agar, showing a deep red or dark yellow colour. They are less acid-fast than the tubercle bacillus. The mist bacillus isolated from dung is believed to be identical with the Timothy grass bacillus.

4. *Johne's bacillus* (*Mycobacterium paratuberculosis* or the bacillus of chronic bovine pseudo-tuberculous enteritis) is an acid-fast organism resembling the tubercle bacillus in morphology. The bacilli occur in large numbers in scrapings from the affected mucous membrane of the small intestine and are pathogenic to goats but not to guinea-pigs. On ordinary media growth does not occur.

Bacillus lepræ

Synonym.—*Mycobacterium lepræ*—the causative organism of leprosy in man, discovered by Hansen in 1871.

Morphology and Staining.—As seen in scrapings of leprous nodules and in section of the affected tissues, the organism appears as a small rod (5μ to 7μ) and resembles *B. tuberculosis* in morphology and staining. It is acid-fast, non-motile and non-sporing.

Culture.—The leprosy bacillus has not been successfully cultivated on any artificial medium.

Occurrence.—*B. lepræ* occur in enormous numbers in the spherodial cells where they often lie parallel to one another in the bundles which compose the leprous nodules. They are also found in the internal organs, *i.e.*, liver and spleen in advanced cases and occasionally in nerves and nerve cells. Their spread is by lymphatics though distribution by blood may also occur.

Pathogenesis.—Attempts to transmit leprosy to animals have failed. Recently, however, inoculation of monkeys with tissues from infected human beings was found to cause a nodule at the site of inoculation which contained leprosy bacilli. A leprosy-like disease occurs naturally in the rat wherein nodules are found in the skin and internal organs which contain large numbers of acid-fast organisms. The disease can be experimentally transmitted from rat to rat by inoculation with the tissue juices containing the bacilli.

Vaccines.—A large number of vaccines have been tried in the treatment of leprosy but with only partial success.

Diagnosis.—1. A leprous nodule is clamped, pricked and films made from the juice. Films of the discharge from any ulcerated nodule or from nasal secretion can be made. Ziehl-Neelsen's stain is used. The occurrence of large numbers of acid-fast bacilli and alcohol-fast within the cells is conclusive of leprosy.

2. In stained sections the bacilli should be found in the "leprous cells."

3. In distinguishing *B. lepræ* from *B. tuberculosis* it may be remembered that *B. lepræ* cannot be cultivated by methods which are employed for the successful cultivation of *B. tuberculosis*. Moreover, leprosy cannot be transmitted to guinea-pigs by inoculation.

Bacillus mallei

Synonym.—*Pfeifferella mallei*—discovered by Löffler and Schutz in 1882—the causative organism of glanders in horses, asses, mules and rarely in man.

Morphology and Staining.—*B. mallei* is a minute, straight or slightly curved rod with round ends measuring 3 to 4 μ by 0·5 to 0.7 μ; non-motile and non-sporing; stains readily with basic dyes and is Gram-negative.

Culture.—Aerobe, grows readily on ordinary media at 37°C. In *agar* (*Glycerin agar*) the growth is white in colour and slimy in consistence. In *serum* growth is similar to that on agar but more transparent. In *gelatin* growth is scanty, greyish-white; no liquefaction. In *broth* at first a uniform turbidity; In *potato* growth is characteristic. On potato which is not too acid the growth is rapid and by the third day covers the surface as a yellowish, transparent, glistening layer, like clear honey in appearance.

Serological Reactions.—The sera of glandered animals contains agglutinins capable of reacting with *B. mallei* and a distinct flocculation within 36 hours with a serum dilution of 1 in 1000 may be considered diagnostic. The *fixation of the complement test* in the diagnosis of glanders has given valuable results.

Pathogenesis.—Spontaneous infection occurs in horses, asses and mules. Infection in these animals takes place through the broken skin (inoculation), the mucosa of mouth (ingestion) and nasal passages (inhalation). The disease in horses occurs in two forms; acute and chronic. In acute glanders there is ulceration of the nasal mucous membrane and swelling of the neighbouring lymph glands which may ulcerate with sinus formation. Rounded, firm or shotty nodules, consisting of a collection of necrosed leucocytes and young connective tissue usually develop in the lungs and death takes place within 4 to 6 weeks.

In the chronic form with a gradual onset known as "Farcy" the inflammation of the nose is attended with subcutaneous swellings all over the body. These swellings break down and ulcerate. The lymphatics become involved and the disease may last for several years and, rarely end in recovery.

Cutaneous wounds and abrasions in men coming in contact with horses may be infected with "farcy" pus or the nasal discharge of a glandered animal. At the site of infection an inflammatory swelling appears accompanied by lymphangitis, ending fatally in 8 to 10 days. The chronic form in man resembles that in horses. Inoculation of animals with cultures of *B. mallei* reproduces all the features of glanders in horses. Of the small animals, field mice and guinea-pigs are extremely susceptible; house mice and white mice are immune. The injection of a glanders culture into the subcutaneous tissue of a field mouse causes death within eight days with the formation of minute nodules in the spleen, liver and lungs. In the guinea-pig the infection is less acute, an inflammatory nodule appears at the site of

inoculation, ulcerates, the corresponding lymph glands become involved and the animal dies within 2 to 3 weeks.

Toxins.—*B. mallei* produces a powerful endotoxin (mallein) which is extremely resistant to heat and to prolonged storage. Mallein is prepared from virulent cultures of glanders bacilli in glycerin bouillon. Such a culture after being allowed to grow for 4 to 5 weeks at 37°C. in flat flasks is autoclaved for 15 minutes at 115°C. or steamed and filtered through a Chamberland filter. The filtrate which constitutes "mallein" is evaporated to $\frac{1}{3}$rd its original volume on a water-bath. To the filtrate is added an equal quantity of 0·5 per cent. carbolic acid as a preservative. The injection of 1 c.c. of the mallein into unglandered horses has little or no effect; on the other hand, in glandered animals the injection is followed within 6 to 8 hours by a sharp rise of temperature to 104° to 106° F. At the point of inoculation a large, painful swelling appears which may last 3 to 9 days. The focal reaction is shown by the increased swelling of the affected lymph glands. Injections are made into the breast or side of the neck.

Diagnosis of Glanders.—

(1) Films from the pus or discharge are stained with Löffler's methylene-blue.

(2) Cultures on blood serum and potato are made.

(3) Animal inoculation is required to confirm the diagnosis; an intraperitoneal injection of a quantity of the discharge or of the material is made into a male guinea-pig: a characteristic swelling of the testicle occurs in 3 to 5 days and *B. mallei* will be found in large numbers in the tunica vaginalis.

(4) *Mallein test.*—A few drops of mallein are instilled into the eyes. After 24 and 48 hours a discharge from the eyes and a swelling of the eyelids occur.

(5) *The agglutination test* is fairly reliable in animals but not in man. Animal serum agglutinates the organism in high dilutions (1 in 500).

Bacillus whitmori

Synonyms.—*Bacterium whitmori; Pfeifferella whitmori*—the causative organism of melioidosis in man, described by Whitmore in 1913.

B. whitmori resembles closely *B. mallei* except that it is actively motile, grows on gelatin at 20°C. with liquefaction of

the medium and forms a pellicle in bouillon. It grows well on ordinary media. On agar it forms a wrinkled or mucoid growth and the strains of the latter type produce on potato a growth not unlike that of glanders. It ferments glucose and sometimes other sugars with gas formation.

Introduced into susceptible animals such as rats and rabbits by feeding or scarification or by the application of cultures to the nasal mucosa, it produces a discharge from the eyes and nose and numerous nodules in the internal organs. The horse appears to be immune. *B. whitmori* is serologically identical with *B. mallei.*

Actinomycosis

1. *Nocardia bovis*

Synonyms.—*Actinomyces; Streptothrix; Oospora bovis; Streptothrix actinomyces.*

Morphology and Staining.—On cutting into actinomycotic tissues such as the tongue, a grating sensation is felt and on examining the section with the naked eye or under the low power little yellow masses or colonies are seen. When fully developed these colonies which are of *Nocardia bovis* may be of the size of a small pin's head and may lie free in pus or in the absence of suppuration may be embedded in the granulation tissue. Under the high power the colonies are found to be composed of a dense felted mass of filaments which, about the periphery, become gradually thickened to form elongated pear-shaped bodies ("clubs") which are arranged radially. The filaments show true branching. At a later stage, when the growth becomes dense, no filaments may be seen and the centre presents a structureless appearance. The filaments and the club-shaped bodies in the young colonies are Gram-positive. In culture the filaments become segmented into round spores or conidia, which are more resistant than the filaments. They are Gram-positive and are regarded as reproductive structures. Cultures of the organism isolated from cases of actinomycosis in man do not show conidia.

Culture.—*Nocardia bovis* is an ærobe and grows well on ordinary media at 37°C.

On *agar* or *glycerin agar* transparent colonies are formed in 3 to 4 days. These enlarge gradually, become nodular and crateri-form in appearance, reddish-yellow in colour and tough

in consistency so that they can with difficulty be picked off the medium. On *maltose agar* the growth has a fawn colour, later turning to yellow or even black. On *gelatin* small yellowish masses of growth with slow liquefaction of the medium begin from about the 7th day. The liquefied medium has a brownish colour and a syrupy consistence. *Broth* remains clear with a dry pellicle of growth on the surface or with a deposit of colonies at the bottom. On *potato* a rounded raised hard adherent growth at first white but later turning to yellow, brown or even black occurs.

Pathogenesis.—*Actinomyces bovis* causes infection in cattle, swine, horses and rarely in man. In these animals it produces large tumour-like masses of granulation tissue containing colonies of the organism and slight pus formation. The nodules may become calcified in swine. Guinea-pigs and rabbits are susceptible to intraperitoneal inoculation of the organism. It is believed that the infection occurs through abrasions, the organism being present on grasses and in grain.

2. *Streptothrix actinomyces*

Synonym.—*Cohnistreptothrix israeli;* ray fungus—the causative organism of actinomycosis in man. Occasionally the infection is due to *Nocardia bovis.*

Cultural Characteristics.—The organism occurs in short or large rods. It grows well anærobically at 37°C. but poorly in the presence of oxygen. On agar, surface colonies under anærobic conditions look like dew-drops and later become yellowish. In a shake culture in glucose agar a dense zone of growth occurs about half an inch from the surface. No growth occurs in gelatin at the room temperature. In broth, growth occurs as a deposit of scaly particles at the bottom. When grown in a medium containing serum or other animal fluid the organism shows branched filaments with clubbed ends.

The disease in man occurs as a chronic inflammation with suppuration. Occasionally, as a result of the abundant formation of granulatin tissue, the nodules may feel hard. The internal organs such as the liver present a honeycombed appearance owing to the formation of numerous foci of suppuration at the periphery of the lesion. In the pus the parasites are visible to the naked eye. Intraperitoneal inoculation in rabbits and guinea-pigs is followed in 4 to 7 weeks by the formation of

granulomatous nodules of a non-progressive nature in which the characteristic actinomycotic colonies are found. In many human cases the organism gains entrance through a decayed tooth, the crypts of the tonsil or an abrasion in the mucous membrane of the intestine. Abscesses may occur in the liver, spleen, kidney and bones. It has been suggested that the organism may occur in the alimentary canal, penetrate the mucous membrane and settle in the tissues. The original source of the organism is believed to be grain, especially barley.

3. *Actinobacillus*

Synonym.—*Nocardia lignieresi* has been described as occurring in a form of actinomycosis of cattle in Argentina and also in England. The organism is a small (1.5 $\mu \times 0.4\ \mu$) Gram-negative, non-motile bacillus which grows well both ærobically and anærobically on ordinary media and does not liquefy gelatin. In primary cultures on agar it produces discrete, semi-transparent colonies. On subculturing a continuous layer of similar growth is produced. In glucose peptone it shows club-formation. Introduced subcutaneously into sheep and ox and intraperitoneally into guinea-pig it produces lesions in which the characteristic actinomycotic granules with clubs are present.

Other pathogenic forms of actinomyces occur in man.

4. *Nocardia asteroides*

Synonyms.—*Cladothrix asteroides; Streptothrix eppingeri,* isolated from a brain abscess, is a Gram-positive, acid- but not alcohol-fast fungus which grows on ordinary media and is pathogenic for laboratory animals. The growth has a yellowish-orange to brick-red colour.

Diagnosis.—*Microscopic examination.*—The pus or discharge is spread in a thin layer in a Petri dish and examined for any yellowish or other granules. If present, a few are picked off with the needle and placed on a clean slide in a drop of 50 per cent. glycerin; if not the pus is examined as a thin film by the $\frac{2}{3}$-in objective. The mycotic granules, if present, are seen as yellowish or brownish, ovoid or reniform masses. The grains are crushed between two slides in a drop of glycerin and examined under high power, when the central part is seen to consist of a tangled mass of filaments with perhaps stunted rods. (In the bovine variety rosettes of clubs occur).

Films of the discharge after fixation in alcohol-ether mixture are stained with Gram's stain and counter-stained with eosin. The filaments stain violet and the clubs yellow or pink. (The clubs in many cases of the human disease do not stain with Gram's method). Paraffin sections of the actinomycotic tissue may be stained by Gram's method with eosin or orange rubin as counterstains or by Ehrlich's hæmatoxylin method with orange rubin as a counterstain.

Culture.—A few grains are picked out with a platinum loop, washed in several changes of sterile normal saline, then shaken in absolute alcohol to kill off the other organisms and again washed in sterile normal saline. A grain is then planted into blood- or serum-broth and cultivated under paraffin to secure anærobiosis if necessary. The culture may be plated on glycerin- or glucose-agar media under ærobic and anærobic conditions.

5. *Madura disease*

The madura disease, otherwise known as mycetoma, madura foot or the " fungus disease of India " is not unlike actinomycosis. It was originally described in India by Carter in 1874, and is fairly common in various other parts of the tropics. The lesion produced by this disease is of the nature of a chronic inflammation which generally affects the foot, less often the hands and very rarely the other parts of the body. Hypertrophy and swelling of the soft structures, nodular swelling later resulting in ulcers and sinuses and caries of the bones occur. In the cavities, the spaces between the fibrous tissues and in the purulent fluid which exudes from the sinuses, characteristic granular bodies are present. Two principal varieties have been described depending upon the colour of the granules.

Pale variety.—The granules are not unlike those of actinomycosis, may have a yellowish or pinkish colour resembling fish-roe and show a tangled mass of branching filaments. Occasionally club-shaped structures may be seen at the periphery. The parasite of this variety, known as *Streptothrix maduræ* (synonyms—*Actinomyces maduræ, Nocardia indica, Oospora indica, Nocardia maduræ*) is identical with that of actinomycosis in morphology, differing in cultural characters only. It grows on the ordinary media but only under ærobic conditions. The colonies on agar are large, raised and knob-like in appearance, adhere firmly to the medium and are first white but later on red in colour. Gelatin is not liquefied. Animal inoculation with this parasite has so far been unsuccessful.

The black variety is now regarded as a distinct disease caused by *Madurella mycetomi.* The granules when cleared

show a dense meshwork of mycelial threads or hyphæ, many of which are thick and swollen and show branches. At the periphery the hyphæ are arranged radially. The old granules are markedly degenerated and present an amorphous appearance. Spores have not been observed.

The organism grows well on ordinary media. On agar the growth appears within a week as a thick meshwork of widely spreading hyphæ of a greyish colour. In old cultures black granules appear among the mycelial masses. In *broth* there is at first a rapid growth of long hyphæ which later assumes the appearance of a powder-puff. On *potato* a dense spreading velvety membrane results, the centre being of a pale brown colour and the periphery white. In old cultures small brown droplets of a coffee-coloured fluid appear on the surface and the medium becomes brown. Microscopically the cultures consist of large branching hyphæ but no spores. Experimental inoculation of animals with cultures has so far given negative results.

Diagnosis.—The granules are placed between two slides in a drop of sodium hypochlorite or of a strong sodium hydrate solution for ten minutes by which time the pigment is dissolved. The slides are gently pressed together and examined under the microscope.

Bacillus anthracis

This bacillus has been described by Koch in 1877 in pure cultures; it is the causative organism of anthrax in animals and in man.

Morphology and Staining.—The anthrax bacillus is a straight rod, 6 μ to 8 μ in length and 1.3 μ in width with squarish ends. In artificial media the bacilli form tangles of long threads. As seen in preparations from animal tissues or blood, a capsule round the organism may be seen by special methods. Non-motile. It forms oval, central spores on artificial media. The bacilli easily stain with ordinary basic dyes and are Gram-positive.

Culture.—*B. anthracis* is ærobe and facultative anærobe and grows readily on all culture media at from 32° to 37°C.

Agar plates.—Colonies slightly wrinkled, irregular in outline, show under the microscope wavy wreaths like locks of hair. Under high power these wreaths are found to consist of bundles of long filaments lying parallel with one another. Each filament consists of a number of bacilli lying end to end in the form of a chain.

Agar slopes.—On this medium a thick felted growth appears.

Blood serum and potato.—Growth is abundant with no special characteristics.

Gelatin plates.—Colonies appear as on agar plates. Gelatin is liquefied.

Gelatin stab.—Growth at first appears as a thin white line along the line of puncture. In about two days thin spicules or filaments of growth radiate out into the medium from the needle track. These filaments are more abundant and larger at the top than at the bottom, giving the appearance of an inverted fir tree. The gelatin is later liquefied.

Bouillon.—Growth is rapid without any diffuse cloudiness. Apart from isolated irregular threads the fluid is clear and there is a flocculent deposit.

Serological Reactions.—*Ascoli's thermo-Precipitin reaction.*—The suspected material, blood or tissue is boiled for a few minutes in 5 to 10 volumes of normal saline to which acetic acid has been added (1 in 1000). The mixture when cool is filtered through a filter-paper. A little of the filtrate is then run on to the top of anthrax-immune serum when a white ring immediately appears at the junction of the fluids. Some normal sera may give a positive reaction but only after fifteen minutes.

Pathogenesis.—The anthrax bacillus is pathogenic for cattle, rabbits, mice, rats and guinea-pigs. Anthrax is essentially a disease of sheep, cattle, horses and deer in which it occurs naturally. It occasionally causes infection in man through the handling of skins, hides, wool, bristles or hair shaving brushes. The most notable feature on post-mortem examination of an ox is the enlargement of the spleen, hence the name "spleen fever." The injection of a small quantity of the culture into the subcutaneous tissues of a guinea-pig causes death within two days The tissues around the site of inoculation are the seat of an inflammatory gelatinous œdema and hæmorrhages. Smears from the affected tissues show numerous bacilli. Hæmorrhages in the internal organs are sometimes seen. The disease spreads in nature through the ingestion of spores by animals while feeding.

In man the disease occurs in two main forms depending upon the method of entrance of the organism into the body.

Malignant pustule.—If infection takes place through cuts or abrasions or through the hair follicles on the feet, hands and forearm, there develops 2 to 7 days after infection a cutaneous pustule resembling a carbuncle.

Woolsorter's Disease is a general infection occurring through the lungs or stomach.

Vaccines.—Anthrax bacilli attenuated in virulence by growing at 42° to 43°C. have been successfully employed for immunizing animals against anthrax.

Serum.—Asses immunised with cultures of *B. anthracis* yield serum which has been found to possess protective and to a certain extent curative properties if used intravenously.

Diagnosis.—(*a*) *Microscopical examination.*—Blood from the auricular vein of the animal is examined bacteriologically. A post-mortem examination of an animal dead of anthrax is rarely done owing to the risk of dissemination of infected material. If necessary, the abdomen is opened and the spleen examined for enlargement. To confirm diagnosis smears from spleen are stained with Gram and McFadyean's methylene-blue methods. Smears from scrapings of the malignant pustule and sections of the excised pustule may be stained in a similar way. *The carcase after examination should either be burnt or buried in quicklime in a deep pit.*

(*b*) *Culture.*—The suspected material is inoculated on agar plates, which are examined after 24 hours for anthrax colonies with their wavy margins.

(*c*) *Animal inoculation.*—A little quantity of the suspected material is mixed with some sterile saline and injected into the subcutaneous tissues of a guinea-pig which dies within 2 days.

Detection of B. anthracis in Wool, Hair, etc.—A suitable quantity of the suspected material is placed in 50 to 100 c.c. of boiled water in a flask and 3 to 5 c.c. of 5 per cent. solution of caustic potash added. The mixture is kept at 37°C. for several hours if it is blood-stained, then poured into a flat dish and the hair or wool thoroughly teased. After heating at 80°C. for 2 to 3 minutes, tubes of melted agar are inoculated with ½ c.c. of the mixture and poured into plates which are examined after 24 hours for the characteristic deep-lying colonies of anthrax.

Anthrax-like bacilli

B. anthracoides morphologicallly resembles anthrax bacillus but with the ends more rounded. Non-pathogenic.

B. subtilis (Hay bacillus) resembles *B. anthracis*. This is non-pathogenic and is dealt with on page 200.

B. vulgatus is identical with *B. subtilis*. The growth on artificial media, however, shows a marked wrinkling and folding.

B. mycoides is morphologically indentical with *B. anthracis*. The colonies of *B. mycoides* on agar differ from those of *B. anthracis* in presenting a spiked or feathery appearance (See page 201).

Bacillus of the Colon-Typhoid Dysentery group

This group includes the following chief organisms:—

1. *B. coli.*
2. Bacilli of the typhoid and paratyphoid fevers.
3. Food poisoning bacilli.
4. Dysentery bacilli.

The general characters of the group are as follows:—The bacilli are non-sporing and Gram-negative. They are mostly motile, generally possess flagella and do not liquefy gelatin and grow easily on ordinary artificial media. In their growth characters they are practically identical.

Bacillus Coli Group

This group comprises a large number of different types possessing many points of morphological and biological similarity though differing in biochemical reactions and in other features.

Bacillus coli

Synonyms.—*Bacterium coli; Escherichia coli; B. coli communis*—described by Buchner in 1885 and Escherich in 1886.

Morphology and Staining.—The *Bacillus coli* is a short plump rod of varying sizes (1·5 μ by 0·5 μ). Small coccoid or long forms up to 10 μ may be seen. It is motile but motility varies with different types and media. The flagella (eight or more) are arranged round the organism. It stains with the usual aniline dyes, stains well with weak carbol-fuchsin and is Gram-negative.

Culture.—Aerobe; temperature range 20° to 40°C.; optimum 37·5°C. Grows well on ordinary media.

Agar.—The colonies are large, circular or irregularly circular discs of a glistening white, greyish or brownish colour.

Gelatin.—In gelatin stab growth takes place along the needle track and spreads as a disc over the surface of the medium; no liquefaction but bubbles of gas develop from the fermentation of dextrose present. On plates the growth is similar to that on agar.

Broth.—This becomes uniformly turbid.

Potato.—Growth varies, usually greyish-white at first, later turning to a moist yellowish-brown if the potato is acid.

Culture Reaction on Special Media.—The organism is easily isolated from mixed cultures such as in fæces, etc., by plating upon special media including lactose, litmus agar, Conradi-Drigalski and malachite green broth.

Biochemical Reactions.—A.—*Fermentation of Sugars.*—*B. coli* produces acid and gas in glucose, lactose, lævulose, maltose, mannite, galactose, raffinose, arabinose, dulcite and sorbite.

B.—*Voges-Proskauer's Reaction.*—This reaction is carried out by growing the organism in 2 per cent. glucose broth for 3 days. A strong caustic potash solution is than added and the tube allowed to stand for 24 hours at room temperature. A pink flourescent colour develops. This reaction is given by certain atypical varieties of *B. coli* and is not given by the true *B. coli* types.

C.—*Indol production.*—The *B. coli* forms indol in peptone water or broth usually within 2 days.

D.—*Curdling of Milk.*—Acid is produced in litmus milk with subsequent clotting.

E.—*Methyl-Red Reaction.*—This is a reaction which is given by the common types of *B. coli* found in excreta and sewage. The reaction is carried out as follows:—

Grow the organism in the following medium:—

Peptone	..	0·5 gramme.
Glucose	..	0·5 gramme.
Dipotassium hydrogen phosphate		0·5 gramme.
Water	..	100 c.c.

After a few days' incubation 5 drops of a methyl-red solution (0·1 gramme in 300 c.c. of alcohol and 200 c.c. of distilled water) are added to the culture. If the colour changes to red, the reaction is positive. Yellow colour indicates a negative reaction.

F.—*Reduction of Nitrates to Nitrites.*—*B. coli communis* reduces nitrates to nitrities (see page 165).

Serological Reactions.—Agglutinins appear in the blood of persons suffering from *B. coli* infections. Animals artificially immunised give a serum of a very high agglutinating titre.

Pathogenesis.—An intraperitoneal or intravenous injection of *B. coli* into a rabbit or guinea-pig frequently causes a fatal septicæmia. The *B. coli* is a pyogenic organism and is frequently associated with a number of pathological processes such as cystitis and pyelitis, appendicitis, peritonitis, cholecystitis and possibly pneumonia and pleurisy. It is believed that a few of the organisms which are constantly present in large numbers in the intestines of healthy persons enter the lymphatics and even the blood stream and though these are usually readily eliminated, they may establish themselves and multiply in certain tissues of the body such as kidney. *B. coli* is said to be the causal organism in calf diarrhœa.

Occurrence.—*B. coli* is constantly present in the intestinal and urinary tracts of man and in those of the lower animals and birds. It may be recovered from the blood in *B. coli* septicæmia. It can be isolated from the discharges of the suppurative processes caused by this organism.

Vaccine.—*Prophylaxis.*—As a preliminary to certain operations on the genito-urinary tract, it is advisable to immunise the patient. This is preferably done by an autogenous vaccine, failing which a vaccine made from a colon bacillus of the same group may be tried. The vaccine is given in three doses of 100, 500 and 1000 millions at intervals of one week. It is necessary to make sure that there is no obstruction to urinary outflow otherwise vaccines aggravate the condition.

Treatment.—Treatment of *B. coli* infections with vaccines should begin as soon as the *acute symptoms have subsided.* Subcutaneous injections of graduated doses of an autogenous vaccine containing 25, 50, 100, 400, 600, 800 and 1000 millions of bacteria are given. The maximum dose should not exceed 2000 millions.

Antisera.—*B. coli* antisera have not proved of value.

Varieties of the Colon bacillus.

B. coli communior.—(*Escherichia communior*) is common in the fæces of men and animals and differs from *B. coli communis* in that it ferments saccharose and dextrose. It produces indol and does not give the Voges-proskauer reaction.

B. lactis aerogenes is constantly present in the human intestine and in milk and is one of the organisms responsible for souring of milk. It is less motile than *B. coli* and on agar it forms large white sticky colonies. When grown in milk it possesses a distinct capsule. It is a facultative anærobe and produces no gas in fermentation reactions. It produces acid and gas in saccharose, adonite and inosite, thus differing from *B. coli* (Escherichia).

B. acidi lactici is found in milk, water and in the intestine and is without any pathological significance. It is non-motile, non-sporing, occurs in pairs or fours, and varies in size from 1 to 2μ long by 0·3μ broad. It breaks up milk sugar into lactic acid, the fermentation ceasing when the acid has accumulated to a certain amount and recommencing on neutralising the media. It does not ferment dulcite and gives acid and gas in adonite, thus differing from *B. coli.*

B. fæcalis alcaligenes inhabits the human intestine, especially after bacillary dysentery. It does not coagulate milk nor produce indol, nor acid in any of the sugars. It is Gram-negative, usually motile and grows like the typhoid bacilli.

B. neapolitanus, the cause of an outbreak of choleraic disease in Naples, is non-motile, produces indol and ferments saccharose and dulcite.

B. cloacæ occurs in grave yards, sullage farms, slaughter-houses, etc., is motile, liquefies gelatin, ferments saccharose and gives the Voges-Proskauer reaction. Dulcite is not fermented.

B. acidophilus, B. bifidus occur in the fæces of infants. These and **B. bulgaricus** are Gram-positive, do not form indol nor produce gas.

Bacillus typhosus

Synonyms.—*Bacterium typhosus; Eberthella typhi*—the causative organism of typhoid or "enteric" fever, discovered by Eberth and Gaffkey in 1884.

Morphology and Staining.—The bacillus is a short rod (1 to 4 μ by 0·5 to 0·8 μ) with 12 or more long wavy flagella arranged round the sides and ends. Under cultivation it becomes markedly pleomorphic and forms up to 10 μ may be noted occurring singly or in pairs. On repeated subcultures involution forms up to 30 μ may occur. In young cultures it is actively motile. It stains with the usual aniline dyes, is Gram-negative and does not form spores.

Culture.—Aerobe and facultative anærobe, grows well on ordinary media. Temperature range 40°C. to 46°C.; optimum 30°C. to 37°C.

Agar.—The colonies are at first small transparent discs and later become opaque. Upon agar slants it forms a thick loosely-attached moist greyish uniform layer.

Gelatin.—Grows readily without liquefaction. In stabs a thick white growth takes place along the needle track and on the surface of the gelatin.

Potato.—On fresh potato after several days it forms a thick, moist grey layer which is hardly visible. If the reaction of the medium is neutral or alkaline the growth is more vigorous and may become yellowish.

Broth.—A general turbidity occurs.

Biochemical Reactions.—In milk slight acidity but no curdling occurs. Indol is not formed in peptone water. Lactose and saccharose are not fermented; acid but no gas occurs with glucose and mannite. Colonies on MacConkey's medium are colourless because lactose which is one of the constituents of the medium is not fermented by *B. typhosus.*

Serological Reactions.—The agglutination test is described on page 166. It was first utilised by Widal in the diagnosis of typhoid fever and is frequently referred to as the "Widal reaction."

Normal serum may cause agglutination of *B. typhosus* in a dilution of 1 in 10 but serum of a typhoid fever patient will agglutinate the organism in a much higher dilution, *e.g.*, 1 in 30 to 1 in 100 or even more. The reaction is generally obtained after the first week of illness.

The serum of a person who has had typhoid fever and of one who has had T.A.B. inoculation may for some months or even a year give a positive Widal. An agglutination of 1 in 30 by the microscopic method or 1 in 100 by the

macroscopic method may be regarded as diagnostic if it occurs within half an hour and the patient has not been recently inoculated.

Intravenous injections of rabbits with *B. typhosus* produce sera of a very high titre, *i.e.*, 1 in 10,000 or more, and such sera are used in the identification of a suspected bacillus. Precipitins and opsonins are also formed in animals immunised with the organism.

Pathogenesis.—In animals typhoidal infection does not occur either spontaneously or on experimental inoculation. In man the organism causes typhoid fever and a number of other diseased conditions.

Occurrence.—In man the organism gains entrance by the mouth in water, milk, vegetable shell or other food or through contact with contaminated fingers. Food may be contaminated by flies which carry the organisms on their legs and also may pass bacilli unaltered in their fæces. In the early stage of the disease it produces a septicæmia. Blood cultures in early cases of typhoid yield, in competent hands, positive results in all cases but with the progress of the disease the organisms become less numerous in the blood. The bacilli are found in the Peyer's patches, mesenteric glands, spleen and, less frequently, in the rose-spots of the eruption, the sweat, the sputum and lungs. Organisms may act as a nucleus for the formation of gall-stones. The bacillus is found in pure culture in the complications and sequelæ of typhoid fever, *e.g.*, broncho-pneumonia, empyema, abscesses, osteomyelitis, acute suppurative osteitis, abscesses of the kidney, etc. The bacilli can be recovered from the fæces in every case and from the urine in about 25 per cent. of cases.

Carriers.—A " carrier " is an individual who harbours and transmits pathogenic organisms without himself showing the usual evidence of infection. " Carriers " are very important from the point of view of the bacteriologist, the sanitarian, the epidemiologist and the physician. Among diseases which human carriers transmit are: " enteric " fevers, diphtheria, dysenteries, pneumonia, cholera, undulant fever and meningococcal infections. Various types of carriers are described:—

1. *The convalescent carrier* is an individual who harbours the organism for a few weeks during convalescence from some disease. These are divided into *temporary convalescent carrier*—those harbouring the organisms only for a short time—and *chronic carriers,* those who have harboured them for a long period (arbitrarily given as 12 weeks in the case of diphtheria).

2. *The contact carrier* acquires the germs of the disease from a sick person or from another carrier of the disease but does not manifest the disease himself.

Normally the bacilli disappear from the excreta of typhoid patients a few weeks after convalescence but in 4 to 5 per cent. of cases they persist for indefinite periods—often years. The reservoir of the bacilli in these cases, who represent 93 per cent. of all typhoid carriers and are usually women, is the gall-bladder, while in urinary carriers the focus is in the kidney.

Vaccines.—The mixed vaccine ("T. A. B.") containing 1000 millions of *B. typhosus* and 750 millions each of *B. paratyphosus A* and *B* in 1 c.c. is now generally employed in prophylaxis Eighteen to twenty-four-hour agar cultures of the bacilli are emulsified in normal saline and the emulsion standardised to contain the required number of bacilli. The bacterial suspension is then heated at 55°C. for one hour and 0·25 per cent. tricresol is added. The usual tests for sterility are made. For prophylaxis half a c.c. of the vaccine is injected subcutaneously, followed from 7 to 10 days later by a one c.c. injection. Vaccines have not been successful in the treatment of typhoid fever but may be employed in cases of persistent bacilluria and pyuria. The dosage in treatment is the same as for *B. coli* vaccine.

The Paratyphoid and Food-poisoning Bacilli

Bacillus paratyphosus A.—Synonym: *Salmonella paratyphi.*

Bacillus paratyphosus B.—Synonym: *Salmonella Schottmülleri*—the causative organisms of paratyphoid fevers, which clinically resemble enteric fever.

B. paratyphosus A is more frequently met with in the East and *B. paratyphosus B* in Europe.

In their morphological and cultural characters these two organisms resemble each other and *B. typhosus* but they differ in their biochemical and serological reactions. *B. paratyphosus A* does not ferment xylose which is fermented by *B. paratyphosus B.* These organisms are distinguished from *B. coli* in that they are non-lactose fermenters, and from *B. typhosus* and *B. dysenteriæ* in that they produce acid and gas in glucose, mannite, dulcite, maltose, etc. They do not produce indol. The two organisms may show cross agglutination, *i.e.*, the antiserum for the A type may agglutinate *B. paratyphosus B* though only in low dilutions.

B. paratyphosus C which is indistinguishable from A and B has also been reported to produce an enteric-like fever. Serological reactions serve to identify these closely allied organisms.

Bacilli of Food-Poisoning

The bacilli associated with food poisoning are *B. enteritidis* (Gärtner), *B. suipestifer* and *B. œrtrycke*. These organisms resemble *B. paratyphosus B* in their morphological and cultural reactions and can only be distinguished by serological means.

B. enteritidis (*Salmonella enteritidis*)—isolated by Gärtner from an epidemic of meat poisoning in 1888 is so serologically distinct from others that it may be regarded as a separate species.

The foods which usually cause poisoning are preserved foods, especially meat, and toxins once formed are relatively heat-resistant.

The organism is virulent for mice, guinea-pigs and rabbits.

Agglutination differentiates *B. enteritidis* from *B. paratyphosus B*, an antiserum for one of these organisms having little effect on the other organism. For biochemical reactions see page 256.

B. suipestifer.—Infection with the organism is extremely rare. Swine fever is now known to be caused by a filterable virus, infection with *B. suipestifer* probably being secondary.

"Aertrycke" Bacilli (isolated by De Nobele in 1898 from an outbreak of epidemic food-poisoning at Aertrycke in Belgium) are the most common cause of food-poisoning. The bacilli include a number of types which differ from one another in their serological reactions only. These various types are: "Mutton," "Hirschfield," "Newport," "Stanley," "Reading," etc.

Rat and Mouse Viruses—The *Danysz's bacillus* isolated by Danysz from an epidemic in field mice is sold as a rat exterminator. The epizootic produced in rats by the use of this and allied 'viruses' has a varying mortality rate and rat apparently develop an immunity to the bacillus. The *B. typhi murium* is an allied organism and both are related to *B. enteritidis*.

B. psittacosis.—This bacillus is closely related, if not identical, with *B. œrtrycke* and causes septicæmia with enteritis in parrots imported from the tropics. The disease may be communicable to man causing broncho-pneumonia with a mortality rate of 30 per cent.

Morgan's No. 1 Bacillus.—(*Salmonella morgani*).—This is a Gram-negative bacillus belonging to the *B. coli group*. It is pathogenic to rabbits, rats, monkeys and is frequently found in summer diarrhœa of infants. It does not liquefy gelatin nor does it ferment lactose, saccharose, mannite, maltose or dulcite. It produces indol but does not give the Voges-Proskauer reaction.

Other organisms found in summer diarrhœa are the *B. paracolon, B. fæcalis alkaligenes,* the dysentery bacilli and *B. proteus.*

The Dysentery Bacilli

B. dysenteriæ (Shiga).—Synonym: *Eberthella dysenteriæ*—the causative organisms of dysentery in man, discovered by Shiga in 1898.

B. dysenteriæ (Flexner).—Synonym: *Eberthella paradysenteriæ.*

Morphology and Staining.—*B. dysenteriæ* is morphologically identical with the typhoid bacillus: short rods with rounded ends showing a tendency to the cocco-bacillary forms; non-motile, no spores; stains with aniline dyes and is Gram-negative.

Culture.—The cultural characters resemble those of *B. typhosus.* On differential media like those of Endo and Conradi-Drigalski, etc., the growth is more delicate than that of *B. typhosus.*

Biochemical Reactions.—(*See* page 256). It is important to remember that *B. dysenteriæ* (Shiga) does not ferment mannite and *B. dysenteriæ* (Flexner) does it.

There are two main groups of dysentery bacilli:—

1. Those producing acid in mannite—"mannite fermenters"—Flexner-Y and Strong.
2. Those not developing acid in mannite—"non-mannite fermenters"—(Shiga).

All the dysentery bacilli produce no gas; they all ferment glucose and all the typical varieties do not ferment lactose.

The classical Flexner-Y type includes four strains, the Flexner V, W, X and Z organisms, which are distinguishable serologically.

Serological Reactions.—The biochemical reactions of the various types are found to correspond with the differences in their serological characters. The serum of a patient may agglutinate the dysentery bacilli in a dilution of 1 in 50, but the test is of no particular value in diagnosis as the agglutination phenomenon is indefinite and appears late in the disease. The test is, however, of distinct value in identifying the organism isolated from the stool of a dysentery case, employing a high titre serum.

Pathogenesis.—Of all the types of dysentery bacilli the Shiga is the most toxic. Introduced intravenously and subcutaneously into the peritoneum the dysentery bacilli are pathogenic to guinea-pigs, mice and rabbits which may die in 24 to 48 hours but do not reproduce the characteristic lesions of dysentery in man. Feeding *per os* has no effect on these animals.

An exotoxin has been obtained from Shiga cultures and has been shown to produce hæmorrhagic enteritis in rabbits, etc.

Occurrence.—The organism is found in the stools, intestinal contents and in the ulcerated mucous membrane in acute dysentery.

Vaccines.—A difficulty attending the use of vaccines is the violent reaction obtained by the injection of killed cultures. To overcome this difficulty Shiga employed sensitised vaccine. Polyvalent non-sensitised vaccines containing mixed strains of Morgan No. 1, Flexner and Shiga were used during the Great War.

Serum—The results of serum treatment of dysentery vary considerably. It appears that the use of large doses (50 to 100 c.c.) in the early stages of the disease is beneficial. The polyvalent serum is antitoxic and is given subcutaneously, intramuscularly or preferably in severe cases intravenously and the administration is preceded by small injections of ½ c.c., 1 c.c. and 5 c.c. to guard against anaphylaxis.

Diagnosis of Infections due to the Colon-Typhoid Dysentery group of Bacteria.

1. Examination of Stools.—(*a*) Macroscopically the stools are at first red and consist of blood streaked over with whitish exudate and thick mucus. The stool is odourless. Later, the stool may be yellow, greenish or contain greenish sloughs.

(*b*) Microscopically, the stool consists almost entirely of mucus, red cells, pus cells and macrophages. These macrophage cells are large and refractile and may contain ingested red and white blood cells and hence are often mistaken for *Entamœba histolytica.*

2. Blood Cultures.—During the early stages of the disease this is the most reliable method. Except in the case of *B. dysenteriæ* the organism recovered is identified by cultural, biochemical and serological tests.

3. Agglutination Test.—This is usually carried out after the first week of illness. The serum of the patient is diluted

with varying amounts of saline and tested against a number of organisms. (The data on which a positive diagnosis may be made are given on page 170).

4. Culture from Fæces.—Culturing from fæces is a procedure of great diagnostic value in the diseases under consideration. *B. typhosus* is most numerous in the stools in the third week. In paratyphoid A infection the maximum occurs about the 12th day and in paratyphoid B at the end of second week.

Collection and transmission of a sample of fæces.—One c.c. of a loose motion is selected. This is packed in a sterile wide-mouthed bottle about 7 cm. by 3 cm. provided with a cork into which is inserted a small spoon of white metal having a bowl large enough to contain 1 gramme of the fæces. If the sample has to be sent to some distance it may be preserved with double the volume of 30 per cent. glycerin in 0·6 per cent. of saline solution, added as soon as possible after evacuation.

A drop of liquid fæces or of an emulsion of the fæces in saline is used for inoculating plates of MacConkey's or Endo's medium. Drigalski and Conradi medium is not to be recommended in the case of *B. dysenteriæ*. At the same time a 10 c.c. tube of peptone water containing brilliant green 1 in 20,000 (brilliant green enrichment medium) is sown with one or more loopfuls of fæcal emulsion and incubated at 37°C. After 9 to 24 hours' incubation, subcultures from these are made on MacConkey's medium. The plates made direct from the fæces as well as those made from the enrichment medium are examined after 12 to 24 hours at 37°C. Several colourless colonies are selected for microscopic examination, for subcultures on agar and for further tests, which include:

(*a*) Morphological characteristics—morphology and staining. These are unimportant.

(*b*) Biochemical reactions—(*i*) fermentation tests, (*ii*) effect on milk, (*iii*) production of indol from peptone water, and (*iv*) liquefaction of gelatin.

(*c*) Immunity reactions, especially agglutination. The organism is tested against known specific sera for the limit of agglutination.

Culture from the Urine.—In about 25 per cent. cases of typhoid fever the bacilli are found in the urine, especially late in the disease. The sample of urine may be centrifugalised and first cultures made from spreading the deposit on MacConkey's medium.

REACTIONS OF THE COLON-TYPHOID GROUP

	Motility.	Glucose.	Lactose.	Maltose.	Saccharose.	Mannite.	Dulcite.	Litmus Milk.			Indol.
								1st day.	3rd day.	12th day.	
B. typhosus	+	A	—	A	—		—	A	A	A or Alk	—
B. paratyphosus A	+	AG	—	AG	—	AG	AG	A	A	A	—
B. paratyphosus B	+	AG	—	AG	—	AG	AG	A	Alk	Alk	—
B. dysenteriæ (Shiga)	—	A	—		—	—	—	A	Alk	Alk	∓
B. dysenteriæ (Flexner)	—	A	—	A	—	A	—	A	Alk	Alk	∓
B. dysenteriæ (Flexner Y-Hiss-Park)	—	A	—	—	—	A	—	A	Alk	Alk	∓
B. dysenteriæ (Strong)	—	A	—	—	A	A	—	A	Alk	Alk	∓
B. enteritidis (Gartner)	+	AG	—	AG	—	AG	AG	A	Alk	Alk	∓
B. typhi murium	+	AG	—	—	—	AG	—	A	Alk	Alk	∓
B. fæcalis alkaligenes	+	—	—	—	—	—	—	Alk	Alk	Alk	—
B. Morgan No. I	+	AG	—		—	—	—	—	—	Alk	+
B. suipestifer	+	AG	—	AG	—	AG	A	A		Alk	—
B. coli	+	AG	AG	AG	—	AG	AG	AC	AC	AC	+
B. coli anærogenes	—	A	A	—	A	A	A	A	A	A	+
B. acidi lactici	—	AG	AG	AG	—	AG	—	AC	AC		∓
B. lactis aerogenes	—	AG	AG	AG	AG	AG	—	AC	AC	AC	∓

A = Acid Alk = Alkalinity C = Clot G = Gas.

\+ = Presence of motility or production of indol.

∓ = Production of indol indefinite.

Bacillus Diphtheriæ

Synonym.—*Corynebacterium diphtheriæ*—the causative organism of diphtheria, described by Klebs in 1883.

Morphology and Staining.—Polymorphic, small, delicate rod-shaped organisms straight or slightly curved: 3 to 4 μ in length, but large or shorter forms may be obtained; commonly occurs in groups of 2 or more which lie parallel to one another or at different angles so as to resemble the letter V or L. Club or bottle-shaped involution forms occur in cultures over 24 hours old. Non-motile, non-sporing, Gram-positive, metachromatic granules, stains distinctly with Löffler's methylene-blue but more distinctly with Neisser's or Pugh's stains. The granules stain blue-black while the rest of the organism shows a light brown discoloration with Neisser's stain.

Culture.—Aerobe and facultative anærobe, temperature range 25° to 40°C.; grows well on ordinary nutrient media but best on serum media.

Löffler's medium.—Colonies appear as smooth, raised, whitish, translucent, circular spots with a moist shining surface.

Agar.—Two types of colonies occur—one small greyish white and numerous and one larger variety which is whiter and has a smooth circular outline and a large thick central portion.

Broth.—Broth is uniformly turbid at first with subsequently a thick deposit and a surface film of growth; clumps also adhere to the sides of the tube.

Gelatin is not liquefied.

Milk becomes acid without clotting.

Biochemical Reactions.—*B. diphtheriæ* ferments glucose, maltose, galactose and dextrin with acid formation but no gas. Saccharose is unaffected. Hiss's serum water medium should be employed in demonstrating these reactions. No indol is produced.

Serological Reactions.—The organism is agglutinated by the sera of diphtheria patients or by a specific diphtheria serum produced by immunising animals but the test is difficult to apply on account of the difficulty of obtaining a uniform emulsion of the organism.

Occurrence.—The organism occurs in large numbers in the "false membrane" and throat secretions but very rarely in the

blood, glands, the pleural exudate and abdominal organs. It is found in local lesions of the conjunctiva, middle ear, genitals and skin, all of which are extremely rare. Local nasal diphtheria is relatively common.

Viability.—The organism retains its vitality in culture for a month but is killed instantaneously by carbolic acid, while in the membrane it is very resistant.

Pathogenesis.—Guinea-pigs, rabbits, dogs, cats, sheep, goats, horse, monkeys and birds are all more or less susceptible. Rats and mice are immune. A subcutaneous injection into guinea-pigs produces a small patch of greyish membrane with necrosis at the site of inoculation and an extensive inflammatory œdema in the tissues around; death generally occurs in 36 hours. Multiple sublethal doses injected into rabbits may cause fever and paralysis of the hind legs.

Toxins.—Broth cultures of *B. diphtheriæ* are highly toxic to animals even after the bacteria have been removed by filtration, and when injected into animals the filtrate produces the same effects as the living bacilli. Such a toxin is called an exotoxin.

Preparation of the toxin.—The production of a potent toxin is essential in the manufacture of anti-diphtheria serum. The development of toxin depends upon a great many factors, namely, the virulence of the strain, a free supply of oxygen, the presence of a large proportion of peptone or albumin in the medium, the absence of acid-producing substance and an initial reaction of P_H 7·5 to 8 of the medium. Various broth media are used for toxin production. Martin introduced a medium composed of "peptone" and glucose-free bouillon. Hartley's broth or the ordinary meat infusion with two per cent. peptone and 0·5 per cent. salt can be used, caustic soda being then added to render the media alkaline to phenolphthalein. Half to 1 litre of media continued in an Erlenmeyer flask is inoculated with the culture and incubated at 37°C. At the end of seven days 5 per cent. phenol is added to each flask to give an approximate strength of 0·5 per cent. The broth is then filtered through paper pulp and then through a filter candle. The toxin having been tested for sterility is standardised as follows:—

Number of dilutions of the toxin are made with saline and one c.c. of each of these dilutions is injected under the skin of the thorax of guinea-pigs of 240-260 grammes weight. The

quantity of toxin present in the smallest dose which proves fatal within 96 hours is called the "minimum lethal dose" (M.L.D.)

Properties and nature of the toxin.—The toxin is relatively unstable and loses its potency when exposed to air and sunlight. It has been suggested that the toxin is not actually destroyed but is converted into a toxoid which is still capable of combining with antitoxin. Heating at 50°C. for two hours destroys the toxicity.

Preparation of antitoxin.—The M. L. D. of a toxin employed for antitoxin production should be at least 0·1 c.c. Young and healthy horses after being subjected to the mallein and tuberculin tests are given subcutaneous injections of the toxin at weekly intervals. The dose and intervals are determined by the severity of the reaction. The initial dose may be 0.5 c.c. and be mixed with an equal quantity of Lugol's iodine solution. Or the toxin (20 c.c.) may be combined with 100 units of antitoxin, for the first, second and third doses. Subsequently the toxin alone is injected until 1 c.c. of the serum contains about 250 to 800 antitoxin units.

Standardisation of the antitoxin.—An antitoxin unit is the smallest quantity of antitoxin that will entirely prevent toxic effects arising from an injection of 100 M.L.D. L+ dose is the least amount of toxin which, when mixed with a standard antitoxin unit and injected subcutaneously, kills a guinea-pig weighing 250 grammes within 4 to 5 days. L_0 is the greatest quantity of toxin which when mixed with one antitoxin unit and injected subcutaneously produces no ill effects in a guinea-pig of 250 grammes weight.

An antitoxic serum is standardised by comparison with a standard antitoxic serum. The strength of a toxin is first estimated by working out its L+ dose by mixing a unit of the standard antitoxin with varying quantities of the toxin and injecting these into a series of guinea-pigs. Suppose the L+ dose of a toxin is 0.23 c.c. (*i.e.*, 0.23 c.c. mixed with one standard antitoxic unit just kills a 250 grammes guinea-pig in 4 to 5 days). 0·23 c.c. of the toxin is mixed with varying quantities of the antitoxin to be tested and injected subcutaneously into various guinea-pigs each weighing 250 grammes. Measure out 99. 199, 299, 399, 499, 599, 699, 799, 899, 999 c.c. of sterile physiological saline solution into flasks and to each flask add 1 c.c. of the antitoxic serum to be tested, *i.e.*, make dilutions of 1|100, 1|200, etc. Shake the mixture thoroughly in each flask. Pipette out 2 c.c. from each flask into separate conical

glass vessels and add to each one L+ dose of the toxin (*i.e.*, 0·23 c.c. in this example). Make each up to 4 c.c. with saline solution and mix well. The total volume to be injected therefore is 4 c.c. One c.c. of the diluted serum contains respectively, 1|100, 1|200, 1|300, 1|400, 1|500, 1|600, 1|700, 1|800, 1|900 and 1|1000 c.c. of the original sample. Therefore 2 c.c. (quantity taken for injection) contains 1|50, 1|100, 1|150, 1|200, 1|250, 1|300, 1|350, 1|400, 1|450, 1|500 c.c. of the original serum. Inject the series of mixtures into a series of 250 grammes guinea-pigs. If the animals which received 1|500 c.c. of the original serum dies in 4 to 5 days, whereas all the others survive, the serum contains 450 to 500 units of antitoxin per c.c. The presence of a definite local reaction in the guinea-pig receiving 1 in 450 c.c. shows that the strength of the serum is 450 units. More exact determination may be similarly made by the use of more graded quantities of the serum.

Measurements of antitoxin in vitro.—That it is possible to estimate the antitoxins of serum without using animals has been recently demonstrated by Ramon, who has shown that the toxin when mixed with a sufficient amount of the antitoxin yields a flocculent precipitate. For this purpose graded quantities of antitoxin are added to a given volume of toxin of not less than 500 M. L. D. per c.c.

Hæmolysins.—The broth cultures of the bacillus have a hæmolytic action on red corpuscles. The lysin is thermolabile as it is destroyed if exposed to 58°C. for ½ an hour.

Diphtheria Carriers.—*B. diphtheriæ* may persist for 2 to 4 weeks in the throats of convalescent diphtheria cases—"convalescent carriers" and in the throats of healthy individuals or "carriers" (contact carriers). A small percentage may become "chronic carriers." It is generally believed that individuals harbouring a virulent type of the bacillus do not spread the infection, hence the testing of the virulence of the organism isolated from "carriers" is important.

The Schick test.—*Indications.*—This test is used to determine whether an individual is susceptible to diphtheria or not, so that susceptible members of a community exposed to diphtheria may be picked out and immunised. The test also distinguishes diphtheria carriers from cases of diphtheria; the former react negatively and the latter give a positive reaction. Schick negative cases may become Schick positive on losing some of their protection while another dose of Schick toxin may suffice to render Schick negative again.

Technique.—The diphtheria toxin used for the test is a 6-day culture in sugar-free broth. It is then carbolised (0.5 per cent.), filtered and allowed to stand for 18 months. The M. L. D. of the stock is then determined in the manner described above. Before use the toxin is so diluted with normal saline that 0.2 c.c. contains 1|50 of a M. L. D. With a 1 c.c. syringe 0·2 c.c. is injected intradermally in the arm. Part of the diluted toxin is heated at 70°C. for 5 minutes and injected (0.2 c.c.) with another syringe into the other arm to serve as a control. The diluted toxin should not be used after 24 hours. The test is best carried out with a toxin obtained from a reliable firm.

Reading and Interpretation of Results.—Readings should be taken on the 4th and 7th day after inoculation. Four types of reactions have been described:—

1. *Negative* in which there is an entire absence of any reaction in either arm, showing that the patient is immune.

2. *Positive* in which the control arm remains normal but the other becomes red and indurated at the site of inoculation after 12 to 24 hours and the redness reaches its height in four days. A positive reaction indicates that the patient is susceptible to diphtheria.

3. *The negative and pseudo-reaction.*—This appears in 24 hours in both arms as a less intense and less circumscribed areola than that given by a positive reaction. The patient is immune.

4. *The positive and pseudo-reaction.*—The pseudo effect appears rapidly on both arms and as the redness fades the true positive appears on the test arm only. The patient is susceptible.

Preventive inoculation.—500 to 1000 units of the toxin should be given subcutaneously to the contacts and those exposed to infection. The immunity, however, disappears within 3 to 4 weeks. In order to avoid this drawback as well as the risk of anaphylaxis, successful attempts have been made to induce active immunisation by injecting a mixture of the toxin and antitoxin in which the toxin is not quite neutralised. This method of immunisation is particularly useful in children with a positive Schick reaction. Three injections are given at weekly intervals.

Pseudo-Diphtheria and Diphtheroid Bacilli

These organisms are similar to *B. diphtheriæ* in morphology but differ from it in their virulence and biochemical reactions.

Bacillus Hofmanni

Synonyms.—*Corynebacterium hofmanni;* Hofmann's bacillus; Pseudo-diphtheria bacillus—described by V. Hofmann Wellenhof in 1888.

Morphology and Staining.—*B. hofmanni* is a short, straight oval rod with rounded ends (average length 1·3 μ—1·8 μ). Unlike *B. diphtheriæ* and *B. xerosis,* which grow out into long forms in cultures it remains constant in size. It is non-motile and does not form spores, stains readily and regularly with Löffler's methylene-blue, may show a single median unstained transverse septum and is Gram-positive. Unlike *B. diphtheriæ* it shows no blue-black granules with Neisser's stain.

Culture.—Aerobe, and the growth on serum, agar, gelatin and in broth is similar to that of the *B. diphtheriæ. B. hofmanni* produces profuse growth on the surface of the medium in a stab culture and there is no growth along the line of inoculation, whereas the growth of *B. diphtheriæ* is confined to the track with little growth on the surface.

Biochemical Reactions.—*B. hofmanni* has no action on carbohydrates. Indol reaction is positive with nitrite and acid.

Pathogenesis.—It is non-pathogenic to guinea-pigs. It does not form hæmolysins.

Immunity.—Immunisation with *B. hofmanni* confers protection against virulent *B. diphtheriæ.*

Corynebacterium xerosis

This is a diphtheroid organism, originally obtained from the conjunctiva. It is non-motile, Gram-positive, morphologically identical with *B. diphtheriæ.* It shows metachromatic granules which are, however, inconstant.

Aerobe; the growth in blood serum is delicate, dry and scaly. The principal biochemical characters of *B. diphtheria* and of some of the important diphtheroids are given in the following table:

Organism.	Glucose.	Mannite.	Sucrose.	Maltose.	Dextrine.
B. diphtheriæ	A	A	O	A	A
B. hofmanni	O	O	O	O	O
B. xerosis	A	A	A	A	O

A=Acid-formation. O=No reaction.

Note.—Hiss's serum water medium should be used for testing these reactions.

Occurrence of Diphtheroids.—Diphtheria-like organisms are frequently present on the skin, in the nose and throat, in nasal sinuses, the eyes and the genito-urinary tract. They have also been described in certain diseases such as leprosy, general paralysis of the insane, typhoid fever, Hodgkin's disease and vaccinia, but their presence in these diseases is now regarded as accidental.

Diagnosis.—*Collection of Material.*—If possible, the bacteriologist himself should remove a portion of the "false membrane" with a sterile forceps or a platinum loop bent at a right angle. In practice the material is usually obtained by persons unskilled in bacteriological technique. Such persons should be instructed to employ a swab of cotton-wool which is made as follows:—A stiff wire long enough to reach the bottom of the culture tube to be inoculated is selected. Non-absorbent wool is twisted into a smooth uniform pad, not too large at the end of the wire on which marks are made for the secure attachment of the cotton-wool. For convenience in handling, the other end may be looped. The swab with the wire is introduced into a test-tube plugged with cotton-wool or provided with a cork and the whole sterilised in a hot-air steriliser or autoclave. When the above swabs are not available fix a piece of cotton-wool to a wire or wooden stick and heat it in boiling water. The swab is enclosed in a bottle which has been rinsed out with boiling water. In swabbing care should be taken that the swab comes in contact with the faucial exudate.

Transmission of material to the laboratory.—Material should be sent as soon as possible to the laboratory. When it is to be sent by post the test-tube containing the swab should be very carefully packed in a box and labelled "pathogenic specimen."

Immediate Presumptive Diagnosis of the Diphtheria Bacillus.—A presumptive diagnosis can be given within 24 hours by two methods, *viz:*—

1. *Microscopic examination.*—Much depends upon the experience of the observer; a film from the swab may be stained with Löffler's methylene-blue, or Neisser's stain or one of its modifications and examined for the characteristic metachromatic granules.

2. *Culture.*—If the swab is dry it is moistened with sterile salt solution or better in the condensation water in the medium tube and rubbed thoroughly over the surface of one or

two tubes of blood serum, care being taken not to injure the surface of the medium. After eight hours' incubation at 37°C. the cultures are examined either by selecting and staining individual colonies or by preparing a film from a sweep of the mixed colonies on the surface of the medium.

3. *The immediate virulence test.*—The intracutaneous or the subcutaneous injection method may be used. After the microscopic examination the cultures on the Löffler's medium are emulsified in saline. About 20 millions of bacilli should be present in 0·1 c.cm. and this quantity is injected intracutaneously into two large guinea-pigs, one of which had received 250 units of antitoxin on the previous day. These guinea-pigs may suffice for testing six cultures. In the case of virulent diphtheria bacilli rose-red swellings of varying intensity or even superficial necrosis of the skin occur in the unprotected animals, while the control animal, *i.e.*, the protected guinea-pig, is normal at the end of 72 hours.

The complete diagnosis of the Diphtheria Bacillus.—1. *Isolation.*—The growth on the original serum medium is diluted and plated on agar, or preferably on Douglas' medium. On the latter medium the colonies of *B. diphtheria* and the diphtheroid bacilli after 18 to 24 hours' incubation are hemispherical with a regular outline and granular in structure. The centre is grey black while the periphery is greyish white in colour.

2. *Tests for identification.*

(*i*) *Staining.*

(*ii*) *Fermentation.*

(*iii*) *The virulence test.*

Vibrio Choleræ and Allied Organisms

Vibrio choleræ—the causative organism of Asiatic cholera, discovered by Koch in 1884.

Morphology and Staining.—The vibrios are curved or comma-shaped rods with rounded ends measuring about 1·5 μ to 2μ in length and 0·5 μ in thickness; most occur singly but some are united in pairs, forming an " S " shaped curve. They possess active motility which is due to a single terminal flagellum. In-

volution forms occur in old cultures. The *V. choleræ* are Gram-negative and form no spores.

Culture.—Aerobe and facultative anærobe. Temperature range 12°C. to 40°C.; optimum 37°C. Grows readily on ordinary media.

Agar.—Colonies look like white circular discs, semi-transparent with well defined circular margins, becoming brownish when old.

Blood serum.—Growth occurs with slow liquefaction.

Gelatin plates.—Colonies at 22°C. are small cream-coloured and accompanied by liquefaction, the plate then showing small sharply marked rings around the colonies.

Gelatin stab.—There is at first a white line of growth along the needle track, thicker above than below. Liquefaction commences at the upper part of the needle track about the second day and a bell-shaped depression forms which gives the appearance of an air bubble; liquefaction then spreads downwards in a funnel-shaped form.

Potato.—Thin, dry, greyish-white growth occurs, turning dirty brown in a few days.

Bouillon.—General turbidity with a characteristic thin pellicle on the surface.

Milk.—Coagulation not constant.

Biochemical Reactions.—The *V. choleræ* produces acid only in glucose, saccharose, mannite and maltose. Dulcite is unaffected.

The cholera-red reaction depends upon the formation of both nitrite and indol in peptone water by *V. choleræ*. The medium should be sugar-free. Dunham's medium consisting of a solution of 1 per cent. pure peptone and 0.5 per cent. sodium chloride in water is recommended for this test.

Test.—Add a few drops of sulphuric acid to a 24-hour old culture in broth or peptone; in the case of *V. choleræ* a reddish-pink colour is produced.

Hæmolysis of red blood-corpuscles.—The *V. choleræ* is non-hæmolytic while most of the other vibrios found in water or fæces are strongly hæmolytic.

Serological Reactions.—*Agglutination tests.*—From the serological standpoint *V. cholerœ* represents a well marked homogenous species. There is no cross agglutination between this and other vibrios. The agglutination test serves to identify unknown strains. A very high titre agglutinating serum can be obtained from artificially immunised animals.

The serum of persons suffering from cholera may agglutinate the organism in a dilution of 1 in 200.

Pfeiffer's reaction.—The test is described as follows in the Medical Research Council Special Report No 51:—

For this test the following reagents, etc., are required:

1. The serum of an animal immunised with strains of known *V. cholerœ*.
2. Three guinea-pigs, A, B and C, each about 200 grammes weight.
3. A 16 to 24-hour old agar culture of the vibrio to be identified.
4. Pipettes, etc.

The test is carried out in the following manner:—

An emulsion of the vibrio to be identified is made by rubbing up a two-milligramme loopful of the culture in one cubic centimetre of broth; to this emulsion 0·001 cubic centimetre of the cholera-immune serum is added and the mixture is then injected into the peritoneal cavity of guinea-pig A.

As a control, guinea-pig B is inoculated intraperitoneally with one cubic centimetre of an exactly similar emulsion of the vibrio to which has been added 0·001 cubic centimetre of normal serum. Guinea-pig C is inoculated with ¼ of a loopful (*i.e.*, 0·5 mgm.) of the culture emulsified in one cubic centimetre of broth.

Samples of the peritoneal fluid are withdrawn by means of capillary pipette 20 and 60 minutes after the injections have been made and are examined either as hanging-drops, or by dark ground illumination, under a cover-slip sealed with vaseline.

If the vibrio under investigation is a true *V. cholerœ* the samples taken from guinea-pig A should show that the vibrios have lost all movement and have become granular, agglutinated into masses and are undergoing bacteriolysis. Those from guinea-pigs B and C should show numerous normally-shaped

activety motile vibrios. On the next day guinea-pig A should be quite normal while B and C should be either dead or dying of acute peritonitis. If the vibrio under investigation is not a true *V. choleræ* the peritoneal exudate of guinea-pig A will show actively motile and otherwise normal vibrios.

The cholera-immune serum employed in Pfeiffer's reaction must have such a titre that 0·0002 cubic centimetre injected into the peritoneal cavity of a 200-gramme guinea-pig causes complete bacteriolysis of 2 milligrammes of an 18-hour old agar culture of *V. choleræ* emulsified in one cubic centimetere of broth in 60 minutes, and also protects the animal from a fatal peritonitis.

Occurrence.—The vibrios are present in the fæces of cholera patients. They may also be found in the lungs, spleen and rarely in the blood. In " carriers " the gall bladder appears to be the focus of proliferation.

Vaccines are made in the usual way from cultures and have been found useful in cholera prophylaxis though not in treatment. The dose is ½ c.c. (5000 millions) followed by 1 c.c. (10,000 millions) subcutaneously in 10 days, but as a broad measure one injection of 1 c.c. at the commencement of the cholera season is sufficient. The vaccine retains its potency in cold storage for six months.

Diagnosis.—*Isolation of V. choleræ from the fæces of an acute case of the disease.*—Pick out an epithelial flake from a rice-water stool with a platinum loop, make a smear, fix, and stain with 1 in 10 carbol-fuchsin. Hanging-drop preparations may be examined for motility. If large numbers of comma-shaped organisms are observed, pick out another epithelial flake, wash in several changes of sterile salt solution and plant it on the surface of a plate of Dieudonne's medium. At the same time inoculate tubes of alkaline peptone water with washed epithelial flakes. Incubate the plate for 18 to 24 hours. If, on examination, large numbers of distinct colonies of vibrios are observed the peptone water culture may be rejected. In case the plates are not satisfactory take a loopful of material obtained by skimming the surface of peptone water tubes by means of a platinum loop and plate on Dieudonne's medium. Incubate and carry out the necessary tests with subcultures to identify the culture as one of cholera. A trace of the colony may be agglutinated with a high titre anti-cholera serum in a dilution of 1 in 200 or higher. If the test is positive the rest of the colony is planted on agar and further tests are carried out with these as under. In this way several colonies can be tested. For rapid diagnosis,

make the plates at intervals of 6, 12 and 18 hours directly from a peptone water tube.

The following tests are employed for the differentiation of the *V. choleræ* from other vibrios:—

1. Rapidity with which gelatin is liquefied.
2. Cholera-red reaction.
3. Hæmolytic test.
4. Pathogenicity for animals.
5. Agglutination test.
6. Pfeiffer's reaction.

Isolation of V. choleræ from the Fæces of a "Carrier."—The focus of multiplication of vibrios is frequently the gall-bladder and the vibrios are looked for in the fæces. The liquid fæces obtained after mild purge are examined as above for *V. choleræ*. Violent purgation is to be avoided as it is likely to precipitate an attack of cholera.

Cholera-like vibrios

Epidemic cholera is now believed to be due to a single species of *V. choleræ*. Vibrios found in isolated outbreaks of a disease having the general symptoms of a mild type are serologically distinct and have been termed *V. paracholeræ*.

El Tor vibrios which were isolated by Gotschlich from the bodies of Mecca pilgrims who died at El Tor in Arabia differ from the true cholera strain in being strongly hæmolytic.

V. paracholeræ which include such strains as *V. massauah, V. nasik, V. paracholeræ* A and B are not agglutinated by the specific cholera serum and react negatively in Pfeiffer's reaction. Serological differences amongst these have been noted.

Vibrio metchnikovi.—This is associated with an epidemic disease of fowls in which it causes gastro-enteritis. It is identical with the *V. choleræ* except that it causes a more rapid liquefaction of gelatin, is hæmolytic, is not agglutinated by cholera serum and is more pathogenic to guinea-pigs and pigeons. In pigeons subcutaneous inoculation of small quantities of the organism causes a rapidly fatal septicæmia whereas inoculation with the same quantity of cholera vibrio is not followed by any result.

Vibrios of Finkler-Prior and of Deneke.—Culturally these are identical with *Vibrio cholera* except for a more rapid liquefaction of gelatin. They do not give the cholera-red reaction nor are they agglutinated with a specific cholera serum. They do not give the Pfeiffer's reaction.

Influenza bacillus and other organisms of the Hæmophilus Group

Bacillus influenzæ

Synonyms.—*Hæmophilus influenzæ; Pfeiffer's bacillus*—described by Pfeiffer in 1892.

Morphology and Staining.—*B. influenzæ* is a minute (1·5 $\mu \times 0{\cdot}3\ \mu$) non-motile, non-sporing organism. The organism may occur singly or in pairs and in sputum masses are clumped together. Staining with dilute carbol-fuchsin for about ten minutes is satisfactory. It is Gram-negative.

Culture.—Aerobe; does not grow on ordinary media; grows at temperatures between 26° to 42°C., the optimum being 37°C.

For its cultivation the most suitable media are those containing blood, such as agar smeared with sterile rabbit's blood. On blood serum or blood agar the bacilli appear within 24 hours in the form of minute, discrete, transparent colonies looking like drops of dew. There is no growth on potato.

A most suitable liquid medium is rabbit's serum into which a certain amount of hæmoglobin has been allowed to diffuse by leaving the serum in contact with the clot.

Resistance.—*B. influenzæ* has a feeble viability outside the body and is very sensitive to heat and drying.

Biochemical Reactions.—The majority of the strains produce indol and reduce nitrates to nitrites.

Serological Reactions.—Sera of persons suffering from influenza do not agglutinate the *B. influenzæ*. The sera of animals immunised with the bacillus agglutinates it in dilutions of 1 in 200 to 1 in 500.

Pathogenesis.—No disease resembling influenza occurs in animals under natural conditions.

Occurrence.—The bacilli are frequently present in the respiratory tract, and in lesions complicating influenza such as otitis media and meningitis. They are, however, rarely found in blood.

Ætiology of Epidemic Influenza.—There is no satisfactory evidence that *B. influenza* is the causative organism of epidemic influenza, though the organism frequently occurs in the epidemic disease. It has been suggested that epidemic influenza is due to increased virulence of the organism normally present in the respiratory tract.

Olitsky and Gates in 1921 cultivated an organism which they termed *Bacterium pneumosintes* (Synonym—*Dialister pneumosintes*). This is exceedingly minute (0·15 μ to 0·3 μ long) in size and is ovoid in shape, occurs, singly, in pairs or short chains. It is Gram-negative, stains with the ordinary dyes but is most easilv demonstrated in culture by staining with polychrome methylene-blue. It is a strict anærobe. In Smith-Noguchi medium it produces cloudiness which commences first round the tissue after 3 to 4 days at 37°C. and then diffuse into the medium. On blood agar under anærobic conditions it forms small transparent colonies after several days of incubation. *B. pneumosintes* does not attack carbohydrates and is present in cultures filtered through Berkefeld V and N filters. On intratracheal inoculation into rabbits it produces fever, leucopenia and œdema and hæmorrhages in the lungs. Other anærobic filter-passing Gram-negative organisms similar to this occur in the throat but these according to Gates and Olitsky are serologically distinct from *B. pneumosintes* and can be distinguished by agglutination tests. They believe that *B. pneumosintes* is the causal organism of epidemic influenza but the relationship of this organism, or of any other, with epidemic influenza, has not yet been completely established.

Diagnosis.—Secretions of throat or material from the affected tissues such as the lungs, brain, etc., are cultivated on blood agar.

Koch-Weeks Bacillus

Synonym.—*Hæmophilus conjunctivitidis*—the causative organism of a form of acute contagious conjunctivitis described by Koch in 1883 and Weeks in 1887.

H. conjunctivitidis is morphologically similar to *B. influenzæ* and is a minute (1 μ in length), non-motile, Gram-negative organism which may occur singly or in pairs, both free and

within the pus cells. It grows well on serum agar on which it produces small transparent colonies like drops of dew. The bacilli do not grow on ordinary media and are not pathogenic to animals though they produce a typical conjunctivitis in man. The organism can be demonstrated in the muco-purulent secretion, films of which are stained with 1 in 10 carbol-fuchsin.

Morax-Axenfeld Bacillus

Synonym.—*Hæmophilus lacunatus*—the causative organism of a form of chronic catarrhal conjunctivitis, described by Morax and Axenfeld in 1896.

As seen in smear preparations of the pus, *H. lacunatus* is a small plump ($2\mu \times 1\mu$) Gram-negative, non-motile non-sporing bacillus, which occurs singly or in short chains usually of two organisms placed end to end. In cultures it is pleomorphic and shows involution forms. It has rounded ends. It is stained with ordinary aniline dyes.

It can be grown only on media containing blood or serum. Solidified serum becomes pitted with areas of liquefaction produced by the round colonies of the organisms. It is non-pathogenic to animals but sets up a subacute conjunctivitis in human beings by inoculation with pure cultures.

Bacillus of Ducrey

Synonym.—*Hæmophilus ducreyii,* described by Ducrey in 1889 as occurring in the purulent discharge from the ulcerated surface of soft sore (Chancroid) and in buboes.

H. ducreyii is a small ($1{\cdot}5\mu \times 0{\cdot}5\mu$) oval, Gram-negative, non-motile non-sporing organism which may occur in short chains or in parallel rows or in small irregular groups. In stained sections (dehydrate by aniline oil method and not by alcohol) it is found in the granulation tissue forming the floor of ulcer, arranged in long chains or in parallel rows between the cells. With ordinary aniline dyes, the ends stain more deeply than the rest of the organisms.

Ducrey's bacillus can be cultivated on blood agar. The primary cultures are grown in rabbit's blood heated at 55°C. (see below). On blood agar it forms small greyish colonies which attain their full size (1 to 2 mm.) in about 48 hours. It is not inoculable into lower animals. Inoculation of the human

subject with pure cultures has been found to produce the characteristic lesions.

Diagnosis.—*Microscopic examination.*—Stain films from the discharge with Löffler's methylene-blue.

Culture.—*Collection of material.*—A stiff iron wire gauze about 5½ inches long, bent upon itself at one end for about ⅛th inch and contained in a test-tube (6 or more such wires may be placed in a test-tube) is sterilised in hot air. In taking a sample of the discharge from a sore the bent end of the wire is rubbed gently over the floor of the ulcer or under its undermined edge. The pus so picked up is transferred to the medium described below.

Medium.—Rabbit's heart blood, aspirated with a 20 c.c. syringe is distributed in amounts of 1 c.c. in dwarf test-tubes. The blood when coagulated at room temperature is heated for 5 minutes at 55°C. and preserved in ice-box or used immediately. An alternative method is to keep the blood in the ice-box 3 to 4 days before use without heating. The tube is incubated at 37°C. after inoculation with the discharge. After 24 hours a loopful of the serum around the clot is taken, smear made and examined after staining for the characteristic chain of small Gram-negative organisms.

Bacillus pertussis

Synonyms.—*Hæmophilus pertussis;* Bordet-Gengou bacillus, Bacillus of whooping-cough.

The organism in the sputum is a minute, oval rod which is scattered among the pus cells or sometimes within the cells. It is Gram-negative, non-motile and non-sporing. The bacilli are most numerous early in the disease. In cultures it shows similar characteristics but is less pleomorphic than *B. influenzæ.* It stains with dilute carbol-fuchsin.

B. pertussis is a strict ærobe. The most suitable medium for its cultivation is glycerin-potato-blood-agar (Bordet-Gengou medium) of P_H 6·6 on which it forms small, greyish, thick colonies after 48 hours. After a few subcultures on the medium it grows readily on plain blood agar or serum agar media. As compared with *B. influenzæ* culturally the growth of *B. pertussis* is favoured by hæmoglobin media and it is thicker, less transparent and tenacious with the margins more sharply marked off.

Though the disease cannot be reproduced in the lower animals the bacillus forms an endotoxin which is fatal to rabbits and guinea-pigs. Locally the bacillus causes necrosis. Monkeys and young dogs are said to have been infected by intratracheal injections of pure cultures.

Specific agglutinins can be demonstrated in immunised animals which give also the complement-fixation reaction.

Diagnosis.—*Microscopic examination.*—The sputum expectorated during the paroxysm of coughing in the earliest stage of the illness is examined in films stained with Gram's method and carbol-thionin blue.

Culture.—Tubes or plates of potato-blood-agar (Bordet-Gengou medium) are inoculated.

Bacillus Pestis

Synonym.—*Pasteurella pestis*—the causative organism of plague is man and rodents, discovered by Kitasato and Yersin in 1894.

Morphology and Staining.—Typically it is a short oval bacillus (1·5 μ by 0·5 μ) with somewhat rounded ends. In cultures its size varies considerably; it grows as chains and pairs in broth. Involution forms including globular, pear-shaped, elongated or irregular, are present in old cultures and are easily produced by cultivation on media containing 3 to 4 per cent. of sodium chloride. In tissues or when freshly isolated deeper staining occurs at the poles than at the centre, which may even be unstained. This appearance is very well marked when stained with Leishman's or Carbol-thionin. Gram-negative, non-sporing; non-motile.

Culture.—Aerobe; optimum temperature is somewhat lower, about 30°C.; grows on ordinary media.

Agar.—Small transparent colonies which first appear as dew drops, later becoming white.

Gelatin.—No liquefaction.

Bouillon.—Growth appears in the form of a granular flocculent deposit leaving the fluid clear. If a flask of bouillon is richly sown with the organism and kept undisturbed at room temperature, long delicate filaments composed of masses of the bacilli are seen to grow down from the islands of growth which

appear underneath the surface of the medium into the fluid. This is called *stalactite* growth and is greatly facilitated if a little sterile oil or "ghee" is floated on broth.

Milk.—Growth with slight acidity but no clotting.

Indol.—None.

Biochemical Reactions.—The organism produces acid without gas in glucose, maltose, mannite, dextrin, lævulose, galactose, but does not ferment lactose, saccharose or dulcite.

Serological Reactions.—Specific agglutinins may appear in the blood of patients (1 in 10 or 1 in 50) after a week's illness from plague and can be produced artificially by inoculation into animals.

Pathogenesis.—The organism is pathogenic to rats, guinea-pigs, rabbits, squirrels and other rodents. The birds are not so susceptible. The disease in these animals takes the form of an acute and rapidly fatal septicæmia.

The Diagnosis of Natural Rat-Plague.—For purposes of diagnosis naked eye examination by a trained observer is more satisfactory than microscopical examination alone.

1. Subcutaneous congestion may be general or limited to the neighbourhood of the bubo; œdema is present around the glands.

2. Lymphatic glands, particularly those of the neck, are enlarged. Bubo on section shows necrosis, and a yellowish centre even if only the size of a pin-head is in favour of its being a primary bubo.

3. Abdominal organs.—(*i*) Liver.—Small necrotic foci scattered on its surface and throughout its substance. (*ii*) Spleen. —Enlarged and congested with well marked granules or nodules. Sometimes a large wedge-shaped portion is converted into a cheesy mass in which the plague bacilli can be demonstrated. (*iii*) The stomach and the intestine show no change but they may be congested. (*iv*) Pluræ and lungs show hæmorrhages. Presence of pleural effusion either clear or blood-stained is of great diagnostic value.

Attention may be drawn to "chronic" plague in rats. The characteristic feature of the condition is the presence of circumscribed abscesses in lymph glands and spleen containing plague bacilli in rats caught alive, the animals usually showing no sign of ill health. The spleen may show scars. The Indian Plague

Commission gave it the name of " resolving " plague. The evidence as regards the condition becoming acute is not yet conclusive.

Occurrence.—Bacilli are found in large number in buboes, in blood in septicæmic cases and in sputum and pulmonary lesions in pneumonic plague. The organism is also found in the internal organs, especially spleen and liver.

Vaccines.—Haffkine's vaccine is prepared by growing *B. pestis* in neutral broth to which a few drops of sterile oil have been added at room temperature (25°C.) for six weeks. During this period the flasks are shaken every two or three days so as to throw down the stalactite growths and to induce fresh crops of the bacilli. After the culture has been thoroughly shaken and tested for purity by growing on agar and by direct smears, it is sterilised by heating at 65°C. for one hour. Carbolic acid is then added in the proportion of 0·5 per cent. A subcutaneous injection of 4 c.c. in adult confers immunity for a period of about six months.

Serum Therapy.—The serum treatment appears to be of value. An intravenous injection of 80-100 c.c. of serum in adults and of 40 c.c. in children (it may be given intraperitoneally if an intravenous injection cannot be given in children) is followed by another injection in six hours if the temperature has not fallen and in 12 to 18 hours if this has occurred. The amount of serum to be given subsequently and the interval between injections depends upon the temperature and the general condition of the patient. The total quantity of serum required to complete the treatment varies from 150 to 200 c.c. and is given in 6 to 8 injections.

Diagnosis.—*Bubonic Plague.*—Naked eye examination as described before. For bacteriological diagnosis, the exudate is withdrawn from a bubo by means of a hypodermic syringe and (*i*) sown in broth, placed on agar and (*ii*) smeared on a slide which is stained with Leishman or carbol-thionin blue and (*iii*) injected into a guinea-pig or a susceptible rat.

Pneumonic Plague.—The organism may be found in the sputum and isolated as described before.

Septicæmic Plague.—The bacillus may be found in blood and isolated by blood cultures.

In cases where the organism is mixed with other organisms such as in sputum, the exudate is rubbed into the scarified skin

of guinea-pig or the mucous membrane of the nose of a rat.

Plague bacillus resembles a number of organisms from which it has to be differentiated:

1. *B. pseudo-tuberculosis rodentium* resembles it most closely. The plague bacillus causes a characteristic disease in white rats which are, however, immune to *B. pseudo-tuberculosis.* Recently Arkwright has drawn attention to the motility of *B. pseudo-tuberculosis* when grown at 22°C. and in his opinion the test may be employed for distinguishing it from *B. pestis* which is non-motile.

2. Bacilli of the hæmorrhagic septicæmic group including *B. avisepticus* (of fowls), *B. cuniculisepticus* (of rabbits), *B. bovisepticus* (of cattle) and *B. suisepticus* (of pigs), etc., are inhibited in their growth on a taurocholate medium on which plague bacilli grow well.

3. Bacilli of the *Enteritidis* (Gärtner) group are readily distinguished, because they produce no stalactite growth and ferment sugars with the formation of acid and gas.

4. *Bacterium tularense* has been found to cause a plague-like disease in rodents, *e.g.*, in ground squirrels. It does not grow on ordinary medium. A medium consisting of pure egg yolk is required for it. It is a minute (0·3 μ —0·7 μ) non-motile, Gram-negative organism which sometimes takes a coccus form. The bacillus is best stained with Giemsa in tissue preparations, but smears of cultures are readily stained with aniline gentian-violet.

II. ANÆROBIC

This group includes a number of organisms varying in their tolerance to the presence of oxygen. Some of them are facultative anærobes, others cannot multiply in the presence of oxygen at all.

Bacillus Tetani

Synonym.—*Clostridium tetani*—the causative organism of tetanus in man and horse, isolated by Kitasato in 1889.

Morphology and Staining.—The *B. tetani* is a slender bacillus (4 to 5 μ by 0·3 to 0·8 μ). Filamentous forms may also occur. Motility is due to numerous peritrichial flagella which are best seen in preparations made from surface anærobic cultures. After 24 to 48 hours' incubation spores appear situated at one end of the bacilli, thus giving the characteristic drum-stick appearance. The tetanus bacillus is readily stained by the usual aniline dyes and is Gram-positive.

Culture.—Obligatory anærobe; optimum temperature 37°C. The cultures give rise to a stable-manure-like odour. In ordinary media such as broth or agar growth takes place only in the absence of oxygen. The addition of 1 to 2 per cent. glucose, maltose or sodium formate to the media mentioned below renders them more favourable for growth.

Agar.—Surface colonies are small and filmy with finger-like projections. Deep colonies have a central nucleus surrounded by a loose meshwork of fine filaments.

Agar stab.—The growth along the needle track consists of nodules from which fine filaments of growth pass out into the medium. Gas is formed but there is no liquefaction of the medium.

Glucose gelatin.—From the growth occurring along the track of the wire, fine radiating processes grow out in all directions.

Meat infusion broth.—Blood-serum.—Growth occurs within 24 to 36 hours under anærobic conditions.

Minced meat medium.—Growth can take place without the exclusion of air.

Milk.—Milk is a favourable medium and is not coagulated.

Biochemical Reactions.—*B. tetani* has no action upon alcohols, carbohydrates or glucosides.

Serological Reactions.—Agglutinins can be developed by intravenous injections of washed and heated organisms into rabbits. By means of the agglutination test it has been shown that strains of the tetanus bacilli fall into five serological groups.

Occurrence.—The organism is found in richly manured soil, dung heaps, on the skin and in the intestines of animals as a saprophyte. It can also be recovered from the pus of wounds in tetanus.

Pathogenesis.—The disease occurs naturally in man and horses. Tetanus can be reproduced in rats, guinea-pigs and rabbits by subcutaneous or intravenous injections of tetanus cultures.

Toxins.—The pathogenic effects of tetanus bacillus are mainly due to the soluble toxin which it produces. Injections of the filtered cultures of the bacilli into rats and guinea-pigs produce tetanic spasms with death of these animals. The toxin is readily destroyed by exposure to 80°C. for 5 minutes and at lower temperature for a longer period. Drying has no effect but various chemicals such as pyrogallol and sunlight destroy it. The toxin therefore should be kept in a cool, damp place with toluol floated on its surface. The toxin is composed of a spasm-producing substance which is called "tetano-spasmin" and a hæmolysin called "tetanolysin" capable of causing hæmolysis of the red blood corpuscles of various animals.

Serum.—Horses, immunised with the tetanus toxin, yield an antitoxic serum which has been extremely useful in the treatment of tetanus. The serum is standardised by a method similar to that adopted for diphtheria antitoxin. The American antitoxic unit is defined as ten times the least amount of serum necessary to save the life of a 350-gramme guinea-pig for 96 hours against the official test dose of the standard toxin which is furnished by the Hygiene Laboratory, Public Health and Marine Hospital Service, U.S.A. The test dose of this toxin consists of 100 minimum lethal doses for a guinea-pig of 350-gramme weight. This is the L+dose of the tetanus toxin. The antitoxic sera generally contain from 150 to 800 units (U.S.A.) per c.c. In the treatment of tetanus 20,000 to 30,000 units should be given intrathecally as early as possible and repeated if required.

In the prevention of tetanus the antitoxin is of the greatest value and is given as a routine measure in hospitals to patients who have sustained fractures and severe wounds. The dose consists of a subcutaneous injection of 1500 units and is repeated at weekly intervals till 3 doses have been administered.

Diagnosis.—1. *Microscopic examination.*—Several smears of the pus or discharge from the wound are stained by Gram's method and examined for the drum-stick bacilli.

2. *Inoculation.*—The granulation tissue is scraped from the suspected wound and a subcutaneous injection of this material made into a mouse. Death occurs with symptoms of tetanus in 24 to 48 hours.

3. *Isolation.*—Two methods are available.—(*i*) The first method depends on the fact that spores are resistant to the action of heat. The material from suspected wound, soil or fæces is inoculated on inspissated serum or into a deep tube of glucose agar. These are incubated anærobically at 37°C. for 48 hours. The culture is then heated at 80°C. for ¾ of an hour to kill the non-sporing bacilli after which subcultures are made and grown anærobically.

(*ii*) Fildes, taking advantage of the growth characters of *B. tetanus,* has recommended the following method:—A tube of freshly boiled blood broth is inoculated with the suspected material and incubated anærobically for 2 to 4 days. This culture is then added to the condensation water of a sloped peptic or laked blood agar tube and incubated anærobically at 37°C. for 24 to 48 hours. At the end of this period the extreme edge of the spreading film of growth which is extending beyond the growth of other organisms is seen as a tangle of extremely fine filaments, which on examination may prove to consist of *B. tetanus* only.

The Anærobic Organisms Associated with Septic Wounds and Gas Gangrene

These anærobic organisms may be divided into two general groups:—1. The saccharolytic: 2. The proteolytic. This classification is not a rigid one inasmuch as the proteolytic bacteria act on some of the sugars.

The *saccharolytic* or non-proteolytic organisms do not liquefy coagulated serum and grow abundantly on carbohydrate media with the evolution of acid and gas. In bullock's heart medium (page 153) containing both carbohydrates and protein

they form acid and gas but no digestion of the medium. In milk they produce acid and gas. The cultures give out a slightly rancid smell and the meat is turned pink by the acid produced. This group is more important than the proteolytic because the organisms which produce spreading inflammatory lesions belong to this group and the stage following this is the proteolytic state due to proteolytic organisms.

The proteolytic organisms are characterised by the digestion of protein. When grown in bullock's heart medium they form proteolytic ferments which break up muscle fibres with the production of foul smelling sulphur compounds which combining with the iron in hæmoglobin form black compounds. Members of this group are distinguished from the saccharolytic group by the fact that the meat is digested and blackened with the evolution of foul smell. The proteolytic bacilli digest milk with the production of alkali. The chief organisms belonging to the two groups are:—

Saccharolytic.	Proteolytic.
1. *B. welchii.*	1. *B. sporogenes.*
2. *Vibrion septique.*	2. *B. histolyticus.*
3. *B. œdematiens.*	3. *B. tetani* (vide page 277).
4. *B. tertius.*	
5. *B. falax.*	

Saccharolytic group

1. *Bacillus welchii*

Synonyms.—*Clostridium welchii; Bacillus ærogenes encapsulatus,* Welch and Nuttall, 1892; *B. phlegmonis emphysematosæ,* E. Fraenkel, 1893; *B. perfringens,* Veillon and Zuber, 1897—the causative organism of gas gangrene, described by Welch and Nuttall in 1892.

Morphology and Staining.—*B. welchii* is a large bacillus (4 to 8 μ in length) occurring singly, in short chains or in clumps and occasionally in long threads. Non-motile, sporing organism. The spores of *B. welchii* are large, oval and central or subterminal. As seen in tissue fluids and when grown in serum media it is seen to possess a capsule which can be demonstrated by special methods (page 130). Stains readily with basic dyes and is Gram-positive.

Culture.—*B. welchii* grows well on all the ordinary culture media but only under strict anærobic conditions. Optimum temperature 37°C.

Agar.—Greyish-white colonies. *Serum agar.*—Superficial colonies are circular moist with smooth margin. *Gelatin* and *solidified serum.*—No liquefaction. *Glucose broth.*—At first general cloudiness but later the fluid becomes clear and a whitish viscid deposit forms. *Cooked meat medium* becomes pink and there is a considerable amount of gas, but without putrid odour or blackening. *Milk.*—*B. welchii* produces a characteristic reaction identical with that produced by *B. enteritidis sporogenes.* Milk is coagulated and the clot is torn by gas bubbles, the so-called "stormy reaction." Finally there is separation of shreds of the clot from clear whey.

Biochemical Reactions.—*B. welchii* ferments glucose, maltose lactose, saccharose, starch and glycerin with the production of acid and large amounts of gas.

Serological Reactions.—Agglutinins produced in response to injections of *B. welchii* in rabbits are very weak.

Pathogenesis.—*B. welchii* is pathogenic to guinea-pigs, pigeons but much less so for rabbits and mice. Bacilli obtained from a surface growth on a solid medium have no pathogenic action but subcutaneous injections of fluid cultures such as the whey of a milk culture into a guinea-pig causes necrosis and gangrene of the subcutaneous tissues with the formation of gas and exudate, ending fatally in 48 hours.

Toxin.—*B. welchii* produces a powerful soluble toxin under anærobic conditions in broth to which fragments of sterile skeletal muscle of the pigeon or rabbit have been added. The filtered toxin produces a massive destruction of red cells when introduced intravenously. A subcutaneous injection of the toxin produces œdema and sloughing of the part and death of a guinea-pig in 3 days: Rabbits immunised with the toxin yield an antitoxic serum capable of protecting guinea-pigs against many times the lethal dose of the culture.

Occurrence.—Besides being present in the wounds it is a normal inhabitant of the intestinal tract of man and animals.

Isolation.—1. *Culture.*—Milk is boiled in a sterile test-tube, allowed to cool and inoculated with 5 c.c. of a fæcal emulsion in saline. The mixture is heated at 80°C. for one hour and incubated. The development of the "stormy reaction" indicates *B. welchii.* To obtain pure cultures anærobic plate subcultures are made from the milk culture.

2. *Animal Inoculation.*—A rabbit is killed 5 minutes after an intravenous injection with the suspected material and placed in the incubator for 5 to 8 hours. At the end of this, the animal on autopsy will be found to be distended with gas bubbles which are present in all the organs, especially liver. *B. welchii*, if present, can be recovered from the liver and the heart blood.

2. *Vibrion septique* (Pasteur)

Synonyms.—*Clostridium œdematis maligni; Bacillus œdematis maligni* (Koch); *B. septicus* (Mace); Bacillus of Ghon and Sachs and Bacillus III of von Hibler—first described by Pasteur in 1877.

Morphology and Staining.—*Vibrion septique* is a large motile organism with rounded ends, occurring in single rods (3 to 10 μ). It may grow out into long filaments which may or may not be segmented both in the tissues and in fluid culture media. On solid media it occurs as a short rod with rounded ends. It forms oval spores which are central or subterminal in 24 to 48 hours at 37°C. It has no capsule. Stains with basic dyes and is Gram-positive.

Culture.—Obligatory anærobe, optimum temperature 37° C. It grows well anærobically on ordinary media. The cultures give out a peculiar heavy odour.

Glucose agar.—Surface colonies consist of a tangle of filaments which radiate out from the centre and are wooly in appearance. In deep tubes and shake cultures the colonies are similarly wooly showing gas formation, which is most marked in a shake culture. *Gelatin.*—Liquefaction. *Cooked meat medium.* No change in colour. *Inspissated serum.*—No liquefaction. *Milk.*—Coagulation without digestion of casein.

Biochemical Reaction.—*V. septique* produces acid from glucose, maltose and lactose; saccharose, inulin, glycerin and starch are not fermented.

Pathogenesis.—*V. septique* is pathogenic for guinea-pigs, rabbits, rats, mice, sheep, goats, and horses. The ox is immune. A lethal dose of the culture injected into the subcutaneous tissues of a guinea-pig causes the death of the animal within 12 to 24 hours with a widespread gelatinous œdema, and blood-stained serum exudation from the affected part. The underlying muscles have a characteristic deep red colour and are softened with a little formation of gas and no putrefaction.

Occurrence.—*V. septique* has been isolated from milk. Spontaneous infection by the organism occurs in sheep, horses and dogs.

Toxin.—*V. septique* produces an extracellular filterable toxin.

Serum.—Horses and sheep immunised with *V. septique* yield a potent antitoxic serum.

Bacillus chauvœi resembles closely *V. septique* in morphology and cultural characters. In a stab culture the lateral offshoots of growths are larger and more numerous in the case of *B. chauvœi* than those of *V. septique. B. chauvœi* ferments saccharose and not salicin whereas *B. septique* ferments salicin and not saccharose. Liver smears of guinea-pigs dead of *Vibrion septique* infection show long swollen filaments which are absent in *B. chauvœi* infection. *B. chauvœi* is less pathogenic to laboratory animals and produces less gas in the tissues than *V. septique. V. septique* is Gram-positive whereas *B. chauvœi* is considered to be Gram-negative.

3. *Bacillus œdematiens*

Synonym.—*Clostridum œdematiens*—first described by Weinberg and Séguin.

Morphology and Staining.—This is a large bacillus 0.8μ long, occurring in chains in cultures. As seen in the wound exudate it possesses flagella and is motile. It forms oval subterminal spores readily in all media. Gram-positive.

Culture.—Obligatory anærobe; optimum temperature 37°C., grows well on ordinary media but only in the absence of oxygen.

Meat broth.—Gas and pinkish colour which fades rapidly are formed. *Gelatin.*—No liquefaction. *Inspissated serum.*—No liquefaction. *Agar.*—Shake cultures are delicate and wooly.

Biochemical Reaction.—*B. œdematiens* produces acid from glucose, lactose and maltose but does not attack any other sugar.

Pathogenesis.—*B. œdematiens* is pathogenic to guinea-pigs. rabbits, rats, and mice. The lesion in the guinea-pig produced by the subcutaneous injection of the culture or the toxin is characterised by a rapidly spreading gelatinous œdema with little or no gas formation and death of the animal in 6 to 30 hours.

An antitoxin has been produced by repeated injections of the toxin into horses and sheep.

4. *Bacillus tertius*

Synonyms.—*Clostridium terium;* Bacillus IX of von Hibler; Bacillus Y of Fleming.

Morphology and Staining.—It is a long and thin bacillus; non-motile. The spores are terminal. Gram-positive, but this characteristic is lost on cultivation.

Culture.—Anærobe. Optimum temperature 37°C. Grows well on ordinary media under strict anærobic conditions.

Agar.—Surface colonies look like round semi-transparent discs. The deep colonies are lenticular. The growth tends to spread in a film on a moist surface. *Milk.*—Gas and clot. *Cooked meat medium.*—Acid and gas and pinkish colour. *Gelatin* and *inspissated serum.*—No liquefaction.

Pathogenesis.—Non-pathogenic but is present with other organisms in the tissue.

5. *Bacillus fallax*

B. fallax is a slender bacillus. It has a capsule and is slightly motile. Spores formed on inspissated serum are oval in shape and subterminal in position. Gram-positive in exudate and young cultures, it tends to become Gram-negative in old cultures.

Culture.—Anærobic, deep colonies are of lenticular shape. *Milk.*—Curdled. *Gelatin and inspissated serum.*—No liquefaction.

Biochemical Reactions.—*B. fallax* ferments glucose, maltose, galactose and lævulose with the evolution of gas.

Pathogenesis.—Pathogenic for guinea-pigs, mice but not for rats. A powerful toxin has been obtained.

Proteolytic Group

The organisms of this group are unable by themselves to produce gas gangrene without the presence of one or more types

of bacilli of the saccharolytic group. They are not pathogenic and do not produce toxins. Two methods are available for the separation of proteolytic and saccharolytic groups:—

1. Rapid transplantation in sugar media causing the proteolytic organisms to be outgrown by the saccharolytic ones.

2. Animal inoculation.—The organisms of the saccharolytic group being more pathogenic frequently invade the blood stream after intramuscular injection and can be isolated from heart's blood.

B. sporogenes.—(*Clostridium sporogenes*).—Gram-positive bacillus, actively motile, forming oval subterminal, rarely terminal spores in all media and in the animal body.

Culture.—Anærobic, the cultures have a putrid odour. *Glucose agar.*—Surface colonies have a granular centre with an arborescent appearance at the margin. *Gelatin and coagulated serum.*—Liquefaction. *Cooked meat medium.*—Development of gas with rapid digestion. The meat turns dirty purple, ultimately black. *Milk.*—No coagulation but there is precipitation of the casein which is rapidly digested.

Biochemical Reactions.—Glucose, lævulose and maltose are fermented.

Pathogenesis.—Non-pathogenic.

Bacillus histolyticus

Synonym.—*Clostridium histolyticum.*

Gram-positive anærobe, motile, forming oval terminal spores in all media.

Culture.—No gas and no odour in cultures. *Gelatin and coagulated serum.*—Liquefaction. *Milk.*—Same as *B. sporogenes. Cooked meat medium.*—Rapid digestion with foul odour with separation of white balls of acicular crystals of tyrosin.

Pathogenesis.—No soluble toxin, non-hæmolytic. Injection of large dose of the culture causes a rapid digestion of the tissues.

Bacillus botulinus

Synonym.—*Clostridium botulinum*—the causative organism of meat poisoning, discovered by van Ermengem in 1896.

Morphology and Staining.—The bacillus is large (4 to 9μ by 0·9 to 1·2 μ) with rounded ends and may occur singly, in pairs or in short chains. The bacilli are motile and possess from 4 to 8 flagella, peripherally arranged. Spores, usually subterminal, are formed when cultivation is carried out at 20° to 25° C. The bacilli stain readily with ordinary aniline dyes and are Gram-positive.

Culture.—The organism can be readily cultivated on ordinary media but only under strictly anærobic conditions. It grows in a neutral or slightly alkaline medium, acidity inhibiting its growth. Optimum temperature 25° C. The cultures have a sour odour due to the development of butyric acid.

Glucose agar stab.—Growth occurs as a thin white column commencing a little below the surface of the medium. Soon the medium is split up in various directions by the abundant development of gas.

Agar.—The colonies on plates are yellowish, opalescent and round with a fringed periphery.

Gelatin.—At 20° to 24° C. the growth is similar to that on agar except that liquefaction with abundant gas formation occurs.

Glucose-Gelatin plates.—The appearance of the colonies is regarded as diagnostic. The colonies are round, yellowish and semi-transparent, consisting of granules which show constant motion along the periphery in the zone of liquefaction.

Glucose broth.—General clouding with formation of gas.

Milk.—Not coagulated. Coagulated serum is not liquefied by the *B. botulinus* which is non-proteolytic.

Biochemical Reactions.—Amongst the various sugars, glucose and to a less extent maltose, lactose, glycerin and starch alone are fermented with the evolution of gas.

Pathogenesis.—Mice, guinea-pigs and monkeys are extremely susceptible and rabbits, cats, dogs and rats are relatively resistant to the action of the *B. botulinus* and its toxin. The pathogenic effects of this organism are due to the extracellular toxin which it produces. A guinea-pig of 250 grammes weight may on subcutaneous inoculation with 0.0001 c.c. or less of the culture-filtrate die with symptoms of paralytic nature. The properties of the botulinus toxin are similar to those of the tetanus toxin. It has been shown that there are two types of the

B. botulinus (A & B) which produce distinct toxins; the antitoxin to A does not neutralise toxin B and vice versa.

Occurrence.—The organism occurs in the soil and also in infected meat and canned vegetables which transmit the disease to man. Botulinus may attack chickens and produce a condition known as "limber-neck."

Serum.—A potent antitoxic serum has been produced which has been used with great benefit in the treatment of botulismus.

CHAPTER VIII

THE SPIROCHÆTÆ AND RICKETTSIA AND THE FILTER-PASSING VIRUSES

SPIRONEMA PALLIDUM

Synonyms.—*Spirochæta pallida; Treponema pallidum*—the causative organism of syphilis, described by Schaudinn and Hoffmann in 1905.

Morphology and Staining.—*Spironema pallidum* is a spiral-shaped organism, having close, sharp, regular twists 6 to 14 in number and presenting a corkscrew appearance. It is 4 to 14 μ long and 0·25 μ thick. In fresh specimens, *e.g.*, in a chancre exudate with a dark-ground illumination, it is actively motile, showing movements of flexion, rotation and gliding to and fro. There is, however, very little actual movement. There is a delicate single terminal flagellum at each end. It stains with difficulty but various staining methods have been adopted of which Becker's is to be recommended.

Culture.—It grows with difficulty and does not grow on ordinary media. The most suitable medium is that devised by Noguchi, consisting of 2 parts of 2 per cent. agar, 1 part of sterile ascitic or hydrocele fluid, to which is added a piece of sterile organ, *e.g.*, rabbit's kidney. The medium is placed in deep tubes and a thick layer of sterile paraffin oil or vaselin floated on the top. The medium is inoculated through the oily layer with the exudate by means of a capillary pipette. Both the spironemata and the bacteria form a thick line of growth along the line of inoculation but later the spirochætes spread out into the surrounding medium showing a haze of growth. To obtain pure cultures the tube is cut and subcultures are made in the Smith-Noguchi medium from those portions of the deep tube which are apparently free from bacterial contamination.

Serological Reactions.—*Wassermann Reaction.*—The theoretical considerations and the technique of this test have been described on page 174. It is necessary to discuss the significance of the test as applied to the diagnosis of syphilis. A strong positive reaction is undoubted evidence of syphilis. The reaction is positive in all cases of primary and secondary syphilis, in about 80 to 90 per cent. of tertiary syphilis and in parasyphilitic affections such as tabes, and in about 50 per cent. of cases

of Yaws and leprosy which are the only other two diseases in which a strong positive reaction has been recorded. It must be remembered that syphilis may be associated with one of these diseases. Weak positive reactions are occasionally present in scarlet fever, in malaria during the febrile periods, in some cases of relapsing fever, in acute tuberculosis and urticaria pigmentosa. A weak positive reaction is of no diagnostic value. The conditions under which a negative reaction may be obtained in a case of syphilis are: (1) the examination of blood before the 4th or 5th week after infection, (2) in some cases of late syphilis, notably tabes dorsalis, (3) after consumption of a certain amount of alcohol by the patient immediately before his blood is taken or in chronic alcoholism, (4) after the administration of chloroform as an anæsthetic, (5) in pregnancy, (6) during the puerperium and (7) in the presence of an antiseptic in the syringe with which the blood is withdrawn from a vein. The foregoing remarks apply to untreated cases only. In treated cases it is, in addition, important to remember that as a result of anti-syphilitic treatment the Wassermann test becomes negative. In treated cases a weak positive reaction gives valuable indications, whereas in the untreated cases no importance is attached to a weak positive reaction. In cases of early, late and latent syphilis, the reaction may be emphasised by a small provocative injection of one of the arsenical drugs provided the blood is tested 2 to 6 days after the injection. In cases of congenital syphilis the test may be negative during the first week of life and may again become negative at the age of 17 to 20 years.

Flocculation tests.—Several different methods have been described for carrying out the test. The various tests known as the Sachs-Georgi reaction, the Sigma reaction of Dreyer and Ward, Kahn's reaction or Meinicke's reaction, are all flocculation tests. Only one of these, *i.e.*, Kahn's, has been described herein. These tests correspond closely with the Wassermann reaction in their results but are not likely to completely replace the latter.

Pathogenesis.—Syphilis does not occur naturally in the lower animals. Chimpanzees and other monkeys are susceptible, the former being more susceptible than the latter. Inoculation of the material containing spirochætes by scarification on the skin of the eyebrows or genitals is followed by an indurated papule or papules in about 30 days with enlargement and induration of the corresponding lymph glands. Secondary lesions of a mild nature with no subsequent tertiary lesions have been observed. Spirochætes can be demonstrated in the local lesions and by inoculating fresh chimpanzees with the material from these lesions the disease can be transmitted. The subcutaneous

and other methods of inoculation, with the exception of intra-testicular and intravenous, have no effect but rabbits can be infected by introducing the virus into the anterior chamber of the eyes. Levaditi and Marie hold that there are two distinct strains of *S. pallidum*: one the "dermotropic" form having a marked affinity for the skin and the other "neurotropic" form which causes tabes and general paralysis of the insane in the human subject. The absence of tabes in India and other tropical countries is explained as being due to the fact that the "dermotropic" is the predominant form in these countries.

Occurrence.—The organism is met with in most of the pathological lesions produced by it in man, *e.g.*, it is frequently present in the primary sore, in the related lymphatic glands, in the papular and roseolar rashes, in condylomata and mucous patches. It has occasionally been found in the spleen and liver. In congenital syphilis, it occurs in large numbers in the pemphigoid bullæ, blood and in the internal organs, *e.g.*, spleen, liver, suprarenals, lungs and even in the heart.

Diagnosis.—A.—Microscopic Examination.—This should always be made prior to arsenical treatment.

Collection of Material.—In collecting serum from a syphilitic ulcer, rubber gloves should be worn. After thoroughly washing the ulcer with a piece of gauze soaked in saline the base of the ulcer is squeezed firmly until serum exudes from the surface or edge. Avoid hæmorrhage, and if blood appears it should be mopped off with gauze. A Harrison's spud or a needle may be used to scarify the edge of the ulcer which is then squeezed. The serum obtained from the edge and from close to the faint pink ærola contains the largest number of spirochætes and this should be collected. If the ulcer has been treated with an antiseptic or if there is a superadded pyogenic infection, a saline foment is applied for 24 hours and the sample then collected in the manner above described.

In taking serum from a *papule or a pustule* of the secondary rash the superficial epithelial layers are removed by scarification and a few scratches made on the indurated base which is then squeezed.

When it is not possible to obtain a sample from the ulcer owing to the narrowness of the prepuce or the complete healing of the ulcer or the cauterisation of the ulcer with strong antiseptic, an attempt should be made to collect fluid from a *gland* provided it is enlarged and of a semi-solid consistency. With the patient in the recumbent position the area over the gland and

the skin around it is cleaned with ether or alcohol. A hollow needle of about 56 gauge is inserted at one pole of the gland and pushed into the substance. A small syringe containing 0.1 to 0.2 c.c. of distilled water is fitted to the needle and this injected into the gland. The syringe is disconnected, the gland massaged, the syringe again fitted to the needle and the mixture is used in making slides or collected in a capillary pipette for transmission to a laboratory. The puncture made by the needle is sealed with collodion.

In some cases the prepuce cannot be retracted owing to the risk of balanitis and the ulcer is situated on the under surface of the prepuce or on the coronal sulcus. To expose the ulcer in these circumstances operative procedures, involving the removal of a piece of the prepuce or even complete circumcision, are necessary.

If a specimen is to be obtained from lesions of the mouth, the inclusion of saliva in the specimen is to be avoided as this may contain other spirochætes which are not easy to distinguish from *Spironema pallidum.*

Transmission.—It is important to bear in mind that the collection in capillary tubes with the subsequent heating in sealing and delay in transmission of the specimen, lessens the chances of finding the spirochætes and examination should, if possible, be made on the spot.

1. *Examination by Dark-Ground Illumination.*—If possible a cover-slip held in a cornet forceps is applied to the freshly exuded serum, and the serum is spread in a thin uniform layer by means of a platinum loop. The cover-slip is applied to a special slide of suitable thickness and examined by dark ground illumination as soon as possible.

2. *Indian-ink Method.*—A drop of the serum to be examined is mixed with one drop of Indian ink on a slide and spread in a thin film. The film is dried in the air and examined under the 1|12 in. oil-immersion. The *Spironema pallidum* appears as a white undulating thread against a dark (yellowish or dark brown) back ground.

3. *Stained Films.*—The best methods are those of Giemsa and Becker's. *S. pallidum* is coloured rose-pink. In Fontana's method silver nitrate is used for staining the organism.

Morphologically *S. pallidum* resembles other spironemata given below, from which it has to be distinguished. The diagnosis rests on the number of spirals a spirochæte has, compared to the length of a red blood cell. *S. pallidum* should have seven coils

to the width of a red cell. The counting of the spirals contained in the length of a red blood cell is facilitated by the use of an eye-piece graticule which introduces a number of artificial blood cells into the field of view.

Spirochæta refringens is a comparatively coarse organism with irregular spirals and blunted ends, very highly refractile, has a greyish colour, few spirals in the length of a red blood cell, and is more actively motile than *S. pallidum.* It occurs in smegma and in ulcerating lesions of the skin and stains blue with Giemsa.

Spirochæta gracile is much thicker than *S. pallidum* and is more highly refractile and is more motile. The spirals, which are regular, are neither so close nor so numerous.

Spirochæta minutum was isolated by Noguchi from the secretions of the external genitalia.

Spirochæta balantidis is a short thick organism with only 2 or 3 twists: the coils are open. It is highly refractile and very actively motile.

Spirochæta pertenuis (Synonyms.—*Spirochæta pallidula, Treponema pertenue Castellani*) of Yaws (Framboesia) is more refractile and the curves are flatter and less regular. The disease is inoculable into monkeys. Immunity reactions in monkeys and rabbits infected with syphilis and framboesia have shown that the two diseases are distinct as animals cured of syphilis were susceptible to framboesia but not to syphilis.

Spirochæta Microdentium is met with in the mouth. Its spirals are narrower than those of pallida. It has a rusty appearance in contrast to the dead white colour of the pallida.

Spirochæta of Vincent's angina is found in association with fusiform bacilli in mouth lesions, occasionally in vulvar and vaginal lesions. The undulations or curves are fewer in number and are of wide amplitude.

B.—Serological reactions.—1. *Wessermann test* (See page 174).

2. *Collodial Precipitation tests for syphilis.*

A number of tests have been devised to simplify the serum diagnosis of syphilis, and are based on the fact that a syphilitic serum when mixed with a Wassermann "antigen" gives a white precipitate or flocculation. Of the various tests probably the most commonly used is that devised by Kahn.

Apparatus Required.—

(1) Dwarf test-tubes preferably Dreyer's agglutination tubes, (2) Pipettes (3) Rack.

Reagents Required.—

1. *Preparation of the extract* is an important element in the test. Remove the fat, fibres, tissue, vessels, etc., from beef heart; mince it thoroughly in a meat grinder and pound it with sand. Spread the mince on a plate and dry it in the current of air from an electric fan. Powder the dried meat and pass it thrice through a coffee mill. Place 50 grammes of the dry powder in a 500 c.c. Erlenmeyer flask and cover it with ether. Keep the flask in the ice box for 24 hours, pipette off the ether carefully, add some more ether to the flask and allow it to remain as before. Repeat the washing with ether once more and on the fourth day the last ether wash is filtered off. Spread the extracted muscle out on a plate to dry at room temperature until no odour of ether can be detected. Add 100 c.c. of absolute alcohol to 20 grammes of the dry muscle and allow the mixture to stand in the ice-chest for nine days and for one day at room temperature. Filter the alcoholic extract. The filtrate is usually 20 c.c. To half the quantity of the filtrate, add 140 mgms. of cholesterin and shake the flask vigorously. To the other half of the alcoholic extract no cholesterin is added.

2. *Preparation of the patient's serum.*—The serum is inactivated in the manner described under "Wassermann reaction."

Method:—Arrange a number of agglutination tubes. Two tubes are required for each specimen of serum. Place 0·3 c.c. of the clear inactivated serum into each of the two agglutination tubes. For use the antigen is diluted as follows. Place 1 c.c. of the cholesterin antigen in a test-tube and 3 c.c. of saline in a 10 c.c. cylinder. Pour the saline into the tube containing the antigen and transfer the mixture from the tube back to the cylinder. The antigen is now ready for use. Non-cholesterin antigen is also prepared in the same way but only two parts of saline to one part of the alcoholic extract are used.

To one tube add 0·05 of the diluted cholesterin-antigen; to the other tube add an equal quantity of the non-cholesterin antigen. Controls with positive syphilitic and normal sera are also put up.

Place the tubes in a rack and shake for three minutes. Incubate at 37°C. overnight and read the results next morning.

With strongly positive sera the precipitation is very marked and the following notation has been recommended for recording the various grades of flocculation. Readings may be made with the naked eye or with a reading glass against a slanted back ground.

+++=(Strongly positive)—heavy flocculation and clear supernatant fluid.

++=(Moderately positive)—diffuse, finely flocculent precipitate.

+=(Weakly positive)—slight amount of precipitate.

—=(negative)—no precipitation, a slight amount of deposit may be present which is easily dispersed in the supernatant fluid.

It is advisable to test the serum with two or three dilutions of the same extract or with two or three different extracts. In the case of cerebro-spinal fluid, 1 c.c. of the fluid is used.

Leptospira Icterohæmorrhagiæ

Synonym.—*Spirochæte icterohæmorrhagiæ*—the causative organism of spirochætal or infective jaundice (Weil's disease) discovered by Inada in 1915.

Morphology and Staining.—As seen in the blood and tissues *L. icterohæmorrhagiæ* is 6 to 9 μ long and 0·25 μ thick; Larger forms are met with in cultures. It is a spiral filament with numerous fine elementary spirals with the ends incurved or "hooked." The individual spirals are in close apposition. Occasionally secondary spirals are also seen. In stained preparations the elementary spirals are seen with difficulty.

Culture.—Noguchi's medium (modified) is the most suitable. Neutral or slightly alkaline agar is melted; when cooled to 60° to 65°C. add 1 to 2 parts of the agar to a mixture of rabbit's serum 2 parts, Ringer's solution (or 0·9 per cent. NaCl solution) 6 parts, citrated rabbit's plasma (or sterile defibrinated rabbit's blood) 1 part. Shake the tube thoroughly so as to get a uniform mixture. The medium is covered with a layer of sterile liquid paraffin.

Pathogenesis and Occurrence.—The organism occurs in the blood of patients in the first 4 to 5 days of the disease and in the internal organs such as liver, spleen, kidneys, adrenals, and is passed in the urine. The largest numbers are found in the liver, sections of which may be stained by the Levaditi method. The spirochæte is a natural inhabitant of both house and field rats and field mice which show no signs of ill health.

The intraperitoneal inoculation of blood taken during the first 3 to 6 days of illness and of an emulsion of organs rich in the leptospira causes infection in guinea-pigs with symptoms of jaundice, albuminuria, anæmia and fever, with a fatal result in 7 to 12 days. The *leptospiræ* invade the peripheral blood about the 4th day of the disease. At autopsy the infected animals show hæmorrhages which may be generalized or confined to the lungs, intestinal walls, etc. In the organs of the infected animals the organisms occur in the interstitial tissues in contrast to the human disease in which the organism is intracellular. Infection in man probably occurs through the alimentary canal, but it is possible that infection may occur through the unbroken skin. *Leptospiræ* similar to *L. icterohæmorrhagiæ* occur in water, mines, etc. but they are non-pathogenic.

Immunity Reactions.—The blood of patients and animals experimentally infected contains agglutinins (Agglutination reaction) and bactericidal substances (Pfeiffer's Reaction).

Serum.—Immune serum has been extensively employed in the treatment of the disease with great benefit.

Diagnosis.—1. *Microscopic Examination.*—Blood should be examined by dark-ground illumination before the third day of illness: leptospiræ are seldom found—or their numbers are unusually very small in peripheral blood and are practically never found after the 12th day. The urine may show leptospiræ from the 9th day till convalescence, especially in the 3rd week of the 9th day till convalescence, especially in the 3rd week of the disease. The deep green colour produced by adding acetic acid to urine is said to be of diagnostic importance.

2. *Animal Inoculation* is carried out by injecting 3 to 10 c.c. of the blood of a patient within the first 7 days of illness into the peritoneal cavity of a guinea-pig. If the fever has subsided the centrifuged sediment from urine obtained by a catheter should be injected into the peritoneum of the guinea-pig. On the guinea-pig dying about the 5th to 8th day its kidneys are dissected out and macerated in some normal saline which is then examined by the dark-ground or after staining with Fontana's or Giemsa's stain.

From the 15th day onwards, the diagnosis may be made by allowing 1 c.c. of the patient's serum to act on several times the lethal dose of the leptospira for 15 minutes. A guinea-pig injected with this mixture will not develop the disease whereas the control guinea-pigs, which have had no immune serum, will die with the characteristic symptoms of the disease.

3. *Agglutination Test.*—The agglutinins appear in blood of patients about the 7th day of illness and persist for about two years. A culture of leptospira on a solid medium is agglutinated by the patient's serum in a dilution of 1 in 500 or even as high as 1 in 1000. Instead of the culture, the centrifugalised deposit of a specimen of the urine rich in leptospira can be used for the agglutination test.

Leptospira Icteroides

This was described by Noguchi in 1918 as the causative organism of yellow fever. There is evidence, however, to support the view that a true filterable virus is the cause of the disease. (See filterable viruses).

This is similar in morphology to the *L. icterohæmorrhagiæ* from which it differs in certain immunity reactions and pathogenic properties. An immune serum against *L. icterohæmorrhagiæ* does not afford protection against infection with *L. icteroides* although recent work has proved the serological identity of the organisms. With regard to pathogenicity, it may be mentioned that *L. icteroides* causes fatty degeneration of the parenchyma of the kidney which is not a marked feature of the *icterohæmorrhagiæ infection,* hæmorrhages in the latter occurring more frequently in the serous membranes. The organism occurs in the blood of patient's only during the first 3 to 5 days of the illness. Infection to man is conveyed by *Stegomyia fasciata* (*Synonym.—Aedes calopus*). The organism has been cultivated on Noguchi's medium and is filterable. Protective antibodies in the blood of infected animals can be demonstrated by the Pfeiffer's reaction. The common monkey, Macacus rhesus, is susceptible to yellow fever.

Diagnosis.—The methods employed in diagnosing this condition are the same as for *L. icterohæmorrhagiæ.* In the blood of man it is demonstrated with difficulty.

Leptospira Hebdomadis

This was described by Ido, Ito and Wani as the causative organism of seven-day fever in Japan.

L. hebdomadis is morphologically identical with *L. icterohæmorrhagæ* from which it differs in pathogenicity and in serological reactions. *L. hebdomadis,* though pathogenic to guinea-pigs of 250 grammes weight on intraperitoneal inoculation, is not so to larger animals and the hæmorrhages and icterus are not a characteristic feature of the disease produced. The sera of convalescents of seven-day fever and of animals immunized

with *L. hebdomadis* give a positive Pfeifferis reaction with *L. hebdomadis* but react negatively with *L. icterohæmorrhagiæ*. The organism can be demonstrated in the blood of infected human subjects and of animals, and, *post-mortem,* in the liver and kidneys. It is a natural inhabitant of wild mouse (*Microtus montebelli*) in which it occurs in the kidney and is excreted in the urine. It is probably conveyed in the same manner as the *L. icterohæmorrhagiæ*. It is not yet certain if the seven-day fever of India is the same as this disease.

Spirochæta Morsus-Muris.

Synonym.—*Spirillumminus.*—Described by Futaki in 1915 as the causative organism of Rat-bite fever.

The organism is found in the skin lesions, blood and lymph glands. The *Sp. morsus muris* occurs as a short (2 to 6 μ in length), thick spiral filament, having a flagellum at each end. As seen with dark-ground illumination the organism possesses active motility of "darting" type. It moves rapidly by active rotatory movement. It is easily stained by means of a Romanowsky stain.

Diagnosis.—*Microscopic Examination.*—Fluid aspirated from an enlarged gland in the neighbourhood of the bite, or blood from a vein, is examined by dark-ground illumination or stained with Giemsa and examined.

Animal Inoculation.—3 to 5 c.c. of the blood obtained from a patient during the pyrexial stage by venous puncture, or, preferably the fluid obtained from an enlarged gland, is injected into the peritoneal cavity of a guinea-pig or a white rat. A more reliable method is to excise an enlarged gland and to inject an emulsion of the gland into the peritoneum of a guinea-pig. The spirillum can be demonstrated in the peripheral blood by dark-ground illumination or staining.

Spirochæta Obermeieri

Synonyms.—*Treponema obermeieri; Borrelia recurrentis; Spironema recurrentis*—the causative organism of relapsing fever, described by Obermeier in 1873.

Morphology and Staining.—The organism as seen in the blood during the febrile paroxysms is a very thin and delicate spiral filament, measuring 10 μ to 30 μ in length and 0·35 μ in

thickness. The organism has fairly regular coils which vary in number according to the length of the organism. The extremities are pointed. The organism possesses a single terminal flagellum and is actively motile. It multiplies by longitudinal as well as transverse fission; the latter phenomenon is often seen in the blood when a long spiral filament shows portions which are thinner than the rest of the organism and are less deeply stained. It stains faintly with aniline dyes. It is best stained with the Romanowsky stain or one of its modifications. It is Gram-negative.

Culture.—Growth on ordinary media does not occur. Noguchi's method is the most satisfactory one. A piece of sterile tissue, *e.g.*, rabbit's kidney, is placed in a sterile test-tube, a few drops of citrated blood from the heart of an infected animal, *e.g.*, mouse or rat and 15 c.c. of sterile ascitic or hydrocele fluid are then added. To prevent evaporation, a layer of sterile paraffin oil is poured on the surface. Incubation is at 37°C. The greatest multiplication takes place about 7th to 9th day at the junction of the ascitic fluid and blood.

Serological Reactions.—Immunization of an animal such as a rat yields sera rich in agglutinins and in bactericidal bodies. The test has been extensively used in distinguishing the different species of the spirochætes of relapsing fever. The serum of a rat immunized against a given spirochæte will only agglutinate that species and cause it to undergo dissolution (Pfeiffer's reaction), but has little or no action on other species. In this way a number of under-mentioned species have been recognised.

Spironema Duttoni.—Synonym.—*Treponema duttoni.* The relatively mild disease caused by this organism is prevalent in Africa and the relapses though of shorter duration are more numerous. It is transmitted by a tick, *Ornithodorus moubata.* The organism is more pathogenic to monkeys than *Sp. obermeieri* and a larger number of laboratory animals are susceptible to it. Immunity produced by *Sp. obermeieri* affords no protection against *Sp. duttoni* and *vice versa.*

The spironema of relapsing fever in India and in North America are respectively known as *Spironema carteri* and *Spironema novyi* and are identical with *Sp. obermeieri.*

The parasite of the Persian form of the disease is known as *Sp. berberum.* Gerbilles and monkeys are susceptible to it. It is probably carried by ticks. Immune serum does not agglutinate *Sp. recurrentis.*

The South American relapsing fever is caused by *Sp. venezuelense* which is carried by ticks (*Ornithodorus venezuelensis*).

The specific immune serum of this organism does not agglutinate *Sp. duttoni.*

Pathogenesis.—Monkeys, mice and white rats can be infected by the inoculation of blood containing *Sp. recurrentis* and in mice intraperitoneal injection is followed by a typical attack of fever with two or three relapses. Rabbits and guinea-pigs are immune. The disease is conveyed to man by infected lice. Infection does not occur through their bites. The lice are crushed and their intestinal contents and fæces are rubbed into abrasions caused by the victim's scratching.

Occurrence.—The organism is present in the blood during the fever and in the internal organs during the fever-free periods.

Diagnosis.—1. Blood films are made and stained with Giemsa or Leishman.

2. Dark-ground illumination may be employed to examine for the organism in a fresh condition when it is seen to be motile.

3. *Animal inoculation.* Rats or mice may be inoculated with the blood by the intraperitoneal route.

Rickettsia Infections

Under this heading a group of diseases have been included the etiological factor of which is believed to be a minute organism called *Rickettsia* which is transmitted by insects. These diseases are:—Typhus fever, Trench fever, Rocky Mountain spotted fever and Japanese river fever. It should however be borne in mind that the relationship of *rickettsia* to these diseases has not yet been completely established. The term *Rickettsia* has been applied to certain minute organisms (0.3 to 0.5 $\mu \times 1.5$ to 2.0 μ), which may occur in the shape of cocci, diplococci or short bacilli . They stain rather faintly with aniline dyes, do not retain Gram's stain and are not acid-fast, but stain well with Giemsa, when they appear as small dots, double cocci or bipolar staining bacilli of a reddish or purplish colour like that of the nucleus of a leucocyte. They are non-motile.

Culture.—There is no satisfactory evidence that the *rickettsia* have ever been successfully cultivated on artificial media though it is stated that Nöller was successful in cultivating *R. melophagi* on a blood-agar medium.

Occurrence.—*Rickettsia* occur specially in blood films and are best seen in de-hæmoglobinised thick drops taken during the periods of fever. They are found in enormous numbers in the stomach and mid-gut of lice which have been fed on patients during the height of the fever a few days previously. They also occur in the fæces of the insects. On the other hand similar bodies have been found in the various blood-sucking insects without causing any kind of infection in their hosts.

The Nature of Rickettsia Bodies.—Prowazek and Rocha-Lima regarded the rickettsia as being protozoa on account of their transmission by insects, the relapsing character of the fever, and the peculiar staining properties of these bacilli. As regards staining reactions it has been shown that with Giemsa the staining reaction is very much like that of bacteria. There is a tendency now to regard *rickettsia* as a class by itself possessing distinct characteristics.

Their Relationship to the Disease.—It has been shown that lice fed on a typhus fever patient show enormous numbers of *rickettsia* in their stomach. Lice so infected are able to give the disease to guinea-pigs when crushed and injected subcutaneously. Lice fed only after the defervescence do not contain rickettsia and are not infective. Lice kept at a temperature of 23° C. do not show rickettsia and are not infective. A temperature of 32° to 35° is the optimum for producing infection and rickettsia in the lice. The presence of small bodies in lice fed on healthy persons and in the various blood sucking insects apart from the occurrence of any disease in their mammalian hosts was regarded at one time as militating against their relationship to any disease. It was, however, pointed out later that there were many different species of *Rickettsia*, some of which were pathogenic and some non-pathogenic.

Typhus Fever

Etiology.—*The virus of typhus fever*, whatever its nature, is present in the blood of typhus patients during the febrile period because the injection of blood into the peritoneum of chimpanzies and guinea-pigs is followed, after an incubation period of 7 to 12 days, by an illness characterised by fever and loss of weight. The fever lasts for 7 days and rarely ends fatally. In man the disease is very fatal; the incubation period averages 12 days; the temperature rises on the 3rd day of onset and lasts about 12 days and the rash appears on the 5th day and lasts up to 10 days.

The virus is present during the fever in the blood and internal organs. There is now a considerable support for the view that *R. prowazeki* is the ætiological organism of typhus fever. The *R. prowazeki* occurs exclusively in the cells of the stomach of the louse.

Proteus-X 19 was at one time believed to be the causative organism but this view has now been discarded. It is a remarkable fact that *Proteus*-X 19, a strain isolated by Weil and Felix from the urine of a typhus patient, shows marked agglutination with the blood of typhus. The agglutination test as applied to typhus fever blood with X 19 is known as *Wel-Felix Reaction.* Some normal sera may react with *Proteus*-X 19 in a dilution of 1 in 50 but typhus-blood serum agglutinates the organism in a dilution of 1 in 100 or even 1 in 1000. The reaction appears about the 7th day of illness and is so constantly present in typhus patients and absent in other diseases that it is of distinct value in the diagnosis of typhus fever. Attempts to identify the Plotz bacillus with this disease have not been successful. Tabardillo of Mexico, Brill's disease of U. S. A. and the so-called tick-typhus of India are local forms of typhus fever, the last two being less virulent than true typhus.

Trench Fever

Also known as *Wolhynian Fever.* This is a disease characterised by relapses of fever at intervals of 7 to 10 days. The disease was prevalent on the Western Front during the Great War, and is carried by the bites of infected lice and by their excreta when rubbed into abrasions. The virus is filterable and is present in the blood so that the disease can be transferred from man to man by intravenous and intramuscular injections of whole blood. The urine of patients is also infective. The virus is rendered inert at a temperature of 70°C. but not below this.

Rickettsia quintana described to be the causal organism is present in blood as well as in the infected liver.

Rocky Mountain Spotted Fever

The disease is prevalent in U. S. A. and is transmitted by a tick *Dermacentor venustus.* The disease is inoculable into monkeys and guinea-pigs. The rickettsia bodies responsible have been named *Dermacentroxenus rickettsi.*

Japanese River Fever

Also known as *Tsutsugamushi* is another disease prevalent in the river valleys of Japan and Formosa during harvest time and

is caused by rickettsia bodies. The disease is transmitted by the bites of the larval forms of an acarus or mite (*Trombicula akamushi*). The injection of the blood of patients reproduces the disease in monkeys and guinea-pigs.

Filter-Passing Viruses

Whilst a large number of infective diseases are caused by micro-organisms which cannot pass through the pores of a porcelain filter and which can be seen under the microscope, there are others the causative organisms of which cannot be demonstrated by ordinary microscopic methods nor can they be retained by a filter. Such organisms have been called filterable or ultra-microscopic viruses.

The principal characters of the filterable viruses are:—

1. Filterability.—If the infective material, such as fluid of a vesicle from foot-and-mouth-disease, is filtered through a Berkefeld filter the filtrate is still infective, though no organisms can be demonstrated either by microscopic examination or by culture methods. It must be remembered, however, that filterability depends upon the size of the organism, on the plasticity of the organism, *e.g.*, (leptospira of yellow fever though of considerable dimensions, by virtue of their plasticity alone can pass through certain filters), on the fineness of a particular filter, and also on the pressure employed during filtration. The inability of a particular virus to pass through a filter therefore does not necessarily mean that it is too large to pass through the pores of the filter.

2. Invisibility.—Micro-organisms less than 0·25 μ are generally invisible and readily pass through a filter; certain of the filterable viruses, *e.g.*, the organism of bovine pleuro-pneumonia, can just be seen but their shape cannot be made out.

3. Non-Culturability.—The majority of the filterable viruses have not been successfully cultivated on artificial media. The viruses of bovine pleuro-pneumonia and bacterium pneumosintes of influenza (Gates and Olitisky) have been grown on the Smith-Noguchi medium.

4. Resistance to Glycerin.—The filterable viruses retain their vitality for a considerable time in 50 per cent. glycerin. This property has been utilised in the preservation of calf lymph for smallpox vaccination.

5. Sensitiveness to Heat and Chemicals.—They are all destroyed at comparatively low temperatures and by weak antiseptics.

6. Great Infectiousness.—The virus diseases are very highly infective and spread rapidly. This is seen in smallpox epidemics when inspite of strict precautions the infection continues to spread. A striking example of the rapidity of their spread is furnished by the Influenza pandemic of 1918. It has been shown that 0·001 c.c. of a filtrate through paper of a 10 per cent. emulsion of brain from an animal infected with herpes is enough to reproduce the infection in a rabbit.

7. The Production of an Active and Lasting Immunity.—Recovery from an infection due to a filterable virus is followed by a permanent and high degree of immunity. This property has been utilised practically in the immunisation against smallpox and rabies. In the case of hog cholera and cattle plague specific sera have been prepared and employed with great benefit.

SMALLPOX

Synonym.—*Variola.*

Etiology.—Guarnieri described certain bodies as occurring in the deeper layers of the epithelium covering the pustules of smallpox and in the cornea after experimental inoculation, the significance of which is still doubtful. They are small bodies which are readily stained by hæmatoxylin, safranin and carmine, and were regarded as protozoa.

The *virus* of smallpox is transmissible to man, the calf and monkey; vaccinia to the rabbit in addition. Attempts to cultivate the virus on artificial media have been usually unsuccessful. The virus is extremely susceptible to heat, a temperature of 55°C. for an hour destroying its virulence completely. The virus remains intact in 50 per cent. glycerin or 10 per cent. ether though chemicals such as potassium permanganate readily destroy it.

The Relation of Smallpox (Variola) to Cowpox (Vaccinia).—This is still a matter of uncertainty. Some observers maintain that the two diseases are essentially different because of the fact that with few exceptions variola has not been shown to be inoculable to cattle. It is, however, generally believed now that cowpox is only an altered and attenuated variety of variola. There is no doubt that vaccinia can be transmitted to man in whom it produces the typical lesion. Variola when inoculated into a calf produces, if at all, only a slight reaction. By passing the virus of variola through a series of calves, it becomes so altered in character and reduced in virulence that it produces the lesions of vaccinia only and such a virus, when inoculated into man, produces the typical localised lesion of

vaccinia. This virus renders man and animals immune to an inoculation of cowpox and, in the case of man, from smallpox as well. Further evidence in support of the identity of the two viruses has been obtained by immunizing animals with vaccinia; the sera of such animals give agglutinating and complement-fixing reactions not only with the vaccinia virus but also with the viruses of variola and alastrim. The sera of smallpox patients give these serological reactions with the viruses of smallpox, cowpox and alastrim.

Alastrim is a highly infectious, variola-like, non-fatal disease characterised by the relative absence of constitutional disturbances and an eruption which may sometimes be so severe as to be confluent. The viruses of smallpox, alastrim and cowpox show cross agglutination and cross complement-fixation.

The Preparation of Vaccine Lymph.—Healthy calves which have been kept under observation for at least a week are washed and cleaned. Healthy cow calves 6 months to 2½ years of age are well suited for this purpose. They should be passed as free of tuberculosis. The abdomen of the selected calf is clipped and shaved over an area extending from the ensiform cartilage to the pubic region and thoroughly washed with soap and water and dried. 20 to 40 vaccinations are made with a scarifier using glycerin paste consisting of 50 per cent. glycerin and crude lymph on the prepared skin. This is seed lymph. Vesicles are produced and the lymph is taken from these, after 120 hours in winter and 96 hours in summer, as the lymph loses strength in the vesicles in high temperatures after 96 hours. This crude lymph is ground up in a lymph-grinding machine with glycerin and distilled water in the proportion of 1 part of lymph to 2 parts of distilled water and 2 parts of glycerin. This is then kept in the cold weather for at least three weeks and then issued for use in either glass capillary tubes containing one dose or in collapsible tubes (made of mixed metals) containing 25 doses. The glycerinated virus may then be submitted to bacteriological examination for quantitative estimation of the bacteria by the plating method, for the tetanus bacilli by anærobic cultures and, by injecting large quantities of the virus into the subcutaneous tissues of guinea-pigs and mice, for *B. welchii* and streptococci.

Only good vesicles are used and a stock of glycerin paste is kept at the same time. The whole vesicle is scraped off with a Volkmann's spoon drawing as little blood as possible. The wounds are dressed with boric acid and zinc powder and the calves are kept till the scales have dried.

Calf lymph will keep and retain its potency for months at a low temperature. If stored at 4°C. it will keep for long periods. Lymph stored in tubes for 6 months at an average temperature of 20°C. is perfectly good at the end of that time. For the purpose of improving and maintaining the quality of the seed lymph which undergoes deterioration by repeated transference from calf to calf, it is necessary to pass it periodically through a rabbit by cutaneous inoculation.

Rabies

Synonym.—*Hydrophobia.*

Definition.—Rabies or canine madness is an acute disease of the central nervous system occurring in dogs and allied animals, transmitted from animal to animal by means of bites. Infection of man occurs when the saliva of a rabid animal gains entrance into wounds from bites and scratches.

Etiology.—The causative agent of rabies has not been identified but the disease is no doubt due to a living virus, "rabies virus".

Occurrence of the Virus.—In the body of a rabid animal the virus is found in the brain, spinal cord, large nerves and salivary glands and in certain of the internal organs such as the pancreas and suprarenal glands.

The Characters of the Virus of Rabies.—Emulsions of the brain, etc. of rabid animals, filtered through coarse Berkefeld filters, are infective. Like the variola virus it can also pass through a celloidin membrane. It is destroyed by one hour's exposure at 50°C. and is attenuated by drying. Attempts to cultivate the virus have failed.

Negri bodies.—In 1903 Negri described certain bodies as occurring in the nervous system especially in the grey matter of the hippocampus and in the Purkinje cells of the cerebellum of animals dying of rabies. They are of varying size, measuring 0·5 to 25 μ; (in the dog 4 to 10 μ). They are round, oval or somewhat angular in outline, taking a pink colour in smears stained with eosin and methylene-blue. The smaller ones are generally structureless but the larger ones are seen as pale blue with pink granules of varying size and number when stained with Giemsa. The Negri bodies are found in practically 98 per cent. of cases of street rabies in dogs and hence their presence forms a rapid and simple means of diagnosis. Negri himself regarded them as protozoa; the organism has been named by Calkins *Neuroryctes hydrophobiæ*. Their absence from the infected brain and the infective saliva of some animals dying of

'fixed' virus, and the fact that the virus can pass through a coarse filter are all against their protozoal nature. The filtrability of the virus led Prowazek to believe that the Negri bodies represented a tissue reaction to invasion by the parasite which was extremely minute and contained in the Negri body itself. The parasite, according to him, belonged to a group of Protozoa termed *Chlamydozoa.* The question is still a matter of controversy though Prowazek's view appears to have considerable support.

Diagnosis.—The methods used for diagnosing rabies in an animal are:—In the case of dog bite the best procedure is to tie up the animal, if it is not obviously rabid, for 10 days. If the animal shows no symptoms of rabies within this period it can be taken that there is no danger of rabies affecting the person bitten.

I.—Examination for Negri Bodies in the Brain.—The method of removal of the brain of a suspected rabid animal and its transmission to the laboratory for examination.—For the following method we are indebted to Greig:—

"The diagnosis of rabies in animals is made in the laboratory by a microscopical examination of the brain: before this can be carried out the brain must be sent up properly preserved, packed, etc. Many of the brains sent for examination arrive at Pasteur Institutes in such a state of emulsification or putrefaction that it is impossible to say what the tissue is, apart from attempting to make a diagnosis.

1. The first procedure is to open the skull and expose the brain. The head of the dog is washed with an antiseptic, such as carbolic acid, phenyle, etc. Then with a hammer a few sharp blows will fracture the skull into many pieces, the skin being kept intact. The skin is then reflected and the fractured bone removed carefully, in this way exposing the brain.

The membranes covering the brain are incised and the brain equally divided down the centre into two longitudinal halves.

If the skull vault is well broken, each half of the brain can now be lifted out with the aid of a knife and forceps.

In removing the brain of a rabid animal the greatest care must be exercised, as the brain substance and saliva are infective, and should never be allowed to come into contact with the hands. An old pair of riding gloves should be worn when carrying out this operation.

Preservation of the brain.—For the microscopical method a special portion of the brain is required, *viz.*, the *hippocampus major.* To those who are unacquainted with the anatomy of

the brain, it is best to divide the brain into two longitudinal halves, as directed above. One half only need be sent for examination in the case of large animals. The brain is lifted out between the forceps and knife and placed in a large wide-mouthed bottle, with a capacity of at least two pints. A layer of cotton-wool should previously be placed at the bottom of the bottle. In no case should the brain be wrapped round with cotton-wool, lint or other wrapping, as this prevents penetration of the fixing fluid.

The bottle is now filled up to the brim with rectified spirit, methylated spirit, 3·0 per cent. bichromate of potash in water, 10 per cent. formalin or Zenker's fluid.

Large quantities of preservative fluid are always required in order to ensure full fixation of the brain: at least ten times as much fluid as brain volume is necessary.

The lid of the jar is now sealed and the jar is carefully packed in a box with sawdust, etc.

A descriptive account of the illness of the animal should *always* accompany the brain, and also the full address of the person to whom the result of the examination is to be sent."

Thin slides of tissues, *e.g.*, of hippocampus are placed in Zenker's fluid or 10 per cent. formalin.

Examination for Negri bodies.—1. Smears, impression smears and paraffin sections are made from the hippocampus major of the animal and stained, while still moist, in the following staining solution for 10 seconds.

Fuchsin-Methylene-blue Method for Staining Negri Bodies.—

1.	Basic fuchsin (saturated solution in pure methyl alcohol)	2 c.c.
2.	Methylene-blue (saturated solution in pure methyl alcohol)	15 c.c.
3.	Pure methyl alcohol	25 c.c.

Mix (2) and (3) and then add (1).

Negri bodies stain red with blue granules in their interior. The presence of Negri bodies is diagnostic of rabies; a negative finding means nothing.

II.—Corneal Scarification in the rabbit using 2 or 3 drops of a 5 per cent. emulsion of the fresh medulla of the suspected animal reproduces the disease, if the animal was rabid. Its non-development in the experimental rabbit can be taken to mean that the suspected animal did not have rabies.

Treatment.—*Pasteur Treatment for the Prevention of Rabies.*—The wound having been efficiently cauterised, the patient should be recommended to go to one of the Pasteur Institutes, even if there is the slightest suspicion that the animal was rabid and that the patient was bitten by him. Pasteur (1885) showed that the rabies virus as obtained from rabid dogs—"street virus" was usually of constant virulence in that it killed rabbits in 15 to 20 days when introduced subdurally. By passing the "street virus" through a series of rabbits (subdural injections), its virulence was increased till a constant strength was attained so that it killed rabbits in about 7 days. He called this "virus fixe" (fixed virus). On the other hand similar passages through monkeys attenuated the virulence of the virus. He had thus at his command viruses of varying strength and he started the treatment with weak virus and gradually worked up to a strong virus. Subsequently he attenuated the virulence by drying infected rabbits' spinal cords in air over caustic potash. The treatment as carried out in Paris is as follows:—The spinal cords of rabbits dying of fixed virus are removed aseptically and suspended in bell-jars containing caustic potash. The jars are dated and kept in a dark room at a constant temperature of 25°C. Commencing with a cord that had been dried for 14 days, doses of emulsions of cords of increasing virulence are injected till, at the end of treatment which varies according to the severity of the case, emulsions of the cords which had been dried for 3 days only are given. In Paris the emulsion made by triturating 1 c.c. (*i.e.*, 2 mm. of cord) in saline is given in one dose. At the Pasteur Institute, Kasauli, carbolized antirabic vaccines are employed and prepared in the following ways. One per cent. emulsion of a brain of a rabbit which has died from rabies after inoculation with the fixed virus is made with sterile normal saline containing 0·5 per cent. carbolic acid. Five c.c. of this emulsion are injected subcutaneously in the abdomen every day for 14 days. 'Ether' vaccine will probably displace 'carbolised' vaccine.

In Högyes' modification, an emulsion is made with the fresh cord of a rabbit which has died of "fixed" virus. The treatment is commenced with very high dilutions of this virus, *e.g.*, 1 in 10,000 or even more, and ended with lower dilutions of the cord.

Epidemic Poliomyelitis

The virus of this disease has been shown to pass through earthenware filters (*e.g.*, Berkefeld N or V) by Flexner and Lewis and by Levaditi and Landsteiner. The virus has been

found in the central nervous system, the Gasserian and some other ganglia, in the salivary and some of the lymphatic glands; in the tonsils and occasionally in the blood.

Flexner and Noguchi have attempted to cultivate the virus by inoculating a medium consisting of human ascitic fluid and a piece of sterile fresh kidney with an emulsion of the brain of an infected animal and incubating the culture at 37°C. under anærobic conditions. About the 5th day a slight turbidity appears in the medium which on staining with Giemsa's stain shows minute bluish or violet globoid bodies (0.24 μ in diameter) which may occur in pairs, chains, or less frequently in groups. Similar cultures have been obtained from infected filtrates. Introduced into the brain or the sheath of nerves, into the mucous membrane of the nose by scarification, into the peritoneum or subcutaneous tissues of a monkey, the emulsion of the central nervous system of an infected human subject or animal reproduces the disease with the same clinical and pathological features as in man. After a few passages of the virus through monkeys, it becomes so exalted in virulence that an intracerebral injection of 0·001 c.c. to 0·01 c.c. of an emulsion of the material from the central nervous system will reproduce this disease. The filtrate of the brain of such an animal is capable of infecting a monkey. The virus retains its vitality for a considerable time in glycerin and at 2° to 3°C. Exposure to 1½ per cent. phenol for 5 days does not kill it. It is destroyed by heating to 45 to 50°C. for half an hour. Both in man and animals the serum of a recovered case possesses considerable neutralising powers for the virus for, a mixture of serum and virus after a few hours' incubation at 37°C. fails to reproduce the disease in monkeys. The serum of a convalescent case of poliomyelitis has been successfully used to arrest the development of paralysis in a dose of 35 to 120 c.c.

Epidemic Encephalitis

Also known as *Encephalitis lethargica.* The causal organism of this disease has not yet been definitely identified though "minute" globoid bodies similar to those described in poliomyelitis have been cultivated by some workers. Others think it is due to a type of streptococcus.

The virus has been claimed to be filterable and inoculable into monkeys and rabbits by the cranial and ocular routes, the pathological lesions in these animals being the same as in man. Rabbits and monkeys are also naturally liable to a type of encephalitis. It is believed that the virus of herpes is identical

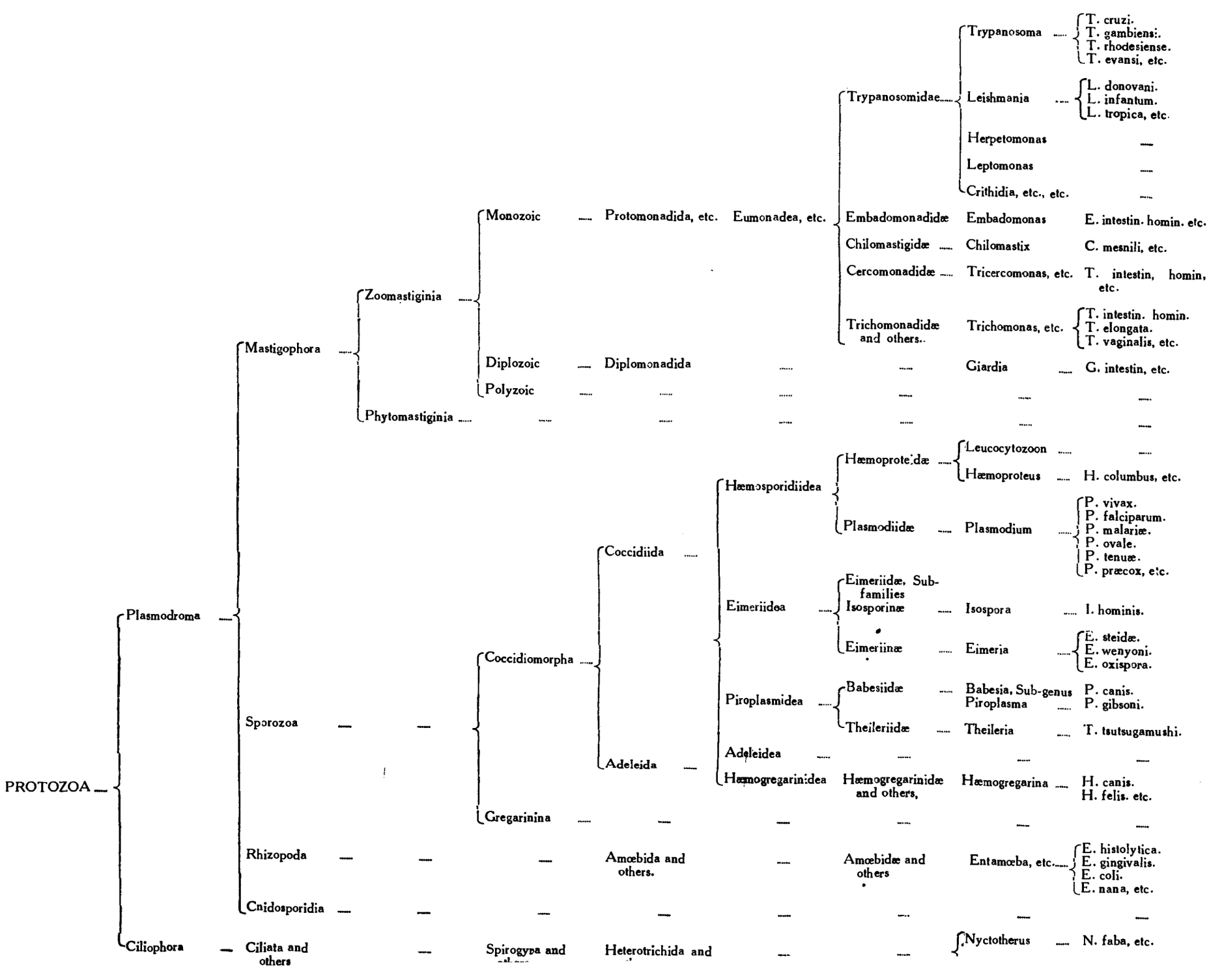
with the encephalitis virus inasmuch as the latter produces herpetic keratitis in rabbits.

Herpetic Viruses

The virus as obtained from the vesicular fluid of *Herpes febrilis* in man or from experimental keratitis in rabbits when inoculated into rabbits by cranial or ocular routes is followed by a fatal result with inflammation of meninges and brain and is transmissible in series to other animals. The herpetic disease in man is, as a rule, harmless and has little or no affinity for the nerve tissues of the human subject. It is killed by an exposure at 56°C. for half an hour or even at 37°C. when kept outside the body for 24 hours. Introduced into the skin or cornea of man, the material from the lesion in a rabbit produces typical herpetic vesicles. Guinea-pigs can be infected with the virus of herpetic keratitis of rabbits. The virus has also been demonstrated in the blood and spleen of experimentally infected animals. After recovery from an infection with herpetic keratitis, a rabbit becomes refractory to further inoculation, intracerebral or corneal, though no antibodies are demonstrable *in vitro* in the serum of recovered animals (or of cases of encephalitis in man). The virus of *herpes zoster* produces a band of vesicles when introduced into the scarified skin of a guinea-pig or rabbit whereas *herpes febrilis* virus is not usually transmissible to the guinea-pig's skin.

Minute granular structures both intracellular and extracellular, stained by Giemsa have been described as occurring in the lesions of the central nervous system and are regarded by some as the specific virus of herpes.

[To Face Page 311.



CHAPTER IX

PATHOGENIC PROTOZOA *

A large number of diseases are caused by protozoa and it is essential that the position of the chief organisms in the classification table be known. The protozoa belong to the animal kingdom, are unicellular and reproduce usually by simple fission and spore formation. In certain classes, however, two life cycles occur—one in which reproduction is by spore formation and the other in which male and female gametes unite to form a zygote, the cycle of sporogony occurring in a separate host, *e.g.*, human malarial plasmodia in anophelines.

TRYPANOSOMA

Trypanosoma gambiense Dutton, 1902.

Synonyms.—*T. eugandense* Castellani, 1903; *T. hominis* Manson, 1903; *T. nigeriense* Macfie, 1913.

Distribution.—Occurs only in Africa.

Morphology.—This has a characteristic trypanosome shape; size 18 to 30 μ × 1 to 3 μ. The anterior end is tapering while the posterior end is blunt; the trophonucleus is situated centrally, the kinetonucleus placed posterior to the trophonucleus; the blepharoplast is near the kinetonucleus; the flagellum passes forwards as the margin of the undulating membrane and ends with the undulating membrane at the anterior end of the body, or, more usually, projects forwards as a free lash. The trophonucleus shows no chromatin dots. Inside the nucleus the prominent karyosome is surrounded by a clear space and between the kinetonucleus and the blepharoplast lies an eosinophilic vacuole.

Life History.—Two methods of transmission of trypanosomes are known. Any blood-sucking insect may cause mechanical transmission (direct). In the "indirect" method a developmental cycle is involved in one particular intermediary host, *viz.*, the Tsetse fly, *Glossina palpalis*. The full development occupies 18 to 25 days, the salivary glands ultimately becoming infected

---

* The standard book on protozoology is that of Wenyon (2 vols.) and this or the excellent Protozoology by Knowles should be referred to.

by the short stumpy forms, which are the forms infective for vertebrates. Transmission by coitus has also been suggested as a possible mode of infection. Reproduction of the trypanosome usually occurs by longitudinal fission, the kinetonucleus, trophonucleus and also the flagellum dividing and the cytoplasm also subsequently by fission commencing at the anterior end and extending backwards.

Culture.—The medium usually employed is N. N. N. medium, but blood agar has been used. In culture trypanosomes tend to assume a crithidial form but later on small trypanosome forms appear.

Pathogenicity.—Most domestic and laboratory animals as well as wild animals may be infected experimentally. Cercopithecus and Macacus monkeys and dogs are very susceptible. The reservoir of infection comprises various species of antelope and also cattle, sheep and goats. In man it produces the disease known as " sleeping sickness " and infection may last for several years.

Trypanosoma rhodesiense Stephens and Fanthom, 1910.

This organism resembles *T. gambiense* morphologically and causes Rhodesian sleeping sickness. It differs from the above chiefly in having a different species of vector, namely *G. morsitans,* and a different geographical distribution, *T. rhodesiense* being limited to the district west and east of lake Nyasa in Northern Rhodesia. When passed through rats the trypanosome may be distinguished from *T. gambiense* by the fact that in some organisms the nucleus is situated at the non-flaggelar end of the parasite. The cycle of development in the fly is somewhat shorter.

Diagnosis.—Both trypanosomes may be demonstrated in the cerebro-spinal fluid, puncture fluid from lymph glands and peripheral blood, specially in early cases. To demonstrate the parasite Leishman or Giemsa stain should be used.

In the blood they may be abundant or (usually) scanty and the red cells are clumped together instead of being in rouleaux. Examination should be made specially during an attack of the fever and the blood may be citrated and centrifugalised. When no parasites can be found, 10 c.c. of blood-serum should be inoculated into a guinea-pig, rat, dog or monkey. In gland puncture, lymph may be drawn off with a hypodermic syringe. Apart from finding trypanosomes in the cerebro-spinal fluid after lumbar puncture the finding of a lymphocyte count of approximately 1000 per c.mm. is said to be of diagnostic importance.

Trypanosoma cruzi Chagas 1909.

This resembles *T. gambiense* morphologically and causes a human trypanosomiasis characterised by irregular fever, swelling of lymph glands, œdema and disturbances of the nervous system seen in Brazil and the northern part of South America. It is transmitted by *Triatoma megista* (Conorhinus megista) and infection probably occurs by the dejecta of the bug rather than by its bite. The disease occurs chiefly in children but also in adults and causes a high mortality. Guinea-pigs, rats and mice are susceptible.

Trypanosoma evansi, Evans, 1880.

This trypanosome causes the diseases known as "surra" in horses, mules, camels, elephants, buffaloes and dogs and occurs frequently in India. Morphologically it resembles the other members of the group of pathogenic trypanosomes. It is transmitted by various biting flies of the genera Tabanus and Stomoxys.

Trypanosoma brucei Bruce, 1895.

This is the cause of a disease of domestic animals in Africa resembling "surra" and called "Nagana." The vector is *Glossina morsitans*. Man is apparently immune. Morphologically it is similar to the other pathogenic trypanosomes.

LEISHMANIA

L. donovani Laveran and Mesnil, 1903.

Synonym.—Leishman-Donovan body—the cause of kala-azar (tropical splenomegaly, Dum Dum fever).

Distribution.—In India: Madras, Bengal, the Valley of the Ganges up to Lucknow, the valley of the Brahmaputra up to Assam and in the extreme south of India; also met with in China and Southern Russia, Asia Minor, the Sudan and Mediterranean littoral.

Morphology.—The organism is spherical or oval, size 1 to 3 μ when spherical, $2{\cdot}5 \times 1{\cdot}5$ to $2{\cdot}0$ μ when ovoid. There is a definite membrane outside the cytoplasm. The latter contains (1) a spherical nucleus flattened where it touches the circumference, (2) a structure known as the kinetoplast, and (3) one or more vacuoles. On staining by Romanowsky the nucleus appears as a mass of bright red granules; in films fixed without drying a nuclear membrane is seen within which is a clear space

containing a spherical karyosome. The kinetoplast is more compact, stains a reddish-purple and is rod-like, its long axis being at right-angles to the nucleus. Christophers (1904) described a red line originating from the blepharoplast (which lies near the centre of the kinetoplast). This is the "axoneme" and from this originates the flagellum of the leptomonas forms in cultures.

The above "aflagellate" form is the form usually met with in human leishmaniasis.

The organisms are practically always found within the cytoplasm of endothelial cells or macrophages. Kala-azar is essentially an infection of the endothelial lining of the blood vessels. In blood films or smears of organs extracellular forms may be seen and are probably due to the breaking up of the containing cells during preparation. The organism does not invade the red blood corpuscles like the malarial parasite but in peripheral blood films the mononuclear or polynuclear leucocytes may contain parasites.

Multiplication.—The only method known is by binary fission.

Culture.—Rogers (1904) obtained flagellates of the leptomonas stage in citrate solution from infected spleen pulp. Nicolle (1908) obtained cultures in the water of condensation in tubes of rabbit blood agar and in the N. N. N. medium at a temperature of 22° C. Subcultures were readily maintained. The stages of development are as follows:—

(1) The organism at first grows in size.

(2) After 48 hours the flagellum appears, commencing from the eosin-staining vacuole which lies in front of the kinetoplast. This vacuole or blepharoplast moves to the future anterior end of the body. The flagellates enlarge to 10 to 20 μ, the flagellum being nearly as long or longer.

In about 4 days various types are seen, *e.g.*, round forms, broad pyriform types with blunt anterior and narrow posterior ends and long sickle-shaped forms. A number of organisms often come together into groups or clusters with their flagellæ directed towards one another.

When the cultures become older, flagellate forms disappear and round forms reappear.

Diagnosis.—(1) Peripheral blood should be examined by the thick drop method (see page 326).

(2) Spleen puncture is perfectly safe and should always be done. It is necessary to exclude the leukemias previously, by examining a thin blood slide of peripheral blood; and also hæmophilias. It is not necessary to obtain blood in spleen (or liver) puncture, the small quantity of tissue cells which are obtained in the end of the needle being sufficient for diagnosis.

(3) The Formol-gel test consists of adding a drop of patient's serum to 2 drops of formalin on a slide. If the diagnosis is positive the serum solidifies. The reaction is not specific.

(4) Culture of spleen pulp on N. N. N. medium is recommended in all doubtful cases.

Canine Kala-azar

In the Mediterranean area dogs are found infected. The clinical picture of the disease is similar to that in man.

The organism is morphologically and culturally indistinguishable from *L. donovani.* Inoculation of dogs with parasites from human sources gives rise to infection. The absence of natural infection in dogs in endemic areas in India is probably due to some factor concerned with transmission which is not understood.

Animal Susceptibility.—Dogs and monkeys have been infected experimentally with material from infected spleen or liver. Culture materials are also liable to produce infection but not so readily. *Old* cultures do not give rise to infection. Intraperitoneal inoculation in animals gives the most satisfactory results.

Transmission of Leishmaniasis.—Patton (1912) first showed that *L. donovani* develops into leptomonas forms in the stomach of the bed bug and persists therein for as long as six weeks. The complete proof incriminating the bed bug is lacking: the distribution of the bed bug and kala-azar do not coincide. Recent work has shown that the female *Phlebotomus argentipes* may acquire a heavy infection after feeding on kala-azar cases. The leptomonas forms of the parasite have been demonstrated in the intestine and pharynx in this sand-fly and the distribution is co-extensive with that of kala-azar. In the Mediterranean form of the disease the dog flea is assumed to be the insect vector and infection may occur not only through inoculation of the insect's saliva into man but also through the insect's dejecta.

Leishmania tropica Wright, 1903.

This organism causes the cutaneous infections known as Oriental sore, Delhi boil, Baghdad boil, etc.

Distribution.—Southern Europe, North Africa, the Near East and the North-Western parts of India, extending as far south as Bombay.

Morphology.—Indistinguishable from the organism of kala-azar, a greater number of elongated, cigar-shaped forms are met with in the cytoplasm of endothelial cells, especially in macrophages.

Culture.—See *L. donovani.*

Growth tends to be more vigorous and a slightly higher temperature is more favourable. Material to be used ought to be bacteria-free; sterilisation of the surface of the ulcer should be done at first, and after making scarification or a puncture with a needle, the material is drawn off with a sterile pipette.

Animal Infection.—In nature, in endemic areas, cutaneous lesions of the *L. tropica* have been demonstrated in dogs.

Experimental inoculation into the skin has been successful in dogs, cats, monkeys, rats, mice, guinea-pigs, etc.

Transmission.—Direct inoculation from man to man is possible and may occur in nature through abrasions, etc. Healthy skin is resistant to infection.

Infection in nature is almost undoubtedly due to Phlebotomi and especially *P. argentipes,* although final confirmation is lacking. *P. sergenti* and *P. papatasii* have also been infected.

Typical oriental sore in humans have been produced by inoculating crushed-up *Phlebotomus papatasii.*

L. tropica can develop in the stomach of the bed-bug which is, however, probably not a natural vector of the disease, as the leptomonas forms do not multiply in the bug.

The type of the disease in the new world varies somewhat from that met with in the old world, the former being of a more chronic nature and mostly confined to the skin, though in about 10 per cent. of the cases mucous surfaces are involved. Nasal and oral leishmaniasis suggest the importance of inoculation by insects as well as of direct contact infection.

Diagnosis.—(1) A film of the serum expressed from the "sore" should be stained by Leishman or Giemsa's method. Blood platelets and yeast cells must be carefully differentiated:

the former occur in groups of 4, 8, etc., are violet in colour, have no nucleus, and are free, or, if they appear to be on an endothelial cell, can be focussed carefully when it will be seen that they are on a higher plane. Yeast cells have a distinct capsule, stain uniformly and may be seen in the process of budding.

(2) Cultures are made on N. N. N. medium as above, or by planting a small piece of the "sore" directly in the medium under aseptic conditions. The surface of the sore should be sterilised with alcohol before incising or scarifying.

Intestinal Flagellates

Trichomonas hominis (Davaine, 1860).

Occurrence.—The trichomonas belong to the monozoic forms of the mastigophora and occur mostly in diarrhœic stools in man. *T. hominis* is usually non-pathogenic but may cause an intractible dysentery.

Morphology.—This flagellate has a pyriform body but may protrude pseudopodia. Its length is 10 to 15 $\mu \times 4\ \mu$ The anterior end is blunt and 3 or more long flagella arise from this. The spherical nucleus is situated near the anterior end just behind the basal granules or blepharoplasts from which the flagella arise. There is an undulating membrane along the dorsal surface of the body; the anoneme (a rod-like structure) is attached to the border of this undulating membrane and may project posteriorly as a flagellum or terminate with the membrane. A small mouth or cytostome is situated anteriorly ventral to the base of the flagella. A broad bar-like structure, the axostyle, begins from the blepharoplasts and runs straight to the posterior end from which it protrudes as a sharp process. The cytoplasm is vacuolated, contains bacteria and food particles and may also contain red blood cells.

Reproduction—by biniary fission. Encysted forms are not known.

Diagnosis is by examination of specimens of fresh stools under the low power microscope.

T. vaginalis Donné, 1837.

Size.—Distinguished from *T. hominis* by its size (10 to 30 $\mu \times 10$ to 15 μ) and the fact that it has four anterior flagellæ.

Giardia intestinalis (Lambl, 1859).

Synonym.—*Lamblia intestinalis.*

Morphology.—This is a paired organism in that both sides are symmetrical. It is pear-shaped and has a sucker-like

structure on the ventral surface of the anterior end. The size varies from 12 to 18 $\mu \times 6 \mu$. The posterior tapering extremity has two flagella and in all 4 pairs of flagella are present. The two oval nuclei are situated near the anterior end. Reproduction is by a complicated process of binary fission. The habitat is the upper part of the small intestine of man. The motile vegetative forms are only found in diarrhœic conditions and normally the forms seen in stools are dead. Cysts occur often in enormous numbers. The cysts are oval, have a characteristic structure and are 10 to 14 μ in diameter. The cyst wall is thick and transparent and inside this is the Giardia, which however does not completely fill the cyst, especially posteriorly. These cysts may be in the different stages an early undifferentiated or undivided stage, a stage with first nuclear division and a stage with complete division. The organism is usually non-pathogenic to man but may cause diarrhœa. It is pathogenic to mice, etc.

Diagnosis is by the examination of fresh stools microscopically and by culture in N. N. N. medium.

Blood Parasites Resembling the Malarial Parasites (Plasmodium)

The order Coccidia has three sub-orders which are blood-inhabiting organisms. The sub-order Hæmoproteidæ has three genera—Hæmoproteus, Leucocytozoon and Plasmodium.

Genus Hæmoproteus Kruse, 1890

The schizogony cycle occurs in the endothelial cells of birds of all types; the gametocytes occur in the red blood corpuscles of the birds and infection originates by the bites of flies. The sporozoites enter the endothelial cells of blood vessels in the viscera, especially in the lung. Inside the cell the nucleus divides repeatedly and a pseudocyst forms and protrudes into the lumen of capillary which is thus blocked. The infected cell finally ruptures into the circulation, the merozoites are liberated and infection of other endothelial cells is started. The lungs, kidneys, liver and spleen are chiefly infected.

Sporozoites (Merozoites) liberated thus may, however, enter the red blood corpuscles instead of endothelial cells and gametocytes result. The early gametocyte is a small ring with blue cytoplasm and a red chromatin dot. Pigment formation occurs. The fully grown gametocyte is doubled on itself and round the cell nucleus. The mature female gametocyte is thinner and longer than the male and is applied round the margin of the cell nucleus, the appearance being like a halter (Halteridium). Multiple infection of a red cell is common.

Infection is conveyed by a biting fly, *Lynchia mœura* in the case of pigeon malaria (due to *H. columbæ*).

The cycle of sporogony is exactly similar to that of the malarial parasite in the anopheles host and infection occurs via the salivary glands and duct during biting.

Hæmoproteus infections also occur in cold-blooded animals, *e.g.*, the tortoise, cobra and lizard.

The stages in the life-history of the parasite in cold-blooded animals have not been worked out.

Genus Leucocytozoon Danilewsky, 1890.

The leucocytozoon resembles the Halteridia but no pigment is formed. The schizogony cycle occurs in the internal organs, *e.g.*, the spleen, and the cells affected are the endothelial cells.

Certain of the merozoites develop into gametocytes as in Hæmoproteus, but the host cell was at first thought to be a mononuclear leucocyte (and not a red cell): hence the name leucocytozoon. Wenyon, however, considers that these are immature red cells.

A feature in this infection is the spindle-shaped distortion of the parasitised cell by the gametocyte. The neucleus of the cell is pressed out against the cell membrane into a ribbon-like stripe of chromatin. The size of a full grown gametocyte is about 25 $\mu \times 5\mu$.

Genus Plasmodium

Plasmodium præcox Grassi and Feletti, 1890.

Synonyms.—*Proteosonia grassi; P. avium.*

Like the human Plasmodium the whole of the schizogony cycle occurs in the red blood corpuscles of the vertebrate host (birds). In its earliest stage, it resembles the small rings of *P. falciparum.* Pigment is soon laid down. As the ring grows, the nucleus of the red cell is pushed to one side or even out of the cell. This distortion of the containing red cell differentiates it from halteridium in which no such displacement occurs.

The period in which maturation of the schizont occurs is said to be from 4 to 5 days. The smaller schizonts have a diameter of 4 to 5 μ and the larger forms may be as much as half the size of the red cell. The smaller schizonts produce about six and the the larger ones from 16 to 24 merozoites.

The infected cell is distorted, pale in colour and the pigment is usually collected in one single dark mass. The gametocytes are slightly elongated, are oval in outline and about as large as the larger schizonts.

Transmission in nature occurs through the culex mosquito. The developmental cycle in the mosquito is similar to that of the human parasite in the anophelines; the time required for infection of the salivary glands is about five days.

The Parasites of Malaria

1. *Cycle of Schizogony.*

A.—The known Parasites of Man.—Three species of the genus Plasmodium occur in man, *viz., P. vivax* (benign tertian parasite) the cause of benign malaria or tertian fever, characterised by resistance to quinine and frequent relapses; *P. malariæ* (quartan parasite), the parasite of the less common but frequently relapsing type of quartan malaria, and *P. falciparum* (*Laverania malariæ;* malignant tertian parasite), the cause of malignant, subtertian, tropical, æstivo-autumnal or pernicious malaria, the type which causes all epidemics and is associated with complications due to the parasites tending to clump in the internal organs.

(*i*) *Plasmodium vivax* (Grassi and Feletti, 1890).—The cycle of schizogony includes the trophozoite or growing phase and the actual schizogony phase, and occupies exactly 48 hours. It commences with the attack on the patient's red cell by sporozoites introduced by the bite of an infected mosquito, or by merozoites (2 to 3 μ long) just liberated in the patient's plasma from a completed schizogony cycle. The merozoite or sporozoite becomes attached to the red cell and may be seen as a small thin ring about one-third the size of the red corpuscle and having a rounded dot of chromatin in the thinnest part of its circumference or just within the circle. As the ring becomes thicker the red cell enlarges and becomes pale.

Although it is occasionally believed that the malarial parasites remain extra-corpuscular and in fact may wander from red cell to red cell in the plasma, it is generally held that the red cell is now penetrated and the active "amœboid" stage then occurs. Well stained thick rings with one or two chromatin dots, distorted rings and pseudopodial forms of all shapes, with more or less pigment appearing as granules, rods or masses, are seen, while the corpuscle becomes more and more enlarged, pale and marked with the fine strippling (Schüffner's

dots) as the parasite grows. The final phase of schizogony now commences; the parasite becomes globular, the nucleus divides into 2, 4, etc. and the final mature schizont appears as a "rosette" of 16 to 18 nuclei (merozoites), each surrounded with its own cell membrane and cytoplasm, while in the centre of the rosette is a mass of hæmozoin.

Forty-eight hours after the initial attack on the red cells, the mature schizont having ruptured, the free merozoites attack fresh red corpuscles. The pigment is absorbed by mononuclear leucocytes, endothelial cells and polymorpho-nuclear white cells.

The incubation period of malaria is due to the time necessary for the merozoites to reach the "fever threshold" (variously placed at from 150 to 250 or even 1000 million parasites) and chronic cases are due to maintaining the parasites at or just below the fever threshold, usually by occasional doses of quinine. In any one blood slide of benign-tertian, once fever of a tertian type has begun all or any phase of the above cycle may be seen (rings, amœboid forms and mature schizonts). Several rings or even parasites in various later stages of development may be seen in one red cell, *e.g.*, a macrogametocyte and schizont, two schizonts or a microgametocyte and schizont, the discovery of which by Thomson led to the overthrowing of Schaudin's theory of parthenogenesis.

Somewhere about the 10th to 14th day after the occurrence of fever (about 3 weeks after infection) almost mature gametocytes appear in the blood, early forms being very rarely seen, and often being practically indistinguishable from schizonts. The mature microgametocyte occurs in a much enlarged pale red corpuscle (which shows Schüffner's dots), is compact and round and stains a blue-greenish colour with Leishman's stain, its nucleus usually lies like a belt partly across the parasite and stains faintly; masses of hæmozoin pigment surround the nucleus. The female gametocyte is larger than the microgametocyte and lies in a much enlarged pale red cell showing Schüffner's dots; its cytoplasm takes on a deep blue stain and throughout this is scattered yellowish brown pigment. The nucleus is a small compact red mass usually lying in a vacuole-like area at one end of the parasite.

(*ii*) *Plasmodium malariæ* (Laveran, 1881).—This parasite is characterised by:—

1. A schizogony cycle of 72 hours;
2. Early "signet" ring forms which appear as oval, well-staining compact rings, the chromatin mass occupying the centre of the ring;

3. Compact angular, comet, ribbon-like or globular amœboid forms containing a large amount of pigment trailing in the tail of the red chromatin "comet" or, in the "ribbon" form, forming a dark band parallel to the red chromatin band stretching across the centre of the red cell.

4. Schizonts which do not completely fill the red cells, which each contain 6 to 12 irregular chromatin masses and later 6 to 12 merozoites and a dense compact mass of pigment, placed excentrically in early schizonts, but usually centrally in older schizonts (*e.g.*, the "rosette" forms).

5. The microgametocytes are compact, greyish-staining, round forms about the size of the containing red cell, filled with pigment which is also congregated in a mass round the periphery or near the centre; their chromatin is a diffuse, badly staining mass usually stretching across the parasite.

6. The macrogametocyte appears as a globular densely blue parasite almost filling the red cell, having a compact red marginal nucleus not lying in a clear "vacuole" and with rods or masses of pigment scattered throughout the blue-stained cytoplasm.

7. In no phase is the red cell enlarged nor marked with "stippling" or "dots" nor is its staining altered from normal.

Double infection of a red cell with "rings" may occur in quartan malaria. This parasite is the most pigmented and well staining of all malaria parasites.

(*iii*) *Plasmodium falciparum* (Welch, 1897, Blanchard, 1905). *Laverania malariæ.* (Grassi and Feletti, 1890).—Only the early ring forms and gametocytes are to be seen in peripheral blood owing to the fact that infected red cells become "clumped" together in the capillaries of the internal organs and schizogony proceeds there: hence the cerebral, intestinal and pneumonic types, etc.

The characteristics of this parasite as seen in Leishman-stained blood slides are:—

1. The blood cells may be very heavily infected with ring forms which are so extremely small and thin as to escape notice. The ring is uniformly thin and is not thickened at any part. The red chromatin mass is relatively large and frequently appears as a bar or blob within the margin of the ring

while two chromatin dots are very frequently seen. "Accolé" ring forms (occasionally met with in benign and quartan infection) are very common in malignant malaria and are seen as minute faint blue semi-circles of cytoplasm on the margin of red cells, the chromatin dot being frequently seen on the free apex of the part of the red cell enclosed by the semi-circle. Multiple infection of red cells with rings occurs more commonly in malignant tertian than in benign or quartan fever. Rings seen in peripheral blood almost never show pigmentation and are invariably minute. The thin blue ring may become stretched in various shapes across the red cell or may assume small "comet" shaped or pseudopodial forms (the "tenue" forms). The red cell is never enlarged, is often "brassy" in appearance, is frequently crenated and may show coarse red-staining clefts (Stephens and Christophers' dots), but never the fine Schüffner's dots of the benign parasite.

2. The further stages of schizogony are seen in slides from spleen puncture or from cultures. The ring forms develop pigment, become irregular or globular and stain a dark blue colour: The chromatin mass divides up into 8 to 32 chunks and later this number of merozoites form. The pigment is very compact and massed excentrically and the complete parasite is only about one-half to two-thirds the size of its red cell.

3. The gametocytes of falciparum are crescent-shaped and only mature forms are seen in slides from the peripheral blood. The microgametocyte is about 10 μ long$\times$3·5 μ broad and has blunted ends; its cytoplasm and diffuse nucleus stain badly and the pigment is diffusely scattered over the middle of the crescent. The macrogametocyte is longer, thinner and more pointed at both ends than the male, the cytoplasm stains a deep blue and the compact red nucleus lies in the middle of a central thick mass of pigment.

Mixed infections with different species of malaria parasites and double infections giving rise to quotidian fever are very frequently met with.

B.—The Doubtful Parasites of Man.—*P. immaculatum* Grassi and Feletti, 1890; *P. falciparum quotidianum* Craig, 1909.—This parasite was reported to cause a quotidian fever associated with extreme cachexia and dropsy and is probably true *P. falciparum*, the daily rigor being caused by a double infection. All the parasites are very minute, the rings being 0·5 μ in diameter, the mature schizonts about 1|10th and the crescents about $\frac{1}{3}$rd the size of their containing red corpuscle.

P. minutum Emin, 1914, Craig, 1909.—The variations noted, *viz.*, oval amœboid stage and appearances resembling *P. malariæ* in an enlarged pale red cell showing Schüffner's dots, are well within the normal variations seen in *P. vivax* specimens; *P. ovale* is considered to be a variation of *P. minutum* Emin, 1914.

P. tenue Stephens, 1914.—Although certain authorities consider this a separate true species, it should meanwhile be considered to be identical with the ordinary *P. falciparum.* The pseudopodial forms are very irregular, "attenuated," and drawn out, the chromatin is more abundant than usual and relatively old schizonts are met with in peripheral blood.

P. perniciosum Ziemann, 1915.—This is true *P. falciparum* and not a separate species.

2. *Cycle of Sporogony.*

The sporogony or sexual sycle of the parasites causing malaria in man occur in certain species of mosquitoes of the genus Anopheles and consists in the fertilisation of the female gamete by the male gamete, the development of a zygote and the production of sporozoites. The separation of the polar chromatin from both male and female gametocytes, the rounding up of the male and female gametocyte to form spherical cells and the fusion of the male and female gametes may be seen in slides of human blood which have been breathed upon before drying, but occurs normally in the stomach of Anophelines. The nucleus of the male gametocyte divides up into eight daughter nuclei, each of which proceeds to the periphery of the cell and becomes liberated as a microgamete, each containing chromatin and a long process of cytoplasm. This is the process of "ex-flagellation." The motile microgametes swim about in the stomach contents, one finally penetrating the rounded stationery macrogamete which has already extruded its excess polar chromatin. The fertilised macrogamete (oökinete or zygote) elongates and travels through the stomach contents of the mosquito until it reaches the mucosa, through which it passes to lie finally encysted between the epithelium and muscular layer of the stomach wall. Up to this stage the zygote is known as a "travelling vermicule" and possesses a pointed anterior end, a blunt posterior end, which contains pigment, and a central nucleus. The encysted zygote (oöcyst) increases in size (up to 60 μ) and its nucleus divides into innumerable daughter nuclei by binary fission. These nuclei become surrounded by cytoplasm and are liberated as free sporozoites into the body cavity of the mosquito by rupture of the oöcyst

wall. The sporozoites wander throughout the body of the mosquito and may be found in any part except the ovaries, but finally congregate in the salivary glands.

The schizogony cycle again commences in man by the injection of saliva containing sporozoites by a bite from the infected female anopheles.

Transmission.—Malaria of man is not transmissible to any animal with the very doubtful exception of the Chimpanzee in which the infection rapidly dies out.

Cultivation.—Malarial parasites may be cultivated by defibrinating 10 c.c. of blood drawn from the median basilic vein and placed in a sterile test-tube containing 0.1 c.c. of a 50 per cent. solution of dextrose in ascitic or hydrocele fluid by stirring with a sterile wire round which the clot adheres. The blood is then placed in a series of sterilised test-tubes and incubated at a temperature of 35° to 38° C. The parasites develop in the layer of red blood corpuscles immediately below the supernatant plasma.

Parasites may be conveniently cultivated by Sinton's method which uses blood obtained from a finger prick (for which see Indian Journal of Medical Research, Vol. X). Blood may be obtained by spleen puncture.

The cultivation of malaria parasites should be carried out if malaria is suspected and blood slides invariably show no parasites when examined by the thick-drop method. No growth may be obtained if the patient has been taking quinine.

The Examination of Blood.—*The preparation of blood films.*—The slide should be perfectly clean. If grease is present, passing the slide through a flame will remove it. Another slide can be used as a spreader. An ordinary hypodermic or other needle, flamed before use, is used to prick the ear or finger (the nail region should be avoided). The oozing blood is touched with the flat surface at one end of the slide so that a small drop adheres to the slide. Too large a drop of blood should not be taken. The slide is placed on a table and the spreader, held at an angle of 45°, is allowed to touch the drop so that a thin film of blood runs along between the edge of the spreader and the slide. The film is next drawn along the slide. The blood must follow the spreader and not be pushed before it. The film is dried in air with the film side down.

Thick films.—After pricking the finger a clean slide is brought into contact with the blood in four places at the corners

of a small square about half an inch in size. The four drops, if large, should be further apart and, if small, near each other. With a round needle the drops are run into each other so as to form an even thick film covering about half an inch square.

A thick film and a thin film should be made on the same glass slide.

Staining of Blood Films.—*Leishman's stain.*—The method of preparation of the stain and staining of thin films are given under "staining methods."

For thick films dehæmoglobinisation was first done by a mixture consisting of glacial acetic acid and tartaric acid in distilled water. It is, however, sufficient to cover the thick film with *diluted* stain (equal parts Leishman and distilled water or dilute Giemsa). These dehæmoglobinise and stain the film. Five minutes with Leishman and 15 minutes or more with Giemsa are required for staining.

A method of staining thick films involving fixation with acetone (Merck) has been described by Brahmachari. The film is fixed with acetone for five to ten minutes, dried and placed in distilled or tap water in which dehæmoglobinisation is complete in about one minute. The film is then washed in methyl alcohol to remove traces of acetone, dried and stained in the usual manner with one of the Romanowsky stains (after Brahmachari and Sen).

The thick film method should be adopted as a routine. The method is much quicker and ensures a much greater percentage of successes than the thin film method. Before accuracy is attained a great deal of practice is required. A thick and thin film should as a rule be made on each slide: the thick film for spotting parasites and their species, the thin film for confirming these if doubtful, for eliminating blood diseases and infections, for examining the pigmentation of leucocytes and performing differential and Arneth's counts.

The Leucocytes in Malaria.—The total white blood count as a rule falls in malaria to between 5000 and 3000 per c.cm. The ratio of white to red cells falls from 1 to 500 to 1 to 900. In kala-azar the leucopenia is more marked. In cured cases of malaria, D. Thomson has found a daily leucocytosis at about the period of the day when the patient was previously having rigors.

In differential counts in malaria the large hyaline mononuclears are of significance. A count of 15 per cent. mononuclears or more, in the absence of kala-azar, is diagnostic of malaria (Stephens and Christophers).

An Arneth's count may show a shift to the left or right according to whether the malaria is acute or chronic.

Pigmented leucocytes are only found in late cases of malaria and are diagnostic of malaria when found. The pigment occurs chiefly in mononuclears and endothelial cells.

Spleen Puncture in Malaria.—Splenic puncture for the purpose of making slides or cultivation is a perfectly safe procedure in malaria or kala-azar and should be performed without hesitation when required. One precaution exists: a blood slide should be first taken to exclude leukæmia.

Where the peripheral blood film shows no parasite and diagnosis is imperative, *e.g.*, in malignant tertian or chronic and relapsing malaria, recourse may be had to spleen puncture. In the stained slide will be seen malaria parasites in different stages of schizogony, macrophages, endothelial cells loaded with pigment, free hæmozoin pigment lying scattered in the field and degenerating parasites lying in the intracellular spaces.

Malarial Dysentery.—The sudden onset of this associated with the fact that it is rapidly fatal if quinine is not given, makes immediate diagnosis imperative. Only four blood-stained stools in 24 hours may be passed and these contain red blood corpuscles in rouleaux and columnar epithelial cells but no pus cells. Malignant parasites may be found in the red cells passed and these may be not only rings but also the further stages of development of the schizont not seen in slides from peripheral blood.

Piroplasma

Babesia canis Piana and Galli-Valerio, 1895.

Distribution.—India, Ceylon, Africa, France and Italy.

Life History.—*B. canis.*—Schizogony occurs in the dog and sporogony in the invertebrate host, a tick.

Cycle of Schizogony.—The parasite is first seen as a pear-shaped body about half the length of its containing red cell and contains a chromatin mass near the pointed end and loose strands of chromatin ending close to a vacuole situated in the blunt part of the body. In the red blood corpuscles the trophozoite while in the ring form has still two unequal masses of chromatin; in the amœboid stage the vacuole disappears and all the chromatin masses coalesce. Later, the chromatin again sub-divides into two, the cytoplasm divides as well as the bigger chromatin mass and thus two pear-shaped bodies (merozoites)

result, each with a vacuole and two chromatin masses connected by a strand. The cell ruptures and the two pear-shaped merozoites invade fresh red blood cells.

Cycle of Sporogony.—The tick is infected on feeding on infected dogs. Hereditary transmission occurs in ticks. *Rhipicephalus sanguineus* in India, *Hæmaphysalis leachi* in Africa, *Dermacentor reticulatus* in France are those which carry the infection.

Piroplasma gibsoni Patton, 1910.

The organism has been found in dogs and jackals, and is like *P. canis* in every respect except that it is smaller and the young pear-shaped forms may appear as rings with one or two chromatin masses.

Theileria tsutsugamushi

This organism is found in the form of spheres, rings or small rods in the endothelial cells at the site of the bite of a mite (*Leptus akamushi*). It then travels to the lymphatic glands and spleen and with increasing severity may be found free in plasma and inside the red blood corpuscles. With Romanowsky the cytoplasm stains blue and the chromatin red.

Hæmogregarines

These are blood parasites occurring in reptiles, fish and mammals but not in man. When found in the nucleated red blood corpuscles of mammals they so closely resemble some of the forms of the malarial parasites of man—especially the crescent—that a mistake can easily be made. As a rule, however, these organisms do not produce any hæmozoin pigment.

The Amœbæ of Man

The amœbæ of man belong to the genus Entamœba of the class Rhizopoda (see chart). The genus includes *Entamœba coli; E. gingivalis; E. histolytica; Endolimax nana; Iodamœba bütschlii.* Of these only *E. histolytica* is pathogenic.

E. histolytica Schaudinn, 1903.

Synonyms.—*Amœba dysenteriæ; Entamœba tetragena.*

The organism was first seen and described by Losch (1875) but Schaudinn (1903) differentiated the pathogenic form, *Entamœba histolytica,* from the non-pathogenic, *E. coli.*

Life History.—Infection is conveyed from man to man through the ingestion of encysted forms originally passed in the fæces of some infected person. The cyst ruptures through the action of the digestive fluids, probably in the small intestine. The escaped amœbæ quickly invade the intestinal wall, crawl into the mouths of the glands in the large intestines and there multiply. Separation and degeneration of the gland cells and blocking of the mouth of the tubules occur.

The next stage is the invasion of the subadjacent intercellular connective tissue while the nodule eventually bursts, discharging its contents, blood, mucus and pus into the lumen of the intestine to be passed out in the fæces. The ulcer which results at the site of the rupture has undermined edges.

The amœbæ which escape invade other portions of the bowel and the infection spreads. The ulcers enlarge, due to the amœbæ, multiplying at the base. Extension to the submucous layer eventually occurs. In the process of extension the amœbæ may bore into the blood vessels and thus secondary foci, notably in the liver and brain, result. Occasionally only plugs of mucus with or without blood may be passed in the fæces and a "carrier condition" arises.

It would appear that this carrier condition is associated with a small type of amœba, the encysting forms or precystic amœba.

The further development of the encysted forms is not supposed to occur in the large intestine of the same host, but animal experiment does not confirm this.

Carriers may be "convalescent carriers", *i.e.*, those recovering from an attack of dysentery or "contact carriers" who pass through life without exhibiting any outward symptoms of the disease. The organisms that find their way into the liver, brain, spleen, etc., undergo a process of development similar to that in the large intestine. Small amœbæ, precystic amœbæ, or cysts, however, have never been demonstrated in these.

Morphology.—Three forms occur.

1. *Active tissue-invading forms.*—These are characterised by (1) the size—the organism varying from 20 to 30 μ; (2) a clear, broad, hyaline ectoplasm sharply marked off from the granular endoplasm, (3) energetic pseudopodial movements, (4) the endoplasm containing food vacuoles in which are seen red blood corpuscles and (5) the endoplasm often assuming a yellowish red tint due to the dehæmoglobinisation of ingested red cells.

Every *E. histolytica* does not show included red cells but it is only rarely that other objects are ingested. *E. coli* on the other hand ingests all kinds of objects indiscriminately excepting usually red blood-cells. The presence of red blood corpuscles is therefore highly suggestive of *E. histolytica.*

The nucleus is spherical, 4 to 7 μ in diameter, its central chromatin karyosome is surrounded by a clear area and chromatin granules are scattered throughout the nuclear membrane. In degenerated amœbæ the karyosome appears larger and is excentric in position while the chromatin may not be distributed so regularly. The position of the nucleus is subject to constant change and in the living *E. histolytica* the nucleus is often obscured by the density of the cytoplasm. In *B. coli,* however, the nucleus is more readily seen as the cytoplasm is less dense.

Multiplication in the tissues is by binary fission though bud-formation and also schizogony is said to occur in degenerating amœbæ.

2. *Precystic ("minuta") forms.*—These forms are small (7 μ to 18 μ) and are produced probably by division from the larger forms. The daughter amœbæ thus formed divide again and again so that increasingly smaller forms result.

Precystic amœbæ otherwise resemble the tissue-invading forms, except that food vacuoles are absent, the chromatin is in masses in the nucleus, a vacuole which colours brown with iodine develops and certain chromatoid bodies staining black with iron-hæmatoxylin are also met with.

The sluggish movements and the larger chromatin granules make it difficult to distinguish these precystic forms from the corresponding stage of *B. coli* and the characteristic cysts must be looked for.

3. *Encysted forms.*—The cyst wall quickly shrinks and hardens into a colourless transparent capsule about 5 μ thick; the shape is generally spherical. The single nucleus divides to form two nuclei and these subdivide and a mature cyst with four nuclei results. Very rarely eight nuclei may be found. The vacuole, if present, gradually disappears and similarly the chromatoid bodies, but the chromatoid bodies when present are of diagnostic significance as they are usually not seen in the cysts of *E. coli.* A fresh *E. histolytica* cyst measures 5 to 10 μ and has a greenish refractile appearance which serves to distinguish it from the cysts of *E. coli.*

Some regard the smallest cysts as belonging to a distinct species of amœba and they have not been proved to be pathogenic. The cysts passed from the body may contain one, two or four nuclei.

The length of the life of a cyst of *E. histolytica* is considerable, specially if in moist condition. After passage and before ingestion by a new host both the chromatoid bodies and the glycogenic vacuole gradually disappear.

Cultivation.—Amœbæ can be cultivated at 28 to 30°C. on egg medium to which a drop of blood is added or human blood medium with 1 per cent. peptone. Recently Boeck and Drbohlav have used a solid blood agar medium with a layer of Locker's solution containing serum or egg albumin.

Pathogenicity.—In certain cases where very few symptoms occur the organisms have apparently only a slight pathogenicity but invasion of tissues may occur whenever the resistance of the host is lowered or the organism gains in virulence.

Animal Susceptibility.—As far back as 1875 Lösch succeeded in infecting a dog with *E. histolytica* but most experimental work has been done with cats, infection being generally produced by injection per rectum of dysenteric stools.

Feeding experiments do not succeed unless cysts are present. Infection in kittens is generally very severe, the whole of the large intestines being involved: the infection first occurs in the lower part of the large intestine. Definite ulcers occur if the animals live long enough but recovery rarely occurs. There is no carrier condition in cats corresponding to that in humans but liver abscess may occur.

Diagnosis.—A piece of mucus from the stool is picked out and preparations in distilled water or normal saline are made. If the stool is fresh, living amœbæ actively throwing out pseudopodia may be seen, especially with the warm stage of the microscope. The main points of distinction from the corresponding stage of *E. coli* are the active motility, a smaller size, clearer differentiation into ecto- and endoplasm, greenish tint as contrasted to the greyish colour of *E. coli* while red blood corpuscles may be present in which case a diagnosis of *E. histolytica* is fairly certain. The nucleus in *E. histolytica* does not stand out so distinctly as in *E. coli.*

Cysts with four nuclei are characteristic of *E. histolytica.* Definite chromatoid bodies are usually also seen.

Iodine solution brings out the details of the cyst much clearer. Eosin stains readily dead amœbæ or cysts while living forms stain slowly.

In amœbic dysentery stools contain large numbers of motile organisms and eosinophils. Several stools must be examined as the excretion of *E. histolytica* is often intermittent.

E. coli (Grassi, 1879).

Synonyms.—*Amœba "Lewis"; Amœba coli; Entamœba coli; Entamœba coli communis.*

Life History.—The organism is normally a harmless inhabitant of the digestive tract of man and does not invade the tissues like *E. histolytica.* It develops on the surface of the intestinal mucosa, its food consisting of yeast, bacteria, cysts of other protozoa, Giardia and even the cysts of *E. histolytica.* Red cells, however, are not ingested.

Morphology.—(1) The adult form.—This is larger than *E. histolytica* and has a diameter of 15 to 30 μ. The ectoplasm is not so sharply defined as in *E. histolytica.* The endoplasm has a number of vacuoles containing chiefly bacteria, but no red blood cells. The colour is slightly greyish as contrasted with the greenish tint of *E. histolytica.* Another point of distinction is that "the organism is much more fluid in consistency" than *E. histolytica.* The nucleus is larger and coarser and is a much more striking object in the living *E. coli.* The chromatin granules are coarser and the clear area surrounding the karyosome is larger than in *E. histolytica.* The karyosome is nearly always excentric.

(2) *Precystic forms.*—Prior to encystment smaller forms are produced as in the case of *E. histolytica.* These resemble those of *E. histolytica* in structure although slightly larger in size.

(3) *Encysted forms.*—A cyst wall forms round a precystic amœba which has become spherical. The nucleus divides and ultimately eight nuclei characteristic of the mature cyst result. Occasionally forms with 16 nuclei are met with.

The size of the cysts varies greatly from 10 to 30 μ the average being about 20 μ. In most cases there occur also a certain number of cysts of a different type usually larger than the others, with two nuclei, a large central glycogen vacuole and cytoplasm which is limited to a thin layer lining the cyst wall. The

structure of the nucleus within the cyst is similar to that of the adult amœba.

Chromatoid bodies are occasionally seen in cysts of *E. coli* but are, however, not so definitely rod-like as those in cysts of *E. histolytica.*

Pathogenicity.—There is no evidence that *E. coli* is pathogenic to man or animals. Infection by the ingestion of cysts has been demonstrated and cysts appear in the stools in from 1 to 11 days.

E. gingivalis (Gros, 1849, Burm, 1910).

This organism is regarded as a saprophyte which lives in the pockets between the gum and the teeth along with numerous spirochætæ, bacteria and trichomonas, in the condition known as pyorrhœa alveolaris. It is not regarded, however, as the cause of pyorrhœa. It is a fairly active organism, 10 to 20 μ in size. A clear narrow ectoplasm surrounds a highly vacuolated granulated endoplasm in which are food vacuoles, occasionally red cells and a nucleus which is distinctly smaller than that of *E. coli.* Reproduction takes place by binary fission and cysts probably do not occur.

E. nana (Wenyon and O'Connor, 1917).

This is of importance because like *E. histolytica* its mature cyst shows four nuclei and the amœbæ may contain many bacteria. It is present in freshly passed normal stools and is found in about one-third of all dysenteric and diarrhœic conditions. The amœba is small, 6 to 12 μ in diameter. The mature cysts are oval, contain four nuclei and some refractile granules but chromatoid bodies and vacuoles are absent. The organism is non-pathogenic.

Note that a clear contractile vacuole is never present in the cytoplasm of human parasitic amœbæ.

Iodamœbæ bütschlii.

This also occurs in about 5 per cent. of human fæces and usually in association with *E. histolytica.* The size is intermediate between *E. coli* and *E. nana,* 9 to 20 μ. Numerous food vacuoles are present. The nucleus has a central, intensely staining, karyosome. The cysts have one nucleus only and are slightly irregular. The amœbæ inhabit the large intestine and though not pathogenic disappear rapidly under emetine treatment.

BALANTIDIUM

This genus contains certain species which occur in various vertebrate and invertebrate animals.

Balantidium coli (Malmsten, 1857).

Distribution.—World wide, especially frequent in the Philippine islands.

Morphology.—This is the largest protozoal parasite of the human intestine. The size is about 50 to 80 μ × 40 to 60 μ, the maximum length being 200 μ. It is probable that different races exist.

The body has a covering of cilia in parallel long rows. The granular endoplasm is surrounded by a thin layer of clear ectoplasm and outside this is a thin cuticle through which the cilia emerge.

At the anterior end is a funnel-shaped depression—the peristome—which has special marginal rows of cilia. Leading from this is the mouth or cytostome from which a very short œsophagus leads into the endoplasm.

There are two contractile vacuoles in the endoplasm—one at the middle and the other at the posterior end. An anal aperture is also present at the posterior end.

The macronucleus is biconvex and lies across the middle of the body. The small spherical micronucleus lies close to this nucleus. Red cells, fœcal and tissue debris are some of the contents of the cytoplasm.

Reproduction is usually by binary fission, though conjugation has also been observed.

The cysts are round or ovoid and measure from 50 to 60 μ. They are by far the largest cysts known in human fæces and as passed in fæces usually contain two ciliates.

Pathogenicity.—This varies from time to time. It undoubtedly possesses tissue-invading properties. Usually, however, no symptoms occur and a "carrier" condition results. When symptoms appear they are generally very severe, with extensive necrosis. Ulceration in this infection is limited to the large intestine.

CHAPTER X

ANIMAL PARASITES

The Helminthes are permanent parasites in man or domesticated animals and represent a biological as against a natural group in systematic Zoology. They are divided into Platyhelminthes or flatworms and Nemathelminthes or round worms. The flatworms are characterised by a complete absence of the body cavity and the presence of hermaphroditism and are divided into three classes: (1) Turbellaria which are terrestial and aquatic free living forms, covered with cilia and possessing suckers but no anus, (2) Trematoda, leaf-like or pear-shaped parasites, possessing suckers for attachment and having an incomplete intestinal system with no anus, and (3) Cestoda, ribbon-shaped parasites in which the body is divided into a series of segments each identical anatomically and which have grooves or suckers but no alimentary canal.

Phylum Platyhhelminthes.—Flat Worms.
Class Trematoda

Genus Schistosoma differs from all other flukes in being unisexual, the male and female being distinct. The ventral sucker is recessed into the body and the genital pore is just behind it. The body of the male is curved ventrally on either side to form a gutter wherein the female is permanently held.

Schistosoma hæmatobium (Bilharz 1852) (*Distoma hæmatobium; Bilharzia hæmatobium; Distoma capense*), the cause in man in Egypt and various parts of Africa and Asia Minor of urinary schistosomiasis (bilharziasis or endemic hæmaturia).

The definitive hosts are man, the monkey, mouse and rat and the intermediary host a mollusc. The adult male measures 1 to 1.5 cm. and possesses a small oral and a large ventral sucker. The surface is covered with small papillæ. The sides are folded to form the gynæcophoric canal in which the female is partly enclosed and carried. The cœcum is short and the testes number 4. The female worm is 2 to 2.5 cm. long.

The adults live in the mesenteric, portal, uterine, vesical and pubic veins. The eggs which are found in the urine and fæces are oval, about 150 μ long and have a spine at one end. The larva, a ciliated miracidium, is contained in the egg, ready to be liberated on the egg coming into contact with water. The liberated miracidium swims about in the water and within 24

hours dies unless it is able to penetrate the tissues (usually the antennæ) of a mollusc of the genus Bullinus. Further development of the parasite occurs in the snail's liver where numbers of daughter sporocysts are formed. Ultimately these rupture, liberating a large number of cercariæ which finally leave the host and enter the water. The cycle in the mollusc takes 14 days.

The free cercariæ die within 48 hours unless they are able to penetrate the skin or mucous membrane of their definitive host. The bifid-tail of the cercaria is shed in passing through the skin and the parasite proceeds to the liver via the lymph and blood stream and matures in about six weeks.

Diagnosis.—(1) The finding of the eggs on microscopical examination (low power) of urine is final—the last few drops of urine to be voided should be examined.

(2) Fairley has described a complement deviation test using an antigen from the livers of infected molluscs. The technique is the same as that for the Wassermann reaction and the reaction is specific, especially in the early stages of the disease.

(3) Cystoscopic examination in the early stages of the disease shows discrete whitish elevations around the orifices of the ureters, these patches becoming hæmorrhagic and inflamed in the later stages.

Schistosoma mansoni.—This was discovered by Bilharz in 1851 and Manson in 1903. The intermediary host which the miracidium penetrates is a mollusc of the genus Planorbis. The disease occurs in South America, Central and West Africa, Egypt and the West Indies.

In this worm the cœcum is long (at least $\frac{2}{3}$ the body length) the testes number 1 to 9 and the miracidia develop within the egg case at the time of laying.

The eggs are long (150 μ), oval and possess a lateral spine near one of the poles. They are found chiefly in the fæces. The eggs give rise to a choleraic diarrhœa and a peculiar cirrhosis of the liver. The adult worm is found in the portal and mesenteric veins in man. A rectal papillomatous growth may occur.

Diagnosis.—Several specimens of solid fæces (as against liquid stools) should be examined for the characteristic lateral spined egg. The urine may occasionally show the eggs. Fulleborn's concentration method consists of rubbing up a small piece of fæces with a glass rod in some $2\frac{1}{2}$ per cent. saline and setting aside to settle for 5 minutes. The supernatant saline is poured off, more saline added and the process repeated. Warm

distilled water is then added and the tube exposed to light. Some perchloride of mercury solution is added and the deposit examined for miracidia.

Schistosoma japonicum (Katurada, 1904)—the cause of Katayama disease characterised by a fatal hepatic and splenic enlargement with ascites—occurs in China, Japan and the Philippines.

This species is distinguishable by its small size, smooth body, short cœcum, having only 6 testes, and the uterus only containing 2 eggs at one time. The definitive hosts are man, dogs, cats, rats and cattle. The eggs are oval, smooth and possess a small lateral spine. The adult resembles *S. hæmatobium* and the intermediate host is a mollusc of the genus Oncomelania.

Diagnosis.—In the early stage a high eosinophilia occurs. The stools should be examined for the eggs. Fairley's complement test may be employed.

Genus Fasciola.—Fasciola hepatica (Linn., 1758). (*Distoma hepaticum; Fasciola humana; Distoma caviæ*), the liver fluke, is found chiefly in sheep and rarely in man. It occupies the liver, bile ducts and portal vein and is said to be the cause of a relatively common pharyngeal disease in man in Palestine.

The adult is leaf-shaped, thin and flat, about 2 to 3 cm. long by 8 to 14 mm. wide and possesses a small cup-shaped oral sucker in its narrow anterior end and a large ventral sucker serving for attachment slightly behind this. The body is bilaterally symmetrical and is provided with numerous rows of rod-like scales. The fluke is a hermaphrodite. The eggs are oval, operculated, brown in colour and measure approximately 130×80 μ.

The eggs on being passed into water develop into ciliated embryos or miracidia. The intermediary host is a fresh water snail of the genus Limnæa and unless the miracidia penetrate these within 12 hours they die. After undergoing metamorphosis the miracidia become cercariæ, possessing a long contractile tail and 2 suckers. The cercariæ on leaving the snails become encysted on grass or other vegetation and infection in the definitive host is caused through eating infected material.

Fasciolopsis buskii, (Lankester, 1857). This fluke occurs in China, India, Assam and the Straits Settlements and is normally found in the pig. Its size is 30 mm.×12 mm. and its ventral surface is covered with transverse rows of spines. The eggs are numerous and are found in the fæces of the person affected, the size being 120 μ ×75 μ. The intermediate hosts are species of Planorbis.

Clonorchiasis, a severe disease associated with diarrhœa and liver enlargement occurring in India, is due to *clonorchis sinensis* a small trematode, 10 mm.×5 mm., the ova of which occur in fæces.

Class Cestoda (Tapeworms).

Tænia solium Linn., 1758.—The adult worm may occur in man. The intermediary host is the pig, and occasionally man, cattle, the rat or dog. The worm is not found in Muhammadans nor Jews.

The adult worm averages 6 feet in length but may be 15 to 20 feet. The head is globular, has four circular suckers and two rows of hooklets which may number 25 to 50. The mature proglottides are longer than they are broad and the genital pore is marginal on one side or the other. The uterus has 7 to 12 diverticulæ.

The eggs are liberated when the ripe segments disintegrate after passing or are eaten by the intermediate host along with contaminated food or water. The eggs are slightly oval, 30 to 50 μ in diameter, are radially striated and contain a small embryo (onchosphere) with six hooklets. The onchosphere passes via the wall of the intestine and blood stream to the liver, eye, brain, muscles, the neck, shoulders, etc., sheds its hooklets and develops as a cysticercus. This *Cysticercus cellulose* varies from 5 to 20 mm. long (in the brain the size may be much greater) and has an invaginated scolex or head possessing six hooklets on a neck connecting it to the cysticercus wall. The cysticercus stage may occur in the muscles of the thorax, shoulders and tongue of man and less commonly in the human brain, lungs, etc., and probably originates by the swallowing of ova passed by the patient himself if he harbours the adult worm. Man becomes infected with the adult worm on eating improperly cooked, infected "measly" pork.

Tænia saginata (Gœze, 1782) (*T. mediocanellata; T. hominis,* etc.) The intermediary host is the ox and the definitive host man. The adult worm may measure 20 feet, the head is larger than that of *T. solium* and the worm is of a semi-transparent white colour. The scolex is square, 1—2 mm. long and has four lateral, often pigmented, suckers but no hooks. The terminal rostellum is replaced by an end sucker. The neck is long and narrow. The ripe proglottides are about 4 times longer than their breadth. The uterus has from 15 to 35 lateral diverticulæ. The genital pore is laterally placed to the right or left margin in alternate segments and at the posterior end of each proglottide. The eggs are oval, about 35 μ by 25 μ in diameter, are surrounded

by a dark brown radially striated border and containing an onchosphere with six hooklets. The eggs from disintegrated proglottides are eaten with grass by the ox, the onchospheres, set free in the stomach, pass through the wall of the small intestine and become encysted as cysticerci in the muscles of the pterygoids especially but also in the heart, tongue and diaphragm. The cysticerci are eaten by man in uncooked meat and develop in the small intestine into the adult worm whose proglottides become mature in 2 to 3 months.

Hymenolepis nana (Siebold, 1852). (*Tænia nana*). This cestode is met with in Southern Europe, the Sudan, America, Japan and in Assam in India. The whole worm is very minute and hundreds may be present in the small intestine of man. The head has a circle of about 30 hooklets and 4 circular minute suckers. No intermediary host is required. The eggs, as seen in the fæces of man, are transparent, oval, 40 μ in diameter, with two membranes and contain an onchosphere with 6 hooklets.

Echinococcus granulosus (Batsch, 1786). (*T. Echinococcus*). Definitive host—the dog and jackal (small intestine). Intermediary host—man, sheep, pigs, cattle, goats, horses and camels (as hydatid cyst).

The adult worm is very small (about 6 mm.) and the proglottides are not more than 4 in number, the distal one being about half the total length of the worm. There are four minute suckers and a rostellum with two rows of hooks. The eggs in the last (mature) proglottide may number over 500 and are oval, averaging 35×25 μ. On obtaining access to the gut of the intermediary host the egg liberates its 6 hooked embryo which proceeds to the muscles, peritoneal cavity, portal vein and liver, lungs or brain, where the cysticercal stage is passed. Daughter-cysts are formed by the inner germinal layer of the hydatid, forming protrusions (brood capsules) by a process of invagination. From the wall of each brood capsule scoleces are given off, each scolex (which is a small complete head like that of the adult worm) having a rostellum and hooklets. The brood capsules now become free from the wall of the hydatid and float as daughter cysts in the clear fluid with which the hydatid is filled.

From one embryo not only daughter-cysts but granddaughter-cysts may be produced as each daughter-cyst may become detached and develop a new typical hydatid itself. Exogenous daughter-cysts may also develop outside the wall of the hydatid from which they originally came. In man, hydatids occur chiefly in children through their association with dogs, etc.

Diagnosis.—This usually depends on diagnosing the physical signs produced. A precipitin reaction using equal parts of the serum of the patient and preserved hydatid fluid has been described by Welch and Chapman, incubation for one hour giving a precipitate in positive cases, but the reaction is not specific. A complement-deviation test using an alcoholic extract of scoleces as the antigen has been described.

Diphyllobothrium latum—(*Dibothriocephalus latus*). Definitive host—the dog, cat and man. Intermediary hosts—fresh water crustacea (cyclops) and fish. The adult worm may attain a length of 30 feet and more than one may be met with. The head has no hooklets but lateral suckers. The proglottides may number 4000, are much broader than their length when mature, and have a rosette-shaped uterus. Mature segments are not passed but the eggs as passed in fæces in great numbers are brownish and have an operculum at one end. A ciliated onchosphere with 6 hooks slowly develops, when the egg is passed into water, and this is ingested by crustaceæ, chiefly of the genus Cyclops, and in about 3 weeks develops in the small intestine of the crustaceæ into a procercoid larva. On the Cyclops being swallowed by fresh water fish the procercoid larva passes into the body cavity and develops as a plerocercoid larva in the muscles, etc., of the fish. Man is infected through eating insufficiently cooked fish, caviare, etc., the parasite becoming mature in about 6 weeks after being ingested.

PHYLUM NEMATHELMINTHES.
(Cylindrical unsegmented unisexual worms).

GROUP NEMATODA.

Filaria bancrofti Cobbold, 1877 (*F. nocturna* Manson); *F. sanguinis hominis*), are thin transparent thread-like nematodes which are found in the lymphatics and glands in man (often as bunches in the varicose lymphatics). The male measures 40 mm. and the female 60 to 100 mm., the genital opening in the latter being near the bulbous termination of the anterior end. The female is viviparous, the embryo or microfilaria developing within the egg in utero. On being passed by the adult worm the microfilariæ proceed through the lymphatics to the blood stream of the host and are thus present in the blood swallowed by mosquitoes feeding on the host. The mosquito cycle occupies about 2 weeks and the microfilaria may be seen in sections in the thorax, head, legs, proboscis, etc., of the mosquito. The microfilariæ are not injected into a new host by the mosquito but ultimately penetrate the terminal end of the proboscis of the mosquito when it is in the act of biting and work their way through the puncture in the skin made

by the mosquito. Certain species of Culex, Anopheles and Aëdes act as hosts to *Filaria bancrofti. F. bancrofti* occurs in India, China, South America, Africa, the Mediterranean coasts, etc.

Loa loa (Guyot, 1778). (*F. loa; F. diurna*). Loa loa differs from the other nematodes in having nodules or protuberances distributed irregularly on its cuticle. The worm measures about 30 mm. and is cylindrical and semi-transparent. The microfilariæ closely resemble bancrofti except that in stained specimens they appear angular. The intermediary host are flies of the Genus Chrysops which remain infective for about a week. The cycle in the fly takes about 14 days. *L. loa* occurs in tropical West Africa.

Filaria ozzardi Manson, 1897 and *Acanthocheilonema perstans* inhabit the mesentery of man—the former occurring in certain parts of the West Indies and South America and the latter in West Africa and tropical Africa generally.

The *Microfilariæ ozzardi* have been found in the blood of man, are sheatheless and have sharp tails in contradistinction to *Microfilaria perstans* which has a blunted tail. Both these microfiliariæ shew no periodicity. The adult *A. perstans* resembles *F. bancrofti;* the male (40 mm. long) is about half the length of the female (75 to 80) and the tail in both sexes is curved upon itself and the extreme tip is split into two. The male possess two spicules one long and one short, protruding from the cloaca.

Dracunculus medinensis (Linn., 1758). (*F. medinensis;* Guinea worm).—This filaria is found in South America, the West Indies, India and Africa and is said to attain a maximum length of about five feet in the subcutaneous tissues of man. The intermediary hosts are species of fresh water cyclops. The female worm is said to average about 80 mm. in length. The worm is cylindrical, of a uniform white colour and smooth. The tail is sharply bent at the end while the anterior end is round and ends in a cephalic shield. The body-cavity of the worm is occupied by a large uterus filled with coiled embryos which each measure about 700 μ long. These microfilariæ have transverse marking and the posterior part of the body is long and thin. The embryo may remain alive up to a week in fresh water and in moist earth life is prolonged for several weeks.

The embryos pass from the water into the body cavity of the cyclops and pass through three moults before maturing.

Onchocerca volvulus Leuckart, 1893 (*F. volvulus*).—This filaria chiefly occurs in the West Coast of Africa and is found

in tumours in the subcutaneous tissues especially in the sub-occipital, axillary and popliteal and intercostal regions in man. The microfilariæ are found just under the true skin and in the blood and show no periodicity. The intermediary host is unknown but is probably one of the insecta.

The female, 35 cm. long, is white and tapering. The male is very small, 30 mm., is striated, the end of the tail is curved once upon itself, 2 unequal spicules protrude from the cloaca and a pair of papillæ exist in front of and a pair behind the anus. The female is viviparous and the microfilariæ which measure about 300 μ are sheathless.

Strongyloides stercoralis (Bavay, 1876) (*S. intestinalis*). This nematode is widely distributed. The definitive host is man, in whom the parasite causes often severe intestinal symptoms since the habitat is the intestinal follicles of the small intestine. The adult worm is 25 mm. long and tapers at both ends. The worms are parthenogenetic. The uterus occupies the larger part of the body and contains 50 eggs, each approximately $50 \times 30\ \mu$ The eggs partly develop in utero and are then passed into the fæces of the host in which they develop into embryos. These embryos resemble those of ancylostoma but have a double bulbed œsophagus and are sexually distinct. After copulation on the ground the female larvæ lay eggs which develop into the filariform larvæ which are infective to man.

Ancylostoma duodenale Dubini, 1843.—This belongs to the family Strongyloidæ. The definitive host is man; the habitat the small intestine. It is a greyish or brownish cylindrical worm, the male being 8 to 10 mm. long and the female 10 to 15. The posterior end of the male ends in an expanded copulatory bursa, divided into three unequal parts, and in the female a median copulatory organ is present in the midline anteriorly. The worms therefore assume a Y shape in copulation. At the anterior end of the worm the buccal cavity points slightly dorsally and this bears 4 hooked teeth in its ventral aspect and deeper pharyngeal teeth.

The eggs which appear in the fæces of the host are oval, about 60 μ long, very transparent and usually show four yolk segments (blastomeres).

Development of the egg occurs in damp ground or fæces. Within 24 hours of being passed in fæces the 2, 4 or 8 blastomeres develop into rhabditiform larvæ. These larvæ moult twice on the third and on the fourth or fifth day respectively and become filari-form larvæ. This is the stage infective to man and in this stage the larvæ may live for some years in damp ground. On

piercing the skin, buccal mucosa, etc., of man, larvæ enter the lymphatics and blood stream, reach the lungs about three days later, and pass via the bronchi to the œsophagus and stomach. A third moult occurs prior to their reaching the œsophagus and a fourth moult on the 7th day after entering the human host when the larvæ are in the intestine. Finally on the 15th day the worm assumes its adult form and it attains sexual maturity in about 3 weeks.

The worm is found in Europe, America, Africa and Asia; about 75 per cent. of the population of the United Provinces in India are affected. The spread of ancylostomiasis depends on suitable temperature and humidity and has been associated in Europe with tunnel construction and mining.

Necator americanus Stiles, 1902.—This occurs in America. Africa, India, Ceylon and the Philippines. It is the common ancylostome of man in India. The adult is smaller than *A. duodenale,* the male being about 9 mm. The buccal grove is irregular in outline, bears one solitary dorsal tooth, and, instead of ventral teeth as in *A. duodenale,* two plates converging ventrally in the middle line. The eggs are large 65 μ by 35 μ to 40 μ.

Ascaris lumbricoides Linn., 1758.—The habitat of the "common round worm" is the small intestine of man and 3 or 4 are generally found together. They are round, the male measures up to 8 inches long and the female up to 12 inches long; the ends taper, the anterior part ending in the terminal mouth surrounded by thin lips, while the anus is on the ventral aspect just anterior to the posterior end of the worm. In colour they are brown with four white vertical lines and the tail of the male is usually curved into a semicircle.

The eggs are slightly oval, large (60 μ to 75 μ), unsegmented and are dark brown in colour when seen in the fæces. The outer rim of the egg is rough, irregular and ill defined but may be broken off, leaving a smooth ill defined outer border. The eggs develop into larvæ when swallowed by rats, sheep, goats, etc., and travel to the lungs and liver, whence they migrate about the 9th day to the alimentary canal. They are passed in the fæces and set up the disease in man if swallowed. Infection may be direct in man without an intermediate host but in this case the larvæ wander through the tissues before settling in the intestine and may produce pulmonary symptoms. It is also possible that the eggs may hatch out in damp soil and man may be infected through the skin direct.

Trichina spiralis (Owen, 1835).—Definitive hosts—the rat, pig and man. The adult worm is just visible to the naked eye as a white thread, the male being 1·5 mm. and the female 4 mm. long. They inhabit the small intestine and the female, which is viviparous, produces about 1000 embryos which are deposited directly into the intestine and lymph spaces of the intestine and thence proceed by the blood stream to the muscles of the host. In 2 to 3 weeks the embryos become encysted. These tiny cysts are chiefly seen in the muscles of the diaphragm, tongue and shoulder and ribs of the pig as small, oval, calcareous gritty nodules and may be examined by pressing them between 2 glass slides or adding some dilute acetic acid to dissolve the lime salts. As many as 4 trichinellæ may be in one cyst. Man is infected by eating improperly cooked pork and about 48 hours after ingestion adult larvæ occur in the intestine. The eggs are about 30 μ and are hatched out in the uterus of the trichina as above mentioned.

Enterobius vermicularis (Linn., 1758). (*Oxyuris vermicularis;* threadworm). The threadworms are small (male 2·5 and female up to 12 mm. long) and white in colour. The male is rare, its posterior third is curved and bears a single spicule. The eggs measure 50 μ by 20 μ, are flattened on one side and have a thick outer shell and thin inner one containing the embryo.

The female worms distended with eggs are passed with the fæces or migrate out of the anus and deposit their eggs containing the partially developed embryo in the nates of the host. In a large number of cases self-infection of the host by contaminated fingers occurs, the egg-covering becoming partly digested in the stomach. The embryos pass through two moults in the small intestine and finally, about 2 weeks after hatching of the egg, are found as mature worms in the cœcum, appendix, etc., of the host. The vulva and bladder of female children may be infected with the worms.

The eggs are rarely found in human fæces.

Trichuris trichiura (Linn., 1771).—(*Trichocephalus dispar;* Whipworm). The whipworm is white or slightly pink in colour and about 2 inches long (male 30 to 45, female 35 to 50 mm). The anterior thin end is twice as long as the thicker posterior portion. The posterior part of the male is curved ventrally and bears a single spicule in a spine-covered sheath. The eggs are brown, barrel-shaped 50 $\mu \times 20$ μ; and thick walled, with a "plug" at each end. The eggs are passed out in the fæces and develop into embryos slowly, up to 1 year being required to attain maturity. The eggs do not rupture, the mature embryo being capable of remaining in the shell for some years or until

the egg is again ingested by man. The adult worms appear in the human cœcum a month after ingestion of the eggs, and apparently attach themselves by pinning themselves through the villi.

Examination of Fæces.—(1) *Microscopic examination.*—(*a*) Ordinary method.—A small piece of the stool is placed on a slide and spread out gently by pressing down a cover-slip. On examining by the high power the following are noted:—The size, colour, shape, striation, operculum and markings on the shell, roughness or smoothness of the envelope, whether the yoke is undifferentiated or has 2, 4 or 8 segments, whether embryonic hooklets are visible. Examination for ancylostoma should be carried out when the stool is fresh, as the blastomeres rapidly develop and the embryo leaves the egg. When examining stools dry preparations are useless.

(*b*) Clayton Lane's original *Direct Centrifugal Flotation* method for ancylostoma uses a small glass centrifuge tube the mouth of which is ground, so that a cover-glass can be used to close the mouth during centrifuging. 1 c.c. of fæces are shaken up in water and centrifuged. The supernatant fluid is removed and replaced by salt solution (sp. gr. 1150) which completely fills the tube so that it touches the under surface of the cover-glass used to close the tube. The cover-glass is examined after centrifuging for 30 seconds and the eggs are found to be adhering to it.

This D. C. F. method is the most reliable method available for discovering mematode infections of the intestinal tract.

(2) *Sugar method.*—A small piece of fæces is shaken up in an equal bulk of a six per cent. solution of ordinary cane sugar in water in a narrow glass vessel; the solution is allowed to stand for 1 hour and the surface of the fluid touched with a cover-glass. The eggs adhere to the cover-glass and can be examined by the low power microscope.

Cultivation of Ancylostoma from soil and fæces.—About 2 c.c. of fæces are mixed gently with ½ c.c. powdered charcoal and ½ c.c. water in a corner of a small dish and kept in a dark, warm, moist place for from 2 to 4 days. About 20 c.c. of sterile water are then gently poured into the Petri dish and the embryos will be seen to emerge and swim in the clear water.

The diagnosis of ancylostoma by finding the adults after administering a vermifuge is more satisfactory than the ordinary methods of examination for eggs.

CHAPTER XI

THE EXTRA-CORPOREAL HOSTS OF PATHOGENIC ORGANISMS, Etc.

Ticks

The ticks belong to the phylum Arthropoda, class Arachnida, family *Ixodidæ*. The sub-families Ixodinæ (hard ticks) and Argantinæ (soft ticks) require some consideration. The following are known to convey disease in nature:—

(*a*) *Ornithodorus moubata.*—This species of the Argantinæ is found chiefly in East and West Africa. It has a rounded body of a brownish colour formed from the fused cephalothorax and abdomen, is marked above and below with grooves, grows to about 8 mm. in length and 7 mm. in breadth and has 8 legs but no eyes. The habits of this tick are similar to that of the beg-bug. It lives in cracks in the walls of the houses and in the roofs, and feeds slowly and at night. The eggs are laid in batches of from 50 to 100 and hatch out in 3 weeks, liberating nymphs as the larval stage occurs in the egg. The nymph goes through several stages of development. While feeding, the adult passes a whitish excreta which is probably concerned in its capability of conveying infection to man and animals.

It transmits the *Spirochæta obermeieri,* the organism of relapsing fever.

(*b*) *O. savignyi.*—This species differs from *O. moubata* in having eyes. It is also suspected of transmitting relapsing fever.

(*c*) *O. lahorensis.*—This species is very similar to *O. moubata* and is suspected of carrying relapsing fever in Persia.

(*d*) *O. tholozani* is a similar tick to the above, commonly, known as the "Persian tick," and is suspected of transmitting *Spirochæta berberum.*

(*e*) *O. venezuelensis.*—This tick is smaller than *O. moubata,* but has similar characters. It measures 5 to 6 mm. by 3 to 4 mm. and conveys relapsing fever in South America.

(*f*) *Dermacentor venustus.*—This tick is common in the Rocky Mountains, and frequently feeds on men and animals, especially rodents. It is involved in the transmission of "Spotted Fever" of the Rocky Mountains.

The female tick, after feeding, drops off the host. She lays some 5000 eggs which hatch out into 6-footed larvæ on the 26th day. These larvæ are especially found on the ground squirrel, and, in turn, drop off their hosts after feeding. After moulting the nymph emerges. This nymph may survive without food for 10 months but ultimately attaches itself to a host—usually the squirrel—feeds, then drops off the host. The adult emerges from the nymph after a further ecdysis and may survive for 2 years without feeding. A tick, as yet unidentified but probably a Hyalomma, is suspected of transmitting Typhus fever in the Kumaun hills of India. For a full description of these ticks a reference should be made to books on entomology.

Sarcoptes Scabiei (Linn., 1758).

Acarus scabiei, the itch mite, belongs to the order Acarina of the class Arachnida.

The female is twice the size of the male and measures 0.4 mm. in length. The abdomen and the cephalothorax are fused together and are provided with dorsal spines. There are four pairs of legs and the four legs in front are provided with "suckers." In the female the four posterior legs have bristles. The female burrows into the skin; the male remains on the surface of the burrow and soon dies after mating. As the female burrows she lays about 50 eggs which are oval and measure about 120 μ long. The eggs hatch out into 6-legged larvæ in under 8 days and the larvæ moult several times, becoming 8-legged nymphs. The whole cycle usually takes about 4 weeks. The female dies in the burrow as she is unable to recede owing to the presence of the dorsal spines.

Sandflies.

Sandflies belong to the class Insecta, order Diptera, family Psychodidæ, genus *Phlebotomus.*

Phlebotomi are small hairy flies (1·5 to 3 mm. long) with six slender legs, a spindle-shaped abdomen which has 10 segments and is covered with long yellow hairs, a short proboscis and long antennæ which have 16 joints and are "bushy" in both sexes. Their geographical distribution coincides with the occurrence of sandfly fever and *P. papatasii* has been definitely proved to carry this infection: the cycle of the parasite (a filterable leptospira) requires 6 days to reach completion in the sandfly. Only the female sandflies suck blood and they only feed at night.

P. papatassi has a wide distribution in Southern Europe, North and East Africa, Java and India and lays about 40 eggs, one at a time, in damp and dark places. A high humidity is necessary for the development of the larval stage. After about a week the eggs hatch out into small caterpillar-like larvæ which pass through four moults in about 4 to 6 weeks before becoming mature.

P. argentipes—the "silver-footed sandfly" is strongly suspected of being the vector of the parasites of Kala-azar. The Kala-azar Commission in India have traced the development of Leishmania to the thorax of the sandfly, but have not as yet observed the life cycle further and the organisms have not been found in the salivary glands.

Tabanidæ

The Diptera are divided into *Nematocera* and *Brachycera*, the latter having, among other differences, antennæ of dissimilar segments includes the family Tabanidæ or "gadflies" which have 3 segments in the antennæ. These flies are easily recognised and the genus *Chrysops* has been proved to be the transmitter of Tularæmia in Central America and the intermediate host of Filaria *Loa loa* in West Africa. The Tabanidæ also transmit "Surra" to animals.

Muscidæ

This family includes the housefly, blue bottles, etc., and the blood-sucking flies, which include the genus *Glossina* or tsetse fly, Stomoxys or stable fly and the Cleg (Hæmatopota).

Tsetse flies are narrow-bodied insects from 8 to 13 mm. in length with a short thick, rigid, proboscis enclosed by the palpi. They have large brown wings which overlap on the back when the fly is at rest. In the genus Glossina the fourth longitudinal vein is distinct. These flies are widely distributed in Africa and also occur in South West Arabia, and both sexes suck the blood of man and animals. *G. palpalis* and *G. morsitans* are known to be the carriers of *Trypanosoma gambiense* and *T. rhodesiense* respectively.

Tsetse flies are viviparous and the fully grown larvæ are deposited by the fly in debris, loose sand, etc., one at a time at the rate of about one every 3 weeks. The larval skin hardens at once to become the pupa case and the pupa stage lasts from 3 to 9 weeks.

Musca domestica.—The common housefly, which is well known, is the carrier of many pathogenic organisms, such as the

amœbæ, the eggs of worms, the organisms of cholera, enteric and dysentery, etc. It is also the intermediary host of a nematode worm, the definitive host of which is the horse. In the case of diseases of man the fly is a mechanical carrier, as organisms are carried on the external surface of the legs and body and also regurgitated and excreted during feeding.

The eggs are laid in bundles of 120, are white, shiny and sticky, and hatch out in 1 to 4 days into maggots which feed on debris for about 5 days when they mature. The larvæ then burrow into the ground, become barrel-shaped pupæ and remain at any depth down to 10 feet for about 5 days when the pupa emerges as a fly.

The lesser housefly, *Fannia canicularis,* and the latrine fly, *F. scalaris,* resemble the common housefly but are smaller and usually infest latrines.

Lice.

Lice belong to the class Insecta, order Anoplura. They are small flattened insects without wings, have a segmented thorax and 6 to 9 abdominal segments. They have mouth parts adapted for sucking. They lay their eggs on the bodies and clothes (especially the seams) of their hosts. There are three species which are parasitic on men, *viz., P. capitis,* the head louse, *P. corporis,* the body-louse, and *Phthirus pubis,* the crab louse. The latter is easily recognised from the two former by having a much broader and flatter body. Lice are the transmitters or intermediary hosts in relapsing fever, typhus fever and trench fever. Lice of the genus Pediculus can survive about 6 weeks when attached to the host. Each louse lays upto 12 eggs daily and these "nits" hatch out in from 1 to 3 weeks, the young lice resembling the adults and passing through 3 moults in the next 14 days. During this period the young lice suck blood. Lice can live without food for about 10 days. The life cycle of Phthirus occupies a minimum of 27 days.

Bugs.

Bugs belong to the order Hemiptera. No disease has definitely been traced to bugs but the herpetomonad stage of the parasite of Kala-azar develops, though it does not multiply, in the bed bug, and from time to time other diseases such as leprosy, tubercle, relapsing fever and plague are suspected of being transmitted by bugs.

Cimex lectularius is the bed bug of northern latitudes and *C. rotundatus* that of the Tropical zone. The adults can live

for some nine months without food, can hibernate in cold weather but are killed off if the temperature rises above 100°F.

In the adult cimex the wings are rudimentary, palps are absent and the proboscis is bent back under the head. There are 3 distinct abdominal segments and the abdomen is flattened.

The eggs are laid in cracks, etc., in batches of 20 or more at irregular intervals. The eggs are oval, have an operculum at the upper end and hatch out in a few days (upto 9 days). The larvæ emerge and feed at once. After 4 days they moult, becoming nymphs, and 4 subsequent moults occur before the adult stage is completed.

Fleas.

Fleas belong to the order Siphonaptera which is subdivided into three families:—

(*a*) Sarcopsyllidæ or Chigger or Jigger fleas,

(*c*) Pulicidæ or true fleas,

(*c*) Ceratopsyllidæ or bat fleas.

Fleas are small wingless insects with a laterally compressed body. Their mouth parts are adapted for piercing and sucking, The body is heavily chitinized and the thoracic segments are distinct and independent. Some fleas will feed on any warm-blooded animal, but the majority restrict themselves to one definite host. Fleas are active ectoparasites of mammals and birds.

General Structures.—(*a*) *The head* is unsegmented and roughly conical in shape, and has a tubercle in its upper part projecting backwards. In some fleas the head has stout heavily pigmented spines (or comb) on its lower edge pointing downwards and backwards. Combs are sometimes found on the thorax and abdomen. Eyes may be present or absent. They are simple, *i.e.*, non-facetted. Above and behind the eyes are antennæ which when at rest in head lie deeply in grooves. The antennæ consist of three joints; the third terminal segment is the largest in size, oval in shape and is more or less divided into nine rings.

(*b*) *The thorax* consists of three segments which are quite distinct. Each segment consists of an upper portion (tergum) and two latero-ventral pieces (sterna). The tergum of the first thoracic segment is called the protergum; of the second segment the meso-tergum and of the last thoracic segment the

metatergum. Similarly the ventral pieces are called mesosternum, protosternum, metasternum. There are three pairs of legs, each terminating in five tarsal segments.

(*c*) *The abdomen* consists of 10 segments of which the last three form the sexual organs. In the tergum of 9th segment there is a pitted sensory plate known as the *pygidium.*

Bionomics and Life History.—*Eggs.*—Minute oval bodies, pearly white, just visible to the naked eye. The eggs are dropped in batches of 12 or more by the female on the ground and hatch in 2 to 4 days.

Larva.—A minute footless maggot of a whitish colour. It moves actively in sand or dust. It has 13 segments including the head. The larva is thinly but regularly covered with hairs. The larva lives on organic matter, *e.g.*, fæces of fleas contained in the dust in which it lives. The duration of larval life is about 10 to 14 days (longer in cold). During development the larva moults twice and when fully grown it spins a cocoon by means of the silk glands in its head. In this it pupates.

Pupa.—The cocoon is generally covered with small gritty particles. The pupal state lasts a fortnight. The pupa does not feed.

Length of life.—The average life of an adult flea varies from 6 to 10 days. The average duration of life is much greater in winter and at very low temperatures they may live for 2 months.

Classification.—The majority of rat-fleas belong to the family *Pulicidæ.* The following key to the identification of the different genera of this family is copied *verbatim* from that compiled by Sinton:—

KEY TO THE GENERA OF PULICIDÆ FOUND ON RATS

Section I.—Club of antenna distinctly segmented only on the hind side. ("hind" side when lying in the groove).

A.—No comb on head and thorax.

1. The internal incrassation, which extends from the insertion of the mid coxa into the thorax, joins the anterior edge of the mesosternite . . *Pulex.*

2. This incrassation joins the upper edge of the mesosternite . . . . . *Xenopsylla.*

B.—With a comb on the pronotum only . . . *Hoplopsyllus.*

C.—With a comb on the pronotum and at the lower edge of the head . . . . . *Ctenocephalus.*

Section II.—Club of antenna distinctly segmented all round.

A.—Eye developed.

1. No comb on head.

(*a*) Pygidium not projecting backwards; frons with tubercle . . . . . *Ceratophyllus*

(*b*) Pygidium strongly convex, projecting backwards; frons without tubercle . . . . . *Pygiophylla.*

2. Two spines at angle of gena . . . . *Chiastopsylla.*

B.—Eye vestigial or absent.

1. Abdomen without comb.

(*a*) Hind edge of tibiæ with about eight short and several long bristles, which do not form a comb:

(*i*) Fifth segment in fore and mid tarsi with five and in hind tarsus with four lateral bristles *Neopsylla.*

(*ii*) Fifth segment in fore and mid tarsi with four, and in hind tarsus with three lateral bristles, there being an additional pair of bristles in all the tarsi on the ventral surface in between the first pair . . . . . *Ctenophthalmus.*

(*b*) Hind edge of tibiæ with about twelve short and three long bristles, the short ones forming a kind of comb . . . . . *Leptopsylla.*

2.—Abdomen with at least one comb . . . *Hystrichopsylla.*

In view of the recent work on the transmission of plague, proving the superior carrying power of *cheopis* as compared with *astia*, the identification of the different species of the genus *Xynopsylla* is important. Over 25 species are recorded but only three, namely. *X. cheopis, X. astia* and *X. brasiliensis* have been found in India. The following key is copied verbatim from that compiled by Sinton.

KEY TO THE SPECIES OF XENOPSYLLA FOUND IN INDIAN RATS

I. Males.

1. With antepygidial bristle on a long pedestal, which projects beyond the apex of the VIIth segment.—*brasiliensis.*

2. With antepygidial bristle on a short pedestal, which does not project beyond the apex of the VIIth segment:

(*a*) IXth sternite club-shaped . . . . . *cheopis.*

(*b*) IXth sternite ribbon-shaped . . . . . *astia.*

II.—Females.

1. The "head" of the receptaculum seminis very much wider than the "tail." . . . . . *brasiliensis.*

2. The "tail" near the constriction is wider than the head:

(*a*) With the "tail" near the constriction, very much wider than the "head," the "tail" comparatively short . . . . . *astia.*

(*b*) With the "tail" near the constriction, distinctly wider than the "head," the tail comparatively long *cheopis.*

Identification and Preservation.—The best method for clearing the specimen is that elaborated by Cragg:—

(1) Place insects in a 10 per cent. aqueous solution of caustic potash overnight.

(2) Transfer the fleas to clean water for two hours, the water being changed at the end of the first hour.

(3) Pass the flea through 50 per cent. and 70 per cent. alcohol into absolute alcohol, allowing about 30 minutes in each of the first two and about 1 hour in the last.

(4) Place in xylol for about an hour or until they become transparent.

(5) Place in a thin solution of Canada balsam in xylol and allow to remain overnight.

(6) "However prepared, when dealing with a large number of fleas, mount them on a slide covered with a thin layer of balsam. Pick up the fleas one by one with a forceps or a platinum loop and arrange them similarly oriented throughout, on the slide in regular rows of ten—five such rows to a slide of ordinary size. Moisten a cover-slip with xylol and lower it gently on to the slide."

Fleas to be sent for identification are best preserved in 70 per cent. alcohol.

Tunga penetrans (*Dermatophylus penetrans;* Chigger) belongs to the family Sarcopsyllidæ. The pregnant female burrows into the skin of man and causes an inflammatory condition of the finger and toe-nails, hands and soles of the feet.

Mosquitoes

The four human diseases which have now been definitely proved to be carried by mosquitoes are Malaria, Yellow Fever, Filariasis, and Dengue Fever.

General Description.—Mosquitoes belong to the order Diptera, the true flies which have only two wings, of the class Insecta of the phylum Arthropoda. The body of the mosquito is divided into three parts—head, thorax, and abdomen. The head is almost entirely composed of two large compound eyes, and bears also the antennæ or feelers and two appendages at the base of the antennæ, known as the palpi, and a long prominent proboscis or beak. The thorax bears the two wings and behind these the two vestigial wings which appear as two knobs at the base of the wings and are known as halteres or balancers. The ventral aspect of the mesothorax bears three pairs of legs. The halteres are characteristic of the true flies. The abdomen is composed of ten segments and bears no appendages except the inconspicuous sexual apparatus at the tip.

Mosquitoes may be distinguished from other two-winged flies by the structure of their wings. The chief points to notice are that (1) towards the apex of the wing there are two bifurcated veins separated from one another by a single simple vein, and (2) all the veins are covered with scales. Male mosquitoes have brushy or feathery antennæ, while the antennæ of the females are not feathery. Some gnats or midges have feathery antennæ, but none has the long proboscis of the male mosquito.

Life history of mosquitoes.—The mosquito passes through four stages during the course of its development (the first three of which are passed in water), namely those of (1) the

egg, (2) larva, (3) pupa, and (4) adult. The eggs are laid by the female mosquito on water, or in moist sand, or at the edge of water. In some cases eggs are laid in dry excavations and hatch as soon as the rain creates pools. The eggs are elongate, blackish, about 1 mm. or less in length. Mosquito eggs are laid either singly (Anopheles) or in cigar-shaped bundles like tiny rafts (Culicines). The eggs of the Anopheles differ from those of other mosquitoes in having special lateral air chambers which help them to float. They hatch out into larvæ in 2 to 4 days, depending on the temperature. The larval stage usually lasts from 5 to 10 days or more, varying with the temperature: high temperature causing rapid development. The larval period is one of growth, during which the skin is cast four times after which the larva becomes a pupa. The pupa stage covers normally from 48 to 72 hours, varying with the temperature. The duration of the adult stage is very variable, some species hibernating through the winter. Usually however, the ordinary life period of the mosquito in midsummer is not more than 3 or 4 weeks. Many species of mosquitoes apparently require a blood meal before their eggs can mature; this necessity causes them to become pests and spreaders of disease. The male mosquitoes do not bite, but live on nectar, fruit, and the juices of plants.

Such, in outline, is the general life-history of all mosquitoes, but, as might naturally be expected, there is a great deal of variation in detail both in the structure and the habits of these insects.

Anopheline Mosquitoes.—The Zoological position of mosquitoes is graphically illustrated as follows:—

Phylum—Insecta.

Order—Diptera.

Sub-order—Nematocera.

Family—Culicidæ.

Sub-Family—Culicinæ.

Tribe Anophelini. Tribe Culicini.

The Anophelini are divided by Christophers into a single genus, *Anopheles,* and five *sub-genera,* Anopheles, Myzomyia,

Nyssorhynchus, Chagasia and Bironella. (Mosquitoes of the first three sub-genera, Anopheles, Myzomyia, and Nyssorhynchus occur in India).

This classification is based on the structure of the male genitalia of the Anopheline mosquitoes. The recorded anopheline species number between 130 to 140 but only a few of them take part in the transmission of malaria.

Within the tribe Culicini there are thirty-one genera, an equal or larger number of sub-genera, and a very large number of species and varieties of these species. The recorded culicine species number between 1500 to 2000.

The principal distinguishing features between the Anophelini and the Culicini are the following:—

Anophelini.	Culicini.
(1) *Adult*—	
Male palpi long; clubbed at tip.	Male palpi long or short; if long slender at tip.
Female palpi as long as proboscis.	Female palpi much shorter than proboscis.
Scutellum uniformly rounded.	Scutellum trilobed posteriorly.
(2) *Pupa*—	
Breathing trumpets short and scoop-shaped, split down the front.	Breathing trumpets conical or tubular, not split down the front.
(3) *Larvæ*—	
Surface feeder, lying horizontally in the water just below the surface film.	Sub-surface feeder, lying vertically or obliquely in the water well below the surface film.
Air-tube absent, represented by five small flaps surrounding the tracheal opening.	Air-tube present, usually long and well developed.
(4) *Egg*—	
Laid singly, more or less boat-shaped, with lateral air floats.	Laid singly or in rafts, elongate ellipsoidal to elongate, conical. No air floats.

The Anopheles Larvæ.—The identification of the Anopheline larvæ is a matter for experts but with little practice the commoner species can be identified nearly as readily as the adults. In case of doubt the best way is to rear them out into adults and then identify them. The distinguishing characters are the form of various hairs or modified hairs borne on the head and the body of the larva. The most important groups of these hairs are as follows:—

1. Clypeal hairs:—These are three pairs of hairs situated at the anterior end of the dorsal surface of the head. The two anterior pairs (inner and outer clypeal hairs) are inserted near the edge of the head and project anteriorly. Both pairs are readily seen in living larvæ (with the aid of an ordinary lens) at times when the mouth brushes are retracted and the head turned dorsal side up. The posterior clypeal hairs are inserted further back on the head and are often very small and difficult to see.

2. Lateral hairs of abdominal segments:—In all anopheline larvæ the abdominal segments bear long or short, feathered, or split lateral hairs. Their structure and branching are of specific importance.

3. Palmate hairs:—These are characteristic fan-like tufts which occur in pairs on the posterior quadrant of the thorax and on the first seven abdominal segments. The presence or absence of these, and the structure of the leaflets are used in the classification of the various species of the Anophelines.

The distribution of the Anopheline Mosquitoes.—There is at present very little known about the various factors which determine the distribution of the Anopheline fauna of the various parts of the world, but it is possible to point to certain special factors such as humidity, altitude and temperature, deforestation, etc., which do play an important part in the distribution of the Anophelines. Covell has recently made a valuable contribution to the literature on this subject (*vide* Ind. Jour. Med. Res. Memoir No. 5, Feb, 1927) and has grouped the different anophelines of India and Ceylon according to their zoo-geographical distribution. Although such attempts have also been made in other parts of the world as yet the various factors delimiting the species distribution are far from being perfectly understood.

The following is a list of the important species of the Anophelines in which infection in nature has been found:—

India and Ceylon

A. culicifacies.
A. listoni.
A. stephensi.
A. ludlowi.
A. willmori.
A. maculatus.
A. umbrosus.
A. minimus.
A. turkhudi.
A. sinensis.
A. maculipalpis.
A. pulcherrimus.
A. barbirostris.

Europe

A. maculipennis.
A. sinensis.
A. superpictus.
A. hispaniola.
A. bifurcatus.
A. plumbeus.

Africa

A. maculipennis.
A. umbrosus.
A. mauritianus.
A. culicifacies.
A. funestus.
A. turkhudi.
A. costalis.
A. maculipalpis.
A. pharœnis.

The New World (U. S. A.)

A. quadrimaculatus.
A. cruciens.
A. punctipennis.
A. pseudopunctipennis.
A. albimanus.
A. argyrotarsis.
A. tarsimaculatus.
A. intermedius.

Australia

A. bancrofti.
A. annulipes.
A. tesselatus.

The synoptic table for the classification of Indian female anophelines is given in the Indian Hygiene and Public Health by the Authors.

Dissection of mosquitoes.—This is imperative in the practical investigation of malaria, in order to determine the

carrier species which is responsible for the transmission of malaria.

There are two sets of dissections required for this purpose—A.—dissecting the stomach to find the Zygotes and Oöcysts and B.—the dissecting out of the salivary glands to find Sporozoites.

With little practice it is an easy matter to find out these structures. The internal anatomy of the adult insects is comparatively simple and as the whole of the alimentary system as far forward as the posterior half of the midgut (stomach) together with all the organs of reproduction may be easily removed in one operation, this may be considered the chief dissection. The only other dissection necessary is the removal of the salivary glands. If it is remembered that the salivary glands are intra-thoracic organs lying over the first pair of legs, there is very little chance to miss them.

A.—*To dissect out the Mid-Gut (stomach)*.—1. Obtain a number of female anophelines from infected areas. Keep them in a mosquito cage in a dark place under proper conditions of temperature and humidity for two or three days until no blood remains in the mid-gut.

2. Pull off, with forceps, the legs and wings.

3. Place the mosquito on its back in a drop of salt solution on a slide and with a needle make a nick as near the tail as possible. Now place one needle on the thorax to fix it and pull gently with the lower needle to draw out the internal structures.

4. Remove the malpighian tubules, ovaries, etc., by cutting across their attachments to the alimentary canal.

5. Mount in a drop of saline and examine under the microscope for Zygotes and Oöcysts.

B.—*To dissect out the Salivary Glands*.—1. Place the mosquito in a drop of salt solution on its side with the head pointing towards you.

2. Place the left hand needle on the thorax to steady it, and place the right hand needle on the back of the head and make steady gentle traction.

3. If done carefully, it will be seen that the head has pulled out a little mass of white tissue from the thorax.

4. Examine this white tissue under a lens; the glands appear as finger-like, transparent, glistening bodies.

5. Break the attachment from the head, clear the extraneous matter from the slide and the six glands will come out with teasing.

6. Crush the gland under a cover-slip and examine it under a microscope for sporozoites. To make certain, stain it with Leishman's or Giemsa's stain in the usual way, when the sporozoites appear as sickle-shaped bodies with blue protoplasm and red chromatin.

Culicine Mosquitoes.—Comparatively few culicines are known to be disease carriers. The numerous genera of the tribe Culicini can be considered as forming a number of groups, characterised particularly by the larval structure and habitat. The following tabulation gives very briefly the characteristics of these groups, which the medical man is most likely to encounter in mosquito survey work:—

1. *The Culicine Group.*—The adult females in this group usually have a broad and flattened abdomen, rather abruptly truncate at the tip, with no cerci or ovipositors visible (the cerci are the two organs projecting from the tip of the last abdominal segment). The larvæ usually live in permanent collections of water. The eggs are in rafts and the larvæ usually have four or more pairs of hair tufts on the air-tube.

2. *Aedine Group.*—The adult females in this group usually have abdomens which taper towards the tip and have two short visible cerci. The eggs are laid singly and larvæ have only one pair of hair tufts on the air-tube. The larvæ usually live in temporary collections of water.

3. *Sabethine Group.*—None of these are of medical importance. They are brilliantly coloured jungle mosquitoes, whose larvæ live in water held by plants, tree holes, leaf-bases of epiphytic bromeliads (palm trees), etc. The larvæ lack the ventral brush on the last segment of the abdomen which is found in all other mosquito larvæ.

4. *Megarhinine Group.*—Mosquitoes whose larvæ live in tree holes and have no pecten (basal row of spines) on the air-tube. These are predaceous larvæ and feed on other mosquito larvæ.

The Culicine mosquitoes, most important from a medical point of view, are the domestic species which breed mainly in artificial water-containers in and about houses.

Short Diagnostic Points to Distinguish the Commoner Culicines.—*Culex pipiens*, the house mosquito of the temperate regions, is a small brownish species known to carry Filariasis in Asia. Proboscis and tarsi are without pale bands. Femora and tibiæ are not longitudinally striped. Abdominal segments have complete basal bands. These resemble Culex fatigans but can be distinguished from it by the colour of the mesonotal scales and the length of the upper forked cell.

Culex fatigans.—This is a nocturnal species which is active only during the evening and night. It is the house mosquito of the Tropics and is a fairly large mosquito of a tawny brown colour. The proboscis and the legs are unbanded, and there is no conspicuous ornamentation on any part of its body. It carries Filariasis and perhaps Dengue.

Culex vishnui is a very common domestic mosquito of India and is a small mosquito, brownish in colour. There is a band on the proboscis and thin white bands on the legs.

Culex bitæniorhynchus is a commoner culicine frequently met with in the Tropics. It has speckled wings, a banded proboscis, and the tarsi are banded (basal and apical extending over the joints).

Culex mimeticus.—The wings are spotted like Anopheles. It resembles *A. culicifacies*, from which it can be distinguished by its conspicuously banded tarsi, scaly cross-banded abdomen, and the short female palpi.

Aedes (*Stegomyia*) *ægypti* (*Stegomyia fasciata*).—The yellow fever mosquito. This mosquito is easily recognised by its black and white colouration, and by the fact that the dorsal surface of the thorax is ornamented by four longitudinal white lines. The proboscis is dark, the legs are black but are conspicuously banded with white. The female abdomen is barrel-shaped with pointed cerci and the female carries Yellow Fever and Dengue.

CHAPTER XII

THE BACTERIOLOGICAL EXAMINATION OF WATER, AIR AND SOIL

WATER

Introduction.—The bacteriological examination of water affords valuable indications as to its fitness for human consumption. Organisms are abundant in water and their number depends upon the source of a given sample. The bacterial flora of water may be conveniently divided into four classes:—

1. *Natural Water Bacteria.*—These include the motile bacilli which are divided into liquefiers and non-liquefiers of gelatin, chromogenic bacteria and iron bacteria, namely *Crenothrix polyspora.* Water, free from bacteria, is not found in nature. The water of wells and rivers usually contain natural water bacteria.

2. *Soil Bacteria.*—These die out rapidly in fairly clean water. These are abundantly found in lakes or rivers which have been flooded. The *B. mycoides, B. megatherium, B. subtilis* and some of the thread bacteria, notably *Cladothrix dichotoma,* are fairly common in water.

3. *Intestinal Bacteria.*—Water supplies are likely to be polluted by human and animal excreta, *e.g.,* by cattle grazing in the vicinity of a river or lake or a cesspool leaking into a shallow well, and from the hygienic point of view the waters of such contaminated wells are not fit for drinking. These organisms are not pathogenic in themselves but their presence is an indication of the pollution of a water supply by animal excreta. These are:—(1) The Coliform group, (2) Spore bearing anærobic bacilli, *e.g., B. enteritidis sporogenes* and *B. welchii* and (3) Streptococci, notably *Str. fæcalis.*

In routine practice *B. coli* alone is taken as an evidence of excretal pollution.

4. *Pathogenic Bacteria.*—These bacteria are also of intestinal origin and their detection in water is frequently of the highest importance.

These are the *B. typhosus, B. paratyphosus, V. choleræ* and *B. dysenteriæ.*

The bacteriological examination of water may therefore be divided into:—

1. *The Enumeration of the Organisms Present*:— Quantitative Examination which includes: A.—Enumeration of bacteria developing at 37°C.; B.—Enumeration of bacteria developing at 20°C.

2. *The Determination of the chief species present*:— Qualitative examination which includes:—

A.—The detection of bacteria of excretal origin (excretal indicators), namely, (*i*) *B. coli* and (*ii*) *Streptococci*, (*iii*) *B. welchii.*

B.—The detection of pathogenic bacteria such as (*i*) *B. typhosus*, (*ii*) *B. paratyphosus*, (*iii*) *V. choleræ* and (*iv*) *B. dysenteriæ.*

N.B.—In routine practice the enumeration of organisms developing on agar at 37°C. and on gelatin at 20°C. and examination for *B. coli* only are carried out.

Collection and Transmission of Samples of Water.—(*a*) *Collection.*—The most suitable vessels for the collection of water sample are small sterilised, ground glass stoppered bottles with a capacity of 250 c.c. (8 oz.) each.

These bottles as a rule are sterilised in the hot-air steriliser. The stopper is inserted with a small piece of paper placed between it and the neck of the bottle, otherwise it will be difficult to remove it after sterilization. The stopper is also covered with paper before sterilization which is removed only just before taking the sample. Failing sterilization by hot-air, the bottle may be boiled for full half an hour in a covered vessel, allowed to cool, the stopper replaced and the bottle used at once for sampling.

The methods employed for the collection of water samples vary and depend upon the place of its collection. Contamination of the water with extraneous bacteria must be avoided and the fingers should not come in contact with the glass stopper or the neck of the bottle while filling it. The bottle should not be quite filled.

(1) If the sample is to be obtained from a *tap* the water is allowed to run to waste for 5 to 10 minutes before the sample is taken.

(2) If the sample is to be obtained from a *stream, tank* or *reservoir,* the unopened bottle is held in the water about a foot

from the surface and away from the edge against the direction of the flow in streams. The stopper is removed with forceps, the bottle withdrawn when full, a few drops of water poured out and the stopper replaced and tied down. The river bed must not be disturbed as the vegetable matter covering it contains many organisms.

(3) If the water is obtained from a *well,* a weighted sample bottle is used. Several special bottles, *e.g.,* Esmarch's, have been devised for this purpose. An inexpensive substitute for these can be made by tying short lengths of string to the neck and the stopper of an ordinary bottle. The bottle is weighted with lead attached to the bottom by means of wires passing round the neck. The whole apparatus is wrapped in paper and sterilised in hot-air sterilizer. To take the sample the bottle is lowered into the well by the string attached to the neck and when it has reached the required depth, the stopper is jerked out by means of the other string.

(*b*) *Transmission.*—The examination of the sample must be commenced immediately, for if allowed to stand for any time a great multiplication of bacteria may take place and the results of the analysis may be altogether fallacious. It is therefore preferable to make the dilutions and to inoculate the media at the spot at which the sample is obtained. It is however usually impossible to carry out these preliminary steps on the spot in India where bacteriological equipment is available in only a few of the larger towns and the sample has to be sent to a distance. In order therefore to prevent the multiplication of the organisms in water during transit the bottle containing the sample is packed in an ice box and despatched to the laboratory with as little delay as possible. Before despatching, the ice used up for initial cooling of the sample should be poured out and replaced by more ice.

(*c*) *Particulars to be supplied with each sample sent to the Laboratory.*—

1. Authority and reasons for sending and name of collector.
2. Source of water, whether from spring, well or standpost.
3. Any possible or obvious sources of pollution.
4. If there is chlorination or other system of purification in use.
5. Date and hour of collection and despatch.

6. Serial number or mark for identification.

7. Other remarks.

Methods of Examination.

I. Quantitative

Apparatus and Reagents required:—

(1) Case of sterile Petri dish;

(2) Case of sterile pipettes, 1 c.c.;

(3) Case of sterile pipettes, 10 c.c.;

(4) Tubes of nutrient gelatin;

(5) Tubes of nutrient agar;

(6) A flask of distilled water;

(7) Water-bath;

(8) Sterile test-tube;

(9) Grease pencil;

(10) Bunsen burner;

(11) Water-bath regulated at 44°C.

A.—Method of enumeration of organisms capable of growing at 37°C.—It is usually necessary to work with a fraction of a c.c. because the number of organisms present in a c.c. is so large as to make an accurate count of the colonies impossible. It is, however, not possible to make accurate measurements of quantities less than 1|10 of a c.c. with the ordinary pipette. To attain this end, preliminary dilutions of the sample of water should be made.

(*a*) *Dilution of the water sample.*—1. Sterile test-tubes plugged with cotton-wool are placed on a rack and marked A, B, C, (and D, E, F, in case the sample is likely to be polluted, *e.g.*, sewage).

2. To each of these tubes 9 c.c. of sterile distilled water are added.

3. The sample bottle is well shaken so as to mix the sample of water.

4. With a sterile pipette the following quantities are added and mixed to the tubes:—

A. 1 c.c. of sample (1 c.c. of A=0·1 c.c. of the sample).

B. 1 c.c. of A. (1 c.c. of B=0·01 c.c. of the sample).
C. 1 c.c. of B. (1 c.c. of C=0·001 c.c. of the sample).
D. 1 c.c. of C. (1 c.c. of D=0·0001 c.c. of the sample).
E. 1 c.c. of D. (1 c.c. of E=0·00001 c.c. of the sample).
F. 1 c.c. of E. (1 c.c. of F=0·000001 c.c. of the sample).

and so on. If the water is comparatively clean two dilutions are sufficient, namely, 1 in 10 and 1 in 100. If, however, it is very much polluted, it is necessary to proceed to further dilutions, *e.g.*, 1 in 1,000,000 or even more.

(*b*) *Plating.*—1. Agar tubes are consecutively marked *a*, *b*, *c*, *d*, *e* and *f* according to the dilutions prepared. The medium is standardised to +10 Eyre's scale, the plugs flamed and rotated to see that they are not adherent to the lips of the tubes.

2. The tubes of nutrient agar are melted in boiling water, cooled to 44°C. and kept at that temperature in a water-bath. The lids of the Petri dishes are labelled with (*a*) the date, (*b*) the identification number and (*c*) letters A, B, C, etc., indicating the particular dilutions of water plated in each Petri dish.

3. 1 c.c. of the diluted water in tube A is added to agar tube *a*. Then the agar tube is rotated on its vertical axis between the opposed palms by rapid backward and forward movements of the two hands so as to thoroughly mix the sample. The agar is poured into Petri dish A.

4. 1 c.c. of the water in B is added to agar tube *b*, and shaken and poured as above to plate B.

5. In a similar manner 1 c.c. of the water from C is added to the contents of agar tubes *c* and plated, and so on.

6. As soon as the agar has solidified, the plates are placed in the incubator at 37°C. with the free surface of the agar facing downwards. By thus inverting the plates the surface of the medium is kept free of the water of condensation and the running together of the colonies is prevented.

(*c*) *Method of counting.*—The plates are examined and colonies counted after 24 and 48 hours of incubation. Only those plates the colonies of which are not crowded together and can be conveniently counted are selected. Colonies may be counted by means of either Jeffer's or Pake's counting disc. These are however seldom necessary and a convenient method of counting is to mark off the glass surface of the agar-containing half of the Petri dish into segments with a grease pencil. If possible the entire plate should be counted. If the colonies

are very numerous, *i.e.*, more than 500 per plate, half or even quarter of the plate may be counted and from these figures the number of the colonies on the entire plate can be estimated. After counting the total number of colonies the result should be expressed as the number of colonies per c.c. of original water. For example, if 100 colonies were counted on the agar plate A (*i.e.*, plate containing 0·1 c.c. of the original water) the number of organisms would be $100 \times 10 = 1000$ per c.c. of the water sample.

B.—Enumeration of the organisms developing at 20°C.— The details of the method are essentially the same as those for counting the number of organisms developing at 37°C. The method is therefore only briefly described here. Tubes of nutrient gelatin are melted and cooled to 30°C. 1 c.c. of each dilution of the water is mixed with each tube of melted gelatin in the same way as to the agar. The gelatin is poured into the Petri dishes and allowed to set. The plates are labelled according to the dilutions and incubated at 20°C. with the free surface of the gelatin facing upwards. The colonies are counted and the results expressed as before. Gelatin is unsuitable in the hot weather in India and agar may be used instead and incubated at 20°C.

II.—Qualitative

A.—Detection and isolation of bacteria of excretal origin.—(*i*) *The detection, enumeration and isolation of B. coli group.*—The *B. coli* is the most convenient bacterial indicator of pollution of water and the methods employed aim at determining the smallest quantity of water which shows the presence of *B. coli.*

Process.—1. 0·5 c.c., 1 c.c., 5 c.c. and 100 c.c. of the water under investigation are taken.

2. Tubes of single strength MacConkey broth (neutral red-bile-salt—peptone—lactose broth, page 157) and provided with Durham's fermentation tubes are used and labelled with marks 0·5, 1, 5 (corresponding to the amount of the water to be added), the date, and the identification mark of the sample. Ten tubes of double strength MacConkey broth each containing 10 c.c. of the medium, are labelled with mark 10 (indicating the volume of the water added).

0·5, 1 and 5 c.c. of the sample are added to the single strength MacConkey broth tubes marked 0·5, 1 and 5 respectively by means of sterilised pipettes, taking care to avoid contamination.

10 c.c. of the water are added with a 10 c.c. pipette to each of the 10 tubes of double strength MacConkey broth.

An alternative method is to add 50 c.c. of the water to a tube containing 50 c.c. of double strength MacConkey and 100 c.c. to 50 c.c. of triple strength MacConkey broth.

N.B.—The double and triple strength MacConkey broth are used with large quantities of water so that the ultimate strength of the medium after the addition of sample does not change to less than the standard strength of MacConkey. In practice it is only necessary to inoculate limited quantities of water.

Afterwards the tubes are incubated at 37°C. and examined on the 2nd and 3rd day, (*i.e.*, 24 and 48 hours after inoculation). The development of acid and gas in the tube is usually regarded as the *presumptive* evidence of the presence of organisms of *B. coli* group.

Example.—Suppose that acid and gas are found in five tubes of 10 c.c., acid only in case of another tubes of 10 c.c. and no change in the rest of the four 10 c.c. tubes or those marked 5, 1 and 0·5 c.c. Coli are presumed to be present in 50 c.c., doubtful in 60 c.c. and absent in 40 c.c.

In order to investigate further the bacteria giving rise to the changes in broth, on the third day a drop is taken on a platinum loop from the last tube showing acid and gas (in this case one of the 10 c.c. tubes) and mixed with sterile broth. A loopful from the broth is plated on the surface of neutral red-bile salt-peptone-lactose-agar. The above process is repeated with the MacConkey tube showing only acid and no gas (the 10 c.c. tube). This is done in case *B. coli* is really present but have had not enough time to give gas as well as acid. The plate is incubated for 24 hours at 37°C. The plates are examined next day and it is noted from which dilution of the original water red *B. coli* colonies appear on the plate. One individual coliform colony is picked off from the plate made from the highest dilution of original water (10 c.c.); if none are available, then from the lower dilution, and subcultured on agar slopes. These slopes are then used for *confirmatory tests.* It is sometimes necessary to differentiate non-fæcal strains of the *B. coli ærogenes* group from the fæcal strains which alone indicate excretal pollution. For this purpose various tests have been recommended. According to Houston a typical *B. coli* produces flourescence (*fl*) in neutral red broth, acid and gas (*ag*) in lactose, indol (*in*) in petone water, acid and clot (*ac*) in litmus milk—the so called "flaginac" reaction.

The agar slope growth or the colonies on the MacConkey plates are emulsified in a drop of sterile water in the Houston's set which consists of five dwarf test-tubes carried in one large tube, only the latter being provided with a cotton-wool plug. From this emulsion each of the following 4 tubes in the set are inoculated:—

1. Neutral red broth (*fl*).
2. Lactose peptone water (*ag*).
3. Peptone water, (*in*).
4. Litmus milk (*ac*).

There are two other tests which may be employed in distinguishing fæcal from non-fæcal strains of organisms of *B. coli ærogenes* group. These are 1. Voges-Proskauer test (*q.v.*) and 2. The Methyl-red reaction.

These two tests serve to separate the lactose fermenting gas-producing bacilli into two types. The predominant type of animal and human excreta gives a positive methyl-red reaction (MR+) and a negative Voges-Proskauer (VP—). On the other hand the MR— and VP+ is rare in human and animal intestine, and is more frequently found in surface water, milk etc.

(*ii*) *The isolation and enumeration of Streptococci.*—Streptococci are abundant in water contaminated with sewage and animal excreta and are extremely rare in pure water, hence the value of their detection. The streptococcus most commonly found in sewage corresponds to the characters of *Str. fæcalis* which produces acid and clot in milk, reduces neutral red, and ferments lactose, saccharose, mannite and salicin. One c.c. of the various dilutions of the sample of water is added to tubes of glucose broth containing an indicator. As the medium becomes acid owing to fermentation of glucose by the presence of *B. coli* the streptococci tend to outgrow the *B. coli.* Lactose neutral red agar plates may be inoculated at this stage and tiny intensely red colonies of streptococci may be distinguished from larger colonies of *B. coli.* The organism thus isolated in a pure culture may be further investigated.

(*iii*) *The detection of B. welchii.*—The different dilutions of water are added to tubes of broth and the broth added to sterile milk tubes which are then heated to 80°C. for 10 minutes. At the end of this time the tubes are incubated anærobically at 37°C. for 48 hours. The development of the "Stormy Reaction" indicates the presence of *B. welchii.* This test is not usually carried out in routine examination of water

as the spores are extremely resistant and may have been introduced indirectly.

B.—Examination for Pathogenic Bacteria.—The important disease-producing bacteria conveyed by water are the bacilli of the dysentery and Gärtner group and vibrio of cholera.

1. Examination of—

B. Typhosus, B. Paratyphosus and B. Dysenteriæ.—Various methods have been devised to overcome the difficulty of isolating these organisms from water by either concentrating or enriching these in the sample of water. They do not, however, give very satisfactory results.

(*a*) *Concentration by alum precipitation.*—5 c.c. of a 10 per cent. solution of alum are added to a litre of the sample of water. After the flocculent precipitate of aluminium hydrate has formed, the bottle is shaken to mix the contents and the mixture is allowed to settle and then centrifuged for 15 minutes at 2000 revolutions per minute. Plates of MacConkey's medium are inoculated with the deposit. The suspicious colonies are investigated by the methods previously described.

(*b*) *Enrichment by brilliant green.* To 900 c.c. of the water sample in a stoppered sterile flask are added 100 c.c. of a 10 per cent. sterile solution of peptone, 5 grammes of sodium chloride and 5 c.c. of 1 in 1000 solution of brilliant green in distilled water. The flasks are incubated for 24 hours after which MacConkey plates are inoculated with this water

2. Examination for V. Choleræ from Water.—Peptone water (peptone. 10 grammes, sodium chloride 5 grammes and water 100 c.c.) is prepared, sterilized and made neutral to phenolphthalein, (alkaline to litmus). To 100 c.c. of the sterile peptone water 900 c.c. of the sample of water are added and this is distributed in stoppered sterile Erlenmeyer flasks so that each flask contains only a shallow layer. The flasks are incubated at 37°C. If vibrios grow they are investigated by the procedure described under *V. choleræ.*

Scheme of the Daily Routine Examination of Sample of Water.—

First day.—

1. Prepare dilutions as required.
2. Plate each dilution on agar. Incubate at 37°C.
3. Plate each dilution on gelatin. Incubate at 20°C.

4. Inoculate neutral red-bile salt-peptone-lactose broth with various quantities of water.

Second day.—

1. Record the colonies on agar plates made on the first day and incubated at 37°C.
2. Record the number of colonies on gelatin plates made on the first day and incubated at 20°C.
3. Record the number of fermentation tubes which show acid and gas.

Third day.—

1. Record the colonies on agar plates made on the first day and incubated at 37°C.
2. Record the colonies on gelatin plates made on the first day and incubated at 20°C.
3. Record the number of additional fermentation tubes which show acid and gas.
4. Plate a drop from the last tube, (*i.e.,* highest dilution) showing acid and gas in MacConkey's plate. Incubate at 37°C.

Fourth day.—

Pick up a few coliform colonies (at least two) from the MacConkey plate and transfer each to a lactose broth fermentation tube and an agar slant. Incubate at 37°C.

If no typical colonies are present incubate the plates for another 24 hours.

Fifth day.—

1. Examine lactose fermentation tubes inoculated on previous tests.
2. Examine microscopically agar slopes and make confirmatory tests.
3. If coliform colonies are now found repeat procedure of inoculating lactose broth and agar slant as done on the fourth day.

Sixth day.—

Any confirmatory tests for slow growing colonies.

The Bacteriological Examination of Sewage and Sewage Effluents

The object of the bacteriological examination of sewage is to determine the efficiency of purification works.

Method.—*Dilution.*—The bacterial content of the sewage being very high it is necessary to work with very minute quantities of it. It is diluted 1 in 100,000 or even more.

The subsequent procedure is the same as that detailed above for the bacteriological examination of water.

The Bacteriological Examination of Ice

It is sometimes necessary to examine the bacterial content of ice in order to determine if it is fit for human consumption.

Collection of Sample.—It is generally the water supply of the ice factory which is at fault. The reservoir of water may also be polluted. The sample is best obtained from the reservoir just before the process of refrigeration is started. The method of taking a sample is the same as for water.

Samples of water used for the production of ice may also be obtained.

In taking a sample of ice for examination wash a large piece of ice in several changes of sterile water. Place it in a sterile towel and crush the piece of ice. Small bits of the ice preferably from the interior of the ice block are picked up with a sterile forceps and placed in a test-tube. The ice is allowed to melt and is then examined in exactly the same way as a sample of water. It should however be borne in mind that the bacterial content of ice is not very high as organisms do not multiply in it. The sample is therefore not to be very much diluted but 0·1 c.c., 1 c.c., 10 c.c., 100 c.c. and 200 c.c. of the raw material should be examined.

Interpretation of the Results

It is not possible to express any opinion as to the wholesomeness of a particular sample of water from quantitative analysis alone. It is obvious for instance that a water containing a large number of saprophytes such as *B. subtilis* is less likely to be harmful than a sample containing even a few pathogenic organisms such as *B. typhosus*.

The ideal method would of course be the detection of pathogenic organisms but as the technical difficulties attending their detection are enormous on account of their feeble viability outside the body and success by the direct method is so uncertain, in practice it is customary to rely on the indirect bacteriological evidence derived from the enumeration of the excretal *B. coli* which point to the contamination of water by human and animal excreta. Whilst the presence of *B. coli* is generally taken as evidence of recent pollution, that of *B. welchii* is not necessarily so, owing to the fact that the resistant spores of *B. welchii* survive in water for a long time. With regard to streptococci, it is believed by some workers that their presence indicates recent pollution on account of their feeble viability outside the body. It must be distinctly understood that *B. coli* and fæcal streptococci are not harmful in themselves but their presence in water is only an evidence of the pollution of water-supply with excremental matter. If human excreta is finding its way into the water supply of a town the risk of the water being infected by pathogenic intestinal organisms is enormous and the water of such a town is hardly fit for drinking. Any standard as to the number of bacteria, which may be permissible in a water-supply is, of course, arbitrary and of limited value as each class of water-supply must be considered separately with special reference to the sanitary conditions of its source and its environment.

In addition to the total bacterial count, useful information may be obtained by a comparison of the organisms developing at 37°C. to those developing at 20°C. Organisms which are normal inhabitants of the intestinal canal naturally multiply rapidly at the body temperature, *i.e.*, 37°C., whilst those normally occurring in water thrive better at 20°C. With a pure water, therefore, in which intestinal organisms are scanty, the ratio of the organisms developing at 20°C. to those developing at 37°C. is usually higher than 10: 1 and with a polluted water this ratio (10: 1) is approached, and frequently becomes 10: 2 or 10: 3, or even less.

The Bacteriological Standards Laid Down for Public Water supplies in the United Provinces of Agra and Oudh (India)

The total number of organisms developing on agar plates incubated for 48 hours at 37°C. should not exceed 100 per c.c. in a potable water.

Water from tube wells, deep wells, storage reservoirs and slow sand filters.—B. coli should be absent in 50 c.c. of these waters.

Filtered chlorinated waters.—B. coli should be absent in 100 c.c.

The Bacteriological Examination of Water Filters

The object of undertaking this examination is to ascertain whether or not organisms can pass through a filter. If their passage through a filter is not arrested the filter is quite useless for the purpose for which it is employed.

The filter to be tested is sterilised in a steam steriliser. A sample of water containing culture of *B. prodigiosus* is passed through the filter for 24 hours. This filteration should preferably be carried out at a temperature below 5°C. with a view to preventing the multiplication of the microbe. Samples of the filtrate should be bacteriologically examined immediately after the filtration has begun and at intervals during the day and at the end of 24 hours. The absence of any organism in the filtrate indicates that the filter is working properly.

Microscopical Examination of Sediment in Water

The sediment should be examined under the microscope (⅓ inch lens) after centrifugalizing or allowing it to settle.

The following inanimate and animate matter may be found by a microscopical examination of the sediment in water:—

1. Inanimate.—(*a*) Mineral.—Sand and flint particles, chalk, iron peroxide, mica, etc., Chalk and iron peroxide can be dissolved by running a drop of hydrochloric acid under the cover-glass; the rest are insoluble in hydrochloric acid.

(*b*) Vegetable.—Parenchymatous cells, dotted ducts, spiral vessels or fibres, vegetable hairs, pollen, woody fibres, fragments of leaves, starch cells of wheat, potato, etc.; macerated paper, linen and cotton fibres, soot, etc.

(*c*) Animal.—Hairs, feathers, down, wool or silk fibres; muscular fibres, fat globules, connective tissue, epithelial scales, shreds of mucous membrane; scales from lepidopterous insects. Reddish brown globular masses are sometimes found in water grossly polluted with sewage.

2. Animate.—(*a*) VEGETABLE.—1. Small and microscopic fungi which may be present as spores, sporangia, or mycelia, the familiar instances being Bacterium termo, Sarcina ventriculi, Beggiatoa alba, Leptothrix, Leptomitus lacteus, Sphoerotilus natans, Crenothrix, moulds, etc.

2. *Algæ.*—The common ones are volvox, oscillatoria, confervaceæ, Protococcus pluvialis raphidium, scenedesmus, desmids, diatoms, etc.

(*b*) ANIMAL.—1. *Protozoa.*—

(*i*) *Rhizopoda.*—Amœba, actinophrys, polyps, Spongilla fluviatilis, cercomonas, Euglena viridis, etc.

(*ii*) *Infusoria.*—Paramœcium, vorticella, coleps, stentor, etc.

2. *Cœlenterata,* such as Hydra.

3. *Annulosa.*—

(*i*) Crustacea, such as Daphnia pulex or water fleas.

(*ii*) Arachnida, such as water bears.

(*iii*) Insecta, larval, pupal or adult forms.

4. *Annuloida.*—Rotifera, næmatoidea worms, anguillulæ or water-worms.

5. *Mollusca,* such as polyzoa.

Human Parasites.—Segments or eggs of tape-worms; guinea worm, (Dracunculus medinensis), small leeches (hirundinidæ), Ascaris lumbricoides or round-worms, Oxyuris vermicularis or thread worms, Schistosomum hæmatobium, Ankylostomum doudenale or hook-worms, Tricocephalus dispar or whip-worms, Filaria sanguinis hominis and the cercaria of Distoma hepaticum.

The presence of any of these parasites in water may be accidental, but if continuous it renders the water unfit for drinking as the collecting area would be liable to continuous gross pollution from human excreta.

THE BACTERIOLOGICAL EXAMINATION OF AIR

There are always bacteria in the air in varying quantities and the quantity varies with the amount of dust in the air.

Bacteria are not in a congenial medium in air; they do not multiply in the air but rapidly diminish owing to the action of the sun and they settle on the surface of the ground, etc., by the action of gravity. The air of sewers contains a certain number of sewage bacteria which are found in large numbers if splashing, etc., occurs.

If agar plates are exposed to air for, say, half an hour, a fairly accurate estimation of the bacteria in the air where the plate is exposed is obtained.

Sedgwick-Tucker tubes are used to obtain a more accurate quantitative estimation of the bacteria in a sample of air. The tube consists of a broad and a narrow portion. It is plugged with cotton-wool at both ends and sterilised at 150°C. A portion of the narrow part of the tube extending from near the cotton-wool plug to the end of the constricted portion is filled with sugar and sterilised for 3 hours at 120°C. A rubber tube is fixed to the narrow end of the tube and connected with an aspirator, the cotton-wool plug at the larger end is removed and a known quantity of air drawn through the tube by letting a measured quantity of water out of the aspirator. The cotton-wool plug is flamed and then replaced.

10 c.c. of liquefied gelatin are then poured into the broad end of the tube and the sugar pushed up into it with a sterile rod which just fits the inside of the tube. The sugar is dissolved, the tube is sloped and a slope culture is made. The colonies are then examined after 48 hours.

The sugar used must be coarse granular sugar, quite dry and it must not be heated to too high a temperature or it will darken and cohere.

Frankland's tubes are also designed to catch the bacteria in a sugar filter. After filtration of a known quantity of air the whole contents of the tube are added to liquefied gelatin and cultivated. Frankland uses glass wool plugs.

Staphylococci are the chief organisms found in air, but streptococci, B. coli, etc., have frequently been isolated. The recent Commission on intercepting traps have reported that pathogenic bacteria cannot be isolated from sewer air and that sewer air is thus not dangerous to health. It is not probable that pathogenic bacteria are to be found in air except in the immediate vicinity of patients suffering from such diseases as tuberculosis, pneumonia, diphtheria and pneumonic plague. The organisms of scarlet fever, chicken-pox and small-pox are also possibly air-borne.

The Bacteriological Examination of Soil

The number of bacteria in the soil vary considerably. According to Houston, virgin sandy soil contains on an average less than 100,000 bacteria per gramme. Garden soils yield from 1 to 2 million of bacteria per gramme. The bacteria found in soil are *B. subtilis,* and *B. mycoides* and allied organisms, streptothrix-like organisms and *B. proteus.* To the sanitarian the examination of soil for the presence of organisms indicative of excretal pollution, *e.g., B. coli, B. welchii* and streptococci yields valuable information inasmuch as he can determine the period at which a dumping area for the refuse of the town can with safety be utilised for building purpose. The soil bacteria are liable to be washed into water supplies. Amongst the pathogenic microbes, by far the commonest are *B. tetani, B. œdematiens,* though other organisms such as *B. typhosus, V. cholera, B. diphtheriæ,* etc., may also occur but these organisms die off rapidly.

Collection of Samples.—If the soil is to be taken from the surface, use a shallow trough (10 inches by 3 inches) which is pointed at one extremity or a soil scoop. These are sterilised and used for collecting samples.

If, on the other hand, the sample is to be taken from some depth, use a special apparatus such as Frænkel's borer.

Total Number of Organisms Present.—A known quantity of the sample of soil is placed into a flask containing a litre of sterile distilled water. The mixture is shaken thoroughly. Various quantities of the mixture are then added to tubes of melted agar and gelatin, which are plated and incubated at 37°C. and 20°C. respectively.

Methods for the Detection of Excretal Organisms.—*B. welchii.*—One gramme of the soil is added to 100 c.c. of bouillon contained in a flask and the contents of the flask shaken thoroughly so as to ensure equal distribution of the bacteria. 1 c.c., 0.1 c.c. and 0.01 c.c. of the mixture are added respectively to tubes of sterile milk. The milk tubes with the soil emulsion are placed in the water-bath at 80°C. for 10 minutes and incubated anærobically at 37°C. The presence of *B. welchii* is indicated by a "stormy clot."

Fæcal streptococci.—Varying amounts of the soil emulsion are added to tubes of glucose broth; as the medium becomes acid the streptococci multiply more rapidly than the *B. coli.* If after 48 hours' incubation glucose broth cultures are plated out on MacConkey, the small and intensely red colonies of strepto-

cocci can be distinguished from the large *B. coli* colonies and picked off for further investigation.

B. coli group.—An emulsion of the soil in broth is plated out on MacConkey's medium. The red colonies are picked off and subcultured for further investigation. In order to estimate the actual *B. coli* content of the soil add varying amounts of the soil emulsion to tubes of MacConkey broth as in water analysis. Subsequent procedure is the same as described in water examination.

CHAPTER XIII

THE BACTERIOLOGY OF MILK, MEAT, ETC.

Bacteria find their way into milk in a variety of ways. Fresh milk as secreted by healthy cows contains few organisms but the "fore-milk" is never sterile, because bacteria gain entrance and multiply in milk which has been left in the milk ducts from the previous milking. The milk of cows suffering from inflammation of the udder due to *B. tuberculosis* and streptococci and also of cows infected with *B. abortus* is rich in organisms. Contamination of milk with excretal organisms such as *B. coli*, fæcal streptococci, etc., may occur from the fæces and urine of cattle or from an insanitary cow-shed. Milking vessels which are not properly cleaned also add to the bacterial content of the milk.

Bacterial contamination continues to increase during conveyance from the farm to the consumer. Milk may be infected with various pathogenic organisms, *e.g.*, *B. typhosus*, *B. dysenteriæ*, *V. choleræ*, etc., either by flies or by the hands of a milker who may be a "carrier" of one of these diseases or by contaminated milking vessels to which these organisms may have gained access.

Bacteria Found in Milk

These are divided into the non-pathogenic (lactose fermenters and non-lactose fermenters) and the pathogenic bacteria. The lactose fermenters include the true lactic acid and coliform bacilli and the acid-forming bacteria, while the non-lactose fermenters include the casein-digesting, chromogenic and other bacteria.

A.—True lactic acid bacteria consisting of *Streptococcus lacticus* and allied organisms are frequently present on the hair of the cow and cause souring of milk.

B. coli and allied bacilli including *B. lactis ærogenes* are derived from animal excreta and their presence is regarded as evidence of excretal pollution. They differ from the true lactic acid bacteria in causing souring of the milk at 37°C. whilst the latter produce this change only at 20°C.

The *aciduric bacteria* comprise a group of organisms which includes *B. bulgaricus*, *B. bifidus* and *B. acidophilus*. These can grow in a very acid medium and thus continue to grow

even when the acidity produced by the true lactic acid bacteria arrests the multiplication of other organisms.

B. bulgaricus is a long, thick bacillus (4·5 μ x 1 μ) occurring in the alimentary tract of the cow. It has a tendency to curl at the ends. The more slender forms are found in chains. It is a non-motile, non-sporing organism, is Gram-positive and shows metachromatic granules when stained with methylene-blue.

It grows readily at 40° to 45°C. under anærobic conditions on ordinary media and forms delicate feathery colonies. Acidity and clotting in milk are produced.

B. acidophilus is similar in morphology and growth characters to *B. bulgaricus* from which it can be distinguished by the fact that it ferments 1 per cent. maltose broth in 48 hours at 37°C. with acid formation, whereas *B. bulgaricus* does not attack this sugar.

B. bifidus is present in the intestine of breast-fed infants and resembles the other two organisms of this group. It is more pleomorphic in cultures and in fæces it may show bulbous ends.

Acid-forming cocci.—Streptococci and staphylococci are fairly common in milk and produce acid but no gas from lactose. Cows may suffer from mastitis due to streptococci, indistinguishable from scarlet fever streptococci in (1) pathogenicity for rabbits, (2) the precipitin test and (3) the formation of a toxin giving a reaction in a Dick positive person.

B.—The non-lactose fermenting bacteria.—Certain casein-digesting bacteria produce clotting in milk owing to the production of an enzyme resembling rennet.

The *chromogenic bacteria* may produce one of the following changes in the colour of milk.

Blue Milk is due to *B. cyanogens.* It is a small, motile multiflagellated bacillus, which does not liquefy gelatin, but stains the media bluish green. It produces alkalinity in litmus milk but no clotting. *B. cyanoflourescens* is similar to *B. cyanogens* but produces a pigment of a flourescent type.

Red Milk.—If milk is coloured red when freshly drawn from the cow, the colour is due to hæmorrhage from the udder. If the change takes place on standing, one of the following organisms may be present in milk: *B. prodigiosus; B. erythrogenes; Torula rosea; Micrococcus roseus; Sarcinæ aurantica,* etc.

Yellow Milk is caused by *B. synxanthus.*

Green Milk is due to the abundant growth of *B. pyocyaneus.*

Bacteria Responsible for various Defects in Milk.—*Bitter Milk.*—This is caused not only by the ingestion of certain plants by the cow but also by certain organisms, *e.g.*, bacillus of Bleisch which is a motile, facultative anærobe, and liquefies gelatin. At high temperatures milk develops a bitter taste within 24 hours. Conn's micrococcus of bitter milk produces slime with subsequent clotting and a slightly sour and bitter taste.

Stringy or *Ropy milk* is caused by *Micrococcus freudenrei chii* and *B. lactis viscosus.*

Soapy Milk, characterised by frothing and a peculiar taste, is caused by *B. lactis saponacci* (Weigmann and Zirn). It grows best at 10°C.

Salty Milk is due to inflammation of the udder.

Pathogenic Bacteria

1. *Derived from the Cow.*—The streptococcus of mastitis; *B. tuberculosis; B. enteritidis* (Gærtner); the viruses of foot-and-mouth disease and of cow pox.

2. *Accidentally Introduced in Subsequent Handling.*—In addition to the above, the following organisms may be present: *B. typhosus, B. paratyphosus* A and B; *B. dysenteriæ, B. diphtheriæ; Str. scarlatinæ* and the bacteria of sore throat and suppurative conditions.

Bacteriological Examination of Milk

The bacteriological examination of milk consists of: 1.—The Quantitative Examination, *i.e.*, the *enumeration of the organisms present,* which includes:—

(*a*) the enumeration of bacteria growing at 37°C.

(*b*) the enumeration of bacteria growing at 20°C.

2. The Qualitative Examination, which includes—

(*a*) Estimation of excretal bacteria, which are:—(*i*) Excretal *B. coli.* (*ii*) *Streptococci.* (*iii*) *B. enteritidis sporogenes.*

(*b*) detection of pathogenic organisms which are—

(*i*) *B. tuberculosis.* (*ii*) *B. diphtheriæ* (*iii*) *B. typhosus, paratyphosus* A & B (*iv*) *B. dysenteriæ,* (*v*) *V. cholera,* (*vi*) *Brucella melitensis* and (*vii*) *Streptococcus pyogenes.*

Collection of Sample.—The sample should be collected in sterile bottles possessing wide mouths and glass stoppers and must be representative of the milk to be examined.

1. If the sample is obtained directly from a cow, the milker must clean the teats, and wash and disinfect his own hands. For routine examination the fore-milk is rejected and the sample of middle milk received directly into a sterile bottle.

2. If the sample is obtained *during transit or delivery,* the milk is mixed in the container either by inverting this several times if possible or by stirring with a sterile rod or pipette. A different sterile mixer must be used for each container to be sampled.

3. If milk is to be obtained from a large can a straight-walled pipette is lowered into the vessel till it reaches the bottom, keeping the top of the pipette open. The top end of the pipette is closed with the thumb, the pipette withdrawn and the milk run into a sterile bottle.

Transmission.—The sample must be packed in ice and sent to the laboratory with as little delay as possible. The following particulars should accompany the sample:—

1. Name and designation of the sender.
2. The identification mark or number.
3. Name and address of the dairy or the address of the producer if possible.
4. Name and address of the distributer.
5. Date and hour of collection.
6. Reasons for sending the sample (epidemic or routine).
7. Source of the sample (Cow, sheep, etc.)

The Enumeration of Organisms Developing at 37°C.—The apparatus and reagents required are the same as for water analysis but in addition glass stoppered bottles for making dilutions and mixing samples are required. Ordinary milk has to be diluted to 1 in 1,000,000 in order to get a satisfactory plate.

Method.—1. A number of glass stoppered bottles with a capacity of about 120 c.c. are placed in a row and marked A B C D. . . . according to the dilutions required.

2. With a 100 c.c. pipette, 90 c.c. of sterile water are added to each bottle.

3. With a 10 c.c. pipette are added—

to bottle A, 10 c.c. of the milk sample, and mixed, 1 c.c. of this contains 0·1 c.c. of original milk;

to bottle B, 10 c.c. of milk A, and mixed. 1 c.c. of this contains 0·01 c.c. of original milk;

to bottle C, 10 c.c. of milk B, and mixed. 1 c.c. of this contains 0·001 c.c. of original milk;

to bottle D, 10 c.c. of milk C, and mixed. 1 c.c. of this contains 0·0001 c.c. of original milk;

and so on.

Note.—The milk in the glass bottle must be shaken vigorously so as to distribute the bacteria evenly.

Plating.—1 c.c. of each dilution is plated as in water analysis.

Counting is done as in water examination.

The Enumeration of Organisms Developing at 20°C.—In ordinary routine examination the gelatin count is not carried out.

The dilution, plating and counting operations of the milk sample are carried out in exactly the same manner as in the case of agar counts except that the plates are incubated at 20°C. with the free surface of the medium facing upwards.

Detection of B. coli.—1 Dilutions of the sample of milk ranging from 1 in 10 to 1 in 1,000,000 as described above are made, the bottles being shaken vigorously. If the quality of the milk is known beforehand it may not be necessary to make all these dilutions.

2. Of each dilution 1 c.c. is added to tubes containing 10 c.c. of neutral red-bile salt-peptone-lactose broth (MacConkey broth) provided with fermentation tubes as in water analysis, using the same sterile 1 c.c. pipette, commencing with the highest dilution and working up to that containing 10 per cent. of milk.

3. The tubes are incubated for 48 hours.

4. The material from the last tube showing acid and gas in the MacConkey broth is plated on MacConkey agar.

5. The plates are examined after 24 hours' incubation and confirmatory tests made with any suspicious colonies.

Detection of B. welchii.—1-2 c.c. of the sample of milk are pipetted into each of ten sterile test-tubes.

2. The tubes are heated in a water-bath to 80°C. for 20 minutes to kill all but spore-bearing bacilli.

3. After cooling the tubes rapidly they are incubated anærobically at 37°C. for 24 hours.

4. On examination in the case of *B. welchii* the milk is coagulated and the clot is torn up by gas bubbles.

Detection of Streptococci is carried out in the manner previously described in water analysis. The estimation is generally omitted from routine examination, owing to the uncertainty as to its interpretation.

Examination for Pathogenic Organisms

B. tuberculosis.—One of the following two methods of concentrating the organism in the specimen should be used:—

(*i*) *Centrifugalisation.*—At least 250 c.c. of milk are distributed among the centrifuge tubes, which are then rotated at 2000 to 3000 revolutions per minute for half an hour. The supernatant cream is broken up by means of a sterile glass rod, as many organisms become entangled in it, and again the tubes are centrifuged for half an hour. The deposit is examined, smears, cultures and animal inoculation being made.

(*ii*) *Method of Douglas and Meanwell.*—In a centrifuge tube provided with a rubber-capped screw top are placed 10 c.c. milk and 0·5 c.c. of trypsin solution (Allen and Hanbury's liq. trypsini Co.). The tube is incubated at 37°C. for six hours. After cooling 5 c.c. of ether is added, the cap screwed on the tube, shaken vigorously at least 200 times and subsequently centrifuged for 20 minutes at 4000 revolutions per minute. The liquid now consists of three layers: the ethereal layer containing dissolved fat at the surface, a clear fluid at the bottom, and between the two a gelatinous disc which contains the acid-fast organisms. Loopfuls of the gelatinous disc are removed with a platinum loop and deposited on slides, a drop of distilled

water added to each specimen and the film allowed to dry at room temperature. The slides are fixed in alcohol-ether mixture for 2 hours at room temperature before staining. This method is not suitable for animal inoculation.

1. *Microscopic Examination.*—Films made from the deposit are stained with Ziehl-Neelsen's method, the organism being acid and alcohol-fast.

2. *Animal Inoculation* is the most reliable method for the detection of tubercle bacilli and should invariably be carried out. A portion of the centrifuged deposit is injected subcutaneously into the inner side of the thigh of one or two guinea-pigs. The weight of the inoculated animal should be recorded weekly and the animal killed after three weeks. The pathological lesions produced in tubercle are obvious and have already been described. Tubercle bacilli should be examined for in smears made from enlarged glands and in sections of the spleen and lungs, as *B. abortus* which may occur in cow's milk produces lesions similar to those of tuberculosis. Other acid-fast organisms may give rise to glandular enlargement but unlike *B. tuberculosis* they grow rapidly on glycerin agar.

B. diphtheriæ.—The sample of milk is centrifuged as before.

1. The deposit is planted on blood serum and incubated at 37°C.

2. On microscopic examination of the culture if any bacilli with the morphological characters of *B. diphtheriæ* are found, the culture is plated on blood-serum agar and incubated at 37°C.

3. Any suspicious colonies appearing after 12 hours' incubation are sub-cultivated upon blood-serum.

4. The diagnosis of the diphtheria bacillus should be confirmed by biochemical and virulence tests.

Note.—An organism identical with *B. diphtheriæ* in morphological and cultural characteristics is found in milk and cheese but unlike *B. diphtheriæ* is non-pathogenic.

B. typhosus, B. paratyphosus A & B, B. dysenteriæ and V. cholera.—1. The centrifuged deposit is plated on one of the special media recommended for the detection of these organisms in water. In the case of *V. choleræ* the deposit is first inoculated

into peptone-water from which plates on Dieudonne's medium may be made after 18 to 24 hours' incubation.

2. The organisms isolated are identified by morphological, bio-chemical and serological examinations.

Brucella melitensis.—1. The centrifuged deposit of the milk sample is planted in broth and incubated at 37°C.

2. Suspicious colonies are picked off and subcultured on agar slopes.

3. The organisms are identified by morphological, biochemical and serological tests.

Streptococcus pyogenes.—The centrifuged deposit is plated on glycerin agar and all suspicious colonies investigated.

The Bacteriological Limits of Designated Milk.—In England the Milk and Dairies (Amendment) Act, 1922, prohibits the sale of milk designated as certified, Grade A, pasteurised or otherwise designated, except under license; prohibits the addition to milk of colouring matter or water or any fluid derived from dried, condensed, separated or skimmed milk and prevents the sale of milk from a cow with a tuberculosis udder. Under the Milk (Special Designations) Order, 1923, licenses are only granted on certain conditions laid down for producers and retailers. The number of bacteria must not exceed 30,000 per c.c. and *B. coli* must be absent in 0·1 c.c. in certified and Grade A milk (pasteurised). In Grade A and Grade A tuberculin tested milk the organisms must not exceed 200,000 per c.c. nor *B. coli* group be present in 0·01 c.c. Pasteurised milk must contain not more than 100,000 bacteria per c.c. *B. tuberculosis* must be absent from certified and Grade A tuberculin tested milk. Certified milk must not be treated by heat and pasteurised milk must only be heated once, retained at a temperature of between 145°F. to 150°F. for half an hour and then cooled below 55°F.

The Bacteriology of Meat

Meat may have to be examined for organisms causing food-poisoning, in addition to examining for cysticerci, trichinæ, tuberculosis, anthrax, etc. Any coloured patches on the meat should be examined and also the gelatin in preserved meats.

The examination of food for *B. botulinus* is carried out as follows:—

1. *Microscopic Examination.*—Films from the food are stained and examined for Gram-positive bacilli.

2. *Feeding experiments on animals.*—Susceptible animals such as guinea-pigs are fed on a portion of the suspected food. If *B. botulinus* is present in the food the animal will die within 24 to 48 hours with the characteristic signs of paralysis, etc.

3. *Animal inoculation.*—An emulsion of the food in broth is made and a portion of this emulsion is heated at 60°C. for an hour to kill the non-sporing bacilli. Both the heated and unheated emulsions are incubated anærobically at 28°C. The cultures are filtered and then injected to guinea-pigs. The death of the animals within 24 hours indicates the presence of *B. botulinus.* In order to confirm the diagnosis two guinea-pigs are inoculated with the filtrate from the broth culture. One of these at the same time is given a protective dose of the botulinus antitoxin. If the organism is a true botulinus bacillus the unprotected animal will die within 24 to 48 hours with the characteristic signs of paralysis while the protected, *i.e.,* the one which received the antitoxin will survive.

Another organism discovered in blown tins of preserved meat is described by Beveridge and Fawcus and is apparently identical with the *Bacillus cadaveris sporogenes* (Klein). This organism is anærobic, motile and forms large terminal spores. It forms gas in neutral-red glucose broth and clots litmus milk. It gives off in all media a putrid odour.

B. subtilis and B. mesentericus group of spore-bearing organisms are repeatedly found in tinned meats. *B. subtilis* (the hay bacillus), occurs in hay, air, water, fæces, etc. *B. mesentericus* is a short, spore-forming Gram-positive organism which liquefies gelatin. There are white, yellow and red varieties. Neither of these bacteria are pathogenic to man.

Sour beef, as has already been stated, is due to *B. megatherium,* 'red-spot' is due to *B. prodigiosus* and 'black spot' to a cladospore and to *Oidium carbis* while 'brown spot' or brown discoloration may be due to *Oidium monhue* or the yeasts.

The Salmonella group of organisms (including B. paratyphosus A and B, B. suipestifer, B. enteritidis Gärtner, (the Aertrycke bacillus) has often been traced to ham and meat pies. Two methods are available for examining food for members of the Salmonella group:—

1. *Culture.*—An emulsion of the food in sterile saline solution or broth is plated out on MacConkey medium. The colonies are identified by biochemical and agglutination reactions. The organism should be tested against specific agglutinating sera of the various food poisoning microbes.

2. *Feeding experiments.*—Susceptible animals, especially mice, are fed on the food under investigation and watched for the characteristic signs of food poisoning such as gastritis, etc. The causal organisms may be recovered by plating out fæces of the experimental animal on MacConkey medium and identified by the usual tests. The method is not free from fallacies inasmuch as the rodents are often carriers of the bacteria belonging to the Salmonella group.

CHAPTER XIV

THE DETERMINATION OF THE GERMICIDAL POWER OF DISINFECTANTS

In addition to the quantity and nature of the tar oils present in a coal-tar disinfectant, the germicidal value depends upon its physical condition also. It is, therefore, necessary to undertake physical examination of disinfectants in association with germicidal tests against a culture of test organism. Two physical tests are prescribed by the Union of South Africa Government, one for homogeneity and the second for stability of the emulsion.

1. Homogeneity and occurrence of sedimentation in bulk and under varying conditions of storage and use is tested by allowing full one gallon drum of the disinfectant to stand without shaking for 14 days. With the help of a 50 c.c. pipette samples are gently removed from the top, middle and bottom layers. Specific gravity of these three samples is determined carefully by specific gravity bottle and comparison made. In a satisfactory sample the figures are uniform.

2. Any agent which destroys or partially destroys the fineness of an emulsion also destroys the germicidal value of the fluid. So the stability of emulsion is tested under various conditions. A 10 per cent. mixture of the disinfectant is made with (*a*) distilled water, (*b*) salt water (32 grammes of Tidman's Sea Salt or sodium chloride dissolved in a litre of distilled water) and (*c*) urine. These mixtures are placed in 100 c.c. graduated burette and the amount of deposit is noted after 24 and 48 hours. A satisfactory emulsion should show little or no deposit in 48 hours.

Of the various methods for finding the disinfecting power of substances, the Rideal-Walker method or one of its modifications is mostly used.

Apparatus and Reagents Required.—

1. A special test-tube rack having an upper tier of 20 holes in two rows, each row containing two sets of five, for tubes of standard broth and a lower tier with 5 holes, 4 for the disinfectant and one for the tube of standard phenol.

2. Sterile test-tubes, 5"×$\frac{5}{8}$" is the size recommended.

3. Case of sterile and standardised pipettes, 10 c.c. (in tenth of a c.c.), 5 c.c. and 1 c.c. and sterile graduated flasks for making dilutions.

4. *Test germ.—B. typhosus* grown in Rideal-Walker broth for 24 hours at 37°C. Half an hour before use the culture is shaken and left in the incubator to ensure even distribution of the bacteria and to break up the clumps. A uniform emulsion should be obtained. Subculturing of the organism from broth to broth every 24 hours produces a culture uniformly resistant to disinfectants. The Rideal-Walker broth for cultivating the *B. typhosus* has the following composition:—

Liebig's extract of Meat	20 grammes.
Peptone (Allen and Hanbury) ..	20 grammes.
Salt (sodium chloride C. P.) ..	10 grammes.
Distilled water	1000 c.c.

The mixture is boiled for 30 minutes then filtered and neutralised with normal soda, using phenolphthalein as an indicator and then standardised to + 15 (Eyre's scale) with normal hydrochloric acid.

Note.—Rideal and Walker emphasise the need for the use of Liebigs extract, as broth prepared from bullock's heart has the effect of depressing the coefficients by about 50 per cent.

5. *Disinfectant to be tested.*—Various dilutions of the disinfectant should be made with sterile distilled water by accurate volumeteric methods. If nothing is known about the germicidal power of the disinfectant, its strength with widely spaced dilutions of the disinfectants (*e.g.*, 1:100, 1:500, 1:1500 and so on) is determined by a preliminary test.

6. A 5 per cent. carbolic acid solution by weight is used as a standard. The solidifying point of the carbolic acid crystals used in its preparation should be above 40°C. The 5 per cent. carbolic acid solution should be standardised by titration with $\frac{N}{10}$ bromine.

7. The platinum loop made of the wire of 27-28 B. W. G. should have an internal diameter of 4 mm. and bent slightly at an obtuse angle to allow of a fair sized drop being taken up for each inoculation.

8. The temperature at which the experiment is carried out should be 15° to 18°C.

9. A square wire basket for the reception of inoculated broth tubes.

10. Sterile distilled water.

Method.—The *Rideal-Walker method* comprises two distinct tests—one of the disinfectants under investigation, hereinafter called X, the other of the standard carbolic acid.

1. Five sterile test-tubes marked A to E are placed in the lower tier of the rack.

2. Into the first four tubes are pipetted 5 c.c. of the various dilutions of the "X" disinfectant, depending upon its strength as worked out by a preliminary test. Assuming from such a

preliminary test that the "X" disinfectant in a dilution of 1: 1500 produces sterility of a typhoid culture in 2½ minutes and that 1: 2000 dilution produces sterility in 30 minutes and that the stock 1: 100 carbolic solution usually kills the stock typhoid culture in 7½ minutes, then the procedure is as follows (lower tier tubes):—

Into 'A' tube measure 5 c.c. germicide X diluted to 1 : 1500.

Into 'B' tube measure 5 c.c. germicide X diluted to 1 : 1600.

Into 'C' tube measure 5 c.c. germicide X diluted to 1 : 1700.

Into 'D' tube measure 5 c.c. germicide X diluted to 1 : 1800.

Into 'E' tube measure 5 c.c. carbolic acid diluted to 1 : 100.

A series of 4 sets of 5 tubes each, one containing 5 c.c. of standard broth, are arranged in the upper tier of the rack and numbered consecutively from left to right. In the tube A (the first tube on the lower tier) 0·5 c.c. of a 24 hours' broth culture of *B. typhosus* is added and the time noted. Into each successive tube (B, C, D and E) in succession at intervals of ½ minute is added 0·5 c.c. of typhoid culture. Half a minute after the last tube (E) has been inseminated a loopful from the tube A is planted into the first broth tube A. Half a minute later a loopful from tube B is inoculated into the 2nd broth tube, half a minute later a loopful from tube C into the 3rd broth tube and this process is repeated at half-minute intervals until the material from tube E has been inoculated into the 5th broth tube. Again the process is repeated in starting with tube A and so on at half minute intervals into the second set of broth tubes, again into the 3rd set, etc. The inoculated broth tubes are shaken and incubated at 37°C. After 48 hours of incubation the broth tubes are examined for the occurrence of the growth. It will be noted that the first set of 5 broth tubes have been inoculated after the exposure of *B. typhosus* to the action of the disinfectant A to D and carbolic acid (E) for 2½ minutes; in the second set of broth tubes after an exposure of 5 minutes; the third set after 7½ minutes, etc.

N. B.—The process of subculturing from the tubes marked A to E into broth tubes is repeated at half minute intervals until the disinfectant had been allowed to act on the culture for 10 minutes. The period of contact of the culture and disinfectant varies with the strength of the disinfectant as estimated by a preliminary test.

The results of the test may be tabulated as follows:—

Culture used: *B. typhosus*, 24 hours' broth culture at 37°C. Room temperature during the test: 15° to 18°C.

Disinfectant. Dilution.	Time of exposure in minutes and result in broth.				Incubation of subcultures.	
	2½	5	7½	10	Time	Temperature.
X 1 : 1500	+	—	—	—	48 hours.	37°C.
X 1 : 1600	+	+	—	—	,,	,,
X 1 : 1700	+	+	+	—	,,	,,
X 1 : 1800	+	+	+	+	,,	,,
Carbolic acid. 1 : 100	+	+	—	—	,,	,,

+ =Growth in the subcultures. — =No growth in the subcultures.

From the table it will be seen that the disinfectant X in a dilution of 1 in 1600 produces sterility in the same time (5 minutes) as carbolic acid in a concentration of 1 in 100. The result is expressed as a coefficient obtained by dividing the weakest dilution of the unknown antiseptic by the strength of phenol which kills the culture in the same time; in the present instance the coefficient is $\frac{1600}{100} = 16$. The unknown disinfectant X is then stated to have a "carbolic coefficient" of 16.

N.B.—The temperature should not vary more than 2°C. between the tubes. A large drop is required in subculturing. Sterile distilled water is used to make the dilution. The broth culture should not be more than 24 hours' old and should be daily subcultured into broth from a stock agar slope which requires subculturing on agar once every month to keep it going.

Examination of disinfectant powders.—In *Robertson's and Severn's process,* as a standard, a 15 per cent. carbolic powder is taken as the unit and owing to the difficulty of maintaining a stable preparation of this, 100 c.c. of 5 per cent. phenol is made up to 332 c.c. and called "Standard powder, 1 in 10." Dilutions are made of this for placing in the 5th tube of the lower rack in carrying out the Rideal-Walker test.

The powder to be examined is mixed in a proportion of 100 grammes powder to a litre and shaken at intervals of 4 hours, then kept overnight. After 24 hours the clear supernatant fluid is transferred to a sterile bottle and used to make the dilutions, each 10 c.c. of the clear fluid representing 1 gramme of the powder.

There are various modifications of the Rideal-Walker method for determining the Phenol coefficients of disinfectant fluids. Some of these are standardised and recognised as official methods in some countries. The underlying principle is essentially the same but the technique differs more or less considerably. The salient features of these methods are indicated below and for detailed standard technique original sources should be consulted.

British Admiralty Method.—The germicidal value is determined in the presence of some organic matter. 10 c.c. of the disinfectant made upto a litre with sterile artificial sea water (32 grammes of Tidman's sea salt per litre) is allowed to stand for 20 hours and then by means of a pipette a portion is removed from the middle layer for test purposes. The test organism is a vigorous *B. typhosus* (Rawlings' Strain) grown for 24 hours at 37°C. in standardised nutrient beef broth containing 1 per cent. peptone and 0·5 per cent. sodium chloride standardised to P_H 7·6. Dilutions are made with sterile solutions of 0·5 per cent. gelatin in distilled water containing 0·5 per cent. rice starch in suspension. The standard is pure crystallised phenol (B.P.) which is also dissolved in artificial sea water as in the case of disinfectant and allowed to stand for 24 hours before proper test is undertaken. The culture is filtered through cotton-wool and 0·25 c.c. is added to 5 c.c. of each dilution of disinfectant and phenol and well shaken at frequent intervals. The organisms are kept in contact for 10 minutes at a temperature between 13 and 18°C. and then a loopful is inoculated in standardised nutrient broth and incubated at 37°C. for 48 hours when result is recorded.

Lister Institute (Martin-Chick) method.—In this method organic matter is introduced in the form of dried fæces. Dried fæces is variable in composition and troublesome to prepare; moreover it does not represent urine, sputum, vegetable debris, etc., so the method fails to represent a practical test. The culture medium is prepared from Brand's Meat Juice (1 per cent.) containing salt (0·5 per cent.), peptone (1 per cent.) and glucose (1 per cent.) and standardised to P_H 6 to 7. 0·15 gramme of finely powdered fæces dried in water-bath at 105°C. are placed in a number of test-tubes and mixed with 2·5 c.c. of distilled water and autoclaved for 10 minutes at 120°C. 2·5 c.c. of various dilutions of disinfectants or phenol are added to these tubes and thus the total volume is made to 5 c.c. fæcal solution. These tubes are kept at constant temperature of 20°C. in a water-bath and then 5 drops of standard 24 hours' culture of *B. typhosus* is added to each, one after another, with a standard pipette. Exactly one minute is allowed between

each inoculation and after a lapse of full 30 minutes after inoculation of first tube duplicate samples are taken with a platinum loop and sown in 10 c.c. glucose broth containing litmus, each tube having a time interval of one minute. Similar experiment is simultaneously made with standard phenol. The highest dilution of disinfectant capable of producing complete sterility in 30 minutes divided by the phenol concentration producing the same effect in the same time is the carbolic acid coefficient in the presence of 3 per cent. fæces.

Lancet test.—In this test Witte's peptone and bullock's heart broth is used and reaction is not standardised to any definite P_H value. Test organism is *B. coli communis,* which owing to its great variation is not suitable for the test. Subcultures are made on MacConkey's broth every 2½ minutes upto 30 minutes. The coefficient is the mean of phenol coefficient obtained from the weakest dilution of the disinfectant which kills the organism in 2½ minutes and that obtained in 30 minutes.

United States Hygienic Laboratory method (Anderson and McClintoc).—Culture medium used in the test is a mixture of Liebig's meat extract (0·3 per cent.), Armour's peptone (1 per cent.), Sodium chloride (0·5 per cent.) in distilled water properly sterilised and standardised to P_H 6 to 7.

Test culture is 'Hopkins' strain of *B. typhosus* grown in the above medium for 24 hours at 37°C. and filtered through filter-paper. Phenol crystal of U. S. A. Pharmacopœia with a solidifying point above 40°C. is used for comparison. Wire loop is specially made by winding a platinum wire (No. 23 B. & S. gauge) as tightly as possible for four complete turns round a steel or other hard wire having a diameter of 0·072 inch. 0·1 c.c. of typhoid culture is added to 5 c.c. of disinfectant and phenol of various dilutions at an interval of 2½ minutes, and then subcultures are made from it at an interval of 5, 7½, 10, 12½ and 15 minutes from various dilutions of the disinfectants. These are kept in water-bath at a temperature of 20°C. during the test and after inoculations they are incubated for 48 hours at 37°C. The coefficient is the arithmetical mean of three coefficients obtained by the highest dilutions of disinfectant and phenol producing complete sterility in 5, 10 and 15 minutes. This is the official method adopted by the Union of South Africa but the physical tests described above are also carried out simultaneously with this germicidal test.

INDEX

B

C

D

E

F

G

H

I

J

K

L

M

N

R

S

T

X

Y

Z