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ADVANCES IN PROTEIN CHEMISTRY

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Editors' Preface

In the last generation, protein chemistry, which was once a relatively narrow branch of organic and biological chemistry, has spread out into the most varied fields of physics, chemistry, and biology. Enzymes, viruses, and many substances of immunological importance, are now known to be proteins. The techniques now used for the study of proteins range from the most elaborate form of X-ray analysis to quantitive measurements of antibodies. Workers in the most diverse fields of science have not only contributed to the development of techniques, but have become interested themselves in applying the techniques they helped develop in the study of the problems of protein chemistry. With the great progress in the knowledge of proteins, the industrial and medical applications of this knowledge have increased greatly and promise to increase far more.

The rapid pace of the advances in protein chemistry, the varied character of the work being done, and of its practical applications to industry and medicine, have given rise to an increasing need for thoughtful and critical evaluation of the results achieved, and of their implications. We hope that this series of volumes will give the opportunity to workers in special subjects to present their views in more organized form than is possible in the regular journals, and also to express their peronal judgment on problems which are still unsettled. We hope, too, that as the reviews accumulate, they will provide a useful and comprehensive picture of the changing and growing field of protein chemistry, and a stimulus to its further development.

In this first volume, special emphasis is laid on proteins as they occur in nature, as components of complex biological systems. In the second volume, which we expect to appear in 1945, there will be a group of contributions which reflect the increased interest in protein nutrition stimulated by the war. These will include discussions of the estimation of amino acids by chemical and bacterial growth methods, of the amino acid contents of protein foods; of protein nutrition in man, and of the relation of protein nutrition to antibody formation.

Since the physical chemistry of protein systems was extensively treated in a symposium which appeared in "Chemical Reviews" in 1942, and has also been discussed in several recent monographs, it has received relatively little emphasis in the present volume. Extensive and critical discussions of recent advances in the physical chemistry of amino acids, peptides, and proteins, however, will appear in later volumes.

The circumstances of war have inevitably imposed severe restrictions on possible contributors at the present time. In many countries, those who might be interested in contributing to such an enterprise are totally inaccessible to us; in the United States and England, others who expressed keen interest in the project have been unable to contribute because of the pressure of more urgent work. The authors whose work is presented here have all prepared their contributions under difficult conditions. We, and we believe our readers also, are greatly indebted to them for their presentation of significant developments in fields with which they are intimately familiar.

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Lipoproteins

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I. Introductory Remarks

This review will deal largely with compounds which are traditionally regarded as impure. It therefore may be appropriate briefly to examine the conception of purity as it applies to many of the complicated substances that are isolated from biological material (compare the review by Pirie, 1940). Although not often clearly formulated, the disparity in the con-

ceptions of chemical purity and biological purity has beset the biochemist from the very beginnings of his science.

Workers are generally agreed on the criteria for the chemical purity of proteins, carbohydrates, lipids, of biological origin, *e.g.*, the constancy of chemical composition, physical characteristics, etc. To these requisites one more usually is added implicitly, *viz.*, that the types of linkages supporting the architecture of the particular substance be clearly definable in terms of our present knowledge. This requirement is obviously not fulfilled in a number of compounds of great biological importance, *e.g.*, certain enzymes, viruses, etc. Yet, many of these substances show a functional homogeneity of the highest degree. If the cytoplasm or certain cytoplasmic inclusions of a cell were to be isolated in their unaltered states, they would certainly be found extremely impure chemically, even though they were to exhibit a high order of biological purity. While it may be hoped that with a more profound understanding of the types of linkages prevailing in these complex structures the borderline between chemical and biological purity will disappear, it is clear that the fear of offending the chemical proprieties has frequently led to the most far-fetched model experiments, in which the true objects of biochemistry (which, after all, is not thanatochemistry) have become obscured. The study of biologically important substances in their natural environment will certainly form a most fascinating subject of biochemical research.

This article cannot attempt to offer a complete treatment of the chemistry of lipid-protein complexes which probably are ubiquitous components of living matter; it will rather be its purpose to point to a number of instances where the occurrence of at least fairly well defined lipoproteins has been shown to be probable. Representative compounds present inside the cell and in the extracellular fluids will be discussed.

Some of the historical aspects of our knowledge of the manner in which the lipids occur in living matter have been discussed by Sandor (1934) and by Macheboeuf (1937). A very adequate review of the present state of chemical information was recently published by Lovern (1942). There exists a vast amount of histological observations on the occurrence of lipid-protein complexes in tissues, and in the innumerable speculations on the nature of protoplasm the function of lipoproteins has often been debated. Some of the monographs and articles mentioned in the bibliography may be found useful in this connection (Biedermann, 1924; Bourne, 1942; Degkwitz, 1933; Guilliermond, 1934; Kiesel, 1930; Lepeschkin, 1938; Lison, 1936; Sponsler and Bath, 1942.)

II. Definition of Term and Classification

The term lipoprotein is used for compounds between proteins and lipids; the latter are in turn defined as a group of naturally occurring fatty acid

derivatives that are soluble in organic solvents. This group comprises the fatty acids, glycerides, glycerol ethers, waxes, phosphatides, phosphatidic acids, cerebrosides, and esters of xanthophylls and sterols. Sometimes the uncombined steroids, carotenoids and related substances are likewise included. A desirable classification of the lipoproteins could be based on the nature of the lipid residue contained in the conjugated protein: one would speak of lecithoproteins, cephaloproteins, etc. Actually, except in the case of a few synthetic lipoproteins which will be discussed later, this cannot yet be done, since the conjugated proteins isolated from natural sources until now were all found to contain more than one lipid species. If the nature of the protein moiety is known, the prefix lipo- could be used in these cases; for instance, lipovitellin for the lipoprotein from egg yolk (Chargaff, 1942, a).

Not infrequently, terms, as *e.g.*, lecithovitellin, are employed for protein preparations which had been freed of phosphatides. This is confusing; it would certainly appear advantageous to reserve such prefixes for the conjugated proteins.

III. Modes of Linkage between Lipid and Protein

A schematic survey of the types of primary valence bonds that could exist between lipids and proteins may be helpful for an understanding of the lipoprotein problem.

1. Fatty acids: Carboxyl group, electrostatic (salt); covalent (ester, amide, etc.).
2. Triglycerides: None. (Mono- and diglycerides could, of course, form covalent ester links through their free hydroxyl groups.)
3. Lecithin: Trimethyl ammonium group, electrostatic (salt). Phosphoric acid group, electrostatic (salt); covalent (ester, etc.).
4. Cephalin: Amino group, electrostatic (salt); covalent (amide). Phosphoric acid group, electrostatic (salt); covalent (ester, etc.).[1]
5. Phosphatidyl serine: Amino group, electrostatic (salt); covalent (amide). Phosphoric acid and carboxyl groups, electrostatic (salt); covalent (ester, amide, etc.).
6. Sphingomyelin: Trimethyl ammonium group, electrostatic (salt). Phosphoric acid group, electrostatic (salt); covalent (ester). Hydroxyl group, covalent (ester).
7. Phrenosin and kerasin: Hydroxyl groups, covalent (ester).
8. Xanthophyll and sterol esters: None.

It is clearly necessary to distinguish between genuine lipid-protein complexes and mixtures or loose adsorption systems which can be separated into their component parts by mild methods of fractionation or extraction.

[1] The phosphatidic acids, glycerol ethers, and acetal phosphatides probably behave similarly to groups 1, 2, and 4 respectively.

The term lipoprotein, therefore, connotes, or should connote, a group of compounds with properties (biological reactivity, solubility, color, optical and other physical constants) different from those of the sum of their components. It is, at the present state of information, not possible to offer a strict formulation of the type of linkages prevailing between a lipid as a prosthetic group and a protein. The introduction of the term "symplex" (Willstätter and Rohdewald, 1934) has, as a purely terminological innovation, by no means advanced our understanding. The inspection of the polar characteristics of the various lipids enumerated above could, however, lead to the following provisional classification (compare also Przylecki, 1939; Lovern, 1942).

I. *Covalent Compounds* (Groups 1 and 3 to 7).
II. *Electrostatic Compounds* (Groups 1 and 3 to 6).
III. *Secondary Valence Compounds* (Groups 1 to 8).

There is little evidence of the occurrence of covalent lipoproteins (*e.g.*, esters, amides, etc.) in nature. It is, of course, true that only very rarely was a search made for the presence of fatty acids in hydrolyzates of thoroughly defatted proteins. There are, however, some indications of the occurrence of compounds of this type in certain species of pathogenic bacteria, *e.g.*, in human tubercle bacilli (Anderson, Reeves, and Stodola, 1937) and in diphtheria bacteria (Chargaff, 1931). It often will be difficult to distinguish experimentally between substances belonging to this class and those of salt-like nature.

The electrostatic compounds would be represented by salts in which the attraction is due to ionic forces between the lipid and the protein. The synthetic lipoproteins and lipoprotamines to be discussed later certainly fall into this group. Substances of this type will be stable within a certain pH range only. It is therefore to be assumed that there will be a significant difference in the reactivities of lecithin and sphingomyelin and of the more acidic phosphatides (cephalin, phosphatidyl serine, phosphatidic acids, etc.).

Lecithin and sphingomyelin, which contain the strong base choline, may be considered as internally neutralized compounds. The isoelectric point of lecithin has been found at pH 6.7, not much lower than the value required by the theory, *viz.*, pH 7.5 (Chain and Kemp, 1934; Bull and Frampton, 1936). The admixture of cephalin appears to bring about a considerable lowering of the isoelectric point of lecithin (Bull and Frampton, 1936; Cohen and Chargaff, 1940). Unfortunately, the information with respect to cephalin is less satisfactory. Most physical measurements on cephalin were carried out with preparations from brain which, as we know now (Folch and Schneider, 1941), contained a large proportion of phosphatidyl serine. This compound is doubtless markedly acidic and it is not unlikely

that most properties assigned to cephalin in the literature, especially those distinguishing it from lecithin, actually relate to the serine-containing compound. This will be particularly true of preparations obtained from brain and lungs, whereas egg yolk phosphatides are practically free of this serine derivative (Chargaff, Ziff, and Rittenberg, 1942). In its polar characteristics, cephalin itself (*i.e.*, ethanolamine phosphoryl diglyceride) is probably much nearer to lecithin than was previously supposed. It may be concluded that at the physiological pH lecithin can hardly be expected to form salts with tissue proteins, whereas cephalin and especially phosphatidyl serine will be able to combine with protamines and certain basic proteins (*e.g.*, histone) by means of ionic bonds to form insoluble products; in other cases (*e.g.*, globin and cephalin at pH 7), the resulting salt may show a higher solubility than the uncombined protein (Chargaff, 1938; Chargaff and Ziff, 1939).

Most lipoproteins occurring in nature probably have to be classified as secondary valence complexes which are held together by van der Waals forces. This assumption certainly is necessary for those lipids (Groups 2 and 8 in the scheme given above) that lack centers of attachment which could give rise to the establishment of covalent or electrostatic bonds. The situation may be further complicated by the possible existence of solid solutions of lipids in the prosthetic lipid portion of a lipoprotein, *e.g.*, glycerides or steroids dissolved in the cephalin part of a cephaloprotein.

The distinction between electrostatic and coordination compounds is probably not sharp in the field of conjugated proteins, as the coordination centers are presumably often represented by electrically charged groupings in the protein and the prosthetic substance. These complexes are stable, and move intact in an electric field, at a pH well above the isoelectric points of their component parts (compare Cohen and Chargaff, 1940; 1941 b; Chargaff, Ziff, and Moore, 1941); on treatment with certain organic solvents, as for instance ethyl alcohol, which would not be expected to disrupt electrostatic compounds, the linkage between lipid and protein tends to break. The action of heparin (Chargaff, Ziff, and Cohen, 1940, b) in displacing the lipids from certain lipid-protein compounds (see Section V) could equally well be explained as the formation of protein salts of a solubility lower than that of the original lipid-protein compounds. Generally, it may be assumed that any action which results in the displacement or the distortion of the centers of attachment in the protein, thereby changing the critical spacing of these centers, will bring about the cleavage of the conjugated protein.[2]

Very little can be said about the nature of the centers of attraction and

[2] The theory of the antigen-antibody reaction has dealt exhaustively with problems of this nature, and reference should be made to recent treatments of this subject (Heidelberger, 1939; Landsteiner, 1936; Marrack, 1938; Pauling, 1940a).

of the forces supporting such secondary valence structures. In some cases the formation of hydrogen bonds may play a rôle (Pauling, 1940, b). The action of alcohol in destroying the links between protein and lipid appears to be irreversible.[3] Because of the lack of reliable amino acid analyses of the protein moieties of naturally occurring lipoproteins, it is not even possible to establish a correlation between the number of basic amino acid residues in these proteins and their tendency to combine with lipids. Such a correlation seems to exist, however, in the salts between basic proteins and lipids which will be discussed in the next section.

Weiss (1941, 1942) has proposed an interesting theory of the structure of organic molecular compounds between substances possessing electronegative groups and unsaturated hydrocarbons (or their derivatives) containing conjugated double bonds. The formation of these essentially ionic complexes is ascribed to an electron transfer from the unsaturated hydrocarbon to the electronegatively charged molecule. Compounds of this type will possibly be encountered among the naturally occurring complexes.

IV. Chemical Properties of Lipoproteins

1. *Synthetic Compounds*

There have been numerous attempts to prepare artificial lipoproteins. Since in most cases very little attention was paid to the polar characteristics of the lipids or the proteins employed, it is not surprising that many of these experiments were not successful. The effort of combining anything with everything will not lead to the creation of a homunculus, even if the resulting mixtures are termed symplexes or coacervates.

In a number of studies the preparation of complexes between serum albumin or egg albumin and lecithin was attempted (Galeotti and Giampalmo, 1908; Liebermann, 1893; Mayer and Terroine, 1907; von Przylecki and Hofer, 1936; Went and von Kúthy, 1934). The competition between lecithin and cholesterol for the serum proteins was investigated by Theorell (1930). The interactions between lecithin and serum globulin (Chick, 1914), zein (Galeotti and Giampalmo, 1908), and caseinogen (Parsons, 1928) were likewise studied. Other workers followed the influence of proteins on the flocculation of lecithin sols (Feinschmidt, 1912; Handovsky and Wagner, 1911; Went and Faragó, 1931). The almost exclusive use of

[3] Macheboeuf and Sandor (1932) have attempted to explain the structure of lipoproteins by the mutual attraction of the hydrophobic groupings of the protein and the lipids. These complexes are assumed to be surrounded by a nimbus of water, held in position by their hydrophilic groups, which prevents the access of ether and other solvents immiscible with water, but not of alcohol. It is doubtful whether this explanation could be applied to the dehydrated complexes.

lecithin in these experiments probably explains the rather inconclusive results since lecithin, in contrast to the more acidic phospholipids, is an inappropriate model substance.

The difference in the behavior of lecithin and the acidic phosphatides of the cephalin group was emphasized in studies on the formation of compounds between these lipids and protamines or basic proteins. With the highly basic protamine salmine (isoelectric point at pH 12) cephalin preparations from brain, which presumably were rich in phosphatidyl serine, formed water-insoluble salts over a wide pH range, *viz.*, from pH 2 to 11 (Chargaff, 1938). These products, which had a P:N ratio of 1:4 or 1:5 and were composed of about 80% of cephalin and 20% of salmine, were soluble in organic solvents, could be recovered unaltered when their solutions in hot ethyl acetate were cooled, and did not change their composition following treatment of their solutions in ether with dilute acids or repre-

TABLE I

Composition of Cephalin-Histone Compounds

(From Chargaff and Ziff, 1939)

pH of reaction	Composition of compounds			Cephalin-binding capacity
	P	N	Cephalin in compound on basis P value	
	per cent	*per cent*	*per cent*	*m.eq. per g. protein*
3.7	2.5	6.7	71	2.78
4.7	2.1	7.9	60	1.71
7.2	1.6	9.4	50	1.03

cipitation with acetone. In one such compound 4.7 milliequivalents of cephalin per g. of salmine were found instead of 5.0, as calculated from the acid-binding capacity of this protamine. With lecithin, on the other hand, no compound formation was observed, except at a very high pH, *viz.*, 10 and 11. The same was true of sphingomyelin.

A study of the compound formation between lecithin and cephalin (rich in phosphatidyl serine) and basic proteins, *viz.*, histone from calf thymus and globin from cattle hemoglobin, showed that histone formed insoluble compounds with cephalin between pH 2 and 7, whereas with globin appreciable formation of insoluble cephalin compounds was observed only below pH 4; lecithin formed no compounds with globin, but did so with histone between pH 7 and 8 (Chargaff and Ziff, 1939). The cephalin-histone and cephalin-globin compounds, whose composition is summarized in Tables I and II were, in contrast to the corresponding protamine salts, insoluble in organic solvents.

The histone complexes formed at pH 4.7 and the globin complexes prepared at pH 3 to 5 doubtless are salts as will be seen from a comparison of the cephalin-binding capacities included in Tables I and II with the calculated acid-binding capacities which are 1.71 and 1.31 milliequivalents of acid per g. of histone and globin respectively. The lower values observed at about pH 7 with both histone and globin[4] may be ascribed to the decreased dissociation of the weakly basic groups in the protein. At a very low pH a larger number of basic groups in the protein react with cephalin, as demonstrated by the higher amounts of cephalin bound. This is probably due to the basicity of the imidazole group at the low pH.

An application of these observations was found in the reaction between cephalin and oxyhemoglobin (Chargaff, Ziff, and Hogg, 1939). Cephalin, in contrast to lecithin, brought about a disruption or a loosening of the

TABLE II

Composition of Cephalin-Globin Compounds

(From Chargaff and Ziff, 1939)

pH of reaction	Composition of compounds			Cephalin-binding capacity
	P	N	Cephalin in compound on basis of P	
	per cent	*per cent*	*per cent*	*m.eq. per g. protein*
2.3	2.3	5.6	66	2.21
3.0	1.9	6.3	59	1.48
3.8	2.0	7.1	57	1.51
4.2	1.9	6.5	59	1.48
5.0	1.9	8.2	54	1.34
7.1	0.88	11.8	28	0.40

hemoglobin linkage, both in oxyhemoglobin and carbon monoxide hemoglobin, in the following manner:

oxyhemoglobin + cephalin $\rightleftharpoons$ cephalin-globin + hematin.

Since this reaction took place at pH 7 and at extremely high dilutions, the cephalin-globin complex remained in solution. Reduced hemoglobin was not attacked.

Very interesting experiments on the behavior of lipid monolayers and on mixed lipoprotein films were described by Rideal, Schulman, and their collaborators (see, Schulman and Rideal, 1937; Rideal and Schulman, 1939). Compounds between proteins and phosphatidic acids were discussed by Wagner-Jauregg and Arnold (1938).

[4] The cephalin-globin complexes formed at pH 5 and 7 are quite soluble and, therefore, obtained in low yields.

2. *Lipoproteins as Part of the Cell Structure*

Cell Nucleus. There is no strict evidence of the presence of lipoproteins in cell nuclei. In fact, the occurrence in these structures of lipids had long been doubted by histologists (compare Kiesel, 1930, page 158). More recently, however, lipids were demonstrated in nuclei by histological methods and by isolation (Dounce, 1943; Stoneburg, 1939; Wegelin, 1928). Even the nucleolus appears to contain lipids (Fels, 1926).

Ground Cytoplasm. A considerable amount of histological evidence for the occurrence of lipoproteins in the cytoplasm and other cell fractions rests on the phenomenon for which the term lipophanerosis was introduced by Noll (1913). This term takes account of the often observed fact that many tissues contain lipids in a masked form in which they cannot be demonstrated microscopically, unless the material has been treated previously with proteolytic enzymes or with agents, as alcohol, that are able to destroy the lipid-protein links (Parat, 1928). Similar conclusions, based on chemical experiments, were reached by numerous workers, *e.g.*, Biedermann (1924), Bogdanow (1897), Dormeyer (1896), Hoppe-Seyler (1867), Schulze (1895). A comparative study of lipoproteins in tissues and isolated lipoprotein complexes with respect to their behavior towards fat solvents and fat stains was carried out by Grundland and Bulliard (1938).

In the early and more optimistic days of biological chemistry, the schools of Hofmeister, Hoppe-Seyler, Kossel, Miescher, and many others carried out vigorous and bold investigations of the chemistry of the cell. But this period soon passed, and the more recent studies of Bensley and a few others, handicapped as they were by the difficultly accessible material, assume the character of pioneering attempts (compare Bensley, 1942).

Bensley (1938) described a fractionation procedure for washed guinea pig liver cells which, while leaving behind the mitochondria and nuclei, permitted the isolation of a fiber-forming easily coagulable nucleoprotein, termed plasmosin. This material is stated to contain a variable but small amount of lipids. There is no indication that the lipids present are anything but a contamination. The material remaining after the removal of plasmosin, mitochondria, and nuclear chromatin was designated ellipsin (Bensley, 1938). This fraction, consisting of the cellular and nuclear membranes, the linin threads, etc., contains about 25% of lipids, but, amazingly enough, is said to be free of phosphorus (Bensley, 1942). Since the common ether-soluble phospholipids appear to be present in the nuclei (Stoneburg, 1939) and the mitochondria (Chargaff, 1942, a) in a rather low concentration, this would leave the submicroscopic particles and, with less probability, the easily soluble proteins of the cell as the main phosphatide repositories.

Mitochondria. The nature, function, and composition of these extremely

sensitive organelles have for a long time been a baffling subject. (See the review by Bourne, 1942, p. 99, and for the cytological evidence, Guilliermond, 1934, Noël, 1923, and Parat, 1928. The possible relationships between mitochondria, secretory granules, and submicroscopic particles were recently discussed by Claude, 1943.) These structures appear to contain proteins and lipids, the latter in a masked form in which they cannot be demonstrated by the usual fat stains (compare Giroud, 1925, 1929; Kiesel, 1930, p. 94). A method for the isolation of mitochondria from hepatic cells was described by Bensley and Hoerr (1934).[5] The lipid content of this preparation (from guinea pig liver) was estimated by Bensley (1937) by a not too adequate technique as: total lipids 35.3, "lecithin" (*i.e.* acetone-insoluble lipids) 4.2, cholesterol 2.2, "glycerides" 28.9%. Vitamin A is present according to Goerner (1937–8). Bensley (1942) later corrected his estimate of the phospholipid content of mitochondria to 45 to 58% of the total lipids.

A more detailed examination of the lipid composition of mitochondria prepared from rabbit liver was carried out by Chargaff (1942, a). A typical preparation was found to contain N 10.6, P 1.3, S 0.6, ash 1.6%. The fresh structures dissolved readily in 0.005 *N* ammonia from which, by acidification, material of practically unchanged composition could be precipitated. The main characteristics of the lipid fraction were the extremely low percentage of ether-soluble phospholipids and the high content in a fraction that was soluble in chloroform, but insoluble in petroleum ether and acetone. The latter fraction contained cerebrosides and substances of a high P content, perhaps lysophosphatides. The composition of the mitochondria was tentatively summarized (in per cent of total dry material) as: phosphatides 4.0, glycerides 5.4, cholesterol (almost all in form of esters) 1.2, cerebrosides 3.3, lysophosphatides (?) 4.3, extraction residue 72.0.

Other Cytoplasmic Inclusions. The evidence of the lipoprotein nature of the Golgi apparatus rests entirely on microscopical observations. It will, therefore, suffice to refer to recent discussions of this subject (Bourne, 1942, p. 113; Hirsch, 1939, p. 156; Kiesel, 1930, p. 98; Kirkman and Severinghaus, 1938; Parat, 1928). There have been innumerable controversies as to the true or artificial nature of these bodies. It has, as in many other phases of the experimental work discussed here, to be clearly understood that the attempt to impress a static pattern on the unceasing alternations of the living cell will always entail dangers. The cell inclusions, while not artifacts, may very well be petrifacts, the perpetuation of a passing phase.

The secretory granules from liver and the zymogen granules from pan-

[5] The homogeneity of this preparation has been questioned by Claude (1943) who assumes it to consist to a large extent of hepatic secretory granules.

creas formed the subject of studies by Claude (1941, 1943). Their composition is summarized in Table III. The hepatic granules were found to contain a ribonucleoprotein carrying between 22 and 24% of lipids.

Submicroscopic Particles. Heavy tissue fractions consisting of submicroscopic particles of a diameter smaller than 200 mμ have during the past few years been isolated from a number of sources by high speed centrifugation (Chargaff, Moore, and Bendich, 1942; Claude, 1938, 1940, 1941; Henle and Chambers, 1940; Stern and Duran-Reynals, 1939). While it is probable that most of these fractions carried lipids, their lipid content was determined in a few cases only. One of these preparations, the thromboplastic protein from beef lungs, will be discussed in the next section. The heavy particles from chicken tumor tissue (Claude, 1940, 1941) were found to contain a nucleoprotein of the ribose type and 40 to 50% of lipids,

TABLE III

Granules and Particles from Liver and Pancreas

(From Claude, 1943)

Organ	Fraction	N	P	S
		per cent	*per cent*	*per cent*
Guinea Pig Liver	Secretory Granules.	12.1	1.3	0.8
	Small Particles	9.1	1.7	0.7
Rat Liver	Secretory Granules	12.1	1.3	0.9
	Small Particles	9.1	1.6	0.7
Beef Pancreas	Zymogen Granules	11.9	1.9	0.7
	Small Particles	9.2	2.1	0.5

mostly phosphatides. There were indications of the presence of phospholipids similar to the acetal phosphatides of Feulgen and Bersin (1939). Analytical data on preparations of small particles from other tissues are contained in Table III.

Thromboplastic Protein. One of the most thoroughly, although by no means yet sufficiently, investigated lipoproteins of animal tissue is the thromboplastic protein from beef lungs (Chargaff, Ziff, and Cohen, 1940, a, b; Cohen and Chargaff, 1940; 1941, a, b; Chargaff, Moore, and Bendich, 1942). The occurrence in living tissue of a powerful thromboplastic agent which induced the formation of thrombin from its precursor was known for a long time (compare Mills, 1921). Cohen and Chargaff (1940; 1941, b) prepared the thromboplastic protein as the fraction precipitable from saline extracts of beef lungs at a saturation with ammonium sulfate of between 10 and 30%, or at its isoelectric point at pH 5.1. This fraction,

which was not as pure as the preparations obtained later by a different method, had an isoelectric point near pH 5.1 and contained about 18% of firmly held lipids that could be removed from the acetone-washed protein by extraction with alcohol-ether, but neither with chloroform nor with ether alone. Some of the properties of this lipoprotein (behavior towards heparin, immunological characteristics, etc.) will be discussed in Section V of this article. Crude electrophoretic experiments in the Theorell chamber showed that, whereas at pH 5.1 a mixture of free lecithin and cephalin moved in an electric field, this was not the case with the phosphatides contained in the thromboplastic protein, and that even at pH 8.8 almost no dissociation took place. The examination in the Tiselius apparatus showed these preparations to be 90 to 95% homogeneous with respect to their electrophoretic behavior; a small, faster moving component presumably consisted of nucleic acid.

The protein component, after the removal of the lipids, had no thromboplastic activity. The lipids proved to be an extremely complex mixture (Cohen and Chargaff, 1941, a). They consisted of alcohol-soluble and alcohol-insoluble phosphatides, both of which showed clotting activity, and of sphingomyelin. Among the split products, palmitic, stearic, and unsaturated acids, choline, ethanolamine, and glycerophosphoric acid could be identified.

The thromboplastic protein preparations isolated by the methods mentioned above, were, on examination in the ultracentrifuge, found to be inhomogeneous with respect to particle size. Considerably more potent and homogeneous (with respect to both electrophoretic and ultracentrifugal properties) preparations of the thromboplastic protein were obtained by the fractional ultracentrifugation of beef lung saline extracts (Chargaff, Moore, and Bendich, 1942). A typical preparation contained N 8.9, P 1.3%. The partial specific volume of the protein was $V_{27} = 0.87$, the sedimentation constant $s_{20} = 330$ S, the diffusion constant $D_{20} = 0.38 \times 10^{-7}$. This corresponds to a particle weight from rate of sedimentation of 167 million. Despite the calculated frictional ratio $f/f_0 = 1.41$, electron micrographs revealed the presence of a large percentage of almost perfect spheres with a diameter of 80 to 120 mμ. The electrophoretic mobility of these preparations was found at 8.0 cm.^{2} volt^{-1} sec.$^{-1}$ $\times 10^{-5}$ at pH 7.5 and at 8.4 at pH 8.6. Their thromboplastic activity was extremely high, as little as 0.008 γ still being demonstrable by clotting tests. It may be of interest to note that the thromboplastic protein preparations isolated by means of the ultracentrifuge exhibited marked phosphatase activity. The thromboplastic protein from lungs is in many respects reminiscent of the submicroscopic particles of tissue cells discussed in the preceding section. It is characterized by a high degree of homogeneity with regard to

particle size which is lacking in most heavy protein fractions isolated from tissue. Whether similar material occurs in extracellular fluids, remains to be established. While circumstantial evidence would seem to assign it a place within the cell, the true relation of the thromboplastic lipoprotein to the mitochondria, the secretory granules, and the submicroscopic particles of the cell and, perhaps, also to the thrombocytes of circulating blood still is unknown.

Egg Yolk. The lipid-vitellin complex present in hen's egg yolk has for many years been considered as the classical example of a naturally occurring lipoprotein (Hoppe-Seyler, 1867; Osborne and Campbell, 1900). It therefore is the more surprising that until recently (Chargaff, 1942, a, b) no attempt was made to characterize the intact lipoprotein and the lipids carried by it. The lipovitellin complex was prepared from egg yolk which, after dilution with an equal volume of saturated sodium chloride solution, had been repeatedly extracted with ether until no more uncombined lipids were removed (Chargaff, 1942, a). Repeated dialyses and ether extractions of the solutions of the resulting precipitate in 10% sodium chloride solution yielded a final product containing N 13.0, P 1.5, S 0.9, ash 3.7, total lipids 23, phosphatides 17.9%. Only a portion of the phospholipids present in egg yolk (18.8%) was firmly bound to the protein. No essential difference in the composition of the phosphatides occurring in the free state and of those combined with vitellin was observed; nor was there any divergence in the rate of formation of these two lipid fractions, which seemed to be in equilibrium, as revealed by experiments with the radioactive phosphorus isotope (Chargaff, 1942, b). The yolk phosphatides were almost exclusively derivatives of choline and ethanolamine; amino acids were absent (compare also Chargaff, Ziff, and Rittenberg, 1942).

Plastids. The plastids of plant cells, *viz.*, the chloroplasts, chromoplasts, and leucoplasts, appear all to contain lipids. (For chloroplasts and the small particles, the grana, see the review by Frey-Wyssling, 1937–8.) Isolated chloroplasts were found to contain up to 36% of lipids, but only a small amount of phosphatides (Menke, 1940). Since most of the lipid fraction could be extracted from the chloroplasts by ether (Menke, 1939) or even by 85% acetone (Neish, 1939), the existence in these structures of lipoproteins is by no means certain. The small particles from carrot and spinach juice were studied by Straus (1942). He isolated a number of fractions of widely different particle size and lipid content. It remains to be established whether these chromatophores may be considered as lipoproteins. The presence of heavy homogeneously sedimenting pigmented particles in plant juices (s_{20} = 77 S for cucumber juice) was observed by Price and Wyckoff (1938).

In this connection, mention should be made of a very important conju-

gated protein, *viz.*, the visual purple. Indications of the presence of phospholipids in purified preparations of visual purple were obtained by Krause (1937) and this was confirmed in a more detailed investigation by Broda (1941). The bond between the phosphatides and the chromoprotein does not appear to be strong: the lipids are removed by treatment with chloroform and by electrodialysis (Broda, 1941).

Cell Membrane. This obviously is not the place to sketch in even the vaguest manner the innumerable discussions on the nature of the cell wall and the plasma membrane in which biological dialectics have long excelled. Very instructive surveys of the chemical and physical evidence with regard to the structure of the plasma membrane will be found in a number of reviews among which those by Danielli (1942), Harvey and Danielli (1938), Picken (1940), and Schmitt and Palmer (1940) may be cited here. The biology of the cell surface is discussed in the monograph of Just (1939). The classical substrate of membrane studies, the erythrocyte, was reviewed by Ponder (1934) and the erythrocyte surface by Parpart and Dziemian (1940).

Whereas much is known about the chemistry of cell surface models, the same, unfortunately, cannot be said of the plasma membrane itself. A monistic theory of so varied a system would appear to present particular dangers, but, inasmuch as natural science progresses by orderly simplification, the old conception of Overton of a lipid layer membrane, with the modification that the lipids are held in place and oriented by proteins, probably still is useful. The existence of a continuous lipid layer (or of a mosaic in which this layer preponderates) satisfactorily explains the permeability conditions prevailing at the oil-water interfaces, whereas the surface tension and elastic properties of the membrane are in agreement with the assumption of a lipoprotein structure. The ability of other lipids or of substances, like heparin or certain detergents, to displace the membrane lipids from their combination with proteins may be of great importance in cytolytic and other phenomena.

The chemical evidence for the occurrence of lipoproteins in red cells is more or less circumstantial. Parpart and Dziemian (1940) showed that between 40 and 60% (varying with the animal species) of the lipids contained in red cell ghosts could not be extracted with ether alone, but yielded to treatment with alcohol-ether. Sigurdsson (1943) isolated, as disintegration product of the stroma, small particles from hemolyzed red cells, which could be sedimented in the ultracentrifuge, containing about one-third of lipid material. The stroma protein examined by Jorpes (1932) had an isoelectric point near pH 5.5; if it really represented the protein of the red cell envelope, the existence of salt-like lipoproteins in the membrane would be unlikely.

Very little is known of the composition of leucocytes. In an as yet unpublished study of the polymorphonuclear leucocytes of the rabbit carried out in the laboratory of this author, no heavy lipoproteins could be discovered. The blood platelets, on the other hand, seem to be lipoproteins and are in many respects reminiscent of the submicroscopic cell particles discussed above (compare Chargaff, Bancroft, and Stanley-Brown, 1936).

3. *Bacteria*

Firmly Bound Lipids. It has been repeatedly observed by Anderson and collaborators in the course of their work on the composition of acid-fast bacteria that the thorough extraction of the bacterial cells with alcohol-ether and chloroform yielded only a part (about one-half) of the lipids present in the material. Additional large amounts of lipids could be removed from these partially defatted bacteria by treatment with alcohol-ether containing 1 per cent of hydrochloric acid. *Mycobacterium leprae* thus yielded 17% of firmly bound lipids in addition to 19% of easily extractable fractions (Uyei and Anderson, 1931–32); *Mycobacterium phlei* similarly contained 9% of firmly bound and 8% of free or loosely held lipids (Chargaff, Pangborn, and Anderson, 1931). The same is true of other bacterial species: *Corynebacterium diphtheriae,* for instance, was found to contain about 5% of free lipids and the same amount of firmly held material (Chargaff, 1931). Anderson, Reeves, and Stodola (1937) performed a more detailed investigation of the firmly bound lipids of the human tubercle bacillus. The isolated lipid-carbohydrate complexes of varied composition clearly were present in the cellular structure in some chemical combination; but it remained undecided whether in the bacterial cells they were bound to proteins or to carbohydrates.

Antigens. A number of bacterial antigen complexes have been described which appear to contain lipoproteins, although the phosphatides are not essential for the antigenic activity. Morgan and Partridge (1940, 1941) isolated a complex consisting of a phospholipid, a conjugated protein, and a polysaccharide from *Bact. dysenteriae* (Shiga). The phosphatide component could be removed without impairing the antigenic properties of the complex. Phospholipid-containing complexes were obtained from *Brucella melitensis* by Miles and Pirie (1939) and from *Bact. typhosum* by Freeman and Anderson (1941).

Bacterial Surface. Curious anti-bacteriostatic and anti-bactericidal effects of phosphatides, possibly due to the formation of lipoproteins at the cell surface, were observed in a number of cases. There was, however, little difference between the phosphatides employed. Lecithin, cephalin, phosphatidyl serine, and sphingomyelin were reported to counteract the effect of detergents on Gram-positive microorganisms (Baker, *et. al.*, 1941; com-

pare also Miller, *et al.*, 1942, for the effect of salmine and histone). Similar results were obtained with gramicidin to which phosphatidyl serine and ethanolamine cephalin were found antagonistic (Dubos and Hotchkiss, 1942), and with a bacteriostatic protein from wheat which was inhibited by lecithin, phosphatidyl serine, and an inositol-containing phosphatide fraction (Woolley and Krampitz, 1942).

4. *Viruses*

It is not certain that lipoproteins form part of viruses. None of the highly purified plant viruses appear to contain lipids. With respect to animal viruses, it is so hard at present to decide whether the macroproteins, some of which do contain lipids, isolated from infected tissue are virus compounds, normal tissue components contaminated with the virus, or substances produced by the action of the virus on the host tissue, that a discussion of the lipoprotein nature of animal viruses is not advisable. There is one possible exception, *viz.*, the elementary body of vaccinia. Hoagland, Smadel, and Rivers (1940) found their preparations to contain an average of 5.8% of total lipids of which 1.4, 2.2, and 2.2% were cholesterol, phospholipids, and glycerides respectively. Cholesterol could be removed by means of ether, the other two fractions only by extraction with alcohol-ether. Similar observations on the presence of lipids in the elementary bodies were made by McFarlane, *et. al.* (1939).

5. *Extracellular Lipoproteins*

Blood Plasma and Serum. The manner in which the lipids are carried in plasma and serum, the limpidity of which was frequently considered as a sign of the existence of special lipid-protein complexes (compare Sörensen, 1930, p. 310) has long been a matter of intense speculation. It will be best to relinquish any attempt at a historical treatment of this problem and to divide the discussion according to the two main lines of approach, *viz.*, the electrophoretic separation and the fractional precipitation of the serum components.

The gentlest separation method for serum proteins probably is the one based on the differences in mobility of the serum components in an electrical field. The separation experiments usually are carried out in the electrophoresis apparatus of Tiselius (1937). The protein fractions from normal human serum thus separated, *viz.*, the albumin and α-, β-, and γ-globulins, were subjected to a lipid analysis by Blix, Tiselius, and Svensson (1941). The results obtained with three different sera are summarized in Table IV. It will be seen that, while the results were subject to very considerable variations, the major part of the serum phospholipids and cholesterol was carried in the α- and β-globulin fractions. The composition of the fractions

was not materially changed by repeated electrophoresis, and the authors concluded that the serum lipids did not occur in the free state but were bound to the proteins in an unknown manner. The β-globulin fraction appeared to contain the lipids in a coarser form than did the other proteins, and usually was opalescent. It may, in view of the effect of heparin on lipoproteins, which will be discussed in Section V, be significant that the "β-globulin disturbance", a tall sharp spike which, on the descending electrophoresis patterns, normally accompanies the β-globulin boundary (Longsworth, Shedlovsky, and MacInnes, 1939), disappeared in human plasma to which heparin was added (Chargaff, Ziff, and Moore, 1941).

The bonds between the lipids and the serum proteins appear in the main to be weak ones. In plasmas from obstructive jaundice and nephrosis cases, large β-globulin peaks are observed which, on extraction of the

TABLE IV

Lipid Distribution in Normal Human Serum Proteins

(From Blix, Tiselius, and Svensson, 1941)

The results are given in per cent.

Serum No.	Albumin		α-Globulin		β-Globulin		γ-Globulin	
	Cholesterol	Lipid P	Cholesterol	Lipid P	Cholesterol	Lipid P	Cholesterol	Lipid P
I	0.99	0.06			4.6	0.31		0.05
II	1.30	0.13		0.30		0.44	0.71	0.03
III	0.92	0.08	4.45	0.28	12.7	0.46	0.11	0.08
Mean	1.07	0.09	(4.45)	0.29	8.65	0.40	0.41	0.04
Calculated as phospholipid		2.25		7.25		10.0		1.0

plasma with ether, are reduced considerably (Longsworth, Shedlovsky, and MacInnes, 1939; Longsworth and MacInnes, 1940). Similarly, Blix (1941) reported that by the precipitation of the human serum proteins with acetone, followed by the extraction with acetone-ether at a low temperature, all the cholesterol and three-quarters of the phospholipids, chiefly lecithin, were removed; the phosphatides remaining in protein combination were mainly cephalin. The action of the acetone may, however, have been quite drastic, because it is known that only little lipid material can be extracted when serum is shaken with ether (Sörensen, 1930, p. 311).[6] It

[6] In this connection, a very interesting observation of McFarlane (1942) should be mentioned. He found that a large proportion of the serum lipids was transferred into the ether phase, when ether-containing serum was frozen to a temperature of below −25°C. and allowed to thaw.

may be stated that little progress can be expected in this difficult field before more is known of the types and quantities of the various lipids contained in serum and of their differential distribution in the serum components. The joint extraction of substances as disparate as, for instance, cephalin and cholesterol often is nothing more than an accident of solubility and connotes neither common occurrence nor common function.

The presence of lipids and steroids in serum fractions prepared by fractional precipitation has often been observed (*e.g.*, Chick, 1914; Bang, 1918; Troensegaard and Koudahl, 1926; Theorell, 1926, 1930; Gardner and Gainsborough, 1927; Macheboeuf, 1929, 1937). Sörensen (1930, p. 308), in a beautiful discussion of these experiments, pointed out that the addition of a precipitating agent to serum could give rise to the association or dissociation of the solutes and the precipitation of insoluble complexes, the composition of which would not give a true picture of the conditions prevailing in the original solution.

It is with this warning in mind that attempts to prepare lipoproteins by the fractionation of serum have to be considered. Macheboeuf (1929) reported that from serum, brought at neutral pH to half saturation with ammonium sulfate and freed of the precipitated globulins, an albumin-lipid complex could be isolated by acidification to pH 3.8. By repeated precipitations of this material from its clear aqueous solution, a fraction of constant composition was obtained. It contained 17.9% of cholesterol esters, 22.7% of phospholipids, 59.1% of proteins, and could only by treatment with hot alcohol-ether be freed of lipids. For further experiments with this complex which, not too aptly, was termed "the lipoprotein coenapse of plasma," the monograph of Macheboeuf (1937) should be consulted.

It may be mentioned here that crystalline albumin from human serum has been found to contain a small amount (2 to 3%) of free fatty acid which could be extracted only after the denaturation of the protein (Kendall, 1941).

Milk. Palmer and collaborators have in a series of studies examined the properties of the stabilizing film on the fat droplets in cream (compare Palmer and Wiese, 1933; Moyer, 1940). This film forming a membrane around the fat globules appears to be a lipoprotein complex between phospholipids and a protein differing in properties from the common milk proteins.

V. Remarks on Methods

1. *Isolation of Lipoproteins*

There are no general methods which could be said to lead to the isolation of lipid-protein complexes from tissue. Each lipoprotein will, for the time

being, have to be treated as a special case and studied individually. The principal methods and the instances in which they were used, discussed more fully in preceding sections of this article, may be summarized as follows:

I. Isoelectric precipitation of tissue extracts (occasionally in the presence of ammonium sulfate): thromboplastic protein from lungs (Cohen and Chargaff, 1940); lipoprotein from serum (Macheboeuf, 1929); plant chromatophores (Straus, 1942).

II. Dialysis of strong salt extracts: lipovitellin from egg yolk (Hoppe-Seyler, 1867; Chargaff, 1942).

III. Electrophoretic separation: lipoproteins from serum (Blix, Tiselius, and Svensson, 1941); thromboplastic protein from lungs (Cohen and Chargaff, 1941, b); milk lipoprotein (Moyer, 1940).

IV. Ultracentrifugal separation: tissue macroproteins (Claude, 1941); thromboplastic macroprotein from lungs (Chargaff, Moore, and Bendich, 1942).

V. Fractional centrifugation of cell organelles: nucleus (Dounce, 1943); mitochondria (Bensley and Hoerr, 1934); granules from liver and pancreas (Claude, 1941, 1943); plastids (Menke, 1939; Neish, 1939); blood platelets (Chargaff, Bancroft, and Stanley-Brown, 1936). These organelles, some of which possess an internal structure, can, of course, only provisionally be considered as lipoproteins.

2. *Shape, Dimensions, Molecular Weight, Electrophoretic Mobility*

The only lipoprotein studied in more detail with respect to these properties is the heavy thromboplastic protein isolated from lungs by means of the ultracentrifuge (Chargaff, Moore, and Bendich, 1942). Electron micrographs of the preparation revealed the presence, together with some aggregated material, of a large percentage of almost perfect spheres with a diameter of 80 to 120 mμ. The particles probably are highly hydrated, as the calculated frictional ratio $f/f_0 = 1.41$ would give an axial ratio of 8 for a prolate ellipsoid. The partial specific volume of the protein was very high, $V_{27} = 0.87$, as would be expected of a lipid-protein complex. The sedimentation constant $s_{20} = 330$ S and the diffusion constant $D_{20} = 0.38 \times 10^{-7}$ correspond to a very high particle weight from rate of sedimentation, *viz.*, 167 million. The electrophoretic mobility at pH 8.6 was 8.4 cm.^{2} volt^{-1} sec.$^{-1}$ $\times$ 10^{-5}.

3. *Fine Structure*

The fine structure of lipoprotein systems is of particular interest in connection with studies on the X-ray diffraction patterns of plasma membranes and the nerve myelin sheath (compare Schmitt and Palmer, 1940). Pal-

mer, Schmitt, and Chargaff (1941) reported X-ray diffraction studies of cephalin-histone and cephalin-globin (see Section IV) and compared the results with those obtained with individual lipids and lipid mixtures (Bear, Palmer, and Schmitt, 1941; Palmer and Schmitt, 1941). The dry cephalin-protein complexes showed diffraction spacings that were, for different proteins, 10 to 15 A greater than the characteristic spacing of cephalin (43.8 A). In the wet state, cephalin showed very long spacings (about 123 A for a 33 per cent emulsion), whereas cephalin-histone, which barely took up water, gave only slightly increased long-spacings. These synthetic lipoproteins were assumed to consist of bimolecular leaflets of cephalin separated by (perhaps monomolecular) layers of protein.

4. *Removal of Lipids*

The treatment of the lipoproteins with alcohol-ether, if necessary with heating, is the method most commonly used for the extraction of the lipids. This procedure does not permit the recovery of unaltered proteins. The much less drastic employment of heparin, discussed in the following paragraph, will under certain conditions be found very useful, but it is not generally applicable. A systematic study of the action of detergents on lipoproteins still is lacking, but reference may be made to the use of soaps for the splitting of serum lipoproteins (sodium oleate and potassium dibromostearate: Macheboeuf and Tayeau, 1938; sodium ricinoleate: Tayeau, 1939).

The freezing to −30° C. in the presence of ether (compare McFarlane, 1942) of a solution of the thromboplastic protein from lungs was found to bring about the disintegration of the lipoprotein into several fragments (Chargaff and Bendich, 1944). This finding tends to emphasize the importance of the lipids in maintaining uniformity of particle size and electrophoretic mobility of the lipoprotein complex.

5. *Action of Heparin*

The effect of heparin on lipoproteins was investigated with the thromboplastic protein, lipovitellin, mitochondria, and a synthetically prepared cephalin-histone complex. The reason for these studies was the possibility that heparin, because of its strongly polar properties and its occurrence in numerous organs, could exert a controlling influence on the attachment of acidic prosthetic groups to proteins. The information gathered so far is by no means sufficient, but it may perhaps permit a tentative classification of lipoproteins with respect to their behavior towards heparin; *viz.*, (1) complexes that are disrupted by heparin with the liberation of the combined lipids and the simultaneous formation of a protein-heparin compound, and (2) complexes which give rise to heparin-lipoprotein compounds without losing the bound lipids.

The first class is exemplified by the thromboplastic protein from lungs (Chargaff, Ziff, and Cohen, 1940, b) and, perhaps, also by some of the extracellular lipoproteins. All the other lipoproteins mentioned in the preceding paragraph belong to the second class: the combination with heparin occurs without the displacement of the bound lipids (Chargaff, Ziff, and Cohen, 1940, b; Chargaff, 1942, a). Whether these differences can be correlated with the nature and frequency of the basic groups in the carrier protein, remains to be seen. It is, for instance, remarkable that lipovitellin, as it is isolated from egg yolk, carries a certain fairly constant proportion of the yolk lipids and appears to be in equilibrium with the uncombined lipids, but is still able to combine with heparin (Chargaff, 1942, a, b).

6. *Antigenic Properties*

Our knowledge of the antigenic behavior of purified lipoproteins is scant. A comparison of the immunological behavior of native lipid-containing serum albumin and globulin fractions with that of the corresponding defatted proteins failed to reveal decisive differences between these antigens or their antisera; the lipids did not appear to influence the specificity (Went and Lissák, 1934). Similarly, the heparin-protein complex produced by the liberation of lipids from the thromboplastic protein from lungs (see the preceding paragraph) reacted with the antiserum to the intact lipoprotein (Cohen and Chargaff, 1940). This seems to indicate that the phospholipids were not essential for the capacity of this lipoprotein to combine with the antibody and that the specificity rested entirely with the protein component.

The author fears that the absence from his article of so-called General Principles will be noted. But at the present state of biological chemistry, general principles, while telling us all about everything, tell us nothing about anything particular. To gain validity, they will have to be based on more than the few observations extant at present.

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Structural Proteins of Cells and Tissues

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Introduction

Proteins and complexes of proteins with lipids and carbohydrates form the basis of the structural components of cells and tissues. Whereas these structural proteins have, in the past, been investigated chiefly by the techniques of classical morphology, advances during the last two decades in the chemistry of these complex molecules and in physical techniques for investigating their internal architecture have laid the foundation for new correlations between structure and function. In this new synthesis, data of analytical chemistry must be related not only to ultrastructural considerations but to the whole body of available information of morphology, physiology, and biochemistry.

In certain tissues, such as muscle, it is possible to extract the structural protein and characterize its chemical and physical properties. When more detailed ultrastructural information is available it will probably not be long before it will be possible to consider the mechanism by which metabolic energy is coupled with the contractile protein mechanism. Since there is considerable similarity in the general properties of fibrous proteins, such as contractility, extensibility, and elasticity, it is probable that when the structure and properties of one protein, such as myosin, are well understood this information will greatly facilitate our understanding of other proteins, many of which are not as amenable to analysis as myosin.

The X-ray work of Astbury, which started with the keratins but soon led to generalizations about physiologically more interesting proteins, is a good illustration of this point. The structure of compact fibers, like the keratins, is still far from being thoroughly understood, and greater difficulties may be expected with the more solvated proteins which are intimately connected with cellular metabolism, such as those of muscle and nerve, the tenuous fibrils of the cytoplasm, and the chromosomes. The present review is meant to be a comparative survey of certain proteins selected as representative of the above range of physiologically interesting structures, chief emphasis being given to results obtained in the last few years.

I. Intracellular Fibers

Many valuable clues concerning the form and chemical properties of the intracellular proteins have been obtained by the classical methods of cytology and histochemistry. However, owing to the instability of these structures to fixing agents and cytological procedures, the danger of artifact formation has made the results suspect to many chemists and physiologists. Needed are methods which can be applied to unfixed cells and which can reveal information about molecular organization of their structures.

There are at present two general classes of methods which provide such information. The first class comprises chemical methods of characterization of proteins extracted from cells and isolated by differential centrifugation. The second class consists of optical procedures, some of which can be applied to the living material, which give information about the submicroscopic organization. These include the ultramicroscope, ultraviolet, polarizing and electron microscopes, and X-ray diffraction.

1. Chemistry of Extractable Proteins

The extraction of intracellular protein components by suitable reagents, such as water and salt solutions, and the isolation of these components by differential centrifugation has proven a powerful tool in determining the chemical composition and probable rôle of these constituents in the cell. While it is not difficult to isolate fractions from tissue cells by this method, considerable confusion resulted when individual fractions were related to structures, such as mitochondria and secretion granules, which have long been objects of study by cytologists. A part of this confusion is doubtless due to alterations in the structures during isolation and to the obvious difficulty of effecting a complete separation of a component, such as mitochondria (which may vary somewhat in composition in different tissues) from all the other complex and interrelated structures in the cell. The results which have been obtained, as well as the opinions of the individual workers, are contained in the symposium entitled "Frontiers in Cytochemistry" (*Biological Symposia* vol. **10,** 1943), held in honor of Prof. R. R. Bensley, a pioneer in this field. In the following resumé emphasis is placed on the isolated fractions themselves, rather than on their supposed relation to recognized cytological entities. Particularly pertinent are the papers by Lazarow, Barron, Claude, Mirsky and Pollister, Hoerr and Bensley.

a. *Salt-extractable Proteins*

Nuclear Chromatin. Claude (1942) and Claude and Potter (1942) isolated chromatin from resting nuclei of leukemic cells. After crushing the nuclei with sand, extraction with water or dilute saline yields strands which resemble, even in some detail, the chromosomes of the intact nucleus.

The analyses show P = 3.6–3.8%, N = 15.3–15.8%, indicating a nucleic acid content of about 40%. The nucleic acid was demonstrated to be of the desoxyribose type. It is to be expected that much may be learned from such preparations, not only about the chemistry of chromosomes but about their ultrastructure as well.

Mirsky and Pollister (1942, 1943) obtained nucleoproteins from sperm heads and the nuclei of tissue cells by extraction with 1 *M* NaCl (2 *M* in the case of marine forms). The extraction yields a viscous solution which shows strong negative stream birefringence. The material contains 3.9% P and 15.5% N and is considered to be a nucleoprotein, the nucleic acid being of the desoxyribose type. The authors are at pains to prove that the material comes only from the nucleus, which is generally considered to be the only source of thymonucleic acid. They state that histones or protamines constitute the sole protein component. The almost complete absence of tryptophan, which is found in most other proteins, is cited as evidence for this view.

TABLE I

Content of Nucleic Acid, Histone, and Chromosomin in Cell-Chromatin

According Stedman and Stedman

Nuclei	Nucleic acid	Histone	Chromosomin
	%	%	%
Cod sperm	28	12	60
Ox spleen	24	16	50
Walker rat sarcoma	26	1.6	72.6

Stedman and Stedman (1943) believe that the above view of the composition of chromatin is erroneous. They state that the principal component of chromatin is an acidic protein to which they give the name *chromosomin.* Though 25% of the constituent amino acids are basic, the protein is acidic because of high concentrations of glutamic and aspartic acids. Contrary to the finding of Mirsky and Pollister, the tryptophan content is found to be relatively high. The content of nucleic acid, histone, and chromosomin in the chromatin of several types of cells is shown in Table I. The reaction of chromosomin with histological stains and with the Feulgen reagent is also discussed.

In view of the importance of this discovery for our views of the properties, composition, and structure of chromosomes it seems best to withhold appraisal of the work until the details of the analysis have been published and the work confirmed independently. Apparent contradictions with experimental facts come to mind at once and some of these have already been discussed (Callan, 1943; Barber and Callan, 1944).

Plasmosin. This name was given to a fibrous protein component isolated from liver by Bensley (1938). After extraction of soluble constituents of separated cells by dilute saline, plasmosin was extracted with cold 10 per cent NaCl buffered to pH 6.8. When placed in water it yields fibrous, contractile strands. Prolonged standing in water causes the protein first to gel and then to pass into solution. In this state it has an isoelectric point at pH 3.2. It is a nucleoprotein, having a P content of 3.7%, and Lazarow (1943) states that the nucleic acid is of the desoxyribose type. Mirsky and Pollister (1943) believe that plasmosin is identical with the nucleoprotein which they extract from the nucleus. They cite the negative Feulgen reaction of the cytoplasm as proof of the absence of thymonucleic acid therein and conclude that plasmosin is not a cytoplasmic constituent as Bensley supposed. Lazarow (1943) agrees that the composition of plasmosin is essentially the same as Mirsky and Pollister's nucleoprotein but disputes their evidence that it is present only in the nucleus. It was Bensley's original thesis that plasmosin represents the protein responsible for sol-gel reversal and for the formation of fibrous structures in cytoplasm. The isolation and characterization of this fibrous cytoplasmic material is highly important but further work is required to determine whether or not it is included in the fraction called plasmosin by Bensley. The problem resembles somewhat that of the structure of the nerve axon (see Section II), in which fibers may be produced by relatively small concentrations of high molecular weight protein.

Submicroscopic Protein Particulates (*Microsomes*). Claude (1941, 1942, 1943, a, b) obtained relatively pure preparations of the submicroscopic particles which make up a considerable portion of the hyaloplasm or ground substance of protoplasm. These granules, called by him *microsomes*, are obtained by water extraction of tissues, in some cases following preliminary grinding of the tissue with sand. They contain 9% N and 1.5% P, and are characterized as a complex of ribonucleoprotein with phospholipids the latter making up 45% of the total and consisting in part of acetalphosphatide. In the living cell they can be observed in the ultramicroscope as refractile bodies having an apparent size of 50 to 300 mμ. They are said to make up about 15% of the dry weight of the cell, or about one-quarter of the dry weight of the cytoplasm. When it is remembered that the cytoplasm of liver cells contains many formed inclusions such as mitochondria, Golgi apparatus, vacuoles, glycogen granules, etc., it would appear that the microsomes must represent the major portion of the protein of the ground substance. Such a system might be visualized as a colloidal sol. However, it is probable that the characteristic non-Newtonian flow, elasticity, high viscosity, and stream birefringence of protoplasm are due to submicroscopic *linear* arrays, perhaps of a reticular nature, as was sup-

posed in one of the earliest theories of protoplasmic structure and documented by more recent evidence (Seifriz, 1938, 1942; Frey-Wyssling, 1938, b). Whether or not submicroscopic particles, such as microsomes, have a structural or functional relationship with the reticular components is not known.

Secretory Granules. Claude (1943, a, b) isolated another fraction from saline extracts of liver and pancreas cells which is said to contain almost exclusively the zymogen or secretory granules long known to cytologists. They have diameters of 0.5–2μ and contain 12% N and 1.3% P. Like microsomes they are ribonucleoprotein-phospholipid complexes, the lipid portion representing 20–24% of the total. The *d*-amino acid oxidase is said to be localized in these granules, none being in the microsome fraction. Claude also finds (personal communication) that practically all of the extractable succinic dehydrogenase and cytochrome oxidase are associated with the secretion granules, very little being present in microsomes. Transaminase activity is also associated with these granules but not with the microsomes.

Relation between Mitochondria, Microsomes, and Secretory Granules. Cytologists have long sought, by observations of morphology and staining reactions, to establish the origin of formed elements in the cell. The apparent disappearance and "*de novo*" formation of mitochondria, and the complex interplay between mitochondria, Golgi apparatus, prozymogen and zymogen granules may be cited as examples. The chemical method provides a powerful tool to investigate such problems. Bensley and Hoerr (1934) reported the isolation of mitochondria from liver. Claude (personal communication) finds that when mitochondria, isolated from lymphoid cells of rat lymphosarcoma, or secretory granules isolated from liver or pancreas, are washed in saline and then in water, they decompose into submicroscopic particulates which cannot be distinguished from microsomes. Both are complexes of ribonucleoprotein and lipids. The lipid portion of each contains about 12% of lipositol, though mitochondria contain 25%, microsomes 45% by weight of lipids. Microsomes and secretion granules are also closely similar in composition, and Claude suggests that the latter may be derived from the former or have a common origin with them.

Lazarow (1943) and Hoerr (1943) agree that the composition of mitochondria and microsomes is similar qualitatively but state that there are quantitative differences. Hoerr believes that Claude's 'secretory granule' fraction really contains mitochondria. Further work is required to establish the reasons for these disagreements. Lazarow (1943) and Barron (1943) discuss the rôle of mitochondria and microsomes as carriers for enzymes involved in cellular metabolism.

Rôle of Protein Particulates in Cellular Enzyme Reactions. In addition to the components mentioned above, various high molecular weight particles having enzymic activity have been isolated (see particularly Stern, 1939, 1943; Korr, 1939; Henle and Chambers, 1940; and Kabat, 1941). Phosphatase, cytochrome oxidase, succinic dehydrogenase, and heterogenetic tissue- and organ-specific antigens have been thus localized.

Considering the relatively mild treatment by which the particulates are isolated (hence the probability that they represent complexes rather than single molecular species) and their great surface, it is not surprising that enzymes have been found associated with them. The interesting point will be whether or not a given type of particulate bears certain enzymes specifically and to the exclusion of other particulates and whether the enzyme, rather than being adsorbed non-specifically, is an integral part of the particulate chemically. In this case there will indeed have been established a bridge between energy liberating catalysts and structure in protoplasm. The differential centrifugation method avoids many of the artifacts encountered by biochemists who have studied extracts obtained by more drastic means. Nilsson (1943) contrasted the characteristics of fermentation processes in intact yeast with those of extracts customarily used by carbohydrate chemists. By controlled differential centrifugation of yeast cells he obtained a partial system which reproduced the chemical fermentation characteristics of the intact cell much more closely. He believes a lipid-protein structure is necessary as a carrier for enzymes in fermentation processes. This structure is not present in extracts obtained by the usual, more drastic means.

b. *Urea-extractable Proteins: Renosin (Structure Protein I)*

Szent-Györgyi (1940) and Banga and Szent-Györgyi (1940, a, b) have described a preparation called *renosin* or *Structure Protein I*, which is obtained by extracting ground tissue with 30 per cent urea in a buffered solution of KCl (0.6 *M*). Its concentration in tissues is given as roughly 30% of the total protein content; hog heart muscle is said to contain more renosin than myosin! It is considered responsible for structure formation in protoplasm. The material shows high viscosity and strong negative stream birefringence and is considered to contain a nucleoprotein. It is difficult to regard such an obviously complex and variable moiety as a chemical entity. Its relation to components, such as plasmosin, isolated by less drastic means, remains to be determined.

c. *Alkali-extractable Proteins*

Ellipsin. After tissues have been thoroughly extracted with water and 10 per cent NaCl their general microscopic appearance is little altered,

though as much as 70% of the protein has been removed. Such residues, consisting of cell and nuclear membranes, linin framework, and so on, may be dissolved by extraction with $M/2$ NaOH. This material has been called *ellipsin* by Bensley and Hoerr (1934), and is stated to be free of P and to contain 25% by weight of lipids (Bensley, 1942). Ellipsin is no doubt a complex of a number of constituents, possibly even including some collagen, but it would be valuable to characterize it further and to investigate the possibility that the structural framework of cells in various tissues and from different animal forms may contain a class of closely related proteins. The relation of ellipsin to the stroma protein of erythrocytes cannot be stated from the present data.

Structure Protein II. This is the name given by Banga and Szent-Györgyi (1940, b) to material which is obtained by extracting the tissue remainder from the renosin extraction with 30 per cent urea plus 2 per cent NaOH with heating at 60°C. for 5 minutes. Like renosin, it shows stream birefringence, but unlike renosin, it retains this property even after boiling for 15 minutes. The material obviously contains a number of constituents, the nature of which has not been determined.

2. Ultrastructure of Intracellular Proteins

Examination of living protoplasm by the ultramicroscope has produced evidence of the existence of submicroscopic granules and fibrils but the information is chiefly of a qualitative sort. Monné (1940) claims that, by a combination of the ultramicroscope and the polarizing microscope, greater phase differences are obtained than with the polarizing microscope alone, making it possible to observe colors with the gypsum plate with objects too thin to give such phase differences with transmitted polarized light. As yet the polarizing ultraviolet microscope has not been developed; this method should provide a powerful tool because of the wide distribution, in cell structures, of nucleic acid which should confer natural dichroism in the ultraviolet.

Plotnikow and Splait (1930) and Plotnikow and Nishigishi (1931) investigated the "longitudinal" scattering of infra red light by liquids and suspensions. When a collimated beam of infra red rays passes through a liquid there is little or no Tyndall scattering; rather the beam spreads out conically, giving an image on a sensitized plate around the central beam, the diameter of which is proportional to the scattering. A beginning at a theoretical treatment of the phenomenon has been made by Neugebauer (1940). Lepeschkin (1939, 1940, 1942) studied the effect in suspensions of cells, both in the normal state and after treatment by narcotics and killing agents. Large effects were observed which were attributed to fibrous proteins. The scattering is said to increase with the mol. wt. of the protein,

though the relation is not linear. It is greater in yeast than in Eledone hemocyanin but less than in certain viruses, from which it is concluded that the effective mol. wt. of the intracellular protein lies between 17 and 20×10^6. He ascribes the scattering to hypothetical vital protein complexes, which he calls *vitaids*, and states that these complexes are broken down by death and adverse environment. The effect deserves further investigation though it is regrettable that another term has been added to the long list of names for hypothetical giant vital complexes. Terms of this sort were in vogue half a century ago but proved to be little more than semantic camouflage for lack of information.

That the optically empty ground substance of protoplasm contains protein ultrastructure has been obvious for many years because of its abnormal viscosity and the behavior of particles moving through it, under normal conditions and in centrifugal fields. Non-Newtonian flow properties of protoplasm have been investigated by Pfeiffer (1937 a, b, 1940) who studied the viscosity and stream birefringence of the protoplasm of plant cells (chiefly *Chara*) in capillary tubes. Longitudinal streaks, observed between crossed nicols at low and medium shear velocities, attested to the presence of asymmetric protein aggregates. At high shear velocities the streaks disappeared and the viscosity dropped, due presumably to disaggregation of the particles.

a. *Polarized Light Observations*

No attempt will be made to cover this large field thoroughly. For reviews see the books and papers by Schmidt (1937, a), Frey-Wyssling (1938, a), Schmitt (1939), and Picken (1940). Particularly valuable for source material are the "Sammelreferate" of Schmidt (1937, b, 1942). After a brief summary of the main facts we shall consider chiefly the papers which have appeared in the last three years.

Many of the fibrous proteins of the cell are very thin or occur in highly solvated systems. Hence, even though they have birefringence as high as that of familiar compact fibers, the retardation, Γ, will be very small.[1] Consequently birefringence can be discovered—not to say measured—in these thin fibers only under special conditions, which include use of a compensator of high sensitivity (such as the Köhler λ/20 or λ/30 rotating mica plate compensator of Leitz) and employment of high intensity of illumination to provide maximum contrast. Where these conditions have been met, birefringence has been found in most of the fibrous protein structures of the cell.

[1] Birefringence $= n_e - n_o = \frac{\Gamma}{d}$, where d is the thickness of the object and Γ is the retardation in the same units.

Intracellular protein fibers behave as uniaxial systems in which the optic axis corresponds with the long axis of the fiber. Their birefringence, as observed in the cell, is very largely form birefringence and, since the sign is positive, they have been considered as composed of submicroscopic rodlets oriented with long axes parallel to the fiber axis. This is based on the theory of Wiener (1912) which, together with Naegeli's micellar theory, gave rise to the concept of isolated rodlets. Such conclusions are not a consequence of this theory, as will be discussed in detail below, and electron microscope (abbreviated EM) observations indicate that the submicroscopic components are more probably very elongated fibrils having thickness of the order of a few hundred Angstrom units. However, the polarized light analysis is important because it can be applied to the fresh cell and is an extremely sensitive indication of preferred orientation.

The intrinsic birefringence (that portion whose magnitude is independent of the refractive index of the immersion medium) is positive in sign in all protein fibers, except those conjugated with nucleic acid, which show a negative sign. The above description holds true for spindle and astral fibers, myonemes of protozoa, cilia, flagella, sperm tails, and many tissue fibers.

Ground Substance (*Hyaloplasm*). Evidence has been accumulating for the existence of a submicroscopic lattice of protein and lipid materials which may be responsible for the non-Newtonian properties of cytoplasm, as well as for contractility and polarity. Peters (1937) refers to the lattice as the "cytoskeleton" and speculates about its possible functions. Earlier polarized light evidence is given by Schmidt (1937, a). Monné (1940) interpreted his birefringence results on pulmonate spermatocytes as indicating a stratification of the cytoplasm in submicroscopic layers concentric with the nucleus. The protein layers are interspersed with lipid molecules oriented with long axes perpendicular to the protein layers, *i.e.*, radially in the cell. Recently Pfeiffer (1942), using a microscope-centrifuge of design similar to that of Harvey, observed the protoplasm of frog eggs in polarized light during application of a centrifugal field. No evidence of orientation in the cytoplasm was found in the normal, uncentrifuged cell. At 10–20,000 r.p.m. weak birefringence was observed at the centrifugal pole; at the highest velocities stratification was observed at this pole, the sign being positive with respect to the direction of deformation. The effects are largely reversible. He concludes that the isotropy of normal cytoplasm is statistical and that the centrifugal field orients the fibrous structures normally present in random array.

Mitochondria and Golgi Apparatus. These lipid-protein complexes, which appear to be essential structures in most cells and are particularly important in secretion processes, have been studied very little as regards

ultrastructure. Giroud (1928) found the fibrous mitochondria of the gut cells of *Ascaris* uniaxially positive and concluded that the protein chains are axially oriented. Grave (1937) found that in the rodlets of the distal convoluted tubule of amphibia the positivity could be reversed by immersion in high refractive index media, suggesting that the protein chains run parallel with the rodlet axis while lipid molecules extend with long axes perpendicular to this direction. The rod-like mitochondria of vitally stained pulmonate sex cells show birefringence which is negative with respect to the long axis, according to Monné (1940). Monné (1939, a) also observed birefringence in the lens-shaped Golgi apparatus in the living spermatocytes of *Helix* and *Tachea* stained with vital dyes. The "substance externum" is lipid-rich and shows birefringence negative with respect to the tangent of the lens, indicating a radial orientation of the lipids. The "substance internum" which contains chiefly the protein complement, showed no sign of preferential orientation.

Cell, Nuclear and Vacuolar Membranes. The plasma and nuclear membranes show chiefly form birefringence which is positive with respect to directions in the plane of the membrane, *i.e.*, negative with respect to the direction perpendicular thereto, the direction of the optic axis (Schmitt, Bear, and Ponder, 1936, 1938; Monné 1939, b; Schmidt, 1939, b). This has been interpreted as meaning that the protein components of these membranes occur as leaflets of submicroscopic thickness with surfaces parallel to that of the membrane. However, the optical data require only that the major axes of the submicroscopic protein components extend in planes paralleling that of the envelope. These components may as well be very thin fibrils, interlaced or oriented at random, as in nets or grids. From an EM study of the envelope of the human erythrocyte, Wolpers (1941) favors the view that the envelope is composed of a lattice of thin protein fibrils with lipid molecules interspersed between the protein particles.

The membranes of vacuoles have a similar structure. Schmidt (1939, c) observed changes in the birefringence of the contractile vacuole of *Amoeba* with the cyclic contraction and filling and suggested that the protein chains oriented parallel with the membrane of the vacuole are responsible for the contraction. This view resembles somewhat that of Seifriz (1938), who believes that protoplasmic streaming in slime molds is due to rhythmic pulsations, which in turn are ascribed to the contraction and relaxation of folded chains of the type pictured by Astbury.

Contractile Fibers. One of the characteristic properties of protein fibers is their ability to shorten longitudinally under appropriate conditions. Since Engelmann (1875) proclaimed his famous dictum "ohne Doppelbrechung keine Kontraktilität" it has been generally believed that the fundamental molecular mechanism by which reversible shortening occurs

will be found to be about the same whether in muscle, spindle fibers, cilia, or elsewhere. The fact that all cellular fibrils show positive form birefringence has been the chief support of this belief. An unfortunate by-product has been the feeling that, like muscle, intracellular fibrils will also be composed of globulins similar to myosin. Enough evidence is now available to show that this is erroneous.

Actually there are relatively few cases of longitudinal shortening, comparable to that of muscle, found among intracellular fibrils. Spindle fibers may qualify (for objections, see Pfeiffer, 1942), but many fibrils which produce active movement (cilia, flagella, and sperm tails) shorten very little *longitudinally*. The contractile stalk of *Vorticella* and related organisms retracts rapidly by coiling of the internal fiber. Schmidt (1941) finds that contraction of the stalk in *Charchesium* results in great decrease in the positive birefringence characteristic of the resting fiber. Likening this to the optical negative variation of muscle contraction (see p. 55) he invokes a collapse of protein chains, similar to Astbury's supercontraction, to explain the coiling.

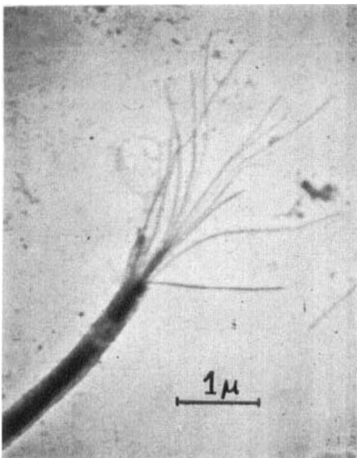
b. *Electron Microscope (EM)*

Contractile Fibers (Cilia, Flagella, Sperm Tails). Motile fibers such as cilia, flagella, and sperm tails, all of which extend from the cell, offer particularly favorable material for ultrastructure analysis, for they can be studied with the EM very conveniently, being obtainable free of protoplasmic material which obscures the electron micrograph (EMG). All of these structures show positive form birefringence (in the case of cilia the lipid may, under certain conditions, reverse the sign of birefringence).

Recent EM studies of Schmitt, Hall, and Jakus (1943) show that the submicroscopic protein structures responsible for this birefringence are not isolated, oriented micellar rodlets with asymmetry ratios of the order of 10 or 20, as has been pictured by analogy with the supposed structure of muscle fibers, but are evenly contoured parallel fibrils 300–500 A wide and extending the full length of the cilium, flagellum, or sperm tail (Fig. 1). In the case of some sperm tails the asymmetry factor for these fibrils would be of the order of 1000 instead of 10 or 20. It is not impossible that smaller units exist within the fibrils, contributing to the form birefringence. Since the fibrils alone account for form birefringence, however, postulation of smaller units seems unnecessary. Subfibrils in sperm tails have also been described by Harvey and Anderson (1943) and by Baylor, Nalbandov, and Clark (1943).

Sperm tails are best suited for such studies because they can be obtained in great abundance under standard conditions. An interesting and probably significant point is that not only have these subfibrils been found in all

vertebrate and invertebrate sperm tails thus far examined, but the number of fibrils per tail is amazingly constant, ranging from 9 to 12. Harvey and Anderson (1943) find a similar number of fibrils in the case of the tails of *Arbacia* sperm. The subfibril seems to be a unit of structure at this level of organization though it is no doubt composed of still finer columnar units; occasionally finer strands are seen, and it is supposed that the cleavage was due to some unrecognized factor in the chemical environment. It is an interesting commentary that Ballowitz (1890) observed the fraying of many types of sperm tails into subfibrils, made visible microscopically no doubt, by adsorption of stains.



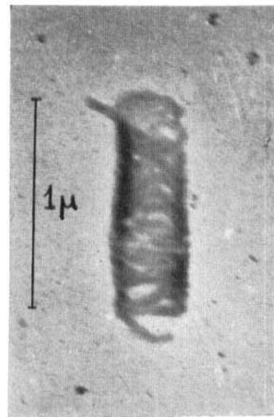


Fig. 1 Fig. 2

Fig. 1. Frayed tail of bull sperm showing subfibrils. × 11,000

Fig. 2. Portion of sheath from human sperm tail fragmented ultrasonically, showing helical structure. × 28,000

In mammalian sperm tails the bundle of fibrils is surrounded by a sheath, the major component of which is a closely wrapped helical fibril, having a thickness also in the range of 300–500 A (Fig. 2). When the tails are fragmented by ultrasonic radiation, portions of this helix can be seen, appearing like miniature solenoids.

This extraordinary system of fibrils has been investigated with respect to chemical properties by EM examination of tails which had been exposed to various reagents, either in solution or after drying the tails on the film of the specimen holder (unpublished work). They are dissolved rather slowly by trypsin, more rapidly by pepsin. Insolubility in water, dilute acid, or salts argues against a nucleoprotein, globulin, or collagenous nature. They are dissolved in relatively high concentration of NaOH (0.2 *M*).

What light do these observations throw on the mechanism of motility in these structures? The carbohydrate metabolism of the sperm tail seems to resemble qualitatively that of muscle (MacLeod, 1941, 1942). Motility ceases when glucose is washed out and returns when glucose is added. However, the situation differs from that of muscle not only in the nature of the fibrous proteins but also in that the motion is an undulatory one rather than a longitudinal shortening. As long ago as 1911, Heidenhain suggested that the central filament is essentially an elastic mechanical structure which is thrown into folds by small contractile regions located at intervals along the edges of the tail. Similar considerations were applied to cilia and flagella. However, EM examination reveals no such structure, except perhaps for the helical sheath, which has been found thus far only in mammals, hence cannot yet be regarded as an essential factor in all sperm movement.

A change in distribution of water between polar groups in fibrous proteins and ionogenic groups in the environment appears to be at the bottom of contractility generally. The submicroscopic longitudinal fibrils here described provide a unique capillary system in which a change in distribution of interfibrillary water might cause the undulatory contortions characteristic of the sperm tail and the pendular beating of cilia and flagella. Since no formed structure other than the fibrils has been found, it may be supposed, for the present, that they not only furnish the rigidity and elasticity required but also play a rôle in the events by which chemical energy from carbohydrate metabolism is converted into mechanical energy.

Trichocysts of Protozoa. Certain protozoa, such as *Paramecium* and *Frontonia*, are provided with ellipsoidal structures, called trichocysts, lying below the surface of the pellicle between each pair of cilia. Under certain conditions of excitation these bodies emit a fibrous dart provided with a very pointed tip. Extrusion is completed under electrical stimulation in but a few milliseconds and in this time the extruded trichocyst has become six to tenfold longer than the resting trichocyst. From ultramicroscope observations Krüger (1930) believed that the explosive elongation is due to solvation of a body which he calls the "Quellkörper." Schmidt (1939, a) found that the extruded shafts show positive form birefringence, from which he suggests that the structure is essentially fibrous and that extrusion may be due to an elongation of previously folded chains.

EM investigation of trichocyst structure has revealed a most interesting and unexpected structure (Schmitt, Hall, and Jakus, 1943). The extruded shaft behind the dense pointed tip (Fig. 3, a) shows a banded structure (Fig. 3, b) which extends to the tapered end. The distance between the bands is strikingly constant in the shafts emitted from any single cell and averages about 600–650 A. The regularity of structure is comparable with

that of collagen (see Section III) but there are differences between the two types.

The chemical nature of the shafts is being investigated by Miss Jakus both by polarization optics and by the EM. She is inclined to regard the highly structured object as an envelope surrounding a colloidal matrix; the swelling of the latter is responsible for the extrusion. The trichocyst represents a highly irritable system which is capable of a violent mechanical response, which apparently involves a rapid translocation of water. All the structural details can be investigated with the EM, and the results should have a bearing both on muscle and nerve physiology.

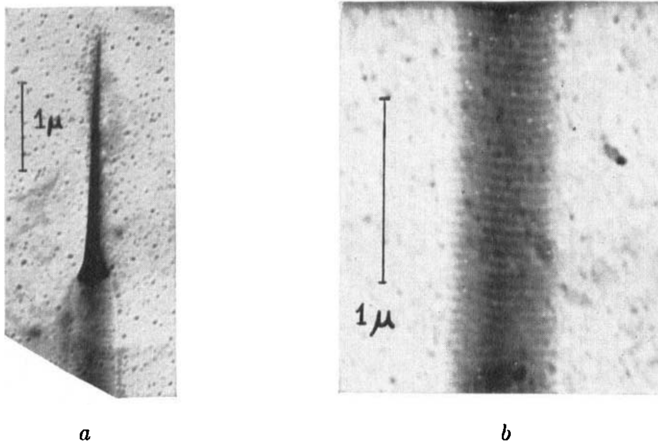


Fig. 3. Extruded trichocyst of *Paramecium*. *a* shows pointed tip, × 11,000; *b* represents a portion of the shaft, × 24,000.

II. Nerve Proteins

Most of the work on the chemistry of nerve tissue has been done on brain, *i.e.*, primarily on nerve cells, whereas ultrastructure analysis has been confined largely to peripheral fibers. Since the peripheral fiber is, in certain respects, simpler than the cell, and since this discussion is concerned more with ultrastructure than with analytical chemistry, this discussion is confined chiefly to the proteins of peripheral fibers.

The nerve fiber is composed essentially of three elements: the axon or axis cylinder (the cytoplasmic continuation of the cell), the myelin sheath (or axon sheath) with its Schwann cells, and the closely or loosely applied fibrous wrappings, thought to be chiefly connective tissue. While the fibrous sheaths present certain interesting problems, only the axon and the myelin sheath will be considered here.

1. Axon

The chief morphological feature of the fixed axon is the system of neurofibrils. The almost century-old debate concerning the reality or artificiality of neurofibrils and their relation to nerve action is a familiar story (Peterfi, 1929; and Schmidt, 1937, a). The normal axon possesses ultrastructure which is very sensitive to the action of reagents and change of environment. This has led to confusion of results and opinions, for it is almost impossible to manipulate the axon chemically or mechanically without altering its structure. Conclusions of investigators have not always been as cautious as desirable with respect to the applicability of their results to the normal axon.

a. *Chemistry*

The literature on brain proteins has been ably reviewed by Block and Brand (1933) and by Block (1937, d, 1938). Block and Brand sum up the situation in 1933 as follows: "... one or more (soluble) proteins can be extracted from the mammalian brain. The mother substance of these proteins is presumably the nucleoprotein of the living cell while the protein or proteins actually isolated are its partial degradation products." This suggested but did not prove that nucleoproteins may be important constituents of axoplasm.

Crustacean nerve is well suited for extraction of axon protein because of its low lipid content. With this material Schmitt and Bear (1935) found that about 65% of the total nerve protein (including connective tissue) is extractable with neutral salt and that the properties of the extract resemble those of the nucleoproteins described by previous workers, the most reliable data being those of McGregor (1917). However, it could not be concluded that the proteins thus isolated were derived solely from axoplasm.

This difficulty has been overcome by the use of the squid giant fiber preparation from which axoplasm, uncontaminated with non-axonic material, can be obtained by simple extrusion. A beginning has been made in the characterization of the proteins by Bear, Schmitt, and Young (1937, b). Except for a small amount of granular material, insoluble even in molar NaOH, axoplasm dissolves in neutral salts isotonic with sea water. The protein content of axoplasm was estimated at about 3–4%. Since the water content is about 90%, the protein accounts for 30–40% of axon solids, the remainder being chiefly salts, free amino acids and lipids (Bear and Schmitt, 1939). The properties of the protein complex agree remarkably with those of the soluble proteins previously described from such different sources as lobster claw nerves, mammalian brain, and spinal cord. It is, therefore, the principal protein complex of the nervous system, and

quantitative considerations suggest that it constitutes the bulk of the material which forms the visible neurofibrils when axons are appropriately treated (or mistreated!). For convenience the complex has been termed *neuronin* by Bear, Schmitt, and Young; it was fully realized that individual components may subsequently be isolated and identified.

That neuronin may be a nucleo- or pseudo-nucleoprotein is suggested by the fact that a histone-like fraction can be isolated from it by alkali treatment, and an acid fraction having certain of the properties of nucleic acid is obtained by acid treatment.

The closer characterization of neuronin awaits further investigation. Caspersson (1940, 1941) and Landström, Caspersson, and Wohlfahrt (1941) have demonstrated the nucleoprotein nature of the Nissl substance (and of the nucleoli) of nerve cells and claim it to be of the ribose type. Gersh and Bodian (1943, a, b) confirmed this in their study of chromatolysis. Bear, Schmitt, and Young (1937, b) observed longitudinally oriented basophilic filaments in squid axoplasm which were prominent near the cell bodies but became sparse in the proximal portion of the axon and absent in the distal portions. The presence of nucleic acid or acidic protein in axoplasm may be significant with respect to some old observations by Bethe (1920) on the effect of the passage of current on the stainability of axoplasmic fibrils with basic dyes such as toluidin blue; anodal polarization decreased the color while cathodal polarization increased it. He termed the substance responsible for the reaction "fibrillary acid" and believed it plays an important rôle in the propagation of the impulse. The great expansion of information and techniques relating to the properties of interfaces and of interfacial films in the last decade has focussed attention on the properties of nerve membranes and sheaths and their rôle in electrical phenomena. However, axoplasm is part of the electrical circuit and doubtless the source of the energy which maintains the ultrastructural integrity of the metastable interfacial mechanism. It is, therefore, highly desirable that further attention be given to its chemistry which, as is obvious from the above, has thus far been sketched only in rough outline.

b. *Ultrastructure*

Polarized Light. Since the polarized light method is coming to be used more by cytologists it may be useful, before relating the results obtained by its application to the axon problem, to indicate briefly the contributions as well as the limitations of the method.

Protoplasmic systems have long been believed to contain submicroscopic particles which are asymmetric and which may or may not contain crystal-like regularity of internal structure (Naegeli's micellar theory). In passing through a system containing such particles the electric field of the

light interacts with the various dielectric phases of the dispersed system. Wiener (1912) considered the analogous case of the effect on the field of a condenser caused by introduction of materials having certain shapes and dielectric properties. From such considerations, and the fact that at optical frequencies the dielectric constant is equal to the square of the refractive index, the following expressions were derived. In the case of a system of submicroscopic rodlets or fibrils oriented parallel with the optic axis, in which the refractive indices of the rodlets and surrounding medium

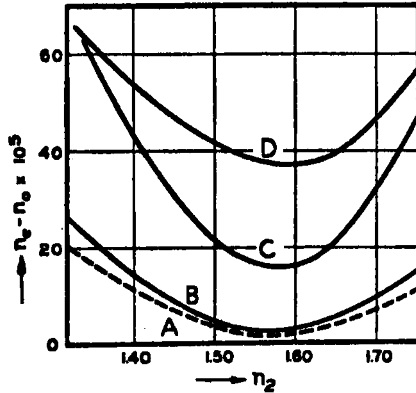


Fig. 4. Form birefringence curves of axis cylinder of squid giant fibers. *A*, calculated curve for fresh axon; *B*, axon fixed in $HgCl_2$; *C*, axon fixed in 35% alcohol; *D*, air dried preparation. Note differences in steepness of the curves with increasing dehydration from *A* to *D*.

in bulk are n_1 and n_2, respectively, and the partial volume of these respective components in the dispersed system are δ_1 and δ_2, then

$$n_e^2 - n_o^2 = \frac{\delta_1 \delta_2 (n_1^2 - n_2^2)^2}{(1 + \delta_1) n_2^2 + \delta_2 n_1^2}, \qquad (1)$$

where n_e and n_o are the descriptive refractive indices of the macroscopic system. If the asymmetric submicroscopic particles are oriented with long axes perpendicular to the optic axis, then

$$n_e^2 - n_o^2 = -\frac{\delta_1 \delta_2 (n_1^2 - n_1^2)^2}{\delta_1 n_1^2 + \delta_2 n_2^2}$$

In the biological case n_2 may be varied by immersing the system in media of different refractive indices and measuring the birefringence ($n_e - n_o$) after sufficient time has been allowed for penetration of each medium. The general practice is to plot $n_e - n_o$ as ordinates against n_2 as abscissae. The result is usually a paraboloid curve, as shown in Fig. 4.

It has been recognized, but not always remembered, that positive or negative form birefringence need not mean that the submicroscopic particles are rodlets or platelets, respectively. Required only is that the long dimensions of the particles be oriented parallel or perpendicular, respectively, to the direction of the optic axis. In the absence of specified evidence from other sources that the particles are indeed rodlets or platelets the theory cannot be used quantitatively.

Another requirement of the Wiener theory is that there be no interaction between particles and immersion medium. Frey-Wyssling's (1940) criterion of such interaction is a tilting of the axis of the form birefringence curve with respect to the coordinates ($n_e - n_o$ plotted against n_2). He noted that, in the lower ranges of n_2, where dipolar media are of necessity employed, polar particles yield lowered form birefringence because of interaction between particles and medium. This causes tilting of the axis of the curve to the left. In the case of apolar particles when immersed in apolar media, necessarily used in the higher ranges of n_2, interaction also occurs, causing decreased form birefringence and tilting to the right. Frey-Wyssling's contribution is valuable, for it provides a ready means of determining whether, in a given system, there is interaction between particles and immersion medium, merely by inspection of the curves for tilting. In a non-interacting system no tilting should occur.

The view of Baas-Becking and Galliher (1931) and Frey-Wyssling (1940) that polar particles cannot conform to the requirements of a Wiener system is not well founded. If the particles are configurationally constrained from disorientation and if the refractive index is that measured at the frequencies of visible light, polarity or apolarity are automatically taken care of.

EM observations on systems whose form birefringence has been studied (muscle, fibrin, sperm tails, cilia) show that the particles responsible for the form birefringence are, in fact, very thin fibrils several hundred A in thickness and of great length (sometimes microns). Such particles may be regarded as reasonably stationary bodies, as required by the theory. The fibrils may be fairly close packed, particularly in the dry state, in which immersion studies are carried out. This close packing may limit quantitative application of the theory to biological cases since the theory requires sufficient lateral separation of the particles to ensure a homogeneous field about each particle.

Another difficulty is the fact that biological systems are not often two-component but rather multi-component systems. However, if one component is anisodiametric and its partial volume is small, the multi-component equation given by Wiener reduces to the same form as that for two-component systems.

In view of the points raised above it is clear that the Wiener theory may be used quantitatively with biological systems only with the greatest caution and any values deduced must be used only as a working guide. However, the following type of information may, in many cases, be obtained. The value of n_1 is determined from the abscissa of the minimum of the curve, for here $n_1 = n_2$. The crystalline birefringence of the system can be estimated from the ordinate of the minimum. From this value the contribution of form birefringence is obtainable. An idea of the value of δ_1 can be obtained from the steepness of the limbs of the form birefringence curve, particularly when δ_1 is small, in which case $\delta_1\delta_2 \cong \delta_1$. Thus determined, δ_1 can be divided into the crystalline birefringence of the whole system to give the birefringence of the individual particles. It must be emphasized again that estimation of these quantities is useful to form an approximate and tentative picture of the main aspects of the system. To use the values more definitively, as was done by Weber (1934) in determining the dimensions of the particles in muscle, may lead to serious errors.

With this brief evaluation of what can and what cannot be determined from the Wiener theory we may consider the results on the axon.

Since the work of Apathy (1897) and Göthlin (1913) it has been known that the axon shows birefringence which is positive with respect to the fiber axis, which is also the direction of the optic axis. Because of its large size (400–700 μ) the squid axon permits measurement of the birefringence. The value of the over-all birefringence in sea water is only about 1.5×10^{-4}. Form birefringence curves obtained by Bear, Schmitt, and Young (1937, a) are shown in Fig. 4.

It will be noted that there is no marked tilting of the curves, hence there was little interaction between the oriented particles and immersion medium. A further feature is the shallowness of the curves, in comparison with those of other fibrous protein systems. This indicates either that the orientation of all the particles is very poor or that there is a small amount of material which is very well oriented and which is responsible for the birefringence. The fact that when visible neurofibrils are formed the birefringence is limited to these well oriented fibrils supports this view and suggests that the small fraction (estimated at 10%) of the total protein, which is well oriented longitudinally in the normal axon, serves as a framework on which unoriented material precipitates when visible neurofibrils are formed under abnormal conditions.

The relation of the neuronin complex to these components has not been determined. The oriented component may be a highly organized form of neuronin, immersed in randomly oriented neuronin components, or it may represent a molecular species different from the unoriented ones.

With a photoelectric cell, in conjunction with a polarizing microscope

and cathode ray oscillograph to record both action potentials and any changes in birefringence of the squid axon, no changes, indicating alteration of orientation or change of state of the oriented submicroscopic components, were observed to occur as the impulse passed over the fiber (Schmitt and Schmitt, 1940). The sensitivity of the method was sufficient to detect alterations of as little as 0.2% of the total birefringence.

Such evidence does not prove, of course, that the specific ultrastructure of the axon does not play a rôle in conduction. The orientation of protein dipoles may provide directional properties of an electrical sort without an oscillation of the protein strands. A speculation based on resonance changes in the oriented axon proteins has recently been offered by O. Schmidt (1943) to explain the propagation of the impulse.

X-ray Diffraction. X-ray studies of the axon of fresh nerves have thus far yielded negative results (Boehm, 1933; Schmitt, Bear, and Clark, 1935). This is doubtless due to the highly solvated nature of axoplasm and the fact that the amount of oriented protein is relatively small. In the squid axon, the axoplasm can be shrunk with alcohol without apparent loss of orientation (from observations of birefringence) to form a highly desolvated cylinder which can be removed from the fiber. However, the pattern of such isolated material resembles that of artificially spun neuronin fibers and shows only the two rings characteristic of denatured proteins (Schmitt, Bear, and Clark, 1939). No evidence of preferential orientation of the chains was found.

Electron Microscope. Richards, Steinbach, and Anderson (1943) examined smears of axoplasm extruded from squid giant fibers. They observed fibrils ranging from about 150 to 500 A in thickness. The thin fibrils frequently show a zigzag structure which, the authors suggest, indicates that the unit of fibril structure is a particle approximately 150 x 500 A in size; the fibrils are formed by linear aggregation of these particles which, in some cases, results in the zigzag appearance. Fibrils were not observed in preparations washed in sea water, which fact is correlated with the observation of Bear, Schmitt, and Young (1937, a) that the birefringence of extruded axoplasm is rapidly lost in sea water. It is concluded that the fibrils about 150 A thick represent the primary neurofibrils, the larger fibers being due to lateral aggregation and coagulation.

EM examination of axoplasm is made difficult not only by the high salt content but also by the presence of large quantities (3%) of free amino acids (Schmitt, Bear, and Silber, 1939) and by the great lability of the structure. Valuable collateral evidence could be obtained by EM examination of various fractions isolated chemically. While the work of Richards, Steinbach, and Anderson is a good beginning in this direction, and it seems possible that the fibrils pictured may correspond to an aspect of the

longitudinally oriented components of the normal axon, further evidence is required to establish the existence and dimensions of the unit of fiber structure. In this connection it is interesting that, in a preliminary experiment made some years ago, a lobster nerve extract prepared by the author and kindly examined with the ultracentrifuge by Dr. E. G. Pickels of the Rockefeller Institute, gave rise to a boundary representing the sedimentation of a homogeneous group of particles. The sedimentation constant was 20 SU. As spherical particles this corresponds to a diameter of 105 A and a mol. wt. = 500,000. Considerable departure from these values may be anticipated since the particles are probably asymmetric. Further investigation in this direction may prove highly informative.

2. Myelin Sheath

The myelin sheath of the fresh fiber shows none of the numerous structures described in preserved fibers in the cytological literature. These structures result from the action of fixatives on a highly organized but very labile system of lipids and proteins. In the present discussion we are interested primarily in the protein components. However, these are so closely associated with the lipids that a clear picture can be obtained only by considering the system as a whole.

a. *Chemistry*

Ewald and Kühne (1877) and Kühne and Chittenden (1890) applied the name *neurokeratin* to the sheath protein because of its insolubility and its resistance to digestion by proteolytic enzymes. However, Block (1932) found that the ratio of histidine, lysine, and arginine of brain neurokeratin is not that of a true keratin and he therefore considered it to be a pseudokeratin. He made a comparative study of the amino acid composition of this protein as well as the effect of age and sex (1937 a, b, c).

It is impossible by chemical analysis of protein fractions of the brain to localize any particular protein in the nerve cell or fiber. Block (1938) later sponsored the view that neurokeratin may not be in the sheath at all but rather in the neurofibrils. From the preceding section it is obvious that this view is untenable. Block agrees with Speakman and Townsend (1937), however, that the chemical properties of neurokeratin result from the customary treatment of brain with organic solvents. He states that a protein having amino acid composition similar to that of neurokeratin can be obtained by water extraction of ground brain. This fact does not necessarily exclude the possibility that neurokeratin is a sheath protein.

It is obvious that practically nothing can be said with certainty about the composition of the sheath protein. This situation is due largely to its close association with lipids; separation of the protein requires the use of reagents which seriously alter the properties of the protein.

b. *Ultrastructure*

Polarized Light. The sheath shows intrinsic birefringence which is positive with respect to the optic axis, which is directed radially (see Schmitt and Bear, 1939, for a review). This is due to lipid molecules which are oriented with paraffin chains extended radially. Extraction of the lipids reverses the sign of birefringence which is then chiefly form birefringence. It is concluded from this that the protein component consists of submicroscopic elements oriented with long axes tangential. These protein elements might consist of networks of very thin fibrils or of laminar nets; the optics give no information about the thickness of the elements or their specific relation to the lipids.

This structure is typical of the axon sheath generally. Invertebrate fibers and very small vertebrate fibers may contain very little lipid, but in all cases the orientation of the lipid and protein components is similar to that in the sheath of highly myelinated nerves (cf. Schmitt and Bear, 1939). The analysis does not rest on a possible artifact due to the action of solvents on the protein, for Bear and Schmitt (1937), in their form birefringence analysis, demonstrated the dependency of birefringence on the refractive index of the medium by employing aqueous solutions of salt and sugar rather than organic solvents.

X-ray Diffraction. The diffraction pattern of medullated vertebrate nerve is due exclusively to the myelin sheath (Schmitt, Bear, and Clark, 1935). The easily observed meridionally accentuated ring at large scattering angles in fresh nerve diffraction patterns represents the interchain separation (ca. 4.7 A) of the lipid molecules which are oriented with chains extending radially.

The clue to the structure of the sheath protein comes from the low-angle, long-spacing patterns. Equatorial diffractions are observed which represent four orders of a fundamental spacing of about 171 A in amphibian nerve and about 184 A in mammalian nerve in the fresh condition. When the nerve is dried this spacing shrinks to 144 and 158 A, respectively, for amphibian and mammalian nerve. In addition, three rings appear which were shown to be due to individual lipid phases which separate out during drying.

A careful study of individual purified nerve lipids as single components and as mixtures both in the dry state and in aqueous emulsions was made (Bear, Palmer, and Schmitt, 1941; Palmer and Schmitt, 1941). From this it was concluded that the patterns of nerve could not be due to the lipid components alone. Quite long spacings were observed with certain lipid-water emulsions, but on drying these reverted to spacings characteristic of bimolecular leaflets of lipids. None of the spacings exceeded about 150 A, whereas the long-spacing of fresh mammalian nerves is 184 A. It was concluded (Schmitt, Bear, and Palmer, 1941) that the long-spacing identity

period is represented by two bimolecular leaflets of mixed lipids plus a layer of protein, the thickness of the protein layer in dried nerve being estimated at about 25 A. The lipids are probably arranged with polar groups oriented towards, and loosely bonded to, the protein layers which, in the fresh state contain considerable water (corresponding to about 26 A in the identity period of 186 A). The sheath is thus pictured as being composed of concentrically wrapped layers of mixed lipids alternating with thin, possibly unimolecular layers of (neurokeratinogenic) protein material.

Nothing more specific can be said about the structure of the protein components. If they consist of a felt-work of fibrils, the individual fibrils must be very thin, containing only a few polypeptide chains. They might consist of laminar grids; those recently pictured by Wrinch (1942) could have the required thickness. It seems improbable that they consist of small globular molecules, for such a view would encounter difficulties with the optical data and with the fact that the specific structure of the sheath is relatively insensitive to heat (70°C.) and to detergents.

The chemical characterization of the sheath proteins will be awaited with interest by nerve physiologists. On the assumption that the critical interfacial membrane consists of a lipid-protein complex, model films which have been studied have contained nerve lipids plus native proteins chosen apparently at random. The use, for this purpose, of proteins known to exist in the sheath and in axoplasm might confer properties more characteristic of those of nerve.

III. Collagen

A thorough investigation of the composition and structure of collagen is highly desirable for at least two reasons: first, collagen can readily be obtained in purified form and is a highly organized fibrous protein which lends itself well to structure analysis; second, collagen forms the chief protein of all the loose and compact connective tissue, hence an understanding of its properties is of considerable biological and medical interest.

The tendency to regard collagen as the stuffing "excelsior" of the tissues, so to speak, may have been blinding us to some of the more active roles of the collagenous tissues. A number of pathological conditions, such as rheumatic fever and disseminated lupus erythematosis, as well as phenomena of aging, have been shown to be closely connected with alterations of collagenous tissues. Klemperer, Pollack, and Baehr (1941, 1942) group certain of these pathological states under the term 'diffuse collagen diseases' and suggest that the major causal factor is an altered colloidal state of collagen, particularly of the ground substance. Biochemical and biophysical investigation of these alterations may prove of considerable clinical value.

1a. Chemistry

In tissues collagen is closely associated with mucoproteins and other substances from which it must be freed. Samples purified by the method of Highberger (1936) or Bergmann and Niemann (1936) may still show a small remainder of mucoprotein and "elastin" as determined by the method of Lowry, Gilligan, and Katersky (1940). According to Salo (unpublished) these remainders may be more apparent than real, depending on the way in which the tests are performed. The very low concentration of amino acids which absorb in the ultraviolet makes it possible to estimate the purity of samples from ultraviolet absorption spectra (Loofbourow, unpublished).

Collagen is characterized analytically by its high content of glycine, proline, and hydroxyproline. Astbury and Atkin (1933) and Astbury (1940) pointed out that glycine accounts for one-third of all residues, while proline and hydroxyproline account for almost another third. Bergmann and Stein (1939) found the ratio of glycine:proline:all other residues to be approximately 7:3:21. Pending further investigation, Bergmann and Stein concluded that it may be necessary to assign a dual frequency to proline to fit the data into the concept of a periodic linear distribution of constituent amino acids.

The high preponderance of imino acids in collagen is doubtless of importance in determining its structural relations. Using chromatographic methods of analysis, Gordon, Martin, and Synge (1943) isolated a number of dipeptides from gelatin and indicated ratios of certain of the amino acids. An important conclusion is that the monoamino acids with the longer fatty acid side-chains (phenylalanine, leucine, proline, valine, and methionine) are not linked to one another in the chains of gelatin. The authors feel that the structure of gelatin is more complex than that visualized by Astbury but that views about the distribution of amino acids by physical classes (Astbury, 1942) may be illustrated by the "spacing out" of the fatty residues along the chains of gelatin. EM and X-ray diffraction evidence (see below) is most easily interpreted on the assumption that the amino acid pattern varies periodically, over distances of 200–400 A, along the fiber axis. Further evidence concerning the distribution of the amino acids in these large patterns, whether from a study of hydrolytic products of collagen or from EM studies, is greatly needed.

1b. Ultrastructure

Polarized Light

The results up to 1933 are reviewed by Küntzel and Prakke. As might be expected, collagen fibers show positive uniaxial form and crystalline birefringence and, as usual, this has been interpreted to signify the presence

of oriented rodlets in the fibrils. Hermann, Gerngross, and Abitz (1930) suggested that the submicroscopic fibrils consist of crystalline regions interconnected anastomotically by fibrils extending through amorphous regions (fringe theory). Actually, EMG's show that the submicroscopic fibrils of varying widths extend unbranched over large distances; this is difficult to reconcile with the original fringe theory. The form birefringence can be explained on the basis of the fibrils seen in the EMG's without necessarily assuming still smaller rodlets. Polarized light methods are very useful in interpreting the changes which occur in thermal shortening, swelling, tanning, etc. (see Schmidt, 1937 a; Küntzel, 1941).

X-ray Diffraction

References to the earlier work are to be found in the paper of Astbury (1940). The features of greatest prominence in the patterns of tissues such as tendon are the equatorial spots at about 11 A and the meridional arc at 2.86 A. The former gives the lateral separation of the chains and is a good indication of the degree of swelling; fully dried fibers give a spacing as low as 10.4 A while in highly swollen fibers the spacing may be as high as 15–17 A. The meridional spacing is believed by Astbury to indicate the length of the amino acid residues along the chains. Since the spacing is less than 3.5 A, characteristic of fully extended chains, it was at first thought that the chains may be folded, but to a lesser degree than in α-keratin. Later Astbury (1940) suggested that the chains are essentially straight, though the high concentration of imino acid residues causes them to be constricted and to take up a configuration in which glycine, proline, and hydroxyproline side-chains lie on one side of the chains. All other side-chains lie on the other side and are restricted, for steric reasons, from approaching the imino acid residues too closely. To stretch the chains to full extension would involve this steric hindrance, and the chains would fall into loops. Actually, EM results show that isolated collagen fibrils are not inextensible (see below) hence it is possible that the chains do not run an exclusively straight and fairly extended course, though the X-ray data suggest that they run predominantly parallel to the fiber axis.

Huggins (1943) has criticized Astbury's structure on the grounds that the unbalanced forces on opposite sides of a chain which has no screw axis tend to bend the chain continuously in the same direction. Huggins extends his conception of the rôle of NHO bonds in the structure of proteins to collagen, suggesting a structure in which the chains can be spirally coiled so as to give NHO bridges between different chains, holding them together in layers. A suggestion is offered to explain the banded structure seen in EMG's. However, as in the case of Astbury's view, additional assumptions must be introduced to explain the great extensibility of isolated collagen fibrils.

Collagen also shows long-spacing fiber-axis diffractions which were described by Clark, Parker, Schaad, and Warren (1935); Wyckoff, Corey, and Biscoe (1935); Clark and Schaad (1936); and Wyckoff and Corey (1936). Considering these spacings, together with the chemical data and interpretations of these from the periodicity theory, Astbury (1940) concluded that each row or chain consists of 288 residues (or multiple thereof); each residue having a length of 2.91 A, a characteristic long spacing of 838 A was predicted. However, Bear (1942) obtained some 20 meridional diffractions from dried tendon corresponding closely to orders of a fundamental spacing of about 640 A (Fig. 5). The first order is much the most intense, and the number and sharpness of the orders indicate that in normal collagen the periods of all fibrils must be very close to 640 A. The spacing

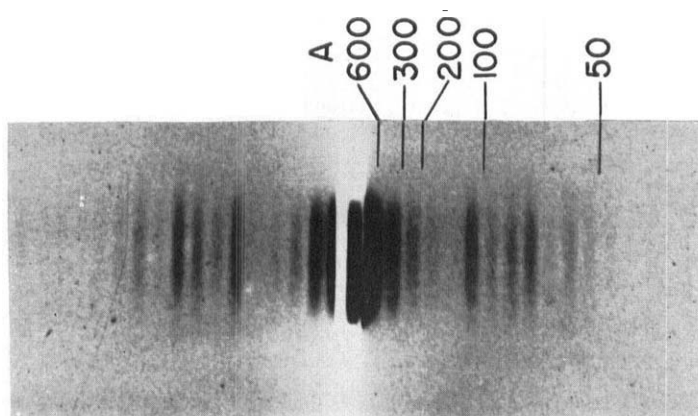


Fig. 5. Small angle X-ray diffraction pattern of rat tail collagen showing orders of the fundamental spacing of 640 A. Scale in A shown above pattern.

is about 5% greater for fresh than for dried tendon, showing that water has little effect on this aspect of the structure. Bear concludes that this spacing is characteristic of collagens generally, for he has found it in the collagen of tendon, skin, cornea, intestine, and bone. Apparently unaware of Bear's finding, Kratky and Sekora (1943) arrived at a value of 642 A for the macroperiod in the collagen of kangaroo tail tendon.

Astbury (1942) readily reconciled this large discrepancy between the actual and the predicted values by pointing out that, from stoichiometry, the basic prediction was that the length of the full pattern must be a multiple of 72 times 2.91 or 209.5 A. If 3 rather than 4 be chosen as the multiple one obtains a figure of 628 A, as compared with Bear's figure of 640 A. Actually, the chemical data were insufficient to form anything but the most tentative notion of the size of the period and X-ray data formed the actual basis. After the correct value becomes available from X rays

(or EM) it is not too difficult to find interpretations from the periodicity theory, particularly if the chemical data are not complete or accurate enough to be too restricting. One does not have to be a stoichiopessimist (Astbury's term) to feel that too much generalization and speculation before the data are in may prove misleading.

Bear (1944) draws attention to the meaninglessness of considering the 2.9 A meridional arc as a high (220th) order of the 640 A period. Under certain conditions the latter may be altered considerably without change of the former. The wide angle pattern is more simply interpreted as related to small periods or pseudocells contained within the macroperiod. If this is the case, attempts to relate diffraction indices to amino acid frequencies derived from analyses of *whole* collagen or gelatin become futile and misleading.

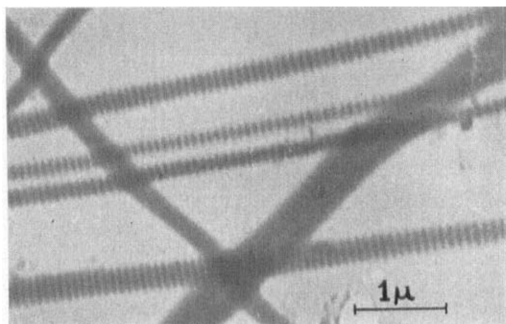


Fig. 6. Fibrils from chameleon tail tendon teased in water. × 12,000

Electron Microscope

It was shown by Schmitt, Hall, and Jakus (1942) that collagenous fibers from loose and compact tissue, from vertebrate and invertebrate sources, present a characteristic structure in EMG's, an example of which is shown in Fig. 6. The structure consists of an alternation, along the length of the submicroscopic fibrils, of relatively dense and light bands, called, respectively, *A* and *B* bands. The distance between adjoining bands of like density is very uniform in individual fibrils and may be called the spacing, *d*. This spacing varies in different fibrils but a distribution curve shows a fairly sharp maximum at d = 620–660 A (Fig. 8). The mean value of all the spacings measured was 644 A. The coincidence of this value with Bear's spacing of 640 A is strong evidence that the same regularity of structure underlies the results of both methods.

The EM results reveal an aspect of structure not yet obtained from X rays, namely, the variability of *d*, depending on the forces acting on the

fibrils. Fig. 7 shows spacings ranging from 400 to 1000 A. In this range the ratio of the lengths of the A and B bands is approximately 2.2. Fibrils show longitudinal cleavability down to widths at the limit of EM resolution with this material. It was concluded, therefore, that the unit of structure is a columnar array, called the *protofibril* which has indefinite axial extension and a thickness in one dimension less than 50 A. The density is greater

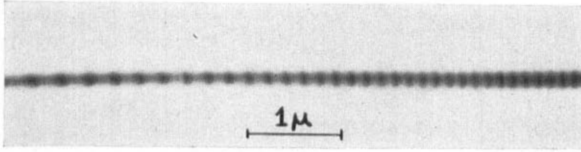


Fig. 7. Stretched fibril from rat tail tendon showing increase in spacing with extension. × 12,500

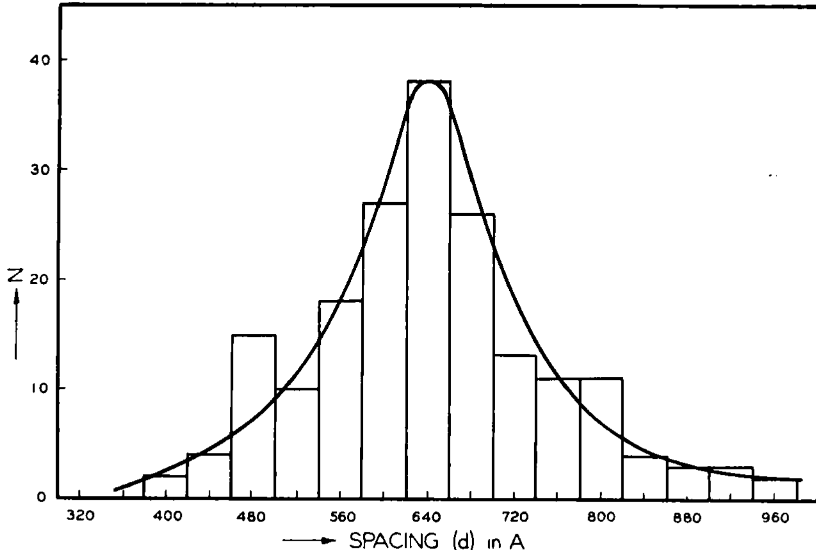


Fig. 8. Distribution of spacings in rat tail tendon fibrils. Ordinates, number of fibrils showing spacings indicated by abscissae.

in the A than in the B regions and the protein configuration is such that longitudinal extension can occur in both bands. Cases of very great extension were noted (Fig. 7); in one case, where $d = 6000$ A, the ratio of lengths of A and B bands was reduced to about 0.6.

This great extensibility of the teased fibrils was unexpected in view of the inextensibility of the whole tissue. The explanation of the effect obviously depends upon the interpretation of the A and B bands in terms of molecular

configuration. Of the various possible interpretations three may be mentioned.

1. The fibrils may be composed of polypeptide chains running in fairly straight lines, as Astbury suggests. In this case the periodic difference in density (roughly estimated at 20–30%) may be due to a specific linear distribution of amino acids having different properties with respect to scattering and absorption of electrons (in the EM). The linear pattern would have to be the same in neighboring chains to produce the cross-striated fibril. To extend such a structure greatly would require lateral slippage of chains and a new distribution of levels within the fibril to explain the continuous increase in lengths of *A* and *B* bands observed in stretched fibrils.

2. The chains may be considerably folded, as suggested by Schmitt, Hall, and Jakus (1942). X-ray and polarized light data indicate that the chains run preponderantly in the longitudinal direction, hence folding might be expected to occur in the form of long, perhaps interconnected longitudinal loops. Extension would cause unfolding of these loops. The density of packing of the loops would be greater in the *A* than in the *B* bands.

3. The spacing may be due to a linear aggregation of particles having a length of 640 A in the normal tissue. Wyckoff and Corey (1936) believed that acetic acid solutions of rat tail collagen contained such particles which could be reaggregated in specific linear array by the action of salt. Such particles have not been observed with the EM, though this may mean only that one dimension is very small.

Various combinations of these possibilities may also be considered. Determination of the actual structure must await further investigation both by X-ray and EM methods. Since periodic structure is characteristic of a number of protein fibers, a solution of the problem in any individual case may greatly assist the analysis of the other cases.

IV. Myosin

The classical work of Muralt and Edsall (1930) pointed to myosin as the protein directly concerned with contraction, and all subsequent work has confirmed this. It is now generally believed that the sole structural protein of the myofibril is myosin, the chains of which are oriented parallel with the fiber axis in the anisotropic bands, while in the isotropic bands they are disposed at angles to the axis or are more highly folded (Schmidt, 1937, a). While other structural proteins may also be of importance in muscle, this discussion will be confined to considerations of the ultrastructure of myofibrils and of myosin models. The chemistry of myosin is discussed elsewhere in this volume (p. 289, 295).

1d. Ultrastructure

Polarized Light

The results of the polarization optical analysis have been reviewed by Schmidt (1937, a), Schmitt (1939), and Picken (1940). We shall consider chiefly the more recent contributions, together with those earlier results which bear closely on the X-ray and EM analysis.

Myofibrils and myosin fibers show positive uniaxial form and crystalline birefringence, the former preponderating over the latter in fresh fibers. Weber (1934), who has contributed so greatly in this field, attempted to apply the Wiener theory quantitatively to the myosin system. Calculations of the partial volume of the particles, together with estimates of particle weight from osmotic pressure data and the apparent non-diffusibility of myogen into the myosin system, led him to suggest that the particles have dimensions of about 50 x 500 A, the oriented rodlets being separated by a protein-free aqueous phase. It has already been pointed out (p. 44) that large errors may result from such use of the Wiener theory. The dimensions quoted do not agree with those obtained from stream birefringence (Edsall, 1942), though this method in turn, may not reflect the status of the molecules as they occur in the muscle. Actually, Weber's estimates have greatly influenced his interpretation of X-ray and EM data, as will be discussed below.

Attempts have also been made to correlate muscle birefringence with chemical and physiological factors. Buchthal and Knappeis (1938) found that birefringence decreases with fatigue and is correlated with the action of lactic acid on myosin. Fischer (1940, 1941, a, b) found that birefringence varies as the log of the contractile power of normal and pathological muscle. Gerendás and Szent-Györgyi (1940) studied the relation of birefringence to mechanical properties of myosin threads.

Muralt (1932) measured the decrease in birefringence which occurs in isometric contraction and assumed it was due to contraction of myosin chains. This effect, now known as the optical negative variation, was shown by Fischer (1936) and Bozler and Cottrell (1937) to depend on changes in length of the fibers rather than on tension. These results may be related to the observations of Marsland and Brown (1942) who believe that the effect of pressure on the state of myosin, which they observed, indicates that the "gelation" of myosin in muscle occurs only when work is done, *e.g.*, when the fibers shorten. Similarly, birefringence change may depend on the performance of work. Schmidt (1942) observed a striking decrease in the birefringence of the stalk of *Carchesium* and *Vorticella* during contraction. Pfeiffer (1942) noted a 60–70% decrease of birefringence in contraction of explants of *Planaria.*

The observations of Needham, *et al.* (1942) on the specific effect of adenosine triphosphate in reversibly reducing the stream birefringence of myosin and the bearing of these observations on the Engelhardt theory are discussed elsewhere (p. 297).[2] In this connection it may be relevant to mention the interesting experiments of Sandow (1943). With a piezoelectric cathode ray oscillograph technique he measured a "latency relaxation" effect, a passive mechanical change due to decrease in elastic modulus of myosin preceding contraction. He believes it due to the interaction in muscle of myosin and ATP and that it is a mechanical indication of the formation of an intermediate complex of enzyme and substrate, during the existence of which, energy is transferred from ATP to myosin. These results have a close bearing on those of Marsland and Brown, previously mentioned.

X-ray Diffraction

Until very recently, the diffraction patterns obtained from muscle have been relatively poor in diffractions, and conclusions based on the analysis of these patterns were concerned chiefly with the distance between the chains laterally and its variation with drying (cf. Meyer and Picken, 1937). The data were interpreted in terms of Meyer's general theory of contraction and of the view that, as in rubber, mechanical changes in muscle are related to alterations in crystalline and amorphous regions.

Astbury and Dickinson (1940) stressed the similarity of the pattern of resting muscle with that of α-keratin and showed that fibers of myosin can be made to display a typical α-pattern which can be changed reversibly to a β-pattern by stretching. The X-ray and elastic properties of myosin resemble those of keratin in which cross-linkages have been broken. Contraction is interpreted as due to superfolding of chains; this is associated with reversible denaturation of the α-form, involving no activation of SH groups (Mirsky, 1936, 1937), since myosin is already "configurationally disposed towards denaturation". A square configuration of the intramolecular fold has recently been proposed by Astbury (1941) for α-myosin as well as α-keratin.

In a highly important paper, Lotmar and Picken (1942) described a singular pattern obtained from the closing muscle of *Mytilus edulis* dried under tension. One particular preparation gave a pattern containing 18 diffractions, this in contrast to the patterns usually obtained which resembled the typical α-type. Like Herzog and Jancke (1926), who had published a somewhat similar pattern, they could not reproduce it at will.

The pattern is by far the best developed short-spacing fiber pattern of

[2] The full paper on the stream birefringence of myosin has now been published (Dainty, *et al.*, 1944).

any protein thus far described. The interferences are said to be considerably sharper even than those of cellulose. Lotmar and Picken deduce that the structure is of the monoclinic type with a two-fold screw axis, the space group being C_2^2. The unit cell has the dimensions: $a = 11.70$ A, $b = 5.65$ A, $c = 9.85$ A, $\beta = 73°30'$. The "backbone" separation is 5.85 A, which the authors believe is consistent with the view that the chains are linked by hydrogen bonds (distance between carbonyl carbons and imino nitrogens of adjacent chains is 4 A). Assuming that the chains, composed of "cis"-type amino acid residues (in contrast to the "trans"-type of silk), are wound about the screw axes, each amino acid residue has a length of $5.65/2 = 2.82$ A along the fiber axis.

This configuration could not be stretched 100%, for the maximum length of an amino acid residue is 3.5 A, as found in silk. It will be remembered that Astbury supposed the length of a residue to be $5.1/3 = 1.7$ A, which, when extended 100%, approximates the maximum length of 3.5 A. Lotmar and Picken suggest that the chains may be more highly folded in the amorphous than in the crystalline regions, hence the total extension of the muscle can be as high as 100%. They believe that the 5.1 A meridional spacing in living muscle, represents the length of two rather than the 3 residues, suggested by Astbury. No comments are made about the β-pattern. The crystalline-amorphous duality emphasizes the analogy with rubber, which shows mechanical properties in many ways similar to those of muscle.

From the sharpness of the equatorial reflections the crystalline regions must have considerably greater extent laterally than the 50 A assumed by Weber (see above), unless it is assumed that, in drying, there is a crystalline aggregation of the fibrillar bundles. It is possible that the well developed structure found by Lotmar and Picken was the result of an atypical process of drying and does not represent the structure of normal muscle. However, even though this particular structure be an artifact, it is highly interesting in itself and should prove of importance in the analysis of muscle structure.

Very little attention has been given to long-spacing diffractions of muscle. In a very recent short note Kratky, Sekora, and Weber (1943) described three equatorial diffractions in myosin having spacings of 66, 42, and 33 A. They suggest that the 66 A spacing reflects the thickness of the myosin fibrils seen with the EM by Ardenne and Weber (see below). Further discussion of the evidence for this conclusion will be awaited. Meanwhile it may not be amiss to point out that the three spacings mentioned are almost identical with those of tissue lipids (cerebrosides, phospholipids, and sterols) as reported by Bear, Palmer, and Schmitt (1941). Bear and Schmitt found lipid long spacings in fibers of extruded nerve proteins, even

when the extrusion was done in 50% alcohol. Kratky, Sekora, and Weber also describe a zone of diffuse scattering at about 100 A which grades off to the center. This they associate with bundles of elementary fibrils of myosin.

Arguing on the basis of the periodicity theory, by analogy with α-keratin and by a questionable interpretation of meridional reflections in muscle patterns, the largest recorded being 26 A, MacArthur (1943) suggested that the true fiber axis period in muscle is 658 A. In the case of clam muscle Bear (unpublished) has demonstrated that the macroperiod is 720 A (see below).

Electron Microscope

Ardenne and Weber (1941) have described EMG's of myosin made by evaporating, on the specimen holder, a very dilute solution of myosin after fixation with osmic acid. They observed fibrils 50–100 A in width and several microns long. The thicker ones are most abundant. From his polarized light analysis Weber had predicted dimensions of about 50 x 500 A for the elementary unit of myosin. Ardenne and Weber conclude that the elementary fibrils are built up by end-to-end aggregation of these units, forming the thinnest fibrils seen in the EMG's. The thicker fibrils represent bundles of these fibrils. The aggregation of the particles is considered due to secondary valence. No fibrils could be found in preparations made from myosin which had been denatured with 2 *M* urea, and the effect was irreversible. This is in agreement with the finding of Edsall and Mehl (1940) that urea irreversibly abolishes stream birefringence.

Evidence from ultracentrifuge data has been given by Schramm and Weber (1942) that, besides the usual myosin, having a sedimentation constant s_{20} = 6.2 SU, a second heavier fraction can be isolated. This component, which is present only in small amounts in the usual preparation, has an s_{20} value of 20 when prepared but, on standing, the value may rise to as high as 36. They believe the heavy component does not arise from the light component but preexists in the solution. In solution only the heavy component aggregates (with this weight a fibril 40 A thick would be 1μ long). Ardenne and Weber prepared EMG's from both components but no difference was found in the fibrils of the two types of myosin. Schramm and Weber, by fractionation, have prepared a truly monodisperse preparation of the lighter myosin and they consider that the elastic and contractile properties of muscle depend on the internal organization of these particles.

Muscle has been used as an object with which to test the possibility of making microtome sections thin enough to use with the EM. Richards, Anderson, and Hance (1942) made EMG's of cockroach muscle which were

0.1μ thick. They found that the dark bands are in reality composed of three bands, an observation also made by Lavin and Hoagland (1943) with

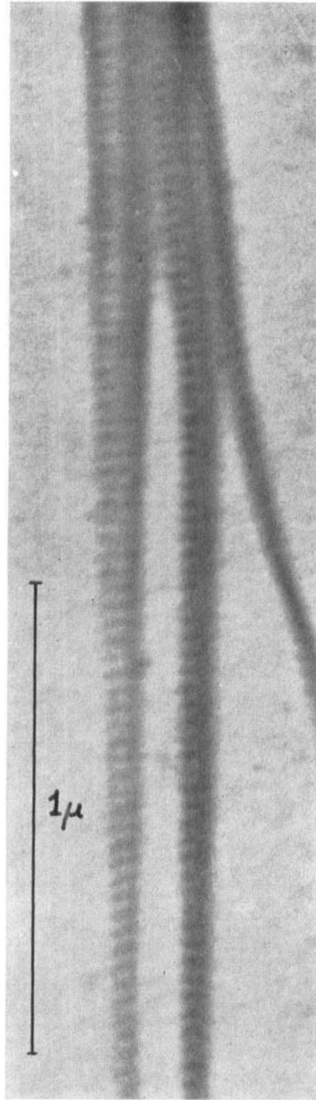


Fig. 9. Fibrils from adductor muscle of *Mya arenaria* treated with osmic acid. $\times$ 62,000

the ultraviolet microscope. No certain conclusions were drawn about the finer structure of muscle from the EMG's of these sections.

Sjöstrand (1943) claims to have made sections of guinea pig *skeletal muscle* 0.05μ thick and shows EMG's of these sections. The thinnest fibrils observed were 50–100 A in thickness. The author states that fibers running longitudinally are divided into lighter and darker segments. The EMG, as reproduced, is not sufficiently clear to make out any details of this banded structure.

A muscle preparation which lends itself particularly well to ultrastructure analysis may be obtained from the *adductor muscles* of marine and fresh water clams (*Mya*, *Venus*, *Anodonta*). These muscles, which are responsible for the closing of the valves, are capable of maintaining great tension for long periods and are usually regarded as smooth muscle. Jakus, Hall, and Schmitt (1944) found that, after maceration of these muscles in 0.2–0.3 *M* KCl, *submicroscopic fibrils* could be isolated by differential centrifugation. In the dark field microscope these fibrils appear long, slender, and needle-like. Suspensions of fibrils show high viscosity and positive *stream birefringence*. The solubility properties in 0.6 *M* KCl and in water resemble those of myosin, and the evidence thus far obtained indicates that the fibrils represent the chief component of the muscle.

In EMG's the fibrils appear very long and evenly contoured. Treated with osmic acid they show a periodic structure consisting of an alternation of relatively dark and light regions (Fig. 9). A statistical study showed the most probable spacing to be about 360 A.

After discovery of this long-spacing periodicity with the EM, an X-ray diffraction investigation of this material was undertaken by Dr. R. S. Bear (unpublished). He obtained small angle diffraction photographs of intact dried muscles showing the majority of the first 40 or more orders of a fiber-axis period of 720 A. The 5th order (144 A) is intensified, as are the 10th, 15th, 20th, etc.

Subsequently, certain stains other than osmic acid revealed greater detail of structure in the EMG's of fibrils. Very thin bands were observed, the spacing being about 146 A (corresponding to the intense 5th order X-ray spacing). Preliminary results indicate variation in the density of the dark bands to form a repeating pattern with a period of about 720 A (the fundamental X-ray period). By a synthesis of the X-ray and EM data it should now be possible to obtain fairly detailed information about the structure of this *protein fiber.*

V. *Fibrin*

The system by which an insoluble fibrous clot, having hemostatic value, is produced under physiological conditions from the various components in blood is of the greatest interest not only to hematologists but also to the student of *protoplasmic structure.* In this system are included, beside

the fibrinogen molecules which form the substance of the fibrin lattice, a series of components whose interplay determines the stability of the system. It may be profitable to consider the formation of fibrous structures in protoplasm as due to a similar interplay of a structure protein with *enzymes*, kinases, and antikinases. At any rate, it is doubtful that the protoplasmic system will prove to be less complex than that of blood clotting.

Few biological systems have proven more difficult to analyze biochemically than that of *clotting*. Reference to recent reviews, such as those of Quick (1942) and Taylor, Davidson, and Minot (1943), reveals how little can be stated categorically. Because of this situation it is impossible, in the scope of the present article, to consider the chemical and structural aspects in detail. Rather, only certain very recent discoveries, which seem to offer promising clues to the puzzle, will be considered.

Amino acid analyses for *fibrinogen* and *fibrin* are available (Bergmann and Niemann, 1937, a). The close similarity of the two shows that the change from the soluble fibrinogen to the insoluble fibrin involves little intramolecular change but rather reactions between a few groups which are externally oriented. Bailey (1943, and elsewhere in this volume) has called attention to a similarity of the amino acid composition of fibrinogen with that of myosin.

Ultrastructure

Polarized Light

Fibrin has been examined with polarized light (v. Dungern, 1937), and positive form and crystalline birefringence demonstrated. Quantitative analysis might prove illuminating but has not yet been done. Fibrinogen solutions show positive *stream birefringence* (Boehm and Signer, 1932; Wölisch and Clamann, 1932; Boehm, 1937). From such data it is clear that the molecules are highly asymmetric; a value of 1800 A was suggested as the approximate length of fibrinogen (see Edsall, 1942).[3] Further information from ultracentrifuge, viscosity, diffusion, and osmotic pressure methods is needed to establish the dimensions and molecular weight more accurately.

X-ray Diffraction

Katz and de Rooy (1933) obtained patterns from fibrin produced by forcing recalcified plasma through a capillary. The essential features are spacings at about 4.5 and 11 A which take the form of equatorial spots if the longitudinal orientation is sufficiently high. The pattern belongs to the β-class.

[3] Recent measurements by Scheinberg and Edsall (unpublished) indicate a length of about 900A for human fibrinogen.

Bailey, Astbury, and Rudall (1943) studied moist strips of film made by drying a fibrinogen sol on a plate. A typical α-pattern was obtained both from fibrinogen and fibrin when sufficiently oriented. By squeezing fibrinogen films laterally, long thin threads were formed which gave typical α-patterns. It was concluded that both fibrinogen and fibrin are normally in the α-form and that fibrin formation consists of an aggregation by end-to-end accretion of fibrinogen units without marked intramolecular alteration. The authors lay great stress on the biological and structural significance of the fact that fibrinogen and fibrin are members of the keratin-myosin group.

Fibrin is undoubtedly far more highly organized than the diffraction-poor patterns thus far described indicate. No long-spacings have been reported, though EM observations indicate (see below) that if fibrin could be suitably prepared such diffractions may arise.

Electron Microscope

Wolpers and Ruska (1939) obtained EMG's of fibrin produced from plasma. They described the smallest fibrils as long, slender, parallel, and unbranched micellar bundles. Comments are made concerning the bearing of the results on v. Dungern's (1937) view that fibrin is an ideal Wiener mixed body. From EMG's of blood platelets, in their relation to the clotting process, it was suggested that activation of prothrombin takes place within the platelets and that adsorption of the fibrin fibrils on granules liberated by the platelets is important for retraction. It is now known, however, that all of the components necessary for clotting may be found in platelet-free plasma. That platelets may be important, especially in the process of retraction, is probable though it is less certain that platelet activation of prothrombin, stressed by Wolpers and Ruska, reflects the actual physiological mechanism.

Ruska and Wolpers (1940) observed striated fibrils in EMG's of the fibrin pellicle which forms in cerebrospinal fluid in cases of tuberculous meningitis. Measurement of their published EMG indicates periodicities ranging from about 500 to 600 A. They could not obtain such striations in clots produced by recalcifying a mixture of plasma and spinal fluid. It was suggested that the very slow clotting in spinal fluid permits a regular organization of the micelles, and that there is a relation between the longitudinal periodicity and the length of the fibrin molecules.

In collaboration with Dr. L. B. Hobson, the author investigated pellicle fibrin from tuberculous meningitis spinal fluid. In these unpublished experiments striations were found in preparations from one case out of seven investigated. To avoid the complexities of this clinical system attention is now being given primarily to the structure of fibrin produced by recalcifying plasma and by mixing highly purified fibrinogen and thrombin.

VI. General Considerations of Protein Fiber Structure

Astbury has developed a general theory of the configuration of the polypeptide chains in protein fibers. He considers two categories: the collagen type in which the chains run in approximately straight lines, and the keratin-myosin type which show the α-β-characteristics. Recently, fibrin has been added to the latter class. Following Neurath's (1940) demonstration of the inadequacies of the original theory of hexagonal α-folding, Astbury (1941) proposed a square folded configuration. Basic is the assumption that the meridional spacing at 5.14 A represents the length of an intramolecular fold comprising three residues. The average length of an amino acid residue is thus about 1.71 A. Keratin can be extended about 100% under appropriate conditions, and it is assumed that the chains are then fully extended, the residues having a length close to 3.5 A.

While the square configuration satisfies stereochemical and certain other requirements, it is not proven from X-ray data as a unique structure. Lotmar and Picken's (1942) interpretation, based on better evidence than was previously available, is that the length of the residue is 2.82 A, which is quite different from 1.71 A and would not allow an extension of 100%. To what extent the singular specimen of dried clam muscle described by Lotmar and Picken is typical of dried muscle generally remains to be determined. While there is certainly something of general application in the α-β-patterns in protein fibers, their real significance is not yet clear. Harrison (1938) and Lotmar and Picken (1942) suggest the possibility of amorphous as well as crystalline phases and that the extensibility data of macroscopic fibers cannot be applied directly to explain changes in spacings measured on the α- and β-patterns.

It has been known for some years that protein fibers show fiber-axis long spacings but little attention was given the matter until the advent of periodicity concepts of the protein molecule. Astbury's interpretations of long spacings have been based on an examination of chemical analytical and X-ray data "with the eye of stoichiometric faith" begotten of the periodicity theory. Thus MacArthur's (1943) data are interpreted to indicate that the fundamental fiber period of α-keratin is 658 A, although the diffraction data in themselves require a spacing no greater than 198 A (Bear, 1943). A spacing of 658 A requires 384 residues along a single chain (if 3 residues are 5.14 A long), whereas the chemical data indicate 576 residues per molecule. To explain this discrepancy, Astbury (1942) suggests that the chains may be folded into long loops of the type which would conform both to 384 and 576. Actually, long axial loops might be invoked to explain the great extensibility of fibrils of collagen (not a member of the keratin class) as observed with the EM by Schmitt, Hall, and Jakus (1942). However, demonstration from X-ray data of the existence of such loops in α-keratin rests for the present on rather precarious grounds.

Ogston (1943) has pointed out that regularities of structure of proteins, as deduced from analytical data, need not require powers of 2 and 3, but may be obtained in many other ways.

The recent EM work has focussed attention on the large unit of structure along the fiber axis in protein fibers. While the procedure necessary for preparing a specimen for EM examination may cause alterations of the spacings in the fibrils, a fairly good approximation of the natural period may be obtained by a statistical study of the spacing in many fibrils. A great advantage of the EM method is that it permits discovery of large periods in material unsuitable for X-ray methods because of such difficulties as poor orientation, irregularities of spacing in extensible fibers, and smallness of specimen. In tissues such as keratin, collagen, and muscle there is semi-crystalline regularity of structure, facilitating X-ray detection of the long spacing. In fibrin, the conditions are not so favorable and in this case periodicity was demonstrated first with the EM. In microscopic fibers, such as trichocysts, sperm tails, and other intracellular fibrils, it may be very difficult indeed to apply the X-ray method though these structures lend themselves well to EM study. Where the X-ray method is applicable, the value of the spacing can be determined with more certainty than with the EM method which involves a statistical study. Also, if the spacing is relatively short, EM detection becomes difficult. A combination of methods provides both the direct visualization of electron densities along the fibrils, with the EM, as well as the possibility of carrying the resolution to greater detail with X rays. Since the difficulties are chiefly technical in nature it may be hoped that both X-ray and EM data may eventually be obtained for each important type of protein fiber, and that a general theory of structure may then become possible.

Attention may also be drawn to the fact that though the short-spacing X-ray patterns of representative protein fibers (such as collagen and myosin) may differ, so that Astbury has placed them in separate categories, they both manifest long-spacing periodicities in which there is an alternation of density of material within the characteristic period. The value of the spacing and the relative densities appear to be different in the two cases mentioned. When the underlying structural meaning of the above facts becomes clear it may be necessary to alter current concepts of the ultrastructure of fibrous protein molecules. The great need for the present remains that of gathering the data; when this is accomplished sound generalizations and unification will inevitably follow.

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Some Contributions of Immunology to the Study of Proteins

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I. Definitions and Methods

1. *Introduction*

The importance of an immunological approach in preventive medicine, in diagnosis, and in therapy is firmly established. Less appreciated, however, is the fact that the same procedures can be utilized to yield valuable information concerning the nature of proteins as chemical substances.

These contributions have been of several kinds. Immunological investigations, often with a practical end in view, have uncovered a number of proteins whose unique properties later engaged the serious attention of protein chemists. Antibodies, complement, and tobacco mosaic virus protein are but a few examples. The problem of characterizing these substances has in recent years greatly stimulated the development and application of physical techniques such as ultracentrifugation, electrophoresis and diffusion. As microanalytical tools, serological methods have found important application in detecting small amounts of proteins in the presence of large quantities of contaminating substances, protein or otherwise. Finally, serological methods have contributed materially to our knowledge of the specificity of proteins, complementing physical methods, and in several cases serving to differentiate proteins where these failed.

2. *Antigens and Antibodies*

If certain substances foreign to the blood are injected into an animal there appears a week or so later in the serum of that animal a new protein—the *antibody*—which can react in a highly specific manner with the introduced material (termed the *antigen*). Although most of the antigens known are proteins, there is direct evidence that at least a few of the bacterial polysaccharides are antigenic in their present state of isolation, and indirect evidence that many of the remainder are antigenic when in proper combination with protein, as is presumably the case in the bacterial cell. The evidence for the antigenicity of lipids is much less certain.

The antigen must be of high molecular weight, at present 10,000 appears to be the lower limit reported for proteins (Seibert, Pedersen, and Tiselius, 1938). It is likely that smaller molecules will not be retained long enough in the circulation to exert antigenic action. The route of injection, whether intravenous, intraperitoneal, intradermal, or subcutaneous may exert a quantitative influence on the antibody response, and possibly a qualitative one as well (p. 103) inasmuch as some proteins do not appear to be effectively antigenic when given by particular routes. This, as well as the increased response obtained with the aid of adjuvents (Freund and McDermott, 1942) is naturally of interest from both a physiological viewpoint and from the standpoint of practical serum production. All proteins

which have been tested are antigenic with the exception of a few degraded forms such as gelatin.

3. *Methods of Detecting Antibody*

In the *precipitin reaction* given by most systems, a flocculent precipitate results when an appropriate amount of antigen is added to the serum. A quantitative significance is often given to the dilution of antigen or more especially antibody at which the reaction fades out. In all systems there is a more or less definite ratio of antigen to antibody at which flocculation is fastest, which again is of some quantitative significance (*optimal proportions method*). A marked excess of antigen not only slows down the rate of precipitation but if sufficient will prevent it entirely. In toxin-antitoxin reactions involving horse serum a complete inhibition is observed with an insufficient amount of antigen as well. In a few cases demonstration of antibody by the precipitin reaction may require a considerable number of tubes containing closely spaced ratios of antigen to antibody. To overcome this the *ring test* has been used—a concentrated antigen solution is layered over the serum, and at some level in the resulting interdiffusion the proper conditions for precipitation will be reached, resulting in a ring of precipitate. The Molisch test for carbohydrate employs the same principle, and is subject to the same limitations.

The surfaces of bacteria, viruses, and blood cells may be regarded as having at least a portion of their proteins and carbohydrates exposed. If the specific antiserum is added these particles will visibly coalesce (*agglutination reaction*), and there is evidence that the antibody acts as a bridge linking the antigens on two or more adjacent particles.

Quantitative absolute methods, suggested in part by Wu and greatly extended by Heidelberger, Kendall and collaborators (reviewed by Heidelberger, 1939), have been used to an increasing extent. Precipitation or agglutination reactions are carried out under conditions applicable to analytical chemistry, and the amount of antibody protein N in the washed specific precipitate or agglutinate is obtained by deducting from the total protein N found the antigen N if any. Antibody determinations corresponding to 0.1–1.0 mg. of N are usually made by a micro-Kjeldahl technique, with a reproducibility of 5% or better. Recently a colorimetric (tyrosine) procedure has been described which permits the estimation of as little as 10 μg. of specific precipitate N with a reproducibility of 2 μg. (Heidelberger and MacPherson, 1943).

A great many antigen-antibody precipitates (or soluble complexes) have the property of removing from serum one or more of a group of normal serum components termed complement (p. 108). The presence of complement can easily be detected by adding to the system an indicator com-

posed of sheep blood cells sensitized with their specific antibody (anti-sheep cell rabbit serum). If the complement has been removed, and therefore the initial antigen-antibody reaction occurred, no lysis of the red cells will be observed. This is a very sensitive procedure, detecting 1 μg. of antibody or less (Heidelberger, Weil, and Treffers, 1941). The familiar Wassermann reaction is a modification of this *complement fixation reaction*. The *anaphylactic reaction* depends on the fact that an animal, usually a guinea pig, given an initial sensitizing dose of antigen may be shocked, with characteristic and often fatal symptoms, after injection of a second and larger dose of antigen (reviewed by Ratner, 1943). Wells (1929) states that as little as 0.05 μg. of crystalline egg albumin will sensitize a guinea pig, and Kabat and Landow (1942) have shown that passive transfer of sensitivity may be accomplished with as little as 0.03 mg. of antibody N.

Under proper conditions the above reactions can normally be demonstrated for any type of protein. However, not all of the protein constituents of bacteria (at least as isolated) give rise to antibodies which have the desirable property of protecting an animal from infection or of killing bacteria *in vitro*, and which are detected by an appropriate biological test.

As the tests described above were developed, the antibodies disclosed were given corresponding names, such as precipitin, agglutinin, complement fixing antibody, etc. It was natural to ask in what manner they were related, and in his Unitarian theory Zinsser (1921) developed the concept that these could all be considered as functional aspects of a single antibody to a single antigen. Experimental confirmation has been given in a number of instances, and while the theory is undoubtedly correct and useful, the application to complex bacterial systems has given rise to some misconceptions. It will again be referred to in connection with the so-called protective antibodies (p. 96).

4. *Limitations of Tests**

It was appreciated as early as 1901 by at least some workers that bacteria and whole serum contain not one but several antigens, each of which could give rise to a specific antibody. Anticipating for the moment a discussion of the evidence, we may say that the antibody response even to a presumably single antigen such as ovalbumin or a bacterial specific carbohydrate may be far from simple. The difficulties in separating protein antigens from the bacterial cell and then from each other and from contaminating polysaccharides or nucleic acid are many. It is also difficult

* Since this manuscript was submitted for publication a review article (Kabat, 1943) has appeared which contains much material of interest for this and other sections.

to be certain that the product obtained is not denatured in some way and that at least a portion of some biological property has not been lost. Much of the early work on bacterial proteins must now be regarded as exploratory and provisional until confirmed with newer, less drastic methods, and until the products have been examined by more rigorous physical criteria of homogeneity (*cf.* the valuable review by Pirie, 1940).

Serological tests such as the usual precipitin, agglutinin, and complement fixation reactions, and the biological technique of anaphylaxis suffer from two defects which often render uncertain the application to protein chemistry of data obtained with them. First, the precision with which the relative precipitating powers of two antigens can be compared, although satisfactory for clinical diagnostic procedures, leaves much to be desired. Antigenic differences important for the immunochemical comparison of two proteins may be masked by titer methods which although sensitive to traces are not well adapted to more quantitative requirements. It may also be difficult to be certain that the positive reaction observed is not due to some minor protein or polysaccharide impurity. In several instances data as to the identity or non-identity of proteins obtained by these methods could have been greatly strengthened by proper combination with absorption methods (p. 74).

Second, these methods permit estimations only in relative, not absolute (weight) units. This may lead at times to quite erroneous conclusions. Statements based on titer estimates are made to the effect that a certain bacterium is a poor antigen, compared to another species. A few elementary considerations make it evident, however, that some bacterial cells are more sensitive to agglutination than are others. They therefore require less antibody, by weight, to effect a visible reaction (Heidelberger, 1943). Although titrations by the dilution method of two antisera to the same bacterium may have a close relation to the relative amounts of antibody in the two sera, such data cannot properly be compared with titrations of antisera to other, unrelated bacteria, unless the various bacterial systems all have the same (but usually unknown) sensitivity to agglutination.

Quantitative absolute methods although somewhat more time consuming enable one to form an opinion as to whether or not the reaction is that of a minor component, by comparison with the known combining ratios of other proteins. The same type of information should also be obtained (although less precisely) from optimal proportions titrations (see however Heidelberger and Kendall, 1935, b) but these have seldom been carried out on carefully defined systems. The quantitative absolute methods are of course especially designed for estimations in weight units. Several typical applications are presented below.

II. The Specificity of Proteins

1. *Cross Reactions*

It was mentioned previously that serological reactions are characterized by a high degree of specificity. This is not absolute however. An antiserum prepared against hen ovalbumin will react markedly with duck ovalbumin (*cross reaction*). If all of the antibody which will thus cross react with duck albumin is first removed by the addition of a slight excess of that antigen, it can be shown that the supernatant will still react with hen ovalbumin (Hooker and Boyd, 1934). The two ovalbumins can therefore be distinguished by the *absorption method*—a useful general procedure.

The extent to which a serum will cross react is the product of several factors. In any species immunized, the percentage of cross reactive antibody increases on extended immunization. It also appears to be influenced by the closeness of the zoological relation between the species whose protein is used as immunizing antigen and the species injected. Landsteiner (1936) discussed the fact that a group of antigens from animals closely related to the species receiving the injections will give rise to sera of sharper specificity than if a quite unrelated species were immunized. This is presumably due to the inability of an animal to synthesize antibodies against those parts of the antigen molecule which are common to its own serum proteins. An interesting problem of this type has been presented by Shope (1939). Cross-neutralization tests were made of seven strains of hog influenza virus with sera from hogs convalescent to the virus. All sera cross-protected, indicating an antibody to some common protective antigen in each strain. When rabbits were injected with the various strains, it was found, however, that only two sera resembled those from the hog in having a general neutralizing action. In contrast, two sera were quite specific, neutralizing only their homologous viruses. The remaining three sera were intermediate in their specificities, neutralizing some but not all strains. Shope suggested that the seven strains of virus had a similar antigenic composition but a different arrangement of groups, and that the rabbit antisera reflected the arrangement, the hog sera being less specific.

2. *Species Specificity*

An analogue of almost every protein can be found in other animals or plants. It is a question of considerable interest from many viewpoints in what respects, for example, the albumins or hemoglobins of various animals resemble each other, or whether pepsin from the hog is the same as pepsin from the chicken, or again whether tobacco mosaic virus protein isolated from a tobacco plant is the same as that isolated from a similar virus infecting cucumber plants.

Although members of a given class of proteins may often be analytically distinguished from each other by such criteria as molecular weight, iron or amino acid content, there is usually among comparable proteins a serological interrelation which parallels their positions in the zoological classifications to a surprising extent. This has naturally aroused the interests of biologists and given rise to a considerable literature (reviewed by Landsteiner, 1936). The unique property of a protein which enables it to be distinguished from serologically related but not identical proteins from other animals or plants is known as the *species specificity*. It is not a factor, however, which is common to all proteins in that individual (although some such factor may exist undetected) since a given protein may show little or no serological relation to other proteins which are found in its immediate environment. As an example, the albumins from horse and goat sera cross react with antisera to each other, and the same relation, quantitatively of the same magnitude (Treffers, Moore, and Heidelberger, 1942) is observed between the globulins of the two species. Nevertheless no crossing has been observed between the albumins and globulins of horse serum, and presumably the same would hold for the pair of goat proteins. In fact the distinct specificities of albumin and globulin from the same species seem to be a general rule.

The identification of a protein or carbohydrate as being derived from a given species is a problem of considerable importance in medical-legal work, as in the characterization of blood found on the clothing of a suspect as of human or animal origin. In cases of this sort, and in problems involving disputed parentage, the probability that a given sample of human blood belongs to a certain individual can be determined with the help of the more than a dozen antigens which have been identified in human blood cells. Our knowledge of many of these, as well as of the basic principles of human blood transfusions is derived from the now classic blood specificity studies of Landsteiner (reviewed by Landsteiner, 1936; Boyd, 1943). The chemistry of these substances, however, has not kept pace with application and technique.

3. *The Chemical Basis of Specificity*

One of the important problems of immunochemistry is to account in chemical terms for the unique specificity of almost every protein. The extensive experiments of Landsteiner over more than twenty years (summarized in monograph, 1936) have done much to clarify the problem. The basic procedure adopted by Landsteiner and by most later workers consists in introducing by some convenient chemical reaction a new group into a protein molecule and then injecting the modified antigen into a rabbit. The antiserum produced will usually react not only with this antigen (homologous reaction) but will also cross react to some extent with the

basic unmodified protein and most importantly, with any other protein modified by the introduction of the new chemical grouping. The latter reaction is significant in that if an unrelated protein base is employed any reaction observed will be due only to the specificity of the newly introduced group. The original protein is employed merely as an antigenic substance (*i.e.* to elicit antibodies); its own specificity does not enter into the test procedure. As an example, the compound *p*-amino-benzoyl-glycyl-leucine (G.L. for short) was synthesized and by means of the diazo reaction linked, presumably through tyrosine, to horse stromata. This compound antigen was then injected into rabbits. As a test antigen the polypeptide derivative was similarly coupled to unrelated protein(s), chicken serum. Similar sets of antigens and also antisera were prepared against leucyl-glycine (L.G.), glycyl-glycine (G.G.), and leucyl-leucine (L.L.) determinants. G.L. antiserum of course precipitated G.L. antigen. It gave less precipitate with L.L. antigen and little or none with G.G. or L.G. Thus, although both amino acids have an influence on the specificity, the terminal one is the more important (Landsteiner and van der Scheer, 1932). The simple determinant compound *p*-amino-benzoyl-glycyl-leucine, although incapable of stimulating antibody formation when not coupled to protein, can react nevertheless with antibody formed by the conjugated protein, and a sufficient excess will completely inhibit the reaction with G.L. antigen.

Such simple chemical compounds Landsteiner termed *haptenes*. Some bacterial polysaccharides are also examples, in that they react strongly with rabbit antibodies to the intact bacillus but cannot elicit them when injected into that animal (although they may be antigenic in other species such as the mouse).

In later experiments polypeptides containing up to five amino acids were prepared (Landsteiner and van der Scheer, 1934, 1939). Here the influence of the kind, order, and number of the amino acids could advantageously be studied. In Table I are given some typical results, expressed in the above terminology.

This table illustrates a number of interesting points, among them how a reactivity toward the antiserum to G_5 is built up with an increase in the number of glycine radicals in the chain, how these may be abolished even for G_4L by a terminal leucine group, and how sharply antisera to peptides of the same empirical composition (G_4L and G_2LG_2) may be distinguished, as in the GL and LG_2 columns. Parallel experiments were also carried out in which the terminal —COOH groups of the polypeptide chains were converted to amides. The most marked cross reactions were often observed with compounds having similar central groups but dissimilar terminal amides, rather than vice-versa. These results contrast markedly with the

extensive earlier results of Landsteiner and his co-workers on the influence of acid (—COOH and —SO_3H) radicals or halogens as determinant groups. Both the nature of the substituent and the position isomerism (ortho, meta, and para) in benzenoid compounds similarly coupled to protein were shown to be important. As in the polypeptide experiments it was found that increase in substituent chain length diminished the sharpness of the determinant specificity. Although phenylacetic acid is quite distinct serologically from benzoic acid, there is less distinction between phenylbutyric acid and phenylcaproic acid (4 and 6 carbon chains).

Limitation of space precludes any further discussion of the many other interesting studies employing diazo coupling reactions. It may suffice to say that they include such variables as cis-trans isomerism (Landsteiner and van der Scheer, 1934) or in the case of carbohydrates, α-β isomerism

TABLE I

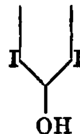
Reactions of Antisera to Determinent Peptides Coupled to Horse Protein With Test Antigens Containing Various Peptides Coupled to Chicken Protein

Antisera to	Test antigens: chicken protein coupled to amino benzoyl derivatives of													
	G	L	G_2	GL	LG	LL	G_3	G_2L	LG_2	GLG	G_4	G_4L	G_5	G_2LG_2
G_5	0	0	tr.	0	0	0	1½	0	tr.	0	1½	0	1½	1
G_4L	0	1½	tr.	2	0	1½	1	3	0	0	1	4	1	0
G_2LG_2	tr.	0	1	0	0	0	2	tr.	2	0	1	0	2	3

The strength of the reaction is indicated by numerals. 0 = no reaction; tr. = trace; 4 = strong reaction. Determinants: G = glycine; L = leucine; G_5 = tetraglycyl-glycine; G_4L = tetraglycyl-leucine; G_2LG_2 = diglycyl-leucine-glycyl-glycine; etc.

(Avery, Goebel, and Babers, 1932) or glycoside formation (Avery and Goebel, 1929).

A number of other types of reactions have been employed for the introduction of determinant groups. Iodinated and brominated proteins cross react with antisera to each other. Both reactions can be inhibited by compounds of the configuration



such as 3,5-diiodo-4-oxybenzoic acid, diiodo-tyrosine or thyroxine but not by $CH_2I \cdot CH(OH) \cdot CH_2I$ (Snapper and Grunbaum, 1936). Other changes in the specificity may be made by phenyl-iso-cyanate (Hopkins and Wor-

mall, 1933); carbobenzoxy chloride (Gaunt, Higgins, and Wormall, 1935); phosphorus oxychloride, (Heidelberger, Treffers, and Davis, 1941); or by formaldehyde (von Eisler and Lowenstein, 1912; Horsfall, 1934). Interesting quantitative immunological data on some of these systems have been obtained by Kleczkowski (1940).

The trend in recent experiment involving the introduction of new groupings has been to use reactions proceeding under as mild conditions as possible in order to avoid secondary denaturing reactions. In other studies, however, the influence of reversible or irreversible denaturation of specificity has been the main point. Blumenthal (1936) found that the precipitating power of serum albumen toward its antiserum was partially destroyed by reduction with thioglycolic acid; it was not restored on reoxidation of the antigen. On the other hand she found that reduction of egg albumin did not affect its serological behavior. The keratins also provide interesting material. In a study of keratins from wool, chicken feathers, and human hair, Pillemer, Ecker, and Wells (1939) found that although each has been reported to have a histidine:lysine:arginine ratio of 1:4:12 there are distinct serological specificities, perhaps due to the arrangements of cystine. Oxidized (air + Cu_2O) and reduced (thioglycolic acid) keratins cross reacted to some extent with antisera to each other.

The antigenic powers of horse and bovine serum albumins regenerated after denaturation in urea or guanidine solutions seem to be less than those of the native proteins. The specificity and the binding capacities are reported to be unaltered, however (Neurath, Erickson, and Cooper, 1942; Martin, Erickson, Putnam, and Neurath, 1942–3).

The experiments on specificity discussed to this point have all involved either an increase in the molecular weight by addition of new determinant groups, or modification by some agency without essential change in weight. Relatively few data are available on what might be termed the size of the unit (or of one such) responsible for the specificity of natural antigens. Pertinent information can be obtained from studies on hydrolytic products actually derived from the antigen. In the case of pneumococcus specific carbohydrates quantitative studies have been made on the reactivity of acid and alkali degraded fractions (Heidelberger and Kendall, 1933; Treffers, Moore, and Heidelberger, 1942). Interesting data on the size of a serologically reactive unit in silk fibers have been presented recently by Landsteiner (1942). Partially purified degummed silk was hydrolyzed with acid and then dialyzed through cellophane for definite periods, and some of the fractions further purified by precipitation. Using the inhibition of the reaction of the unhydrolyzed silk with its antiserum as a test, he found that complete inhibition could be obtained with a 1 per cent solution of a product corresponding to a peptide of 7 amino acids, or a chain

weight of about 600. The strongest inhibition, at a greater dilution, was given by a product corresponding to 12 amino acids.

This section has dealt mainly with experiments on compounds of known structure. Many of the studies considered below, although not concerned with materials as well defined as haptene determinants, have nevertheless a considerable bearing on the problem of protein specificity. It is preferable, therefore, to postpone a summary and application of the data until these have been presented.

4. *Blood Proteins*

The hemoglobins are characterized by a common prosthetic heme group, linked to a protein which has a species specificity. That the properties of the various hemoglobins are not identical can be shown by mixed solubility tests (Landsteiner and Heidelberger, 1923). In a series of serological tests it was shown that a rabbit antiserum to crystalline horse hemoglobin gave a cross reaction only with donkey hemoglobin. Human, dog, sheep, and goat hemoglobins were all inactive toward this serum. That the iron containing portion was serologically inactive was further indicated by the failure of hematin to inhibit the reaction between horse hemoglobin and its antiserum (Heidelberger and Landsteiner, 1923). Hektoen and Schulhof (1927) have reported that globin and hemoglobin do not have completely identical specificities, but as has been seen this is not due to any generalized hemoglobin specificity. It could be shown however, that an excess of dog hemoglobin would completely inhibit the reaction of horse hemoglobin. Others examples of this phenomenon will be met with below.

More complete data are available for the serum albumins. The generality of the cross reactions between albumins of various species has been discussed by Wells (1929), and by Landsteiner (1936). One of the difficulties in investigations of this type is that even recrystallized horse serum albumin may contain enough globulin to incite detectable anti-globulin on injection (Goldsworthy and Rudd, 1935). By using smaller amounts of antigen than did previous investigators, Kabat and Heidelberger (1937) were able to obtain a strong antiserum which gave clean-cut equivalence zones, that is, after addition of a certain amount of antigen, with removal of the precipitate, a supernatant was obtained which did not give a test for either antigen or antibody. As will be seen later (p. 91) this is often a useful test for antigen homogeneity. Tested against this antiserum by the quantitative precipitin reaction, the horse serum albumin alone, or coupled to a dye grouping (R-salt-azo-diphenyl-azo-) gave identical results. This indicated that the tyrosine to which the dye groupings were linked played little if any part in determining the specificity of the horse serum albumin. This is in marked contrast with similar experiments on dye-egg albumin

in which the compound antigen could be fractionated until it no longer reacted with anti-egg albumin (Heidelberger and Kendall, 1935b). Injection of the dye-horse serum albumin into rabbits produced an antiserum which also differed in behavior from the antiserum to the untreated horse protein in that the reaction was not identical for both proteins. The uncoupled protein base removed about 43% of the total antibody to the dye-protein. In this case the unit specificity was considerably larger than the dye molecule alone, since the latter gave only a trace of precipitate and when in excess produced no significant inhibition of the dye-protein reaction.

III. The Properties of Antibodies

Antibodies are an essential component of all serological reactions. In view of the considerable literature on the subject it may be advantageous at this point to summarize without extensive documentation the evidence presented in detail in other reviews (Marrack, 1938; Heidelberger, 1939; Boyd, 1943; Treffers, 1944) and to discuss in the space thus available some topics not as fully considered elsewhere.

1. *Origin*

Buchner tried to account for the origin and specificity of antibodies by suggesting that they contained fragments of the antigen. With the discovery that antibodies, including those to carbohydrates or to proteins containing tracer substances such as arsenic were protein in nature and contained no demonstrable antigen this idea was abandoned. The situation today is illustrated by a quotation from Heidelberger (1938a), "... there is now much evidence that antibodies actually are modified serum globulin. Occasional claims are still put forward that protein-free antibodies have been obtained but their analysis indicates that the oft-repeated demonstration has been forgotten that chemical tests for protein fail at dilutions at which biological reactions such as anaphylaxis and bacterial agglutination readily occur. Until such claims are accompanied by the isolation of weighable amounts of protein-free antibody they can carry little conviction." It may be added that the cautions mentioned are equally applicable to claims for protein-free bacterial toxins, phages, etc.

Interest has therefore been shifted to mechanisms whereby the normal serum globulins might be synthesized. Sabin (1939) has reinvestigated the question and presented new histological evidence based on the use of a dye-antigen as tracer that the reticulo-endothelial cells are involved. Breinl and Haurowitz, and Mudd have proposed that antibodies result from a modification of the normal processes of globulin synthesis. Pauling (1940) after reviewing the data postulated that the fundamental polypep-

tide chains of normal and antibody proteins are the same, the specific biological properties being due to differences in folding induced by the antigen. As a deduction from this he suggested that antibody might actually be synthesized *in vitro* by a reversible denaturation of protein in the presence of antigen, and has recently reported some details of an experiment of this type (Pauling and Campbell, 1942). Further extensions of this important development are naturally awaited with interest.

The relation of plasma proteins, including antibodies, to other body proteins has been investigated with isotopic techniques by Schoenheimer, Heidelberger and their colleagues (1942). After administration of amino acids containing N_{15} it could be demonstrated that the plasma proteins incorporated the isotope at the same rate as liver and kidney, and that all fractions of the plasma were involved to an equal extent. The half-life of an antibody molecule was estimated at about 2 weeks, the same as that of the average serum protein. It could also be shown that an antibody passively introduced into an animal did not take part in this metabolic cycle whereas that actively formed in the animal did.

Most theories of antibody formation do not account satisfactorily for changes in the nature of antibody on extended immunization nor for the persistence of antibody long after both the antigen and the antibody initially synthesized have presumably been broken down. To overcome this, Burnet (1941) has suggested that antibodies are synthesized by intracellular proteases which can be modified by the antigen. When the latter is withdrawn the protease still retains its impress but is slowly modified in its succeeding forms.

2. *Chemical and Physical Properties of Antibodies*

In some respects antibodies are the simplest proteins to isolate—it is only necessary to add the specific antigen to immune serum and collect the precipitate. After washing, the specific precipitates are quite suitable for many purposes, such as amino acid analyses, if proper deduction for the antigen is made, or a non-protein antigen used. The data (tables given by Marrack, 1938; Heidelberger, 1938, a; Boyd, 1943) do not indicate any striking differences in composition for horse and cow antibodies compared to various normal globulin fractions. The analyses were done, however, before antibodies were sufficiently characterized physically (as in Table II) to permit selection of strictly analogous normal globulin fractions. A real difficulty also lies in determining whether the "normal" component selected is not also an antibody, perhaps to an inapparent infection.

Heidelberger, Kendall, and Theorell (1936) showed that under proper conditions of temperature and salt concentration the combining proportions of some antigen-antibody systems could be altered. By starting with

washed specific precipitates a portion of the antibody can be released into the supernatant. Such solutions, in which 90–100% of the total nitrogen is precipitable by the specific antigen have now been obtained in several laboratories.

Antibody solutions have also been obtained by various special reactions, but the properties, especially the percentage of precipitable nitrogen have not been reported in detail. Kirk and Sumner (1934) prepared anti-urease (p. 107) from immune rabbit sera by denaturing the specific precipitate with dilute acid. On bringing the solution to the isoelectric point of urease the denatured enzyme was precipitated, leaving the antibody. The operation could then be repeated, if desired, with this solution. Trypsin had no action on the anti-urease at pH 7, but pepsin rapidly destroyed it at

TABLE II

Physical Properties of Antipneumococcus Antibodies From Various Animal Species

Species	% protein in soln. examined	Sedimentation Constant	Diffusion Constant $D_{20} \times 10^7$	Calculated Molecular Weight	f/f_0
		Svedberg units			
Rabbit	0.19	7.0	4.23	157,000	1.4
Monkey	0.31	6.7	4.06	157,000	1.5
Human	0.39	7.4	3.60	195,000	1.5
Cow	0.64	18.1	1.69	910,000	2.0
Horse	0.22	19.3	1.80	920,000	2.0
Pig	0.58	18.0	1.64	930,000	2.0

Values given are valid only at protein concentrations stated. For details the original: Kabat (1939) should be consulted.

pH 4.3. The presence of considerable amounts of carbohydrate was noted. Antibody can also be released by digestion of the latent mosaic virus antigen contained in antibody-virus specific precipitates.

The usual Tiselius electrophoretic diagram of horse, rabbit, or human sera clearly show a separation into components of different mobilities: albumin and the α, β, and γ-globulins. That these components are not homogeneous can be shown by a more detailed electrophoretic analysis, by sedimentation, by solubility determinations, or by serological tests. The interrelations of some of the fractions identified by these means are discussed by Cohn (1941), Svensson (1941), Boyd (1943), and Treffers (1944). The progress of immunization is marked by a number of changes in these patterns. In view of active research still in progress, and restrictions on publication, developments in the investigation of human serum proteins are not reviewed here.

3. *Rabbit Sera*

In the rabbit, antibodies have been detected only in the γ-globulin (Tiselius and Kabat, 1939), although Seibert (1943) has noted changes in other components after tuberculin sensitization. No distinction, except reactivity toward the specific antigen has been found as yet between antibodies and the normal γ-globulin.

4. *Horse Sera*

Here the situation is more complex. Injection of pneumococci may result in the production of a new component with mobility between β and γ (the so-called β_2 or T component), or the antibody may be found exclusively in the γ-component, or finally both may occur simultaneously (Tiselius and Kabat, 1939; van der Scheer, Wyckoff, and Clarke, 1941). This antibody is of a higher molecular weight, 990,000 than the normal γ-globulin (150,000), although a small amount of the heavier material is found in normal horse serum as well (Heidelberger and Pedersen, 1937). Many other anti-bacterial antibodies seem to resemble the γ-anticarbohydrate just described.

The injection of toxins or toxoids into the horse gives rise to antitoxins, of which those to the diphtheria bacillus have been studied in some detail. The first phase of the response, at least in some animals, consists in an increase in the γ-globulin. The antitoxin in this fraction flocculates (p. 101) relatively rapidly with toxin (Kekwick and Record, 1941). The next phase, and in some animals the only one, is the formation of a new β_2 or T component which increases in amount on immunization. This antibody flocculates more slowly with toxin (Pappenheimer, Lundgren, and Williams, 1940).

The physical properties of this antibody are quite unlike those of antibodies to bacterial cells, with a lower molecular weight, and a more marked water solubility (Table III). The untreated material given for comparison consists essentially of an antibody concentrate (Column 2). Although homogeneous by sedimentation, diffusion, and electrophoresis this protein could easily be shown to be composed of at least two fractions since only one-third of the nitrogen was precipitable by toxin. It is not present to any extent, however, in normal horse serum, and its amount increases markedly on immunization. The properties of this inactive material suggest an antibody function but no antigen to it has been discovered in diphtheria culture filtrates. The third column refers to this antitoxin solution after digestion with pepsin (pH 4.2) and removal of inactive split protein by heat coagulation (58°C.—45 min.). The serologically inactive portion previously referred to has evidently been digested at the same rate

as the antitoxin inasmuch as the per cent of precipitable nitrogen has not changed. Normal horse pseudoglobulin is almost completely split under these conditions (Pope).

Normal antitoxin has a molecular weight of 184,000 and an antitoxic activity of 86,000 units per g. protein, equivalent to $15,800 \times 10^6$ units per mole. After digestion and purification, the antitoxin was precipitated with toxin and the floccules (specific precipitate) washed and again treated with pepsin, this time to remove the toxin. The resulting material (Column 4) had a molecular weight of 113,000 and an activity of 135,000 units per g. protein or $15,255 \times 10^6$ units per mole. The recovery of 98% of the activity as indicated by the last figure demonstrates that the material split off was inactive. The 64% increase in activity per g. of residue is paralleled

TABLE III

Properties of Diphtheria Antitoxin Preparations

	Antitoxic pseudoglobulin[1]			Crystalline Antitoxin[2]
	Untreated	Pepsin-treated	Dissoc. from floccules	
% of precipitable N	33	33	77	92
Antitoxin floc. units/mg. N.	860			700–900
Sedimentation const. $\times 10^{13}$	7.2	5.7	5.7	5.5
Diffusion const. $\times 10^7$	3.9	5.8	5.0	5.8
Molecular weight	184,000	98,000	113,000	90,500
Ratio major: minor axis	7.0	3.3	5.3	

[1] Pappenheimer, Lundgren, and Williams (1940); Petermann and Pappenheimer (1941).

[2] Rothen (1941–42) (Northrop's preparation).

by a 68% increase in its carbohydrate content, compared to an equal weight of undigested material. The change in axial ratios noted in Table III suggests that pepsin digestion split the antitoxin molecule in a plane normal to the major axis (Petermann and Pappenheimer, 1941).

By fractionation of trypsin-digested floccules with ammonium sulfate, Northrop (1941–2) has obtained an antibody fraction from diphtheria antitoxin which was homogeneous by electrophoresis, sedimentation, and by solubility analysis. It could be crystallized—the first antibody to be so reported—although the crystals were rather unstable, and reverted easily to a less soluble form; 92% of the nitrogen could be precipitated by purified toxin. The combining ratios of this antibody with purified toxin extend over a much wider range than is the case with unfractionated antitoxin (p. 102). The physical properties of the highly purified antibody (Rothen, 1941–2) are included in Table III.

5. *Serological Properties of Antibodies. Anti-antibodies*

Since antibodies are proteins they should be capable of inciting antibodies against themselves when injected into a foreign species. As will be seen shortly this is a matter of some importance in therapy. It also permits the application to antibody protein of the serological methods useful in examining other proteins. Preliminary data were obtained by Landsteiner and Prasek (1911) who demonstrated that precipitins for normal horse serum would remove the agglutinin from anti-typhoid horse serum.

Although purified antibody solutions can be used as antigens, specific precipitates are quite satisfactory and can be prepared in greater yield. If the antigen part of the specific precipitate is a carbohydrate haptene it will not give rise to antibodies on injection and any response observed will be due solely to the antigenic power of the antibody part. In an extensive series of experiments on normal and immune horse serum fractions injected into rabbits, Ando (1938) concluded that antigenically there was a relation between normal horse globulin and diphtheria antitoxin and that this was distinct from the specificity of various antibacterial antibodies. These results were extended and made more precise by Treffers and Heidelberger (1941a,b) by means of an adaptation of the quantitative agglutinin method. They showed that antipneumococcus types I and II, and anti-C (group specific), and anti-H influenza antibodies from horse antisera were quantitatively identical in their antigenic reactions toward a rabbit antiserum to one of them (type I pneumococcus horse antibody). Diphtheria antitoxin-toxin floccules, on the other hand, were found to remove only one-half of the precipitable antibody from this anti-antibody serum. The results were confirmed with a similar anti-antibody produced in the chicken. Use of the latter species also permitted the examination of the antigenic behavior of rabbit antibodies, which were found to be identical as far as studied. In a later paper (Treffers, Moore, and Heidelberger, 1942) comparison was made using the same rabbit anti-antibody serum (to a specific precipitate from horse antiserum) of the antigenic properties of salt-dissociated antibody solutions prepared from two different serological types of antipneumococcal horse sera with various fractions isolated by electrophoresis and ultracentrifugation of normal horse and goat sera (Fig. 1). It will be noted that the two antibody solutions, as well as the γ-globulin fraction from an antipneumococcus horse serum all had the same quantitative antigenic properties. They also removed the same final amount of anti-antibody N as did the specific precipitates used as antigens, although the quantitative relations were necessarily different because of the differences in surfaces involved. This was taken to indicate that no detectable denaturation had occurred during the preparation of the purified antibody solutions.

Fractions of the same sedimentation constant (s = 18) found for antibacterial antibodies in horse sera were prepared from two samples of presumably normal horse serum, in which they occurred in small amounts. One such fraction (Curve 2) approached the antibody solutions in antigenic behavior—possibly it represented a so-called normal antibody to an inapparent, sub-clinical infection. The other fraction similarly prepared was quite different in this property (Curve 4), and no satisfactory explanation is as yet available.

Curve 3 is of especial interest. It represents the antigenic reaction with this serum of normal horse γ-globulin. Although the latter has the same electrophoretic mobility as do the antibacterial antibodies, it is a much smaller molecule, with a sedimentation constant of 8 and a molecular weight of 165,000, contrasted with 18 and 990,000 respectively for antibodies used as antigens in Curve 1.

It was shown by Heidelberger and Kendall (1935, b) that protein—antiprotein reactions of the type of Fig. 1, Curve 1, could often be represented by the empirical equation

$$\text{mg. antibody N precipitated} = k_1\,(\text{An}) - k_2\,(\text{An})^{3/2} \qquad (1)$$

where An represents the *amount* of antigen precipitated. Division by An gives

$$\frac{\text{mg. antibody N precipitated}}{\text{An}} = k_1 - k_2(\text{An})^{1/2} \qquad (2)$$

It will be noted that a plot of the left hand ratio against $(\text{An})^{1/2}$ gives a straight line, with ordinate intercept k_1, and slope k_2. Many anticarbohydrate, as well as a few antiprotein systems can be better represented by the equation

$$\text{mg. antibody N precipitated} = k_1\,(\text{An}) - k_2\,(\text{An})^2 \qquad (3)$$

and its equivalent form

$$\frac{\text{mg. antibody N precipitated}}{\text{An}} = k_1 - k_2(\text{An}) \qquad (4)$$

which Heidelberger and Kendall (1935a) and later Kendall (1942) have derived from theoretical considerations so that the constants have an immunological significance. In these treatments antibody was considered as a homogeneous substance for mathematical simplicity only, its actual complexity being explicitly recognized. Both equations have several properties which facilitate treatment of experimental data. Eq. (2) and eq. (4) are determined in principle by two experimental points, although at least three are desirable when dealing with unknown systems in order

that the applicability of the equations may be shown. From the straight line the entire course of the reaction between antigen and antibody, up to an equivalent amount of the latter, may easily be read off. Analogous forms may also be used in the region of antigen excess. The *k*'s provide a sensitive indication, for example, of the effect of continued immunization (p. 90) or of chemical treatment on the combining ratios. The height of each curve is a measure of the total amount of precipitable antibody in the system which in turn is a function of the antigen used, the technique, time, and route of injection, etc. By very simple mathematics (Heidelberger and Kendall, 1935c; Treffers, Moore, and Heidelberger, 1942) each equation may be recalculated to a common maximum, usually 1 mg. N per ml., permitting direct comparison of sera investigated in different laboratories or at various times.

These points have been considered in some detail because, although qualitative methods can yield much interesting information about protein antigens and antibodies, it is equally clear that there are many problems for which they are not suited. Thus it has been reported on the basis of precipitin titers that the antigenic properties of horse antibacterial antibodies are essentially that of the normal γ-globulin (Wright, 1942). A comparison of Curves 1 and 3 (Fig. 1) shows that this is not correct; the cross reaction is so extensive that important differences can not be detected by the qualitative method.

If Curve 3 is recalculated to the same maximum as Curve 1 another relation emerges: both curves are coincident at all points, which is not necessarily a general phenomenon. In fact, it is only possible when the k_1's are equal and the k_2's are a definite function of their respective antibody content. Stated in other words, the low molecular weight normal horse γ-globulin only precipitates a part of the rabbit anti-antibody to the higher molecular weight horse antibody, but it does so in a manner quantitatively identical to that of the homologous reaction. This behavior is exactly paralleled by the reaction of acid-treated pneumococcus type III polysaccharide with an antiserum to the intact polysaccharide (Heidelberger and Kendall, 1933) but not by the alkali-treated type I polysaccharide system. The chemical preparation of the first polysaccharide antigen is known to be merely a hydrolytic splitting of a long chain molecule, the second involves side reactions as well. This suggested that possibly the horse normal γ-globulin reacted as if it were a unit, of which the antibody were a hexapolymer (from the molecular weight ratios). This interpretation, which may have some significance for the structure of antibodies, could not have been derived from qualitative experiments, no matter how carefully carried out.

Active immunity—produced by injection with the appropriate antigen

—is usually more desirable than the passive immunity conferred by the injection of an immune serum, especially if the latter is from a foreign species. This is particularly true with respect to its duration, since in the latter case no new antibody is manufactured to replace that lost. More troublesome, however, is the fact that the foreign antibody or the normal serum proteins which even in purified preparation usually exceed it in amount, are antigenic. After a short induction period a state of hypersensitiveness or allergy may be produced which can give rise to anaphylaxis

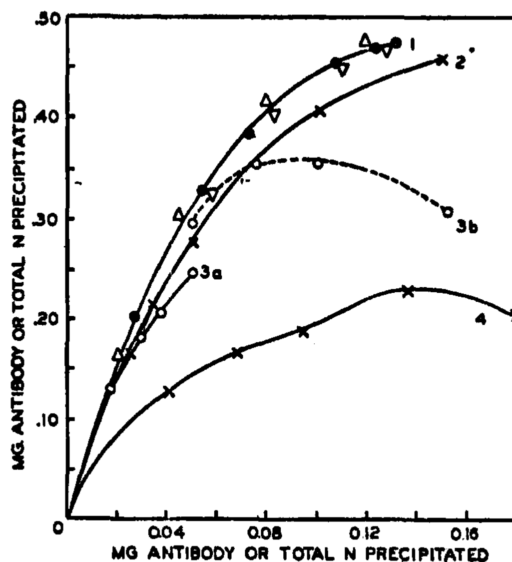


Fig. 1. *Precipitation of an anti-antibody rabbit serum by various antigens.*
Curve 1, triangles = Pneumococcus Types I or II antibody solutions; circles = immune horse γ-globulin solution.
Curve 2, normal horse heavy globulin.
Curve 3a, normal horse γ-globulin solution (antibody N pptd.). Curve 3b, ibid., total N pptd.
Curve 4, normal horse heavy globulin, other preparation.
(Adapted from Treffers, Moore, and Heidelberger, 1942.)

or serum sickness especially if a second portion of serum is administered. This state can last for months or even years (literature in Zinsser, Enders, and Fothergill, 1940).

A number of investigations directed toward overcoming this difficulty have been made, both in England and in this country. Most have employed digestion with enzymes, with or without subsequent heating. Marked changes occur in the electrophoretic patterns (van der Scheer, Wyckoff, and Clarke, 1941) and other physical properties (p. 78) and in the antigenic properties of the treated sera. If carried far enough most of the

original species specificity can be made to disappear (Weil, Parfentjev, and Bowman, 1938). A critical resume of the clinical application of these "despeciated" sera has been given by Ratner (1943); additional references to the chemical and immunological studies will be found in the paper of Kass, Scherago, and Weaver (1942).

6. *Changes During Immunization*

Much interesting and valuable information has accumulated on the qualitative and quantitative properties of antibodies produced by various injection procedures (reviewed in part by Topley and Wilson, 1937). The changes which can be induced in the circulating blood by extended immunization are truly surprising. Bjorneboe (1940) has investigated some of these by the quantitative agglutination method. He found an average total nitrogen of 10.3 mg. per ml. for 11 pooled normal rabbit sera; for 56 immune rabbit sera to the pneumococcus the agglutinin N (principally antibody to the capsular polysaccharide) was given by the equation

$$\text{Agglutinin N} = \text{Total N} - 10.2 \qquad (5)$$

After six months of injection with a combined vaccine containing five pneumococcal types the rabbits gave sera containing about 13 mg. of antibody N per ml. Controls injected with any single type averaged only 3 mg. N per ml., which until recent years was considered quite high for sera. Above 5 mg. of antibody N the above equation did not hold since the non-antibody protein N dropped as much as 50 per cent. These experiments demonstrate that the antibody content, initially almost indetectible can be increased until it reaches 180 per cent of the normal *total* protein content. The number of antigens to which antibodies can be produced simultaneously is apparently almost without limit. Hektoen and Boor (1931) have found antibodies produced to 32 out of 35 antigens injected into a rabbit. If polyvalent sera are produced by the injection of multiple antigens it has been verified in several instances (Heidelberger and Kendall, 1936; Heidelberger and Kabat, 1938; Bjorneboe, 1940) that the amount of antibody precipitated to each antigen is independent of the order in which the precipitation is made. From this it may be concluded that no significant fraction of the antibody contains groupings directed toward both antigens. Experimental tests of this problem, leading to the same conclusion, have been made with azo antigens carrying two determinant groups (Hooker and Boyd, 1933, 1934; Haurowitz and Schwerin, 1943) (*cf.* also the data on vaccinia, p. 104).

7. *One Antibody or Many?*

In addition to these impressive quantitative changes in antibody levels, it has often been demonstrated that there are changes in the combining

power of the antibody as well. This naturally raises the question: how many antibodies to a single antigen are there? Experiments designed to examine this are best done with purified test antigens, as homogeneous as possible, and it is conceivable that our conclusions may have to be modified somewhat as valid criticisms are presented as to the purity of the materials which have been used.

It is common experience that the extent to which a serum will cross react increases with continued immunization. The most probable explanation

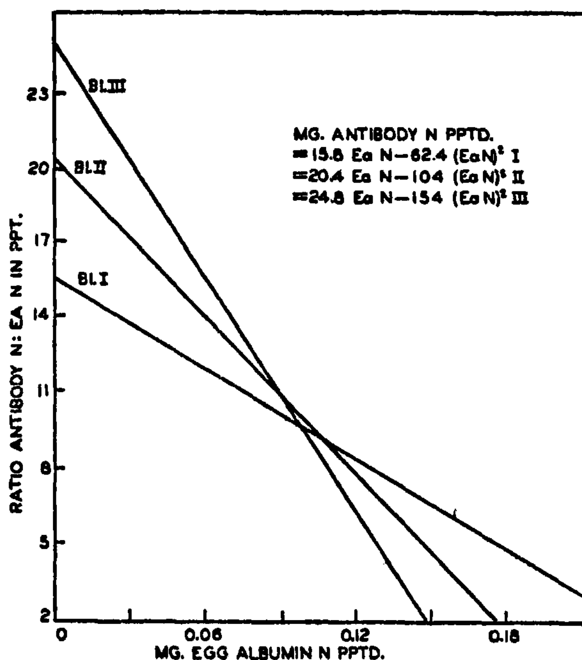


Fig. 2. Changes in the combining properties of a rabbit antiserum to crystalline egg albumin (Ea) over three courses of injections.
(Adapted from Heidelberger and Kendall, 1935.)

is that the antibody formed later reacts with a larger number of distinct groupings on the antigen, with some perhaps for the first time. Related to this is the increase in reactivity toward a single antigen, illustrated by data from Heidelberger and Kendall (1935c). Fig. 2 represents the reaction of hen ovalbumin with its antiserum in the rabbit, for three successive courses of injection. The data are presented in the form of antibody:antigen ratios, corresponding to equation (4) above. They show the variation, from a combining ratio (extrapolated intercept) of 15.8 mg. antibody N per mg. of antigen N for the first bleeding, to 24.8 mg. antibody N for the third bleeding.

The equivalence zones indicated in the figure mark the ratios between which neither antigen nor antibody can be demonstrated in the supernatant above the precipitate. Such zones are deserving of attention (which they seldom receive) for several reasons: it has been shown that a negative test for both components is indicative of antigen homogeneity, at least serologically, if there is no extensive dissociation, or a fortuitous relation between the relative amounts of antigens and antibody (for literature see Kabat, 1943); changes in their breadth mark a variable binding capacity for antigen; the average of the zone, the so-called equivalence point, has been cited as a constant for any one system (see however Heidelberger and Kendall, 1935, c). The effect presented in Fig. 2 was again demonstrated by Heidelberger, Treffers, and Mayer (1940) for a more extended course of injections, although not all antisera to the same antigen displayed it.

The fact that antibody and antigen can combine in various ratios as implied in equations (2) and (4) leads to several interesting consequences. In the experiments of Heidelberger and Kendall cited above it was found that if to one of the sera 0.46 mg. of egg albumin N (Ea N) were added at once—the usual procedure for determining a point on the reaction curve—9.1 mg. of antibody N was precipitated. If however, the 0.46 mg. of Ea N were added in four portions a larger amount of antibody N, 9.4 mg. precipitated. A small amount of added antigen thus removes proportionately more antibody than does a larger amount, and for a serial precipitation the sum is larger. As will be seen, this is true up to a certain point, after which it decreases for other reasons. In this case the effect although definite is not as large as has been observed. It was first noticed in toxin-antitoxin reactions that an amount of toxin which would just neutralize antitoxin if added in one portion, would give an excess of toxin (toxic mixture) if added in several portions (Danysz effect). Although reversible, the latter case is complicated by a slow rate-determining step involving soluble complexes (Pappenheimer and Robinson, 1937).

8. *Evidence from Serial Experiments*

The quantitative experiments just cited are of further interest in that they often demonstrate still another type of antibody. Thus it was found that if the addition of small portions of Ea was continued until a slight excess remained, a total of 11.34 mg. of antibody N was precipitated, compared with 14.55 obtainable if all of the antigen were added at once. Thus 22 per cent of the antibody was not precipitable at all by serial additions of antigen. However, most if not all of the antibody could be recovered by mixing the supernatant solution with a fresh portion of serum and again adding antigen. The resistant antibody—which would not precipitate directly with antigen—could add to the complex newly

formed. In an analogous experiment with horse serum albumin recovery was 97.5 per cent complete (Kabat and Heidelberger, 1937).

One of the major theories of antibody reaction involves the assumption of multivalent antibody, capable of combining with more than one molecule of antigen, as a requisite to precipitation. In this scheme the antibody just considered would be univalent, or at least of lesser valence, incapable of combining directly with antigen but able to attach to multivalent antibody-antigen precipitates (Heidelberger and Kendall, 1935a). This type of antibody has been demonstrated for a number of other systems (Heidelberger and Kendall, 1935b; Pappenheimer, 1940; Heidelberger, Treffers, and Mayer, 1940).

9. *Evidence from Cross Reactions*

We have mentioned previously in connection with the problem of distinguishing between two antigens that an antiserum to hen ovalbumin would cross precipitate to some extent with duck albumin, and that the supernatant would then react only with the homologous antigen. The reaction is equally valuable in demonstrating that there are in the antiserum two antibodies—one which reacts with both duck and hen ovalbumins, and another which reacts visibly only with the latter.

A given antiserum may cross react with many antigens, as is illustrated by data of Landsteiner and van der Scheer (1940). The sera from 22 rabbits injected with hen ovalbumin were tested against a number of other ovalbumins and the relative volumes of precipitates taken as a rough measure of the antibody content. The means and standard errors were: hen ovalbumin, 100; turkey 50 ± 2.4; guinea hen, 42 ± 2.3; duck, 25 ± 1.7; goose, 19 ± 1.4. It is obvious from the extent of the crossing that some of the antibodies involved must be identical or at any rate overlap considerably. That this is so is further indicated in Table IV. The ovalbumin from the guinea hen which gave a 2½+ reaction with the antiserum to hen ovalbumin (bottom control line) reacted similarly, within the rather large experimental error, with the same serum previously absorbed with either duck or goose ovalbumin. Absorption with turkey (and naturally, hen) ovalbumins removed all antibody to the guinea hen protein. Absorption with any of the others removed all antibody for both duck and goose ovalbumins. Experiments of this type are complicated by the fact that a much larger amount of turkey antigen than used in the reactions tabulated would markedly or even completely inhibit the reaction of the homologous antigen. Thus, as was noted also on page 74 antibodies rendered apparently homogeneous by cross-absorption may still have sufficient affinity for a heterologous antigen to block completely the homologous reaction, a

finding whose significance in terms of the structure of antigens or of antibody is not clear at present.

Other results of interest in the present connection were reported by Landsteiner and van der Scheer (1939) in their experiments on glycine and glycine-leucine peptides previously mentioned. It was found that the triglycyl antigen reacted not only with its own antiserum but cross reacted with antisera to G_5 and G_2LG_2 as well. That the antibodies concerned could not be absolutely identical is evident if we consider that each had a second specificity directed toward its own homologous antigen. It might be expected, however, that the groupings directed toward G_3 and G_2LG_2 might be identical. The data show this not to be the case. Thus the antiserum to G_3 was noticeably inhibited by G_2LG_2, while the G_5 antiserum was less affected. Similar reasoning for the other antibodies led Landsteiner and

TABLE IV

Reactivity of Ovalbumins From Various Species With Antiserum Absorbed With Other Ovalbumins

Antiserum to hen ovalbumin, absorbed with ovalbumins of	Test antigens: ovalbumins from				
	Hen	Turkey	Guinea hen	Duck	Goose
Turkey	2½+	0	0	0	0
Guinea hen	3½+	1½+	0	0	0
Duck	3½+	3+	2½+	0	0
Goose	3+	3+	2½+	±	0
Unabsorbed control	3½+	3+	2½+	2+	2+

(Landsteiner and van der Scheer, 1940.)

van der Scheer to the conclusion that if an antigen cross reacted with various antibodies, the antibodies concerned were different. The important elements of similarity are therefore not between the various cross reacting antibodies but between portions of the cross reacting antigen and each antibody. As an illustration, an antigen ABC might cross react with antisera to antigens ADE, BFG, and CHI, the cross reacting antibodies being dissimilar in each case.

An attempt at evaluating this point experimentally has been made by Haurowitz (1942). Antisera, for example, to sulfanil-azo-sheep serum globulin were tested with a number of antigens which among them were designed to contain all of the possible determinant portions (sulfonic acid, phenyl azo, serum globulin, etc.) and from the weights of the precipitates obtained some measure of this influence could be calculated. Although exception can be taken to details of the procedure, the results are in general accord with the scheme presented above.

10. *Cross Reactions of Polysaccharide Antigens*

In addition to providing evidence for the inhomogeneity of antibody protein, serological data with polysaccharide antigens whose structures are at present more accessible than are those of proteins may help in interpreting, if only by analogy, data obtained with protein antigens. An instance has already been given. Other points are raised in experiments on the cross reaction of pneumococcus types III and VIII made by Heidelberger, Kabat, and Shrivastava, (1937), and Heidelberger, Kabat, and Mayer (1942). The chemical structure of the type III substance (abbrev. S III) is known (Heidelberger and Goebel, 1927; Adams, Reeves, and Goebel (1941); it is a polymer of an aldobionic acid containing glucuronic acid and glucose. The type VIII polysaccharide (abbrev. S VIII) contains in addition approximately two molecules of glucose for each aldobionic acid unit, although the complete structural formula cannot be written at present (Goebel, 1935).

The reaction of a horse antiserum to the type VIII pneumococcus with purified S VIII is given in Fig. 3, Curve 1 (p. 102). It follows equation (3) above, the particular values being:

$$\text{mg. antibody N pptd.} = 21.5\text{ S} - 108\text{ S}^2 \qquad (6)$$

The curve for the reaction between this antiserum and the cross reacting S III is different in form (Curve 2), the initial curved portion being followed by a linear region. The equation for the curved portion, recalculated to 1.0 mg. of total precipitable antibody N per ml. of serum is

$$\text{mg. antibody N pptd.} = 22\text{ S} - 120\text{ S}^2 \qquad (7)$$

An antibody solution was also prepared by salt-dissociation of a specific precipitate which contained an amount of S III corresponding to the point at which the curve just begins to straighten out. The reaction curve of this antibody solution was then determined (Curve 3), the constants calculated to 1.0 mg. of antibody being

$$\text{mg. antibody N pptd.} = 21\text{ S} - 114\text{ S}^2 \qquad (8)$$

When equation (6) experimentally found for the homologous reaction is also recalculated to this common basis of 1.0 mg. antibody N it becomes

$$\text{mg. antibody N pptd.} = 21.5\text{ S} - 116\text{ S}^2 \qquad (9)$$

All three equations calculated to a common basis (equations 7–9) may be considered identical within the experimental limits.

All of the cross reacting antibody (Curve 2) is precipitable by the homologous S VIII or the heterologous S III; in fact, a portion (*i.e.* up to the

linear part) is precipitated in a quantitatively identical manner by either (equations 7–9).

The serum supernatant from the specific precipitate used for the production of the antibody solution mentioned should have contained the antibodies responsible for all or a part of the linear section of Curve 2. That this is so may be seen from the reaction of this supernatant with S III (Curve 4). Although the amount of antibody precipitated was directly proportional to the amount of added antigen (a rather unusual serological phenomenon) only one-half of the antibody estimated to be in the supernatant could be thus removed.

It was concluded from the analysis of the reaction curves that the antibody responsible for the linear portion of Curve 2 was of the low grade or valence type revealed by the serial precipitation method, and its precipitation here ascribed as being due to its addition to higher-grade antibody present. The incomplete precpitation observed in Curve 4 can be accounted for by assuming sufficient complete antibody (as an impurity in the preparation) to bring down some but not all of the antibody of lower reactivity.

In summary, the data indicate that a part of the antibody is revealed by the sensitive quantitative precipitin method to be incapable of distinguishing between the two related but chemically distinct polysaccharides. Another part (linear portion of Curve 2) reacts with both, although in different quantitative proportions. Lastly, the residue, amounting to about $\frac{2}{3}$ of the total antibody is type specific, reacting only with the homologous S VIII. By an analysis of the quantitative curves it was possible to make some conclusions about the structures of the antigen molecules, which are in accord with the known arrangements.

IV. Bacterial Antigens

1. *Fractionation Procedures*

The difficulties of biochemical fractionations (including those of bacterial products) have already been mentioned. In the case of bacteria, the reader may well ask: why do it at all? There are several reasons. In the first place, many bacteria are not good antigens, particularly in their ability to elicit effective protective antibodies. Isolation of the particular antigen responsible for the latter may therefore be desirable. This is especially true when the injection of large amounts of the whole bacterial cell is accompanied by toxic symptoms. It may then be possible to separate the antigen from the toxin, if they are not identical. In other cases, a soluble extract such as common tuberculin or Shick toxin (diphtheria) may be needed for diagnostic skin tests on susceptibles. Lastly, but by no means of negligible importance, there has been a certain biochemical

curiosity as to the nature of bacterial products compared to those derived from higher orders.

A number of general methods have been used for the disruption of the cell and the isolation of components. Recent illustrations of these include: dry grinding (Henriksen and Heidelberger, 1941); supersonic vibration (Mudd and Lackman, 1940); enzymatic release (Raistrick and Topley, 1934; Thompson and Dubos, 1938); extraction with dilute acid (Lancefield, 1928); with solvents of graded acidity (Heidelberger and Kendall, 1931); with urea (Walker, 1940); formamide (Fuller, 1938); phenol (Palmer and Gerlough, 1940; Morgan and Partridge, 1941); trichloroacetic acid (Boivin and Mesrobeanu, 1935); diethyleneglycol (Morgan, 1937).

2. *Group and Type Specificities. Protective Antibodies*

The exact characterization of bacteria is rendered difficult by their tendency to vary in almost every respect under the influence of environmental conditions. There is of course a norm, or no classification would be possible, but as every bacteriologist is aware the deviations may be marked and in some cases capricious. The enzymatic properties of bacteria are usually well-defined and frequently serve as a basis for classification (fermentation reactions). Classification by antigenic content is often more reliable. A strain lacking a given enzyme may however acquire it under proper conditions (adaptive enzymes, rev. by Dubos, 1940). Although a good deal is known about the properties of bacterial enzymes (rev. in part by Stephenson, 1939) little can be said at present about the part that they play in infection and resistance. Anti-enzymes can certainly be produced (p. 107) but the action of immune sera to whole bacteria on individual bacterial enzyme systems is not clear at present. Bacterial enzymes can be used, however, as reagents toward materials of immunological interest (Dubos, 1939).

Although an antigen may be present in a bacterial cell the antibody response to it may be diminished by the presence of other antigens more advantageously situated.

Most bacteria can exist in a series of fairly well-defined states, although intermediate transition forms are also found. The more that is known of bacterial variation, however, the more difficult it is to make general statements, but if this is kept in mind, the following will at least serve as a working guide.

Organisms which have been maintained for a long time on artificial media are often found to be in the "rough" state, characterized by certain colonial forms, a minimum number of antigens, and frequently by a relative or complete avirulence for animals. The same species passed through animals or freshly isolated from active cases of disease is usually although

not always in the smooth or the mucoid state, with a distinctive colonial form, enhanced virulence, and contains additional antigens beyond those of the rough form. It is often tempting to consider that these new antigens are associated with the increase in virulence, especially when antibodies directed against them exhibit protective power toward infection by the organism. There is a growing body of evidence, however, that this is not so in every case, but consideration of this interesting subject would lead us too far afield here.

The rough forms of some groups of bacteria such as the pneumococcus or sub-groups of the streptococcus contain one or more protein or polysaccharide antigens in common, so that an antiserum to one member may agglutinate the entire rough group to some extent (group specificity). The more complex smooth and mucoid forms contain additional antigens which are usually chemically and serologically distinct, giving rise to type specificities. The rough forms of the pneumococcus contain a common nucleoprotein and a common polysaccharide antigen; the smooth forms a specific polysaccharide, of which some 50 varieties have been distinguished. The antibodies to these agglutinate the organism, precipitate with the isolated carbohydrates, and protect against infection. In the case of the streptococcus (Group A) the type specific antigen (T) gives rise to an antibody with marked agglutinating power but no protective action. Each type contains, however, another antigen (M protein) which is also type specific although distinct from T, the antibody to which is protective but not markedly agglutinating.

The difficulties of using agglutinin titers, without qualification, as a measure of the protective potency of serum are apparent. The case of the *Salmonella* may be cited as another example. Certain of these important and ubiquitous organisms are quite pathogenic, causing typhoid fever and various forms of gastroenteritis in man and in animals. It is important diagnostically to distinguish them from relatively non-pathogenic forms which may be isolated. The serological relations are most complex; to date over 110 distinct varieties have been characterized, in terms of combinations of a more limited group of antigens (*cf.* the comprehensive review of Bornstein, 1943). The difficulties are aptly illustrated by recent experiments of Ungar, Jenner, and Hunwicke (1942). The principal antigens of the typhoid bacillus, *E. typhosa*, are designated IX and d. Another organism, *S. gallinarum*, pathogenic for fowl, contains antigen IX, while *S. munchen*, an organism causing gastroenteritis in man and in animals contains antigen d among others. By injecting a mixture of the two latter organisms into mice these workers were able to produce agglutinin titers for both antigens IX and d which equalled or exceeded those produced by the typhoid bacillus. When the mice were infected with the typhoid

bacillus, however, 9 out of 10 of those receiving the combined heterologous vaccine died, compared with only 1 out of 11 of those vaccinated with the typhoid bacillus. These details are presented not to discredit the importance of agglutinin antigen analyses for many purposes, but on the contrary to emphasize that a deeper approach, including chemical data when necessary, must be made if we are to understand more fully the mechanism of of protection against infection.

3. *Tubercule Bacilli*

Because of their importance the proteins of the tubercule bacillus have been extensively investigated (see the review of Wells and Long, 1932). Four distinct strains are recognized: human, bovine, avian, and timothy grass, the first two being very closely related. Careful fractionations of the corresponding proteins have been made (Heidelberger and Menzel, 1934; Menzel and Heidelberger, 1938). In the human strain at least three antigenic proteins can be recognized, in addition to a number of carbohydrate fractions. Corresponding protein fractions from all four strains can be distinguished by their chemical or serological properties. Seibert, Pedersen, and Tiselius (1938) have studied the physical properties of tuberculin fractions, finding molecular weights for the bovine, human, and timothy proteins of 10,000, 32,000, and 17,000 respectively. By electrophoresis tuberculin proteins of two distinct mobilities can be distinguished (Seibert, 1943). The field has been reviewed by Seibert (1941).

4. *Streptococcus*

The antigenic complexity of the streptococcus, revealed in great part by the important work of Lancefield and her co-workers (reviewed by Lancefield, 1941) has been briefly mentioned. A number of interesting protein fractions were obtained by Heidelberger and Kendall (1931) from a strain of streptococcus which has since been shown by Lancefield to contain in addition to the M and T protein of type 3, a T but not the corresponding M protein of the type 1. The nucleic acid derived from this organism is of interest in that evidence was presented that it was linked at least in part by an ester rather than a salt linkage to protein (*cf.* also Sevag and Smolens, 1941). Further studies were made by Heidelberger, with Scherp (1939), and with Henriksen (1941), including comparative data on other strains. The utility of the quantitative precipitin reactions with antisera as a method for controlling fractionation procedures is illustrated by an experiment in which it was found that a certain protein fraction precipitated less antibody N than was added as antigen N. After further purification, four to five times as much nitrogen was precipitated as was added in the form of antigen.

Numerous studies on the properties of streptococcus fractions have been made by Mudd and his co-workers (Mudd and Wiener 1941, and earlier papers). The properties of the M protein have been studied in detail by Zittle (1942) and by Zittle and Mudd (1942). It appears to occur in association with nucleic acid. The physical data (Pappenheimer, Williams, and Zittle, 1942) indicate it to be homogeneous by sedimentation and diffusion, and to have a molecular weight of 41,000.

5. *Fusobacteria*

Relatively little information is available concerning the proteins of strictly anaerobic bacteria. Weiss and Mercado (1938) have isolated four type specific proteins from as many strains of anaerobic fusobacteria. Although these organisms are not pathogenic for common laboratory animals, they can be isolated from human carriers and may cause disease in man. The proteins identified were strictly type specific, not crossing with antisera to each other although two of them reacted with antisera to various streptococci. The proteins were antigenic on injection, and this property was destroyed on digestion with pepsin or trypsin.

6. *Staphylococcus*

A protein has been isolated by Verwey (1940) from the type A staphylococcus which is serologically distinct from the specific carbohydrate. Since the phosphorus content was less than 0.1% it is not a nucleoprotein. Its precipitating power toward antisera was also destroyed by trypsin digestion.

7. *Cholera Vibrios*

The antigenic properties of this group have been extensively investigated, especially with reference to the possibility of classification into pathogenic and non-pathogenic types, a problem of considerable diagnostic importance. In a series of papers by Linton and his co-workers (reviewed by Linton, 1940) three specific polysaccharides and two proteins were described. The proteins could be distinguished by the Woodman method of racemization by alkali (*cf.* Marrack, 1938, for other applications of this method). Isolation of amino acids after the racemization procedure demonstrated that some of the amino acids in one protein were optically inactive, whereas the same amino acids in the other protein were not.

8. *Dysentery and Typhoid Bacilli*

The protein antigens discussed in previous sections have been isolated in relatively simple form, although their probable association with nucleic acid in the bacterial cell was noted by some investigators. More complex

antigens have been discovered in recent work on the dysentery and typhoid groups. The reader interested in the background of these interesting studies is referred to the comprehensive reviews of Weil (1943) and of Bornstein (1943). By extraction of the Shiga dysentery or the typhoid bacillus with diethylene glycol or with phenol, Morgan and his co-workers (Morgan and Partridge, 1941, a) have obtained complexes containing phospholipid, protein, and polysaccharide. The entire substance is antigenic as isolated, although the phospholipid can be split off without impairing the antigenicity. By various methods they were able to separate the protein and polysaccharide portions. The proteins of the two organisms are believed to be identical; the polysaccharides are serologically distinct however. The proteins are weakly antigenic, the polysaccharides non-antigenic in the rabbit. If neither has been denatured, they can be recombined to form a highly antigenic complex, the polysaccharide acting as a

TABLE V

Properties of Conjugated and Simple Degraded Proteins of the Shiga Dysentery Bacillus

Protein	$[\alpha]_{546}$	N	P	Alkali solubility	Acid solubility
Conjugated	−50°	11–12%*	0.6–0.8%	soluble	insoluble
Degraded	−84°	14%	none	soluble	soluble only between pH 2–2.5

* 50% amino N.
(Morgan and Partridge, 1941b.)

haptene determining the specificity. By chemical treatments both could be degraded so that they would no longer recombine with the undegraded partner. Some properties of the intact "conjugated" and the "simple" degraded protein are given in Table V.

These finding not only help to clarify the chemistry of these important but confusing groups of organisms, but may be of wider applicability as well. Thus Morgan has shown that the intact protein will combine with a number of unrelated polysaccharide haptene polysaccharides from agar, gums, or blood group A to form active antigens.

9. *Bacterial Toxins*

A number of diseases such as diphtheria, tetanus, scarlet fever, and gas gangrene are characterized by a more or less profound toxemia. Specific toxins can readily be isolated from filtrates of cultures of the organisms, and when injected these will reproduce many if not all of the symptoms of the disease. The toxins are often exceedingly potent. Topley and Wilson (1937) mention that 0.00001 cc. of tetanus filtrate will regularly kill a mouse

and it is calculated that 0.25 mg. of toxin would be fatal for man. The toxin of the botulinus organism (food poisoning) is even more deadly. 0.000001 cc. being fatal for the mouse, and 0.0084 mg. for man. Fortunately for immune prophylaxis and therapy some organisms such as the tetanus bacillus even though existing in different serological types form only one toxin, and therefore require only one antitoxin. In the botulinus group, however, there are specific toxins associated with each of the three or more serological types.

The Gram-negative bacteria (which include the dysentery, brucella, typhoid, and pertussis organisms) are on the whole characterized by being toxic of themselves, although soluble toxins can be isolated from filtrates of older cultures. This group is often referred to as containing endotoxins, as contrasted with the exo-toxins described above, although the distinction is not completely satisfactory and several toxins do not fit exactly into either group. Of practical importance is the property of the typical exo-toxin to be rendered non-toxic without destruction of its antigenicity (*i.e.* power to elicit neutralizing antibodies to the unaltered toxin) by such reagents as formaldehyde or other aldehydes, iodine, and ketene. The chemistry of the reactions is still quite obscure, and much remains to be learned of this important process. Discussions of the chemical and biological aspects of toxins and of detoxification will be found in Topley and Wilson (1937) and Zinsser, Enders, and Fothergill (1940).

Antitoxins such as those to diphtheria toxin, produced in the horse, have the interesting property of flocculating only when the amount of added toxin lies within certain narrow limits. No precipitates are observed if more or less than these amounts are added. The physical properties of this antibody were given in Table III. Rabbit antitoxic sera, on the other hand, do not show the inhibition with excess antibody, and thus behave like other rabbit antisera (Pappenheimer, 1940). The amount of antitoxin in a given horse serum may be determined with some exactness by noting the amount of toxin which flocculates it most rapidly. This bears a more or less constant ratio to the antitoxin content, although deviations from the antitoxic potency determined by animal tests sometimes occur (p. 83).

The quantitative precipitin reaction has been extended to the diphtheria antitoxin-toxin, and the analogous scarlet fever systems (Fig. 4) (Pappenheimer and Robinson, 1937; Hottle and Pappenheimer, 1941). Although it is not immediately obvious from the curves the amount of antibody N (antitoxin N) precipitated is constant throughout the linear region; the increased amounts of total N precipitated are due to toxin which continues to be precipitated as it is added. The curves thus have two important properties: the antitoxin N can be quantitatively estimated from a single nitrogen determination on any point in the linear region, and from the slope

of the line the N value for a flocculating unit of toxin can be determined. The latter is difficult to measure otherwise except on preparations of the

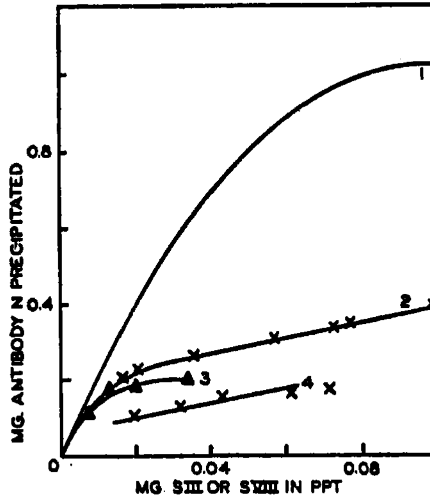


Fig. 3. Reaction of a Type VIII antipneumococcus horse serum with homologous (S VIII) and heterologous (S III) pneumococcus polysaccharides. (Adapted from Heidelberger, Kabat, and Mayer, 1942.)

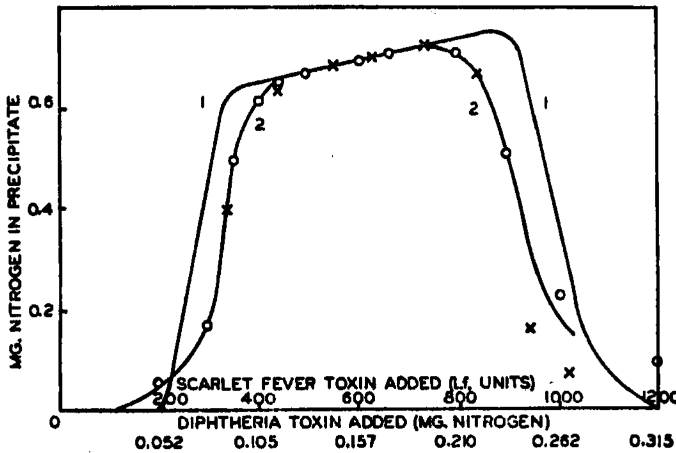


Fig. 4. Flocculation of diphtheria and scarlet fever antitoxins (horse) by their respective toxins. (Hottle and Pappenheimer, 1941.)

highest purity. By the quantitative method even crude preparations give the same final values, since the impurities are not precipitated.

The reasons for the qualitative differences in reactivity and physical properties between antibacterial (anticarbohydrate) and antitoxic anti-

bodies (Fig. 3 and 4, Tables II and III) have naturally been sought. It was first concluded that the antitoxin type of reaction was a characteristic response to all protein antigens since crystalline egg albumin (Pappenheimer, 1940; Heidelberger, Treffers, and Mayer, 1940) and hemocyanin (Hooker and Boyd, 1942) also give rise to antibodies of this type in the horse. It must be noted, however, that the antiprotein antibodies have all been produced by subcutaneous injection, the antibacterial antibodies by intraveneous injection. It was shown recently (Heidelberger, Treffers, and Freund, 1942 and unpublished work) that while subcutaneous injection of rabbit albumin into horses gave rise to the typical "antitoxic" antibody, the intraveneous injection of rabbit globulin produced antibody which was inhibited only by excess antigen. A current working hypothesis is that the route of injection may determine the type of response, but this cannot be conclusively established until the two types of antibody are produced by injection of the same antigen by the two routes. The antigens so far examined have yielded antibody by only one or the other of the routes used.

V. Some Virus Proteins

Most of the pertinent references to this active field will be found in the abstract literature, including the Annual Review of Biochemistry. Two reviews may be mentioned, however, as containing material of special interest to chemists, a general survey (edited by Rivers, 1943, and a review of the physical properties of viruses (Markham, Smith, and Lea, 1942). Attention will be confined here to the two groups of viruses best characterized. The earlier work on the immunological property of the tobacco virus group has been reviewed by Stanley and Loring (1938). It is interesting to note that important data on tobacco mosaic virus could be secured by immunological methods (Beale) before the chemical isolation of the virus. In other studies (Bawden and Pirie; Chester), it was shown that various strains of the virus were closely related but not identical. Knight and Stanley (1941) have shown that the Holmes ribgrass strains and cucumber viruses 3 and 4, differ in tyrosine, tryptophan, and phenylalanine content from those of the remaining numbers of the group, and that these are paralleled by serological differences in reactivity toward anti-tobacco mosaic virus.

These reactions have been studied quantitatively by Kleczkowski (1941), who found that from anti-tobacco mosaic virus antiserum the same amount of antibody could be precipitated by both aucuba mosaic virus and the homologous tobacco mosaic virus, but that the reverse system was quite different. Only one-third of the antibody in the anti-aucuba mosaic virus antiserum could be precipitated by tobacco mosaic virus. With the same amount of antibody present, these two rod-shaped viruses gave about four

times the weight of precipitate brought down by the almost spherical bushy stunt virus.

The elementary bodies of vaccinia are the most thoroughly studied of the animal viruses (*cf.* the comprehensive review of Smadel and Hoagland, 1942). A great many components have already been identified, including protein, sugar, lipids (neutral fat, phospholipids, and cholesterol), nucleic acid which is largely but not entirely of the desoxyribose type, and biotin. Copper also appears to be a constituent, possibly as a component of an enzyme. Several enzymes have been identified as being associated, but it has also been demonstrated that the virus possesses a marked ability to absorb at least some of these from enzyme containing solutions, and thus possibly from host cell tissues. This fact renders it uncertain at the moment whether they are really constituents of the virus. The antigens of vaccinia have also been studied in some detail, particularly by Craigie and Wishart, and by Rivers and his co-workers. The antibodies to four of them are involved in agglutination reactions with the elementary bodies. They can also be absorbed from antisera by the purified antigens. The most interesting is the LS antigen, which has been characterized in considerable detail. It is a protein (N = 15.7%) which contains no lipid, phosphorus, nucleic acid, or glucosamine. From the sedimentation constant of 4.3 and a diffusion constant of 1.5×10^{-7} a molecular weight of 240,000 and an axis ratio of 30:1 are calculated. The name is derived from the fact that two distinct antibodies can be elicited on injection; the two antigenic portions, however, differ considerably in their heat stability, one being labile (L), the other stable (S).

More recent work has demonstrated that a still finer differentiation can be made. Thus the intact protein will remove both L and S antibodies from antisera. If it is heated for a short time it can no longer precipitate L antibody, although it can still inhibit precipitation of the latter with native LS antigen. Precipitation with S antibody still occurs at this stage, but with further heating all reactivity is lost. If the complex is heated with alkali no reaction is observed with L antibody, and inhibition of, but no precipitation with S antibody can be shown. On the other hand, digestion with chymotrypsin results in a protein which does not react at all with S antibody but still precipitates L antibody.

A nucleoprotein is the most abundant antigen in the elementary bodies, comprising about 45 per cent of the weight by isolation. It is relatively stable under a variety of conditions. In addition, a so-called x-agglutinogen has been postulated for the elementary bodies since not all of the agglutinating antibody can be removed from all sera by absorption with the three antigens previously mentioned. Moreover, such an absorption does not result in a reduction of the neutralizing (protective) power of the residual

antibody for infective virus, nor is a protective antibody produced by injection of animals with the purified antigens. The x-agglutinogen may be related to the "protective" antigen but this remains to be proven. Further work on the antigen responsible for protection is obviously necessary. For details and references on the material of this section the reader is referred to the reviews of Smadel and Hoagland (1942) and Smadel and Shedlovsky (1942).

VI. Bacteriophages and Their Antibodies

Bacteriophages are substances which can lyse bacteria. Their biological properties are in many respects parallel to those of viruses and the two are often treated together. Bacteriophage is potentially of therapeutic value, nevertheless, although enthusiastic claims have been made for it, clinical application is not considered unequivocally successful as yet. It has recently been demonstrated, however, that phage, usually grown in broth cultures, can also multiply *in vivo*, and an analysis is presented of the factors necessary in a rational approach to phage therapy (Dubos, Straus, and Pierce, 1943).

Several investigations have been made on concentrated, purified phage preparations. Northrop (1938) isolated from 35,000 liters of staphylococcus phage a nucleoprotein (N = 14.1 – 14.6%, P = 4.6 – 5.0%, glucose 1.5%). The diffusion constant depended on the concentration, but the protein was homogeneous by sedimentation, with a sedimentation constant of 650 Svedberg units. The molecular weight was of the order of 300,000,000; by diffusion smaller particles of 450,000 could be detected as well. Evidence was presented by both methods that the active agent was associated with the protein. Its solubility approached that of a pure substance. Kalmanson and Bronfenbrenner (1940) working with a coli phage also obtained a protein (N 15%) but the phosphorus content was quite low (0.07%). This may be due to strain differences although Schlesinger had previously isolated a coli phage containing 3.7% P. The great sensitivity of phage action may be seen from the fact that the phage isolated by Kalmanson and Bronfenbrenner had 6×10^{-7} g. protein per lytic unit. Molecular weights estimated or calculated varied from 36,000,000 to 25,000, depending on the methods used and the assumptions made.

Many phages are antigenic when injected, and the resulting antibodies inhibit their action. Since the phage can only be grown at present along with bacteria, whose products are difficult to remove, the antigen mixture injected will also give rise to antibacterial antibodies. The latter are serologically unrelated, however, to the phage and may be removed without reducing the antiphage titer, by absorption with bacteria free of phage. Antisera can be thus produced which at a dilution of 1:20,000 will still

produce considerable inactivation of the phage. The lytic action of phage can be completely abolished by heat, phenol, or formalin, but if the conditions are not too strenuous the phage may still be highly antigenic, a situation paralleled by the conversion of toxins to toxoids.

The action of a bacteriophage is characterized by a specificity, the degree of which varies with different strains. By growing a phage on an organism related to the homologous one, a phage active only with the latter may often be trained to lyse the new one as well.

The antigenic specificity of a phage, on the other hand, is not changed under these conditions, and has served as a basis for classification of several groups of phages (Burnet, 1933). It has been found that phages in the same antigenic group may lyse widely different types of bacteria, and, on the other hand, that phages acting on the same bacterium may belong to different antigenic groups. The existence of at least two qualitatively

TABLE VI

Properties of Diphtheria and Scarlet Fever Toxins

	Diphtheria toxin[1]	Scarlet fever toxin[2]
Biological potency, per mg.	2200*	150 million skin test units
Sedimentation constant, Svedberg units	4.6	2.7
Diffusion constant $\times 10^7$	6.0	9.5
Molecular weight	74,000	27,000

* Flocculation units per mg. N.
[1] Petermann and Pappenheimer (1941).
[2] Krejci, Stock, Sanigar, and Kraemer (1942).

different receptor groups on the phage has therefore been postulated. One (A-grouping) reacts with the antiphage antibody; the other (B-grouping) reacts with the bacterial surface antigen.

It is quite likely that there are a number of both A and B groupings on each phage molecule, and that these differ among themselves in degree of reactivity. Killed bacteria which absorb phage without being lysed, can then be agglutinated by antiphage. The latter has no action unless the organisms are thus coated. It has been demonstrated by a number of investigators that the reaction between the phage B receptors and the corresponding groups on certain, although not all, bacteria may be blocked by using a soluble antigen such as the specific polysaccharide. The same type of inhibition has been observed for specific bacterial agglutination. Analogous in some respects too is the inhibition by haptenes of precipitating antibodies. If, however, a phage is first treated with enough antibody to inactivate it by at least 50 per cent, the residue may be no longer susceptible

to further inactivation by the soluble bacterial antigen. This has been taken to mean that some of the A and B receptors are quite close together on the phage surface (Burnet and Freeman, 1937). General discussions of antiphage reactions will be found in the reviews of Burnet, Keogh, and Lush (1937) and Delbrück (1942).

VII. Anti-enzymes

As has been mentioned previously, little is known of the role of bacterial enzymes, and of antibodies to them, in infection and resistance. Data are available, however, on other enzyme-anti-enzyme systems. Sumner and his colleagues were the first to produce a satisfactory anti-enzyme of the antibody type. Rabbits were injected with amounts of crystalline urease increasing from 2.5 to 600 units. Although 20 units are usually fatal to a normal rabbit, 1000 units could be tolerated by the immune rabbits.

Analyses showed that the blood ammonia level of immune rabbits remained low following an injection of urease, while those of unimmunized rabbits rose to fatal levels. Quantitative studies of the inactivation of urease activity by anti-urease indicated an 80 per cent reduction in activity with large serum excess. Complete inactivation was never obtained, demonstrating that the specific precipitate always contained free enzyme reactors not covered by antibody. Soybean urease was inactivated by anti-jackbean urease (and animals similarly protected against it), but the serological data are not complete enough to permit a decision as to whether they are serologically identical or merely closely related. Anti-urease can also be produced in the hen, which can tolerate very large doses (17,000 units), since its blood urea level is too low to form toxic products. The immune hen antiserum protected rabbits against urease. Sumner's various contributions have been summarized in a review (Sumner, 1937).

Antibodies to the enzyme tyrosinase derived from the mushroom *Psalliota campestris* have been produced both in humans and in the rabbit. The precipitin reactions follow equation (3) above quite closely. No cross reaction was given by tyrosinase from another species of mushroom. In the first instance, although the enzyme was precipitated by the antibody no inhibition of its activity was observed in the presence of serum (Adams, 1942).

Urease is inactivated by air oxidation, although up to 80 per cent reactivation can be made by treatment with reducing agents such as hydrogen sulfide or tissues. Ultraviolet light irreversibly denatures it. Pillemer, Ecker, Myers, and Muntwyler (1938) have studied the antigenic properties of normal, oxidized, heated, and ultraviolet inactivated urease. All four cross reacted with the various antisera, except heated urease with sera to oxidized urease. The latter serum protected rabbits against 10 lethal

doses of normal urease. The authors believe this effect to be due to normal urease antigen, since tissues were noted to reduce the oxidized material injected.

The serological reactions of pepsins and pepsinogens from various species have also been examined. The experiments are limited, however, by the denaturation of pepsin above pH 6. The differences found have a significance, nevertheless, in indicating varied responses to denaturation. Pepsins from the cow and from the guinea pig removed all of the antibody in an antiserum to swine pepsin; chicken, shark, and rabbit pepsins were inactive toward it. Pepsin antisera reacted both with pepsin and with pepsinogen, although not with the homologous serum proteins. Pepsinogen antisera on the other hand reacted only with pepsinogen, and not with twice recrystallized pepsin or with the serum proteins (Seastone and Herriott, 1937). Differences between beef and pig trypsin have been demonstrated by the anaphylactic reaction (Tenbroeck, 1934).

Anticatalase has been prepared by injecting rabbits with beef catalase (Campbell and Fourt, 1939). It reacted (ring test) with beef, horse, cat, and dog catalase. Analysis of the supernatant activity showed that it took more anticatalase (beef) to reduce the activity of horse catalase by a given amount than it did for the homologous beef antigen. The washed specific precipitates possessed almost full catalase activity, the slight reduction being attributed to the increased dispersion. The reaction is not inhibited by either hematin or hemoglobin.

Beef ribonuclease has also been found to be antigenic. Like catalase the enzyme is precipitated by the antibody but the complex still retains 70–90 per cent of the activity of the soluble enzyme. No cross reaction was observed between the anti-enzyme and cattle serum (Smolens and Sevag, 1942).

A most interesting and detailed demonstration has been given by MacFarlane and Knight (1941) that *Cl. welchii* (type A) toxin contains a lecithinase which decomposes lecithin into phosphocholine and a diglyceride. It was suggested that the lecithinase is identical with the α-toxin of this organism, although it was noted that conclusive proof must await the isolation of a single substance having all of these properties. The enzyme is activated by Ca^{++} and inhibited by fluoride, citrate, and phosphate. *Cl. welchii* (type A) antitoxin specifically reduced enzyme action, the protocols indicating at least 94% reduction if sufficient serum is used. When properly standardized the inhibition reaction with lecithin could be used as a measure ($\pm$10%) of the antitoxin content of the serum.

VIII. Complement

It was noticed by John Hunter as early as 1792 that fresh blood resisted putrefaction to a marked extent. This property has been shown to decrease

on standing, and to be abolished when the blood was heated at 60°C. for a short time. The fundamental work of Buchner, of Bordet, and of Ehrlich at the turn of the last century demonstrated that there were two factors concerned in the destruction of bacteria by this mechanism: one present in most fresh normal sera, the other even in heated (60°C.) immune sera specific for the organism. The first is termed complement or alexin (protective substance); the other is the specific antibody.

Among the properties of complement now recognized are (a) the lysis of sensitized (antibody coated) red cells; (b) lysis of some although by no means all sensitized bacteria; (c) the capacity to kill certain sensitized bacteria in the absence of lysis; (d) the ability to add to many antigen-antibody complexes (complement fixation).

Four distinct components have been revealed by a combination of chemical and serological methods. All four seem to be necessary for full complement activity. By dialysis of fresh serum against distilled water or by treatment with dilute acid a precipitate is formed (midpiece). The supernatant contains another activity (end-piece). Both activities are abolished in a few minutes at 56°C. Treatment with cobra venom, yeast cells, or an insoluble carbohydrate derived from yeast removes still another activity (third component), while ammonia or a variety of amino compounds remove the fourth component activity. The latter two are relatively thermostable.

Although as indicated above the properties and activities of complement have been extensively explored, until quite recently nothing definite could be said of its chemical nature, nor could the absolute amounts present in sera be estimated as only relative titer methods were available. Pillemer, Ecker, Oncley, and Cohn (1941) have now isolated under rigidly controlled conditions three of the components of guinea pig complement (Table VII). The first component (C′ 1) is relatively stable between pH 5.4–6.0 but it is inactivated at neutrality. The activities of the second and fourth components (C′ 2 and 4) although apparently associated with a single molecule, can be differentially inactivated under various conditions, in a manner similar to that of the LS antigens of vaccinia. They are most stable in slightly acid solution (pH 6.2 to 7). The third component (C′ 3) has not yet been isolated in purified form. It is much more stable in slightly acid than in alkaline solutions. When one considers the small amounts of these components in serum and their conflicting stability ranges the difficulties of earlier workers become evident. A beginning has also been made in the characterization of human complement. The properties of its C′ 1 are included in Table VII.

The estimation of the absolute amounts of complement in human and guinea pig sera has been carried out by Heidelberger (1941) and Heidelberger and Mayer (1942). It was found that specific precipitates prepared

in the presence of fresh guinea pig or human sera would carry down additional nitrogen, and this was related to the disappearance of hemolytic titers. The amount found for the principal combining components of guinea pig complement (0.04–0.06 mg. N per ml.) is in good agreement with that found by actual isolation, 0.06 mg. Citations to the other investigations along these two lines will be found in the recent review of Pillemer (1943).

TABLE VII

Properties of Purified Components of Human and Guinea Pig Complements (C′)

Property	Human[1]	Guinea Pig[2]	
	C′1	C′1	C′2 and 4
Precipitated by $(NH_4)_2SO_4$ of concentration	1.4 *M*	1.4 *M*	2.2 *M*
Electrophoretic mobility (pH 7.7–7.8) × 10^5	2.9	2.9	4.2
Sedimentation constant, Svedberg units	6.9	6.4	
Carbohydrate content, %	3.6	2.7	10.3
Optical rotation, $[\alpha]_D$		−29°	−193°
Destroyed in 30 min. at deg. C.		50	{50 (C′2) 66 (C′4)
Protein nitrogen, per cent	13.9	16.3	14.2
Apparent isoelectric point, pH	6.0	5.3	6.4
Fraction of total serum protein, %	0.8	0.6	0.2

[1] Pillemer, Seifter, San Clemente, and Ecker (1943).
[2] Pillemer, Ecker, Oncley, and Cohn (1941).

IX. Other Applications of Serological Methods

One of the difficulties in the isolation of materials from biological systems is the detection of contaminants. In bacterial fractions these may arise from the media on which the organisms are grown, in animal or plant virus preparations from the normal tissues of the host. Serological methods have in numerous instances permitted tests to be made of the product with antisera to the normal medium. This has been done, for example, with tobacco mosaic virus and definite indication found (Chester) of impurities in earlier but not in later preparations (Stanley and Loring, 1938). The use of anti-agar sera to detect that component in bacterial preparations may also be cited. The progressive purification of pepsin prepared from pepsinogen could be followed by the precipitin reaction (Seastone and Herriott, 1937) (Table VIII). The material left in the crude pepsin solution still gave a reaction with anti-pepsinogen, but this could be eliminated after two recrystallizations.

Serological methods may also be used to follow proteins in the presence of large amounts of unrelated protein. Thus Landsteiner and Parker

(1940) cultivated chick embryo connective tissue fibroblasts for 8 months (35 weekly passages) on rabbit plasma and rabbit embryo juice, and showed that fluids from the culture still reacted positively with anti-chicken sera. Evidently tissues cultivated on a foreign medium do not lose their species specificity. As another example, the iron containing protein ferritin has been isolated from a number of sources. Granick (1943) found that a rabbit antiserum to horse spleen ferritin would react with horse spleen ferritin freed of iron (apoferritin). There was a weak reaction with dog ferritin but none at all with human ferritin. The precipitin reactions were also found useful in locating ferritin in tissues where the amounts were too small to be readily isolated.

Serological methods may be of help in isolating a given protein from a mixture. Their utility in the preparation of purified antibody solutions has already been mentioned. Antigens may be similarly concentrated.

TABLE VIII

Conversion of Pepsinogen to Pepsin, as Followed by Precipitin Reaction

	Titer in pepsinogen antiserum	Titer in pepsin antiserum
Pepsinogen, before activation	5+	2+
Activated pepsin (pH 2, 18 hrs., then adjusted to pH 7.6)	4+	4+
same, after 1 cryst.	+	4+
same, after 2 cryst.	−	4+

(Seastone and Herriott, 1937.)

Thus Kirk (1933) found a 850 fold concentration of urease (specific precipitate activity) when crude soybean meal was treated with anti-urease.

The injection of protein hormones into human subjects sometimes results in the production of a refractory state. While there seems to be general agreement as to the definiteness of this condition, there is still controversy as to whether the anti-hormone is an antibody. Space precludes further discussion of this point here, the reader being referred to the reviews of Collip, Selye, and Thomson (1940) and Thompson (1941). It may be remarked, however, that antibodies to some hormone-like proteins can be produced in animals, and a detailed quantitative study of the serological cross reactions between thyroglobulins from various species is available (Stokinger and Heidelberger, 1937).

X. Some Conclusions and Summary

From the various interpretations that have been offered in connection with the data presented in this review certain regularities are seen to emerge.

Although the applications to protein structure are not as conclusive as the protein chemist might prefer, they are perhaps not less significant than are those from most other lines of evidence.

The concept of serological specificity has a number of features in common with the specificity of reaction exhibited by enzymes. Bergmann's concept of the homospecificity of enzymes, as illustrated for example, by the identical substrate requirements for the physically different proteins hog pepsin and beef spleen cathepsin, is also applicable to antibodies, say to egg albumin, as produced in the rabbit and in the horse. The process of combination between enzyme and substrate is not often visibly evident but some enzymes such as pepsin do give precipitates with proteins. The reversible inactivation of certain antigens by oxidation and reduction has been mentioned above.

It is, however, in the inhibition of enzyme action by compounds which are near enough to the substrate configuration to combine with, although not to be decomposed by the enzyme that the most interesting parallelisms occur. Thus compounds such as thiamin or piperazine inhibit the action of diamino oxidase by a firm union with the enzyme which prohibits further action with a potential substrate (Zeller, 1942). A more extended example is derived from the studies of Quastel and Wooldridge (1928) on toluene-treated *B. coli.* It was found that hydroxymalonic or glyceric acids specifically inhibited the lactic dehydrogenase, and that citric or tricarballylic acids inhibited only the succinic dehydrogenase. From a number of such experiments the requisite configuration of the specific inhibitors could be deduced, and it could be shown that meso-tartaric acid, which possesses both configurations did indeed inhibit both enzymes. The analogous property of simple haptenes to combine with and inhibit antibodies without otherwise visibly reacting has been discussed above and needs no further comment.

The experiments on the chemical basis of specificity have involved mainly the introduction of groupings which do not occur at all, or only exceptionally, in native proteins. The actual determinant portions of proteins are not known with certainty. We do know, however, that as far as examined the prosthetic groups of enzymes, or the carbohydrates naturally linked to serum or egg proteins do not appear to influence serological specificity (Neuberger and Yuill, 1940). On the other hand, many bacterial or other polysaccharides in suitable linkage to protein can determine the specificity; in fact, the original protein specificity may be completely masked.

In view of our present limited knowledge of the fine structure of proteins (derived chiefly from X-ray studies on fibers) we can make but little direct application of the data on the influence of isomeric linkages. It does seem indicated, nevertheless, that there is plenty of material to account for an

almost unlimited number of distinct specificities for even the smallest protein from the various permutations and combinations of, say, 100 amino acid residues. Amino and carboxyl groups have both been shown to function as determinants when introduced on aromatic groups coupled to protein. It is evident, moreover, that the nature and order of amino acids in polypeptide chains may have a decided influence on serological specificity, and it may well be that these are the principal factors, at least in simple proteins which yield only amino acids on hydrolysis. This point has been discussed in some detail by Marrack (1938) and by Boyd (1943). The possibility that the amino acids of proteins occur in regular patterns has been discussed by Bergmann and Niemann (1937); see also Chibnall (1942).

The size of the unit of specificity is a matter of some interest. Experiments designed to determine this must take into account, however, the variable nature of the antibody response even to a single antigen. Thus it will be recalled that the percentage of antibody which will cross react with heterologous antigens increases with immunization. However, antisera from some species may not cross react at all in a given case. In the experiments with antisera to the type VIII pneumococcus reported above it was noted that whereas even relatively weak horse antisera cross reacted with the type III polysaccharide, no cross reaction was observed with rabbit antisera of equal or even greater antibody content. Many other examples might be given. Whether the antibodies from various species reflect different portions of the specificity units (as Shope concluded from his experiments cited above) or whether the differences are due to unequal binding capacities is not always clear.

Several lines of evidence indicate that the unit of specificity is much less than the whole protein molecule. Experimentally it has been shown that low molecular weight hydrolysis products from silk fibers or bacterial polysaccharides may function as inhibitors for at least a part of the antibody. The action of some simple haptenes also belongs in this group. The existence of cross reactions among members of groups of proteins such as the ovalbumins, hemoglobins, or serum proteins demonstrates that these may contain a number of specificities. In fact, one possible concept of the characteristic specificity of a protein is that the latter consists of a more or less unique combination of "standard" building units. Finally, we may cite the data on the multiplicity of determinant groups as bearing on this point.

It is becoming increasingly evident that a protein may have a number of independent functional groups. In the illustrations just considered these were all antigenic. In other cases, however, the presence of additional biologically active but not necessarily antigenically detectible groups can be inferred. Antibodies to enzymes, bacteriophage, antibodies, and toxins

can be produced which have the property of precipitating these substances without inactivation. Thus, an anti-antibody specific precipitate may still bind bacterial polysaccharide, and anti-catalase specific precipitates will decompose hydrogen peroxide. In some instances a quantitative reduction in the activity may occur but as long as complete inactivation is not obtained this may be accounted for by changes in dispersion, or in terms of steric factors. Lack of marked dissociation must of course be shown experimentally if these considerations are to apply.

It is common experience that the toxic properties of diphtheria and tetanus toxins may be abolished by formaldehyde and other reagents without imparing the antigenic properties. The toxicity may also be neutral-

TABLE IX

Molecular Composition of Specific Precipitates

Antigen	Empirical composition				
	At extreme antibody excess	Antibody end of equivalence zone	Floccula-tion point	Antigen end of equivalence zone	Soluble cmpd. in inhib. zone
Cryst. egg albumin	EaA_5	EaA_3		Ea_2A_5	[EaA]*
Dye egg albumin	($DEaA_5$)	($DEaA_3$)		DEa_2A_5	DEa_2A*
Thyroglobulin	TgA_{40}	TgA_{14}		TgA_{10}	(TgA)*
Type III pneumococcus	SA	S_3A_2		S_2A	S_5A*
Cryst. serum albumin	SaA_6	SaA_4		SaA_3	(SaA)*
Diphtheria toxin	TA_8*	TA_4	TA_2	T_2A_3	TA* and T_2A

The diphtheria toxin system was studied in horse sera, the others in rabbit sera. A = Antibody, S = Minimum polysaccharide chain weight reacting. Data in parentheses are somewhat uncertain. Asterisks indicate soluble complexes.

From Heidelberger, (1938,b); Pappenheimer, Lundgren, and Williams, (1940).

ized by antitoxin in proper proportions. On the other hand, the toxic antigens of most Gram-negative antigens are precipitated but only partly neutralized by antitoxin—a fact which often gives rise to troublesome clinical reactions in prophylactic immunizations. That dissociation or non-specific adsorption are not factors in the case of the typhoid antigen has been demonstrated by H. R. Morgan (1941).

It is quite clear that antibodies can combine in more than one proportion. The development of quantitative absolute methods, and the measurement of molecular weights of the purified reactants have permitted the assignment of molecular formulas (Table IX). Immune reactions have been thus stripped of their air of colloidal mysticism and placed in the framework of definite chemical combinations. From the quantitative data a concept of immunological valence can be developed. Although there is general

agreement that antigens are multivalent—that is, they can combine with more than one molecule of antibody—there is some disagreement as to whether or not antibody is multivalent. The consensus of opinion is that it is bivalent in most instances, although vigorous exception has been taken to this. There are also differences of opinion as to the specificity of various stages of the interaction between antigen and antibody. For details the reader is referred to the works of Marrack (1938), Heidelberger (1939), Kendall (1942), Boyd (1943), Hershey (1942, 1943), and of Pauling, Campbell, and Pressman (1943).

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The Interaction between the Alkali Earth Cations, Particularly Calcium, and Proteins

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I. INTRODUCTION

The biochemical literature contains a vast amount of evidence to show that proteins and also the amino acids form complexes with many of the metal cations. In the case of the proteins, it seems probable that, with the exception of the alkali elements, some degree of complex ion formation occurs on the alkaline side of the isoelectric point with all the metals. In many cases, in analogy with well known compounds, the manner in which the union occurs is quite clear. Thus coordination with the unshared electron pairs of amino and other nitrogen groups of proteins and amino acids plausibly accounts for complex formation with silver and mercury ions. The complex formed between cysteine and cobalt implicates the thiol groups of the proteins in the reaction with this element (Schubert, 1931), although from analogy with the cobalt ammines, various nitrogen groups might also be the site of the union.

In the case of many of the metal cations, however, there is no such ready explanation. This is particularly true of the alkali earth elements. A possible explanation of the manner of binding in these cases will be considered below.

The discoveries of recent years, that certain of the metal cations act either as activators or as accelerators of many enzyme reactions, have served to heighten the interest in the problem of metal-protein complexes. Among the cations conspicuous in this respect are manganese, cobalt, zinc, copper, magnesium, and calcium. Abundant evidence exists that in contrast to the iron-porphyrin enzymes, the metal-enzyme compounds of the above elements are readily dissociable.

While, as has been stated above, there is abundant evidence for the formation of metal-protein complex ions, this evidence is largely of a qualitative nature. In rare cases only is there available quantitative data on the stoichiometry, the dissociation mechanism, or the dissociation constants of the protein complexes.

It has not seemed profitable to the writer to sift the vast literature for all the metal ions which are involved in complex formation[1] with proteins, because of the qualitative nature of the evidence. Rather, it has seemed more profitable to confine this review largely to a single group, the alkali earth elements, and in particular to calcium ion, because with the latter, a quantitative approach has been made to the problem.

II. Experimental Methods for Studying the Physical-Chemical States of Metal Ions in Protein Containing Systems

1. *Membrane Distribution*

Combination of ions with proteins can be estimated from the distribution of ions in Donnan membrane systems. An extensive series of membrane distribution experiments were carried out by Northrop and Kunitz (1924, 1925, 1928) on purified gelatin solutions. The method of calculating the amount of ion combined with the protein has been derived from the theory of membrane equilibrium by these authors. The data required for such a calculation is the total ion concentration in the pure salt solution outside the membrane, the ion concentration in the protein solution inside the membrane, and the membrane potential of the system.

The membrane potential (E_M) is related to the ratios of the activity of the ion outside to that inside by the following equation:

$$E_M = \frac{RT}{F} \ln \frac{a_o}{a_i} = \frac{RT}{F} \ln \lambda \qquad (1)$$

In the equation, R is the gas law constant, T the absolute temperature, F the faraday number, a_o and a_i the activities of the ion outside and inside

[1] For a survey of the subject and references to the literature see Schmidt (1938).

the membrane respectively, and λ is the ratio of these activities. The activities are related to the molal concentrations by the relations

$$a_o = \gamma_o M_o \text{ and } a_i = \gamma_i M_i$$

where the letter γ stands for the activity coefficient.

From the above it follows that

$$\frac{\gamma_o M_o}{\gamma_i M_i} = \lambda \quad \text{and} \quad M_i = \frac{\gamma_o M_o}{\lambda \gamma_i}$$

The concentration of ion combined with the protein, M_c, then is given by

$$M_c = M_i - \frac{M_o \gamma_o}{\lambda \gamma_i} \qquad (2)$$

If it can safely be assumed that $\gamma_o = \gamma_i$, then equation 2 simplifies to:

$$M_c = M_i - \frac{M_o}{\lambda}$$

The above authors studied the combination of gelatin with the ions Cu^{++}, Ca^{++}, Mg^{++}, Al^{+++}, La^{+++}, Ag^{+}, and also with the alkali cations and certain anions. No combination between K^{+}, Li^{+}, Na^{+}, NO_3^{-} or SO_4^{--} and gelatin could be detected. Copper was found to have an equivalent combining value of 0.9 *mM* per g. gelatin and La^{+++} and Al^{+++} about 0.5 *mM*. The experiments with calcium yielded results which indicated that there is no combination on the acid side of pH= 3 and that the amount combined rises rapidly between pH 3.8 and 4.7. The maximum combination of the calcium approached the value of 0.9 *mM* per g. gelatin. On the other hand the maximum combination of magnesium only approached values of between 0.4 – 0.5 *mM* per g.

The first experimental observation that calcium in blood serum could be differentiated into several fractions was made by Rona and Takahashi in 1911. By dialyzing serum against saline solutions containing variable amounts of calcium, it was found that about 60% of the total serum calcium was diffusible and capable of passing through a membrane impermeable to proteins. Rona and Takahashi suggested that the non-diffusible fraction of the calcium is bound in some manner to the proteins. These observations have been verified and extended by many investigators employing more refined dialysis and other methods. The results on the partition of calcium in serum will be considered in detail later.

The physical-chemical state of the magnesium of the blood serum has been studied to a lesser degree by the same methods (Stary and Winternitz, 1929, 1932; Watchorn and McCance, 1932; Greene and Powers, 1931). An extensively employed variation of the dialysis method is ultrafiltration.

In ultrafiltration, a portion of the fluid of a protein containing solution is forced through the pores of a membrane impermeable to the protein by a sufficiently high pressure gradient. Greenberg and Greenberg (1931) have published experimental evidence to support the concept that the ultrafiltration of diffusible electrolytes from systems containing electrically charged colloids is analogous in its nature to a Donnan membrane distribution.

TABLE I

Transference Numbers of Sodium and Calcium Caseinate at 30°C.

	0.1 N silver deposited in coulometer	Q	B	QB = K	T_{casein}	T_{cation}	T_{casein} + T_{cation}	i
	cc.							%
Sodium	1.70	1.37	6.52	8.95	0.499	0.542	1.041	
caseinate	4.50	1.09	8.55	9.33	0.462	0.534	0.996	
	4.60	1.02	9.05	9.23	0.454	0.548	1.002	
*		1.73	5.6	9.68	0.453	0.561	1.014	
*		1.34	7.0	9.40	0.455	0.540	0.995	
*		1.04	8.25	8.60	0.430			
Calcium	1.90	0.411	12.23	5.03	0.953	0.331	1.284	48.7
caseinate	1.95	0.331	12.95	4.28	1.185	0.338	1.523	49.3
	1.60	0.300	13.20	3.96	0.968	0.194	1.162	40.5
	1.95	0.2195	14.70	3.23	1.295	0.343	1.638	49.7
	1.90	0.196	14.75	2.90	1.070	0.278	1.348	45.4
	1.60	0.197	15.40	3.02	1.200	0.199	1.399	40.8

B = cc. of 0.1N alkali per g. of casein; Q = electrochemical equivalent per millifaraday; i = fraction of metallic element which exists as ions; T = transport number.

* These data are taken from Greenberg and Schmidt (1926).

Table taken from Miyamoto, S., and Schmidt, C. L. A. (1933), *J. Biol.Chem.* **99**, 335.

2. *Electrical Transference and Conductivity*

Conductivity and transference data demonstrate that there is a striking difference between the ionization of the alkali earth and the alkali proteinates. Greenberg and Schmidt (1926) observed that the equivalent conductivities of the alkali earth caseinates ranged from one-fifth to one-third of the values for similar solutions of the alkali caseinates. Similar differences in conductivity have been found with serum globulin (Adolf, 1923).

More profound evidence of the difference in ionization of the alkali and alkali earth proteinates is given by the transference experiments of Greenberg and Schmidt (1926) and of Miyamoto and Schmidt (1933). The Hittorf transport numbers of the alkali caseinates give a picture of an

ordinary salt-like behavior, much like that observed in the case of a simple fatty acid. The transference data lead to the interpretation that the alkali proteinates are completely ionized in the sense that this term is used in the interionic attraction theory of strong electrolytes.

On the other hand, the transference numbers of the alkali earth proteinates are subject to the interpretation that a considerable fraction of the metal is united to the protein anion and migrates to the anode with the protein in the field of a direct current. Miyamoto and Schmidt explain this by a step wise dissociation of the alkali earth cations. Representative transference data of protein solutions are shown in Table I.

Greenberg and Schmidt found that the incomplete ionization of the alkali earth caseinates could be represented by the following equation:

$$\text{T cation} = \frac{iU - (1 - i)V}{i(U + V)} \tag{3}$$

where i stands for the fraction of the alkali earth metal which exists as cation, U for the mobility of the cations and V for the mobility of the complex metal-protein ions.

3. *Activity Determinations with Electrodes*

A considerable number of attempts have been made to employ amalgam electrodes to measure the activities of the alkali earth elements in protein containing solutions and in biological media. It can readily be appreciated that this offered great difficulty because of the great reactivity of the alkali earth metals. The earlier of these studies have been reviewed by Fosbinder (1929).

The first attempts at electrode measurements of the calcium ion activity in biological media were of doubtful validity. The calcium amalgam electrode is reliable in aqueous solutions containing only calcium salts. However, when the solution contained salts of other cations, Fosbinder found that the potential of the electrode was lowered, probably on account of the formation of a mixed amalgam electrode. Even very small amounts of protein (0.02%) destroyed the reliability of the calcium amalgam electrode of Fosbinder. He suggests that this effect is due to the tendency of proteins to form a film on a metallic surface. The film prevents the ordinary electrode equilibrium from taking place and results in the building up of a large ionic concentration inside the film.

Ordinary amalgam electrodes of the alkali and alkali earth elements were found to be unstable in the presence of amino acids by Joseph (1936). He also found that they were unstable in the presence of ammonium salts.

In order to protect the amalgam from protein, Joseph (1938) introduced the use of a cellophane membrane which is permeable only to electrolyte

and solvent. With this device, successful measurements were made of the activities of calcium and magnesium chlorides in the presence of isoelectric solutions of the following proteins: horse carboxyhemoglobin, horse serum albumin, pseudo-globulin, and gelatin. Unfortunately these measurements gave only the changes in the activity coefficient of the salt caused by the altered solvent properties of the protein containing solutions. In this work, the proteins were practically at the isoelectric point, so the combination between the proteins and ions was quite small. Therefore, the results throw little light on the combination between metal ions and protein. The presence of the protein produced a lowering in the activity coefficients of all of the salts tested. This included sodium and zinc chlorides as well as the alkali earth chlorides. The lowering of the activity coefficient of calcium and magnesium chlorides was much greater than that of sodium chloride.

Calcium amalgam proving too reactive, Kirk and Schmidt (1928) turned to barium amalgam electrodes. With dilute amalgams, it was found possible to make successful activity measurements on solutions of the barium salts of amino acids and the protein, casein. The measurements with the amino acids led to the conclusion that their barium salts behave as strong electrolytes. On the other hand, the measurements with casein showed that there was a low degree of dissociation of barium ions. This is shown in Fig. 1. by the curves plotted from the results of their measurements on barium caseinate.

Use of electrodes of the third kind to measure the calcium ion activity of biological fluids has not had much success. The theory of this type of electrode and the difficulties involved in their use has been thoroughly treated by LeBlanc and Harnapp (1930).

Joseph (1939) has recently employed an electrode of the third kind consisting of lead amalgam in contact with lead oxalate and calcium oxalate to determine the activity of calcium chloride in solutions containing amino acids or egg albumin and similar electrodes to determine the effect of amino acids on the activities of barium and strontium chloride. As in the case of the studies by Joseph mentioned above, these experiments determined the effect of the alteration of the solvent produced by the amino acids or protein on the activity coefficients of the alkali earth chlorides. The magnitude of the lowering of the activity coefficients produced by relatively high amino acid concentrations, (0.5 and 1.0 *M* glycine and alanine), may be very considerable.

4. *Determination of Calcium Ion Concentration by Contraction of the Frog Heart*

The contraction response of the excised frog heart offers an interesting biological method of estimating calcium ion concentrations. This method was perfected by McLean and Hastings (1934). The shortcomings of this

method are that it can only be employed on fluids with an electrolyte composition approximating that of serum and it is limited with respect to the concentration range over which it is sensitive; the upper limit being about 2.0 *mM* Ca^{++}. The great field of usefulness of this method has been in ascertaining the calcium ion concentration of various body fluids under normal and pathological conditions.

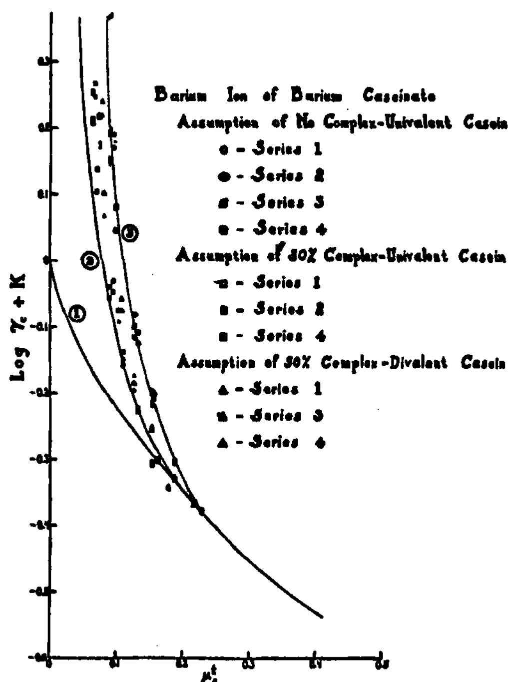


Fig. 1. Comparison of the values for log γ_0 + K for the barium ion of barium caseinate with the typical curves for the barium activity of typical strong electrolytes. Curve 1: Barium ion activity coefficient curve of typical electrolytes taken from Lewis and Randall (1923). Curve 2: From electromotive force measurements with the barium amalgam electrode on barium chloride and barium hydroxide. Curve 3: Barium caseinate. (From Kirk, P. L., and Schmidt, C. L. A. (1928), *J. Biol. Chem.* **76**, 115.)

5. *The Ultracentrifuge*

A unique approach to the study of ion-protein relationships has been made through use of the ultracentrifuge. Calcium and protein containing solutions were exposed to centrifugal forces of sufficient magnitude to produce gradients in the protein and calcium concentrations. From these gradients it was found possible, by a graphical method, to estimate the diffusible calcium concentration and the amount of calcium bound to protein. Chanutin, Ludewig, and Masket (1942) employed a Beams type

airdriven ultracentrifuge. The rotor had a capacity of 66 ml. of solution and the holes to hold the Lusteroid test tubes were bored at a 10° angle. The rotor was run at 1000 revolutions per second and exerted a mean centrifugal force of 200,000 times gravity.

The diffusible calcium is derived from the analytical data by plotting total calcium concentration against protein concentration and extrapolating to zero protein. In solutions prepared from protein and inorganic salts, the diffusible calcium should be equivalent to the ionic calcium concentrations. In solutions containing citrate and other complex forming ions, soluble non-ionic calcium is present in addition, in the diffusible fraction.

6. *Solubility Methods*

The solubility product of a slightly soluble salt offers a valuable method for estimating the activity coefficients of ions in solution. Lewis and Randall (1923) point out that: "When, at a given temperature, a solid salt is in equilibrium with a solution, the activity of that salt in the solution is fixed." "It cannot be changed by any change in the nature of the solution, such as would be produced by the addition of other electrolytes." This principle has received wide application in the estimation of the approximate degree of dissociation of calcium and magnesium proteinates.

Weir and Hastings (1936) have made use of the solubility of calcium carbonate for this purpose. Solutions containing calcium and various proteins, casein, serum albumin, and serum globulin were brought into equilibrium with solid $CaCO_3$ at a definite CO_2 tension at 38°C. The calcium ion concentrations were calculated from the stoichiometric solubility product and the carbonate concentration according to the equation

$$[Ca^{++}] = \frac{Ksp}{[CO_3^{--}]}$$

The calcium combined with protein was calculated by subtracting the estimated $[Ca^{++}]$ from the total calcium concentration.

Other compounds that have been used to estimate calcium ion concentrations are calcium oxalate (Brinkman and Van Dam, 1920; Nordbö, 1936) and calcium picrolonate (Nordbö, 1939,a) and tropeolin 00 for calcium and magnesium ion concentrations (Nordbö, 1938, 1939 b).

III. Application of the Law of Mass Action to the Combination between Calcium and Protein

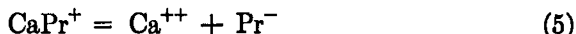
1. *Theoretical Considerations*

An important contribution to the problem of the interaction between protein and metal ions was the demonstration by McLean and Hastings

(1935,a) that calcium proteinate apparently behaves as a weak electrolyte whose dissociation can, as a first approximation, be represented by a very simple type of mass law equation. McLean and Hastings assumed that the dissociation should be represented by a chemical equation in which the protein behaves as a series of divalent ions, thus;

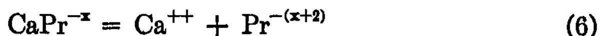
$$CaPr = Ca^{++} + Pr^{--} \qquad (4)$$

The above assumption was soon seen to be unnecessary. Weir and Hastings (1936) and McLean (1938) later pointed out that the dissociation might equally well be represented by the equation:



$$CaPr^{+} = Ca^{++} + Pr^{-} \qquad (5)$$

Actually this equation is just as improbable as the first. It appears quite unlikely that the calcium proteinate, in the solutions that have been studied, carries no electrical charge or that it is positively charged. The transport experiments of Greenberg and Schmidt (1926) with casein and the usual Tiselius electrophoresis pattern of blood serum, on the contrary, show that the calcium proteinate is negatively charged. Therefore, it appears more appropriate to write



$$CaPr^{-x} = Ca^{++} + Pr^{-(x+2)} \qquad (6)$$

From the standpoint of the mathematics involved, the electrical charge on the calcium is a matter of indifference. The important point is that the dissociation of the calcium takes place in single units.[2]

Mathematical equations to represent the calcium proteinate dissociation are obtained as follows. In the derivation the symbols TPr, TCa are used for total protein and total calcium; Pr^{--} represents protein ion, and CaPr, calcium proteinate. Symbols without brackets represent concentrations in milligrams per 100 g. of H_2O and in the case of the protein fractions, grams per 100 g. H_2O. Bracketed symbols designate molal concentrations.

[2] That the mass law equation 7 fits the dissociation of calcium proteinate probably has an at present unrealized significance. A protein molecule undoubtedly is capable of combining with a large number of calcium ions. Logically therefore, the calcium should appear to a higher power than one in the mass law equation thus:

$$[Ca^{++}]^{n} \times [Pr^{-2n}]/[Ca_{n}Pr] = K_{CaPr}$$

A situation similar to the above occurs in the field of enzyme kinetics. In the well known Michaelis-Menten equation, the substrate concentration enters to the first power in all the well established cases of enzyme reactions, although it would appear probable that in many instances the enzyme is capable of uniting with many substrate molecules.

The hypothesis of McLean and Hastings embodied in equation 4 can be represented by the mass law equation;

$$\frac{[Ca^{++}]\cdot[Pr^{--}]}{[CaPr]} = K_{CaPr} \qquad (7)^{3}$$

It is not possible to make an independent determination of the protein ion concentration. Since, [TPr] = [CaPr] + $[Pr^{--}]$ on substitution for Pr^{--} there is obtained

$$\frac{[Ca^{++}]\cdot([TPr] - [CaPr])}{[CaPr]} = K_{CaPr} \qquad (8)$$

Rearranging gives

$$\frac{[TPr]}{[CaPr]} = 1 + \frac{K_{CaPr}}{[Ca^{++}]} \qquad (9)$$

To make numerical use of equation 9 requires the assumption that

$$[TPr] = f\cdot TPr \qquad (10)$$

where f is a factor for converting protein concentration from the usual gram units to molal units when the calcium combined with the protein has its maximum value; namely when $Ca^{++} = \infty$.

When 10 is introduced into equation 9 there is obtained

$$\frac{TPr}{[CaPr]} = \frac{1}{f}\left(1 + \frac{K_{CaPr}}{[Ca^{++}]}\right) \qquad (11)$$

In the form given, equation 11 was derived by Chanutin, Ludewig, and Masket (1942). A similar but less elegant method of deriving equation 11 was first employed by Greenberg, Larson, and Tufts (1935).

To apply equation 11, it is necessary to be able to evaluate the conversion factor (f) by which protein in term of grams may be converted to moles. McLean and Hastings (1935) used the hydroxide combining value of a protein as the basis for calculating the conversion factor. The weakness of this procedure is that no evidence exists to show that the hydroxide and calcium combining values of a protein are the same.

Greenberg, Larson, and Tufts (1935) pointed out that the conversion factor could be derived from the experimental data itself by an extrapolation to $Ca^{++} = \infty$. If equation 11 is obeyed a plot of TPr/[CaPr] against $1/[Ca^{++}]$ yields a straight line. It may readily be seen that when $1/[Ca^{++}]$

[3] On a strictly thermodynamic basis the activities of the components involved, and not their concentrations, should be employed in equation 7. However, it is a characteristic property of weak electrolytes that a reasonably good fit of the equation is obtained when concentrations are employed.

= 0, the intercept on the axis of TPr/[CaPr] = f. This provides a method of evaluating the protein conversion factor which avoids the use of titration data and any assumption as to valency.

Chanutin, Ludewig, and Masket pointed out certain possible interpretations of equation 11 which are useful in practice and are of interest in the light of past efforts to establish mathematical relationships between the levels of calcium and protein in blood sera. These are

(a) *The* $[Ca^{++}]$ *is constant and protein varies.* This leads to a linear relationship between CaPr and TPr which may be written as

$$[\mathrm{CaPr}] = \mathrm{m\ TPr} \tag{12}$$

Introducing this into 11 yields,

$$\frac{1}{\mathrm{m}} = \frac{1}{\mathrm{f}}\left(1 + \frac{K_{\mathrm{CaPr}}}{[\mathrm{Ca^{++}}]}\right) = \frac{\mathrm{TPr}}{[\mathrm{CaPr}]} \tag{13}$$

If

$$[\mathrm{TCa}] = [\mathrm{CaPr}] + [\mathrm{Ca^{++}}] \tag{14}$$

it follows from equation 12 that

$$[\mathrm{TCa}] = \mathrm{m\ TPr} + [\mathrm{Ca^{++}}] \tag{15}$$

In body fluids, the diffusible calcium may exist in unionized as well as ionic form. In such a case equation 14 becomes [TCa] = [CaPr] + diffusible Ca and equation 15,

$$[\mathrm{TCa}] = \mathrm{m\ TPr} + \text{diffusible Ca.}$$

The presence of an undissociated form of diffusible calcium does not alter the equations derived from the mass law.

Equation 15 shows that in the straight line obtained by plotting [TCa] against TPr, m is the slope of the line and $[Ca^{++}]$ is given by the intercept on the [TCa] axis.

Equation 15 has been used in an empirical manner on blood sera by many investigators (Marrack and Thacker, 1926; Hastings, Murray, and Sendroy, 1927; Greenwald, 1931; Gutman and Gutman, 1937). In all cases the deductions that were reached have had only a limited validity. Several of the reasons for this can readily be seen. Probably the most important is that the blood serum contains a mixture of proteins each of which has a different calcium combining value. The proportion of the different proteins is only roughly constant in the blood of healthy individuals and varies greatly in many pathological conditions. Variations in the nature of the protein of the blood serum will cause variation in the factor m. The available experimental evidence indicates that the albumin of the blood has the

highest calcium combining capacity; that of the globins being considerably lower.

Gutman and Gutman (1937) were able to obtain a very good representation of the relation between serum calcium and protein by the use of the equation

$$Ca = 0.8 \text{ albumin} + 0.2 \text{ globulin I} + 7$$

The constant 7 in the above equation contains a value of one mg. Ca per 100 ml. serum assigned to combination with a globulin II in the serum which remains constant in amount.

The second difficulty in the application of equation 15 to blood serum is that the calcium ion concentration or diffusible calcium does not remain exactly constant in the blood of all individuals.

(b) *The* $[Ca^{++}]$ *is not constant.* When the $[Ca^{++}]$ varies and the proportion of the component proteins remain unchanged, it has already been pointed out that a linear relationship exists between TPr/[CaPr] and $1/[Ca^{++}]$. In such a plot of equation 11, the slope of the line is K_{CaPr}/f and the intercept of the line extrapolated to the TPr/[CaPr] axis is 1/f, which also is equivalent to 1/m.

By rearranging equation 13 there is obtained

$$[Ca^{++}] = \frac{m\,K_{CaPr}}{f - m} \qquad (16)$$

This equation can be used to evaluate the calcium ion concentration if the slope (m) and f and K_{CaPr} are known. If the data are plotted in the above manner, from what has been given, it follows that the slope of the straight line obtained is,

$$m = \frac{K_{CaPr}}{f} \qquad (17)$$

Therefore $$K_{CaPr} = m\,f$$

Greenberg and Larson have pointed out that the constant K_{CaPr}, expressed in molal units, is given by the product of the above constants divided by the factor 4×10^3 if the concentration of calcium is expressed in milligrams and proteins in grams per 100 g. of H_2O of the solution.

2. *Dissociation of Calcium Caseinate*

The dissociation of calcium caseinate has been studied quantitatively by three of the best of the known experimental methods, namely the frog heart technique, equilibration with solid calcium carbonate, and fractionation

in the ultracentrifuge. A summary of the dissociation constants found by the several investigators are given in Table II. Plots representative of the ultracentrifugal data are shown in Figs. 2–4.

TABLE II

Dissociation Constants of Calcium Proteinates

Method	Solution	pH	Temperature	Conversion factor	pK	Literature source
			CASEIN			
Frog heart	NaOH-H_2O	7.35	22°C.	0.300	2.38 ± 0.02	(McLean and Hastings, 1935)
$CaCO_3$ solubility	Modified Ringers (μ = 0.160)	7.2-7.7	38°C.	0.293-0.355	2.23	(Weir and Hastings, 1936)
Ultracentrifugal analysis	NaOH-H_2O	6.3-8.5	5.0°C	0.40	2.73	(Chanutin, *et al.*, 1942)
Ultracentrifugal analysis	NaCl-NaOH-H_2O	6.3-8.5	5.0°C	0.40	2.36	(Chanutin, *et al.*, 1942)
			TOTAL SERUM PROTEIN			
Frog Heart	Serum and body fluids	—	22°C.	0.122	2.22 ± 0.07	(McLean and Hastings, 1935)
Frog heart	Serum	—	—		2.17 ± 0.06	(Chu and Hastings, 1938)
Ultrafiltration	Serum	—	25°C.	0.062	2.44	(Greenberg and Larsen, 1939)
			SERUM PROTEINS (a) Serum globulins			
$CaCO_3$ solubility	Modified Ringers μ = 0.160	7.0-7.9	38°C.	081-0.119	2.36	(Weir and Hastings, 1936)
Frog heart	Modified Ringers μ = 0.160	7.4	22°C.	0.05	2.96 ± 0.13[1]	(Drinker, *et al.*, 1943)
Frog heart	Modified Ringers μ = 0.160	7.4	22°C.	0.11	2.70 ± 0.14[2]	(Drinker, *et al.*, 1943)
Frog heart	Modified Ringers μ = 0.160	7.4	22°C.	0.055	2.00 ± 0.10[3]	(Drinker, *et al.*, 1943)
Frog heart	Modified Ringers μ = 0.160	7.4	22°C.	0.145	3.13 ± 0.14[4]	(Drinker, *et al.*, 1943)
			(b) Total serum albumin			
$CaCO_3$ solubility	Modified Ringers μ = 0.160	7.3-7.8	38°C.	0.139	2.11	(Weir and Hastings, 1936)

[1] Constant for pseudoglobulin—γ from normal horse serum.
[2] Constant for euglobulin P_I.
[3] Constant for euglobulin P_{II}.
[4] Constant for euglobulin P_{III}.

The significant result obtained from the analysis of calcium caseinate is that, except at a great dilution of casein, the dissociation obeys the mass law with a high degree of accuracy. The dissociation constant of calcium

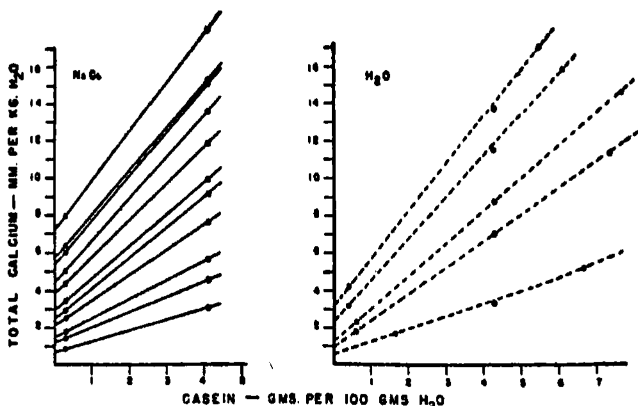


Fig. 2. Dissociation of calcium caseinate in NaCl and H_2O solution. The effect of varying the calcium concentrations of casein dissolved in 0.9% NaCl and in aqueous solutions (pH 7.2). The saline solutions were centrifuged at 1000 revolutions per second for 5 hours, and the top fraction and original analyzed. The aqueous solutions were centrifuged for the same time and divided into two portions for analysis. (From Chanutin, A., Ludewig, S., and Masket, A. V. (1942), *J.Biol.Chem.* **143**, 737.)

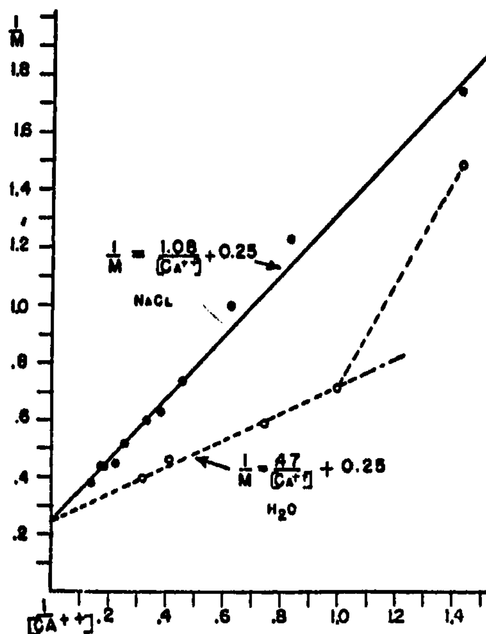


Fig. 3. Analysis of the dissociation of calcium caseinate by method of equation 13. Plot of the reciprocals of the slopes (m) and the calcium ion concentrations of the data in Fig. 2. Solid line, NaCl solutions; dashed line, H_2O solutions. (From Chanutin, A., Ludewig, S., and Masket, A. V. (1942), *J.Biol. Chem.* **143**, 737.)

caseinate was found to be independent of the concentration of casein or calcium. However, it varied somewhat with different batches of casein. As is shown in Fig. 2, the degree of dissociation of calcium caseinate is increased by elevating the electrolyte concentration with sodium chloride. The dissociation constant K_{CaPr} is increased from 1.88×10^{-3} in H_2O to 4.32×10^{-3} in the salt solution. Chanutin and coworkers (1942) suggest that this is caused by the increase in ionic strength of the solution. The extrapolation of the calcium caseinate curves to $[Ca^{++}] = \infty$, yields the

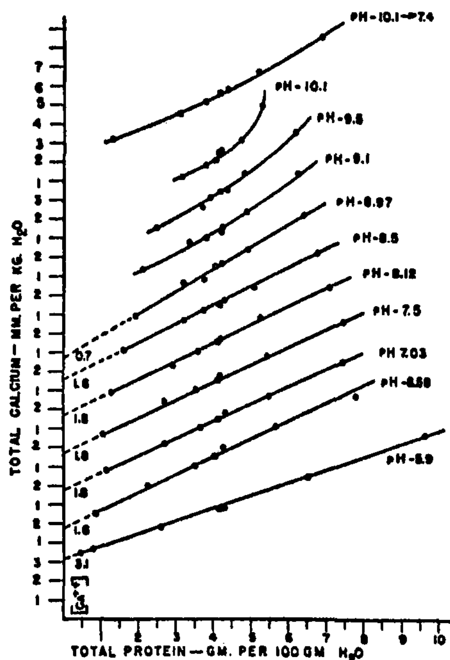


Fig. 4. Variation in the dissociation properties of calcium caseinate at different hydrogen ion concentrations. (From Chanutin, A., Ludewig, S., and Masket, A. V. (1942), *J. Biol. Chem.* **143**, 737.)

value of 0.4 as the factor required to convert grams of protein to millimoles (see Fig. 3). This conversion factor is considerably greater than the values derived by Weir and Hastings (1936) from the base titration curve of casein by Pertzoff and Carpenter (1932). Increase in the ionic strength of the solution does not alter the maximum calcium combining capacity.

Fig. 4 shows the effect of pH on the ionization of calcium caseinate. It is remarkable that both the calcium combining power and the pK remain essentially constant over the range pH 6.3 – 8.5. Below pH 6.3 the degree

of dissociation increased rather sharply as shown by increases in the values of K_{CaPr}. The calcium caseinate solutions at pH 9 or higher no longer show a linear relationship between total calcium and total protein concentrations. Chanutin and coworkers advance the interpretation that the results indicate that the calcium combines with the zwitterion of the protein (NH_3^+–R–COO^-) as well as the protein anion (NH_2–R–COO^-).

When citrate was added to the calcium caseinate solutions, the diffusible calcium increased and the [Ca^{++}] decreased to a degree predicted from a combination of the pK values of calcium caseinate and calcium citrate.

3. *Dissociation of the Calcium Salts of the Serum Proteins*

The state of the calcium in blood serum has been studied by most of the methods cited in the section on experimental methods.

As has been stated above, interpretation of the data on the physical-chemical states of calcium in unfractionated serum is a difficult problem due to the several kinds of proteins present, the presence of magnesium, the possibility that the diffusible calcium represents a number of chemical species, and the possible presence of a colloidal form of calcium phosphate. Nor may the proteins be the only calcium combining components in blood serum. Drinker and Zinsser (1943) have shown that the phospholipid cephalin is capable of combining with calcium in a manner parallel to that of proteins. A dissociation constant of 1.27×10^{-3} was found for calcium cephalinate. The above authors estimate that 30 to 40% of the bound calcium in serum may be united to cephalin.

McLean and Hastings (1935) approached the problem by showing that the dissociation constant, pK_{CaPr}, derived from measurements on serum, ascitic fluid, pleural fluid, and edema fluid of human origin was quite constant with a value of 2.22 ± standard deviation 0.07, if the [Ca^{++}] was estimated by the frog heart method and the calculations carried out according to equation 8. In these calculations it was presumed that the albumin: globulin ratio in the above fluids remained approximately constant at the value 1.8. The factors for converting protein from grams to millimoles were taken from the hydroxide titration curves of the serum proteins (Van Slyke, *et al.*, 1928). The deductions of these authors are summed up in the Cartesian nomogram relating total calcium, total serum proteins, and Ca^{++} concentration which is reproduced in Fig. 5.

Greenberg and Larson (1939) determined the partition of the calcium by ultrafiltration in the sera of animals in which alterations in calcium were produced by such means as treatment with large doses of vitamin D, or parathyroid extract or injection of calcium salts, and alterations in protein content by plasmapheresis. A plot of the data in the form T Pr/Ca Pr against $1/Ca^{++}$, assuming the diffusible calcium to be a measure of the

ionic calcium, seemed to indicate that although there was considerable scattering, the best fit of the points was obtained by a straight line. A statistical evaluation of the correlation between the above variables yielded the highly significant result: $r_{1,2} = 0.728 \pm 0.034$. The magnitude of the dissociation constant derived from the plotted data was $pK_{CaPr} = 2.44$.

The ultracentrifugal analysis of the dissociation of the calcium proteinate of horse and human sera on the contrary did not conform with the mass law equation if there was employed but a single constant for the total serum protein. Upon plotting total calcium against total protein, discontinuities

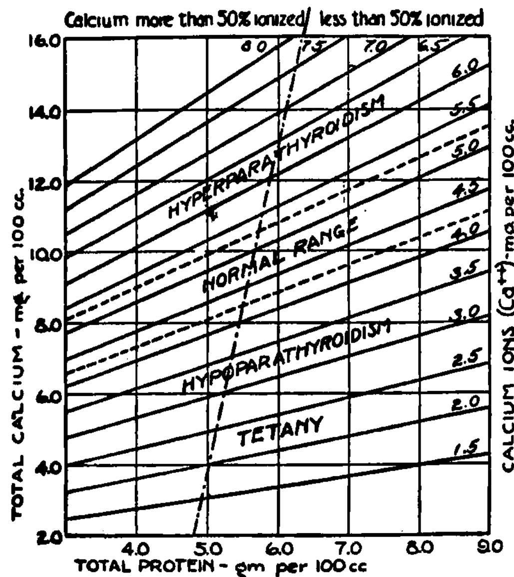


Fig. 5. Cartesian nomogram chart for calculating the calcium ion concentration from the total calcium content of blood serum or plasma. (From McLean, F. C., and Hastings, A. B. (1935,b), *Am. J. Med. Sci.* **189**, 601.)

in the slopes of the curves were observed in the majority of the centrifuged sera (Ludewig, *et al.*, 1942). Because the globulin of the serum, being higher in molecular weight, sediments more rapidly in the ultracentrifuge, marked variations were found in the albumin and globulin concentrations of the respective centrifuged fractions of a single serum. The distribution of the protein components, however, was not helpful in interpreting the slopes or discontinuities of the plots. It is interesting that despite the variations in the protein fractions, the relationship between calcium and protein concentrations was substantially linear over a limited region of the curve. When the respective fractions of a serum were recentrifuged, the

resulting distribution of protein and calcium values yielded substantially linear plots. This finding indicates that the fractionation of the serum proteins is responsible for the failure of the data from the original serum to fit the mass action equation.

Representative plots of the results obtained with the ultracentrifuge on human sera are shown in Fig. 6 and the results of recentrifugation in Fig. 7.

Certain of the serum protein fractions have been studied individually, particularly by Hastings and coworkers. McLean and Hastings (1935) evaluated the individual dissociation constants of albumin and globulin by the frog heart technique, and Weir and Hastings (1936) even more

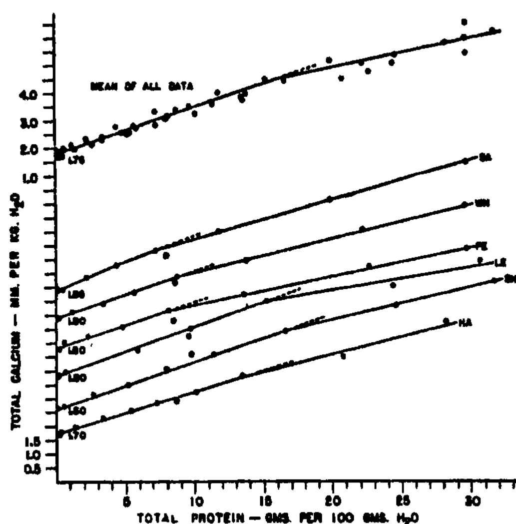


Fig. 6. Partition of calcium and protein in the serum of six hypertensive patients centrifuged for 4.5 hours at 1000 revolutions per second. The broken lines indicate the extent to which the segments differ in slope. Solid circles represent points of centrifuged fractions; open circles represent the original sera. (From Ludewig, S., Chanutin, A., and Masket, A. V. (1942), *J. Biol. Chem.* **143**, 753.)

carefully by equilibration with solid calcium carbonate. Drinker, Green, and Hastings (1939) studied the calcium dissociation of four separate fractions of the serum globulin by the frog heart technique. The calcium combining values and the dissociation constants obtained from these studies are recorded in Table II.

From the discussion given above one can hardly escape the inference that the law of mass action fits the reaction between calcium and individual proteins in solution of known composition quite well. On the other hand, blood serum is too complex a medium to be adequately represented by the simple one constant equation embodied in the law of mass action.

No comparable data exist on the nature of the reaction between proteins and the other alkali earth cations. A mathematical analysis of the kind reviewed here has so far only been carried out with calcium. However, from the close chemical kinship of the alkali earth elements, the comparable behavior observed in transference experiments with casein, and the similarity in the dissociation mechanism of calcium and magnesium and strontium citrates (Hastings *et al.*, 1934), it may safely be concluded that the dissociation of calcium and the other alkali earth proteinates is very similar.

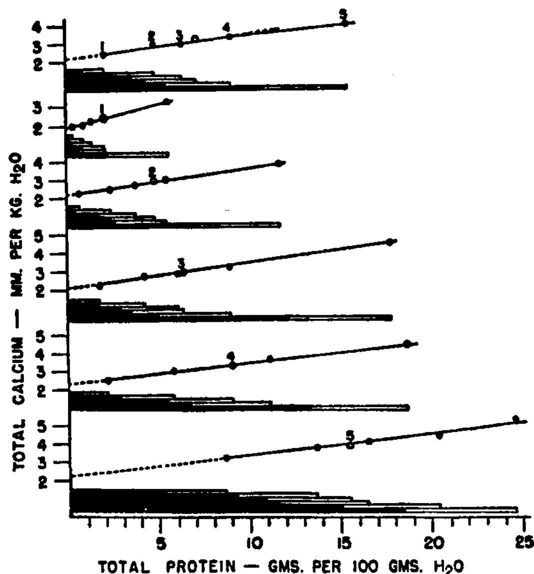


Fig. 7. Plots showing the linear relationship between total calcium and total protein and the albumin-globulin fractions obtained after recentrifugation of serum. The figures on the curves represent the fraction numbers of the serum in the top curve. The solid boxes represent globulin; the clear boxes, albumin. (From Ludewig, S., Chanutin, A., and Masket, A. V. (1942), *J. Biol. Chem.* **143**, 753.)

IV. Structural and Physical-Chemical Evidence of the Nature of the Combination between Alkali Earth Cations and Proteins

An explanation for the interaction between the alkali earth ions and proteins is naturally to be sought for in terms of groups in the protein molecule capable of binding cations and preventing their ionization and the nature of the bonds involved; namely, whether they are covalent or electrostatic.[4] A logical approach to the problem is to seek evidence from

[4] By electrostatic bond it is not implied that a compound so united necessarily ionizes in a suitable solvent (see Pauling, 1939, pages 5 and 37).

the structure of simple compounds of well known composition, whose dissociation is similar to that of the proteins. The problem may also, to a certain degree, be approached in a more empirical fashion by blocking off, through substitution reactions, the reactive functional groups in the protein that could be concerned in the reaction and then testing the product.

The latter method of approach has been employed by Abels (1936). The calcium binding power of crystalline egg albumin and of albumin minus amino and/or hydroxyl groups were estimated by the membrane distribution method. Amino groups were removed by deamination with HNO_2,

TABLE III

	1 Observed Ca *mM/l* in protein soln.	2 Ca in dialysate *mM/l*	3 Cl_d/Cl_p	4 Calcd. Ca in protein soln. *mM/l*	5 Bound Ca in protein soln. *mM/l*	6 Protein *mM/l*	7 Groups of Ca to protein
Egg albumin	5.05	3.20	1.06	3.60	1.45	1.18	1.23
	4.70	3.56	1.04	3.94	0.76	0.60	1.26
Deaminized albumin	4.95	2.75	1.05	3.10	1.85	0.60	3.10
	6.45	3.40	1.08	3.95	2.50	1.16	2.16
Acetylated albumin	4.25	3.80	1.06	4.22	0.03	0.67	0.04
	3.15	2.88	1.05	3.18	−.03	.26	Approx. 0
"Deacetylated" acetylated albumin	4.42	3.27	1.05	3.60	.82	.64	1.29
	4.30	3.05	1.06	3.42	.98	.72	1.36
Gelatin	4.82	3.51	1.05	3.88	.94	.70[a]	1.34
	3.54	2.53	1.04	2.70	.84	.60[a]	1.38

[a] 34,500 was chosen arbitrarily as the molecular weight of gelatin to provide comparison with the ratios obtained for the egg albumin.

From Abels, J. C. (1936), *J. Am. Chem. Soc.* **58**, 2609.

and acetylation was employed to block both the amino and hydroxyl group. Abels' results are recorded in Table III. Abels points out that the calcium is probably not bound through amino groups, since no decrease in calcium binding properties resulted from deamination. Acetylation, on the other hand, caused a complete loss of the capacity to bind calcium. These experiments indicate that hydroxyl groups are important structural elements for the property of complex formation with calcium and other alkali earths. The experimental work also shows that the hydroxyl group is not necessarily a phenolic one, since gelatin, which is lacking in tyrosine, unites with calcium to about the same extent as egg albumin.

Pertinent to the present discussion is the available experimental evidence that bears on the dissociation of the alkali earth salts of organic acids. This experimental work implicates structures related to the dicarboxylic or hydroxy acids as being particularly concerned in the interaction between proteins and alkali earth cations. This evidence has been gathered by diverse physical-chemical methods which include conductivity (Money and Davies, 1932; Davies, 1938, a; Topp and Davies, 1940), the effect on the stoichiometric solubility products of such slightly soluble salts as $CaSO_4$, $CaCO_3$, and $Ca_3(PO_4)_2$ (Davies, 1938, b; Greenwald, 1938), and anomalous titration curves (Cannan and Kibrick, 1938). This evidence tends to show that with but few exceptions, the alkali earth salts of organic acids are incompletely dissociated in solution.[5] Di- and tricarboxylic, and α-hydroxy acid ions are most prominent in depressing the dissociation of the alkali earth cations.

From the data of the anomalous titration curves of certain organic acids in the presence of the alkali earth cations, Cannan and Kibrick (1938) have calculated the strength of the association in terms of mass action constants for the formation of compounds between the carboxylate anions and each cation. The calculations are based on the Hasselbalch equation

$$\mathrm{pH} = \mathrm{pK} + \log \frac{[\mathrm{A}^-]}{[\mathrm{HA}]}$$

In the presence of a cation capable of forming a complex with the anion, the titration curve will be shifted to a more acid region, *i.e.*, the acid titrated will appear abnormally strong. The association constants can be calculated from the shift in the titration curve. In calculating the association constants, the equations given below were used for mono- and dicarboxylic acids respectively.[6]

$$\frac{[\mathrm{MA}^+]}{[\mathrm{M}^{++}][\mathrm{A}^-]} = C_1 \qquad \frac{\mathrm{MA}}{[\mathrm{M}^{++}][\mathrm{A}^{--}]} = C_2$$

[5] A low degree of dissociation of calcium and magnesium salts is quite general according to Greenwald. It has been found to be the case among the sulfates, phosphates, and carbonates and bicarbonates of these elements (Greenwald, *et al.*, 1940, 1941; Tabor and Hastings, 1943).

[6] The reader is referred to the original article of Cannan and Kibrick for the methods of calculating the association constants from the titration data. They are too long to be taken up here. The simplest case, the calculation of C_1 for a univalent acid, involves the solution of the following five simultaneous equations.

(a) $$[\mathrm{TA}] = [\mathrm{HA}] + [\mathrm{A}^-] + [\mathrm{MA}^+]$$

(b) $$[\mathrm{TM}] = [\mathrm{M}^{++}] + [\mathrm{MH}^+]$$

(c) $$[\mathrm{Na}^+] + [\mathrm{H}^+] = [\mathrm{A}^-] + [\mathrm{MA}^+]$$

It will be noted that the logarithms of the association constants are identical with the usual pK method of expressing the dissociation constant.

Values of the logarithms of the association constants of compounds significant in the present connection are given in Table IV.

It is significant that an α-hydroxy group in the molecule decidedly enhances the association constant of a monocarboxylic acid. A β-hydroxy group apparently has only a slight effect. This agrees with the observations of Smythe and Schmidt (1930) in their study of complex formation

TABLE IV

Association Constants of the Alkali Earth Salts of Carboxylic Acids

Acid	Mg	Ca	Sr	Ba
Log C_1 for Monocarboxylic Acids[1]				
Acetic	0.51	0.53	0.43	0.39
Butyric	0.53	0.51	0.36	0.31
β-Hydroxybutyric	0.60	0.60	0.47	0.43
Glycolic	0.92	1.11	0.80	0.66
Lactic	0.93	1.07	0.70	0.55
Log C_2 for Dicarboxylic Acids				
Oxalic	2.55			
Oxalic[2]	3.43	3.00	2.54	2.33
Malonic	1.91	1.46	1.25	1.23
Succinic	1.20	1.20	1.06	1.03
r-Tartaric	1.36	1.80	1.65	1.62
Citric[3]	3.22	3.22	2.70	

Table compiled from Cannan, R. K., and Kibrick, A. (1938), *J. Am. Chem. Soc.* **60**, 2314.

[1] μ = circa 0.2.

[2] Constants computed from conductivity data of Money, R. W., and Davies, C. W. (1932). *Trans. Faraday Soc.* **28**, 609.

[3] Constants determined by Hastings, A. B., McLean, F. C., Eichelberger, L., Hall, J. L., and DaCosta, E. (1934). *J. Biol. Chem.* **107**, 351, by use of frog heart method for estimating calcium ion concentration.

with ferric ion. In the case of the dicarboxylic acids, the association constant, C_2, diminishes with increasing separation of the carboxyl groups.

(d) $$K_1 = [H^+][A^-]/[HA]$$

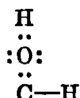
(e) $$C_1 = [MA^+]/[A^-][M^{++}]$$

In the above equations [TA] represents total acid concentration, [TM] total complex forming metal concentration, and $[Na^+]$ the alkali introduced to titrate to a given H ion concentration.

As was found with the monocarboxylic acids, a neighboring hydroxyl group enhances the binding of the alkali earth cations.

Among the tricarboxylates, Greenwald (1938) found that tricarballylic acid, which may be considered as containing two substituted succinic acids, depresses the ionization of calcium more than succinic. Citric acid because of its OH group, reduces the dissociation even more than tricarballylate ion.

To explain complex formation requires the demonstration of the action of some other force in addition to the usual ionic bond between an anion and cation. As has been pointed out by Smythe and Schmidt, this additional force may be derived from the amount of residual charge on certain groups in a molecule due to the attractive forces of unequal kernel charges. This may be calculated approximately from the rule of Latimer and Porter (1930) that when a pair of electrons is shared between two atoms, except in the case of hydrogen, the effect of their charges is distributed between the atoms in the ratio of the positive charges on the two kernels. Thus a pair of electrons between carbon and oxygen contributes 0.4 × 2 electron charges to the carbon atom and 0.6 × 2 electron charges to the oxygen. As an example we may consider the charge on a hydroxyl group which has the electronic arrangement,



The kernel charge of oxygen is +6 of carbon, +4. The 2 electrons between these atoms, it is assumed, will be shared in this ratio. The unshared electrons will react completely toward neutralizing the kernel charge on the oxygen, and hydrogen is considered to be sufficiently close to the center of the negative charges between it and oxygen to just neutralize 1 electron. Summing up the positive and negative charges there is obtained,

$$6 - 4 - (2 \times \tfrac{1}{2}) - (2 \times 0.6) = -0.2$$

Therefore, according to this calculation, the oxygen of the hydroxyl group possess a residual negative charge of 0.2 electron units. In a similar manner one may calculate that the residual charge on the carbonyl oxygen of a carboxyl group is −0.4 electron units. The relatively high dielectric constants exhibited by alcohols afford a physical demonstration of the considerable negative charge on the hydroxyl group.

The spatial distribution of the atoms in a compound as well as the force involved is an important factor in determining whether a complex will be formed. Among the most important of the spatial arrangements are those which favor ring closure. Metal ions which are good electron acceptors, namely the iron, copper, and zinc groups of the periodic table, readily com-

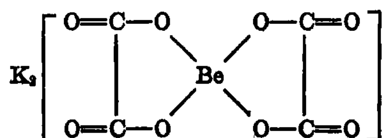
plete ring closure forming chelate rings (Diehl, 1937; Pfeiffer, 1940). In these chelate rings, the metal shares a pair of electrons with each of two atoms in such a manner as to complete the ring. The metal ions are held in the ring by covalent bonds. Compounds with reactive atoms spaced so as to permit formation of 5 or 6 membered rings, chelate most readily, since these are the rings of greatest stability.

In the case of the alkali earth elements, chelate ring formation with the dicarboxylic and hydroxy acids must be ruled out because the alkali earth elements do not share electrons to form covalencies.[7] Pfeiffer (1940) points out that the complex forming force of the alkali earths is generally slight. Pauling (1939) states that magnesium, calcium, strontium, and barium form essentially ionic bonds with the more non-metallic elements. Consequently, mainly electrostatic forces can be considered as acting to prevent or retard the ionization of the alkali earth cations. The residual negative charges and the spatial position of hydroxyl and carboxyl groups in a molecule should play an important rôle in this respect.

Acids with α-hydroxy groups, like lactic and glycolic, and dicarboxylic acids like oxalic should tend to form 5 membered rings. Acids with a β-hydroxy group, and dicarboxylic acids related to malonic, should tend to form 6 membered rings. Tartaric acid is simply equivalent to two glycolic acids. In citric acid there is a tendency to form one six and one five membered ring involving the hydroxyl. A number of these structures are illustrated by photographs of constructions with Hirschfelder atomic models in Fig. 8.

The values for the association constants listed in Table IV are in harmony with the above views with the exception of the case of β-hydroxybutyric acid. A β-hydroxy group in a saturated acid appears to produce only a slight increase in the binding force for divalent cations. This may be explained by the freedom of rotation of the groups about the α-carbon which can lead to a wide separation between the hydroxyl and carboxyl groups. The repulsion of these two negative groups would favor such a separation. Because of this, only a small proportion of the hydroxyl groups present are situated so as to exert their maximum negative attractive force on a metal cation. On the other hand, an α-hydroxy group cannot become so separated from the carboxyl group by rotation.

[7] Beryllium, the first member of the alkali earth elements, is an exception to this rule because of its acid character and can form true chelate rings. An example cited by Sidgwick (1929) is the oxalato-compound of beryllium.



In seeking evidence from the properties of simple compounds, the most important to be considered in the present connection are the amino acids. Amino acids are known to form complexes, particularly chelate ring structures, with many metal ions. The best defined cases are, of course, with the iron, copper, and zinc groups of metals of the periodic table. The amino group of an α-amino acid anion is a potent factor in favoring complex

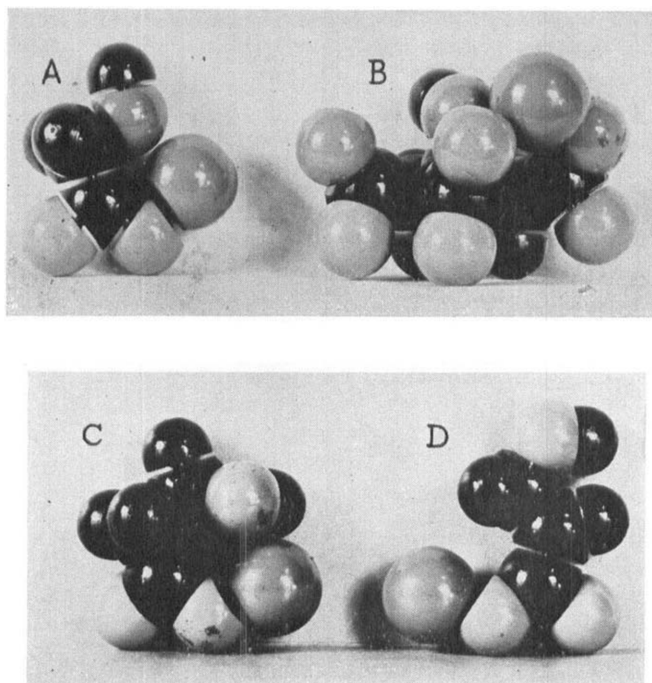


Fig. 8. Atomic models of salts of hydroxy acids. Small light spheres represent oxygen; large light spheres, the metal ion. Hydrogens, dark, can be distinguished from the carbons, also dark, by their half spherical shape.

A. Structure of glycolate ion. The model shows that rotation of the α-hydroxyl group cannot produce any greater separation from the carboxylate group.

B. Structure of citrate showing the possibility of forming one 5 and one 6 membered ring by linking the metal cation to the hydroxyl group and two of the carboxylate groups in the molecule.

C and D. Representation of two structural configurations of a β-hydroxyl acid ion. C shows a configuration which favors 6 membered ring formation. D shows the wide separation possible between the β-hydroxyl and carboxylate groups.

ion formation. The same factors that operate with an α-hydroxy group to retard ionization of cations are equally potent with an α-amino group. In addition, an amino group is a much better electron donor than is a hydroxyl group. However, in a protein, the α-amino groups are combined, for the most part, in peptide linkages so that this group loses its major significance in relation to complex formation by proteins.

The most significant of the amino acids for complex formation between the alkali earths and proteins would appear to be the hydroxy and dicarboxylic amino acids. The hydroxy amino acids that have been established to be widely distributed in proteins are serine, threonine, tyrosine, and hydroxyproline. Serine and threonine are β-hydroxy acids which have been shown to have only a small effect in reducing the ionization of the alkali earth cations. The chief dicarboxylic amino acids in proteins are aspartic and glutamic. These in the combined state might be expected to have an associating effect of the order of magnitude of succinic acid (Table IV).

Experimental evidence on the dissociation of the alkali earth salts of the amino acids is scanty. Greenwald (1939) has calculated the dissociation constants of magnesium aspartate and magnesium glycinate from the anomalous titration curves of Simms (1928). From these values it appears that the logs of the association constants are of the order of about 2.3 for the aspartate and 1.7 for the glycinate. It is of interest that the value for magnesium aspartate is in the same range as the of proteins (Table II). Comparison of the association constants of the two amino acids with the constants of related organic acids in Table IV makes it appear that they are probably too high.

Quite different results were obtained by Miyamoto and Schmidt (1933). These authors found no evidence of complex formation from the conductivity or transference values of the dicalcium and barium salts of aspartic and glutamic acids. No abnormalities were found in the transference numbers. A rough calculation, using the constant of Greenwald leads to the result that about one-third of the calcium and barium aspartate should have remained undissociated in the concentration employed by Miyamoto and Schmidt (0.04 M). If the constant for succinate of Table IV is used in the calculation, it leads to the result that the solutions are nearly completely dissociated. If the unionized calcium and barium are present only in the form of the neutral aspartate and glutamate it is quite comprehensible that the transference numbers would offer no evidence of incomplete dissociation. However, one might expect to derive such information from the conductivity data. The conductivity values, particularly in their treatment by Onsager's equation, closely paralleled the behavior of barium nitrate or calcium chloride. These latter salts are generally assumed to be completely ionized in the sense of this concept in the interionic attraction theory of electrolytes.

The above discussion on structure, in general, is favorable to the hypothesis that the free carboxyl groups of the dicarboxylic acid and the hydroxy groups of the hydroxyamino acid residues in the proteins are involved in the interaction between the alkali earth cations and proteins.

Since what is known about the structure of proteins indicates that practically every α-carboxyl and amino group of the amino acids is linked in a peptide bond in the protein, one must consider other structural relationships than those found in the amino acids to account for the phenomenon. These structural considerations are such that they would bring several carboxyl groups in close proximity to each other and hydroxyls in close proximity to carboxyl groups.

V. Biological Significance

The most widely known of the biological effects of the incomplete ionization of the alkali earth proteinates is in connection with the regulation of the calcium ion concentration or activity of the blood and the other body fluids. The maintenance of a relatively constant calcium ion concentration in the body fluids is of signal biological importance. The proteins, to a certain degree, take part in this regulation. Calcium ion is important for the coagulation mechanism of the blood, for the processes which control the deposition of the mineral matter of the bone and for maintenance of the normal irritability and contractility of heart, skeletal and smooth muscle. The relation of calcium to the subjects of bone mineralization and irritability will not be taken up here.

The calcium factor in blood coagulation has been reviewed by Ferguson (1936). Ordinary blood clotting requires the presence of not less than 13 to 21 mg. total calcium per l., nor more than about 18 g. per l. Amounts in excess of the above preserve the fluidity of the blood, just as does an excess of any neutral salt. It will be noted that the minimum concentration required for coagulation is about one-tenth of the normal blood serum calcium concentration. From a review of the literature, Ferguson reached the conclusion that ionized calcium is essential for the formation of thrombin from its inactive precursors, prothrombin and thromboplastin, in the blood. It is sometimes asserted, without much evidence, that thrombin is a calcium-cephalin-protein compound.

A fascinating aspect of the interaction between metal ions and proteins is the activating effect of certain cations on a variety of enzymes. Both magnesium and calcium belong to this category. A widely accepted explanation of this phenomenon is that the associated metal-protein complex represents the active form of the enzyme. The metal serves as an activating prosthetic group. In a large number of instances the combination between metal and enzyme protein appears to be of the same readily dissociable character as has been described in the preceeding sections.

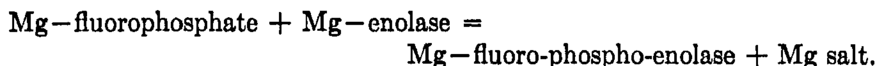
Among the hydrolytic enzymes, magnesium activates phosphatases (Folley and Kay, (1936), and certain peptidases, particularly the leucyl peptidases (Johnson, *et al.*, 1936; Berger and Johnson, 1939). Johnson and

coworkers estimated that the dissociation constant of the magnesium-leucyl peptidase of the hog intestine was about 1.8×10^{-3}. Manganese is an even more potent activator of the leucyl peptidases than magnesium. It is more effective at 1 *mM* than magnesium is at 10 *mM* concentration.

An unusual enzyme phenomenon is the effect of calcium upon the formation of trypsin from its inactive precursor.

McDonald and Kunitz (1941) found that crystalline trypsinogen is completely transformed into trypsin by means of a trace of trypsin in the presence of calcium salts. In the absence of calcium salts, part of the trypsinogen is transformed into an inert protein which cannot be changed into trypsin by any known means. Besides calcium, strontium, and magnesium favor the formation of active trypsin by inhibiting the formation of inactive proteins. Barium has the opposite effect.

Various of the enzymes concerned with biological oxidation are activated by magnesium. The best known cases are carboxylase (Lohmann and Schuster, 1937), phosphoglucomutase (Cori, *et al.*, 1938), isocitric dehydrogenase (Adler, *et al.*, 1939), and enolase (Warburg and Christian, 1942). In all four of the above enzymes, manganous ion is a more effective activator than magnesium. It is generally believed, however, that in nature magnesium is the conjugating metal ion because of its relative abundance as compared to the scarcity of manganese in biological material. Carboxylase has been isolated from brewers yeast as a diphosphothiamine-magnesium-protein (Green, *et al.*, 1941; Kubowitz and Lüttgens, 1941). The most active preparations contained 7 gram atoms of magnesium and 1 mole of thiamine per mole of protein (75,000 g.). Enolase has been isolated in crystalline form by Warburg and Christian, and the association with magnesium conforms quite well to the type of mass law formulation represented by equation 7. The measured dissociation constant was $K = 2.8 \times 10^{-3}$. In low concentrations zinc and manganous ions are better activators than magnesium, but in high concentrations they poison the enolase. The inhibition of the activity of enolase by fluoride is attributed to the formation of a complex magnesium fluorophosphate which reacts with the enzyme according to the following proposed equation



$$\text{Mg}-\text{fluorophosphate} + \text{Mg}-\text{enolase} = \text{Mg}-\text{fluoro-phospho-enolase} + \text{Mg salt.}$$

Inhibition of enolase was found to be proportional to $[Mg^{++}][HPO_4^{--}][F]^2$.

A unique discovery in enzyme chemistry is the observation of Engelhardt and Liubimova (1939) that myosin, the major protein component of muscle, is probably an enzyme which causes the breakdown of adenosine triphosphate to adenosine diphosphate. The great importance of this reaction is that the breakdown of adenosine triphosphate is a reaction most

closely associated in time with the act of muscular contraction and is believed to supply the energy for contraction. The adenosine triphosphatase reaction of myosin is activated by metal ions, calcium being the most efficient (Bailey, 1942). Other metal ions that have an activating effect are Mn^{++}, Ba^{++}, Mg^{++}, and Fe^{++}. Bailey has suggested that the essential feature of the excitation and contraction of muscle is the liberation of calcium ions in the neighborhood of the adenosine triphosphatase groupings of myosin. The calcium, by an almost instantaneous catalysis of the decomposition of adenosine triphosphate, can make available a large amount of energy and set off the contraction. The obscure point in this hypotheses is the lack of a mechanism to explain how calcium ions are liberated when a muscle is stimulated. A hypothetic suggestion based upon observations of Greenwald (1938) to account for this is that a compound which associates strongly with calcium is decomposed by the stimulation of the muscle and liberates a considerable concentration of calcium ions which persists only for a brief interval.

The examples given above illustrate the general importance of metal ion interaction for the field of protein chemistry. Much remains to be done to obtain a clear understanding of the manner in which the metal ions contribute to the catalytic functions of the enzymes. The group or groups in the enzyme protein which link with the cation is not definitely known in any single case. The whole subject definitely invites further investigation and the application of new tools and methods of approach.

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The Purification and Properties of Certain Protein Hormones

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A. Introduction

All the animal hormones which have been isolated so far are either steroids, proteins, or polypeptides. Rapid progress in the isolation of protein hormones, especially the pituitary and the pituitary-like hormones has been made during the past decade. This rapid advance was in a great measure possible because the knowledge that has been gained through the painstaking isolation of protein enzymes has been applied with considerable success in the purification of pituitary hormones. Thus among the eight proteins which Herriott (1942) considered to be pure according to the most rigorous tests for homogeneity (including the solubility test), three have been isolated from the pituitary gland. They are lactogenic hormone (1937), swine and sheep metakentrins (1940), and posterior lobe hormone (1942). Subsequently (1943) adrenotrophic hormone has been shown to be pure according to these rigorous criteria.

Since this review deals with recent advances in the chemistry of protein hormones the discussion of biological aspects will be limited to the extent that it will be helpful in the understanding of the significance of chemical problems and findings. It is beyond the scope of this review to describe in any detail the physiological functions of the hormones, and it is necessary to omit mention of many important papers not contributing directly to chemical investigation.

The pituitary gland is known to secrete a number of active principles. A few of them have been well characterized as chemical individuals while the existence of others in the gland or its crude extracts is based on biological identification only. In this review the discussion will be limited to those protein hormones whose chemical nature is reasonably clear. They include the hormones from the pituitary glands (gonadotrophic, lactogenic, adrenotrophic, growth promoting, and posterior-lobe hormones) as well as gonadotrophins secreted during pregnancy. Other protein hormones such as secretin, insulin, and thyroglobulin have been isolated in relatively pure form but very few chemical data have been added since the publication of numerous reviews and an occasional book; it is for this reason that discussion of these hormones will be omitted.

Many monographs, excellent or otherwise, dealing with the chemistry of hormones of the pituitary gland have been written. Among those recently published are: "Cold Spring Harbor Symposia on Quantitative Biology, Volume V," published by the Long Island Biological Association (1937); "The Physiology and Pharmacology of the Pituitary Body," by van Dyke (1939); "Symposium on the Endocrines. II. The Physiology of the Anterior Lobe of the Pituitary Body with Special Consideration of Gonadal-Pituitary Interrelationships" by van Dyke (1940), in the Bulletin of the Richmond Academy of Medicine, Richmond, Va., "Glandular Physiology and Therapy," published by the American Medical Association (1942); "Extraction, Separation, Concentration of some Anterior Pituitary Hormone," by Bergman and Turner (1942); and "Protein Hormones of the Pituitary Body," published by the New York Academy of Sciences (1943). Recently Fraenkel-Conrat published a review of the chemistry of hormones in the "Annual Review of Biochemistry" (1943).

B. How Is the Purity of a Protein Established?

For detailed discussion of this topic, the readers are referred to the papers of Pirie (1940) and Shedlovsky (1943).

When one deals with complicated molecules like proteins, one has to employ the various physico-chemical procedures which will provide the most satisfactory criteria for estimating the degree of purity of proteins. The important methods are electrophoretic analysis, ultracentrifugal analysis, and the determination of solubilities in various solvents.

In electrophoretic analysis one determines the electric mobilities of protein molecules. Although two protein molecules may have identical mobilities at one pH value, the probability that they remain the same over a wide range of pHs is small. Thus if only one component is observed to be present in the protein solution under examination, at various pH values, the protein may be said to be electrically homogeneous. Ultracentrifuga-

tion of proteins determines sedimentation constants which depend on the size and shape of molecules, although it is possible that two different proteins may give the same sedimentation constant. Solubility measurements provide the most rigid criteria for the purity of proteins. The principle of this test is based on the phase rule of Willard Gibbs. In brief, if the solid under examination is homogeneous, its solubility in a given solvent is independent of the amount of solid phase present. On the other hand, if it is inhomogeneous the solubility will increase as the amount of solid is increased.

Although all the protein hormones under discussion in this review have been analyzed either electrophoretically or ultracentrifugally, some tests have been made under less rigorous conditions than others. In order that readers can evaluate critically the results of the tests, it is necessary to understand the experimental conditions which affect the sensitivity of analyses and the reliability of the conclusion drawn. These conditions may be briefly numerated as follows:

I. Electrophoretic and Ultracentrifugal Analyses

1. *The Optical System*

Probably the most important, single factor which determines the accuracy of electrophoretic analyses is the optical device used to record the complete electrophoretic pattern. As originally adopted by Tiselius (1937), a boundary appears in the focus of the schlieren lens as a dark band —called the "schlieren method." The method does not furnish information as to the variation of the refractive index in the boundary and is useless for the determination of small amounts of impurities. Two other methods were devised based on a plot of the refractive index gradient in a thin layer of solution in the channel as ordinate against the height of the layer as abscissa. One of the methods is the schlieren-scanning device (Longsworth, 1939) and the other is cylindrical-lens method (Philpot, 1938; Svensson, 1940). The Longsworth device yields the most reliable results in detecting components in small amounts.

Similarly, the optical system of an ultracentrifuge is of paramount importance. Results obtained from the schlieren diagram are much less reliable than those obtained from the schlieren-scanning device (Philpot-Svensson). I have been kindly informed by Dr. Rothen that under the most favorable conditions one can detect about two per cent of impurity.

2. *The Range of pH*

The significance of the results will be small unless the analysis is performed over as wide a pH range as the stability and the solubility of the protein permit.

3. *The Choice of Buffer*

Some impurities in small quantities will show up in the scanning patterns very distinctly in one buffer but not in another. Thus, using a two per cent solution of human albumin one can detect with certainty the additional presence of as little as 0.01 per cent of globulin if veronal buffer at pH = 8 is the solvent. If the albumin be dissolved in phosphate buffer of the same pH, 0.04 per cent of globulin cannot be detected with certainty.

II. Solubility-Measurement

1. *The Equilibration*

A state of true equilibrium must be reached before one can measure the solubility of a protein. The equilibrium may be reached either from the undersaturated or the supersaturated side.

2. *The Choice of Solvents*

In order to minimize the possibility that the solubility of the contaminant is very close to that of the protein hormone, measurements should be made in more than one solvent containing a variety of salts and at different pHs.

3. *The Choice of Points of the Solubility Curve*

The critical evaluation of the constancy of solubility of a protein in a given solvent depends to a very large extent on the proper choice of points on the solubility curve. It is not sufficient to determine the solubility in the presence of two or more amounts of suspended solid if these amounts are high relative to the concentration of the dissolved material. It is of the utmost importance to make careful measurements over the region of the curve where the solid phase first appears. If the solubility is the same at this point as in the presence of a large excess of solid, then the preparation has a constant solubility (Herriott, 1942).

Although the relative amounts in a mixture of two or three proteins can be calculated from a solubility curve, it is highly doubtful whether any of the solubility studies so far made on pure protein hormones was capable of detecting impurities of five per cent or less.

C. Pituitary Hormones

Among the pituitary hormones which have been isolated in relatively pure form are the gonadotrophic hormones, lactogenic hormone, adrenotrophic hormone, growth hormone, and posterior lobe hormone.

I. The Gonadotrophic Hormones

The discovery of the gonad-stimulating effects of implants or of extracts of the pituitary furnishes concrete evidence that the gonads are not auton-

omous structures but depend upon pituitary hormones transported in the blood for their development and maintenance. Among the gonad-stimulating principles present in crude extracts of the anterior pituitary are: (1) The follicle-stimulating or gametogenic hormone, which causes the growth of graafian follicles preparatory to the release of ova and stimulates the sperm-forming tissues of the testis. It is also called thylakentrin or FSH (Coffin and van Dyke, 1941). (2) The luteinizing hormone, which stimulates the interstitial cells of the ovaries or testes and causes the formation of corpora lutea from preformed graafian follicles. It is also known as metakentrin or ICSH (Coffin and van Dyke, 1941). (3) The lactogenic hormone, which has been shown to play an important part in initiating and maintaining the secretion of progesterone by the corpora lutea. The question of the greatest interest to endocrinologists about a decade ago was whether the luteinizing and follicle-stimulating hormones were one and the same substance or two distinct chemical entities. The two hormones have since been separated essentially free from one another by chemical fractionation; moreover, metakentrins in homogeneous form have been isolated from both hog and sheep glands.

The separation of metakentrin and thylakentrin has been accomplished by using different solvents or precipitants which range from organic reagents, *e.g.* ethyl alcohol, dioxane, and pyridine, to a variety of inorganic salt solutions (ammonium sulfate, alkali halides, etc.). Regardless of the solvent used metakentrin can be precipitated by a lower concentration of either organic reagents or of inorganic salts than thylakentrin.

The two gonadotrophic hormones mentioned above differ from each other not only in their solubility but also in sensitivity to proteolytic enzymes. Trypsin, as we shall see later, destroys metakentrin at a greater rate than thylakentrin. Utilizing such a difference in digestibility, McShan and Meyer (1940) devised a method for preparing thylakentrin from aqueous extracts of sheep pituitary powder. After digestion the enzyme was destroyed by heat treatment and toxic substances present were removed by dialysis against acetate buffer at pH 4.0. By this procedure the luteinizing as well as lactogenic and thyrotrophic hormones were destroyed.

Since pituitary glands contain a large number of hormones, Fevold, Lee, Hisaw, and Cohn (1940) attempted to isolate other hormones besides the gonadotrophins from sheep glands by "isoelectric precipitation" at different concentrations of ammonium sulfate. They found that the range over which the metakentrin was precipitated by ammonium sulfate at pH 7.0, appeared to be from 1.4 to 2.4 *M*. Only traces of the thylakentrin were precipitated from the original extract by ammonium sulfate at a concentration up to 2.4 *M*, but at 2.8 *M* pH 7.0 the precipitation appeared to be complete.

1. *Isolation of Metakentrin from Hog and Sheep Pituitary Glands*

In 1940 metakentrins from hog and sheep pituitary glands were isolated in pure form in different laboratories.

a) *Hog Metakentrin Isolation.* van Dyke and his coworkers isolated metakentrin from hog pituitary glands in a pure form. Their method[1] is briefly as follows:

(1) Freshly ground hog pituitary glands are extracted with two per cent NaCl solution.

(2) The pH of the suspension is adjusted to about 4.2–4.6. The precipitate which appears is centrifuged off and discarded.

(3) The supernatant solution is saturated with solid ammonium sulfate. The precipitate is removed by filtration and dialyzed against water.

(4) The precipitates which appear during dialysis and after adjustment to pH = 5.1 are centrifuged and discarded.

(5) The supernatant solution is brought to half saturation of ammonium sulfate at pH = 5.5. The inactive precipitate is removed by centrifugation and discarded. Metakentrin remains in solution under these conditions.

(6) The protein in the supernatant solution is precipitated by the addition of ammonium sulfate to saturation, filtered, and dialyzed until salt-free.

(7) To one volume of the salt-free solution are added one volume of 1 *M* acetate buffer, pH = 4.4 and two volumes of 41 per cent sodium sulfate.

(8) The precipitate containing all the metakentrin is filtered and dialyzed. The filtrate which contains thylakentrin is saved.

(9) The dialyzed solution is brought ⅓ to SAS[2] and pH = 7.4. The precipitate is centrifuged and the process is repeated until a pure product is obtained.

The protein so isolated was found to be homogeneous in the ultracentrifuge and in the electrophoresis apparatus over a wide range of pHs and also to possess a constant solubility in the one solvent used. The isoelectric point of the protein was estimated to be 7.45 and the molecular weight to be approximately 100,000.

b) *Evidence that the Protein and the Hormone are Identical.* Although the protein was shown to be homogeneous by all three physical chemical tests, the view that the protein is the hormone could not be taken as proved. Chow, van Dyke, Greep, Rothen, and Shedlovsky (1942) pre-

[1] See Chow, Greep, and van Dyke, 1939; Shedlovsky, Rothen, Greep, van Dyke, and Chow, 1940).

[2] SAS = saturation of ammonium sulfate.

sented further evidence that the protein and hormone are identical. Their approach was to attempt to separate a hypothetically active principle from the pure protein. Should such an attempt succeed, it would appear that the true hormone was an accidental contaminant of the apparently pure protein. Several fractions were obtained after electrophoretic migration or ultracentrifugation of the protein or after the protein had been partially dissolved in a solvent containing sodium sulfate and acetate buffer.

(1) Fractional Electrophoresis. The pure protein solution was electrolyzed in a Tiselius apparatus and the protein was allowed to migrate electrophoretically. Protein molecules, although "pure," do not migrate at the same rate in the electrophoresis cell. Those not migrating with the majority were assayed biologically and the results of assays showed that the two protein fractions had the same activity per unit nitrogen as the original material.

(2) Fractional Ultracentrifugation. Similar experiments were performed using an ultracentrifuge. Here again the protein that sedimented owing to centrifugation as well as a portion of the protein which sedimented more slowly owing to diffusion showed identical biological potency.

If the hormone were not the protein, and had an electrical mobility different from the protein or had a lower sedimentation constant then it would be expected that the active substance should remain behind either after electrolysis or centrifugation and that it should have relatively higher biological activity per mg. of nitrogen. On the other hand the experimental results showed no evidence of increase of activity and they are, therefore, in harmony with the hypothesis that the protein is the hormone.

(3) Fractional Solution. If the protein and the hypothetical hormone were different chemical substances it is probable that their solubilities would also differ. It might therefore be possible to obtain dissolved and undissolved fractions which would possess different biological activity per unit weight of nitrogen. Thus if the hormone were much less soluble than the protein and if a sufficient amount of any solvent were used to dissolve most of the protein (say 90 per cent), the unit activity per mg. of protein N of the undissolved fraction should greatly increase. Likewise the unit activity of the dissolved fraction should decrease. On the other hand, if the hormone were much more soluble than the protein, and if only enough solvent were used to dissolve a small amount (say 10 per cent) of the test mixture, the unit activity of the dissolved fraction should greatly increase and that of the undissolved should decrease correspondingly.

The metakentrin from the hog pituitary was so fractionated with sodium sulfate solution containing acetate buffer, and the biological

potency of all fractions remained the same. Therefore the data of such experiments again furnished no evidence of the presence of a more active substance in the pure protein.

Another test to ascertain whether the hormonal activity is a property of the protein can be made by the determination of unit activity of the remaining protein after a partial destruction by denaturation, digestion, inactivation with chemical reagents, etc. If the unit activity of the unaltered protein remains unchanged it will provide an additional evidence for the identity of the protein and hormone. Although such methods of attack have yielded convincing results in demonstrating the identity of crystalline protein and enzyme, they have not been applied to the study of pure hormone proteins.

It is impossible at the present moment to decide by any one experiment whether the hormonal activity is a property of the protein itself or a property of another molecular species associated with the protein, because of the limitations of our only available physico-chemical tests for the purity of a protein. All the fractionation experiments described above are in harmony with the hypothesis that the protein is the hormone (swine metakentrin). Furthermore, there is no evidence at present to indicate that the biological activity is due to a different highly active absorbed contaminant.

c) *Sheep Metakentrin.* Li, Simpson, and Evans (1940) published a method of preparing pure metakentrin from sheep pituitary glands. The method of purification is summarized briefly as follows:

(1) A 40 per cent ethanol extract of acetone-dried sheep pituitary is extracted with water and adjusted to pH 4.5 and precipitated with an equal volume of acetone.

(2) Fifty g. of the precipitate (dried with acetone and ether) are extracted with one liter of 1 per cent saline solution and the extract is brought to half saturation with ammonium sulfate. The precipitate is centrifuged and the supernatant liquid is saved for the purification of follicle-stimulating hormone.

(3) The half SAS precipitate is dissolved in water and made to 0.2 SAS. This precipitate is removed by centrifugation, discarded, and the supernatant solution is brought to 0.4 SAS.

(4) The 0.4 SAS precipitate is dissolved in water and brought to 0.37 SAS and the mixture is centrifuged in order to remove and discard the precipitate which appears.

(5) To the supernatant fluid (0.37 SAS), SAS is added to bring the solution to 0.40 SAS. The 0.40 SAS precipitate is separated by centrifugation and is dissolved in water and, to a one per cent solution of the

hormone, trichloroacetic acid is added to 2.5 per cent. This precipitate is dissolved in water with the aid of sodium hydroxide.

The precipitation with trichloroacetic acid was necessary "to remove the last traces of FSH." It is remarkable not only that the hormone can withstand repeated treatment with 2.5 per cent trichloroacetic acid but also that the protein itself, which one would ordinarily consider to be denatured by trichloroacetic acid, is homogeneous as judged by electrophoretic or ultracentrifugal analyses.

A high degree of purity of the preparation was indicated by analysis in the Tiselius apparatus and the isoelectric point was found to be 4.6. The sedimentation constant was found to be 3.6×10^{-13} and the molecular weight was estimated to be about 40,000 by the colloid osmotic pressure method (Li, Simpson, and Evans, 1942).

Solubility studies were also made and the results indicated the presence of only one chemical compound. The hormone was found to contain 4.5 per cent tyrosine and 5.4 per cent cysteine.

2. *Comparison of Physico-Chemical and Biochemical Properties of Metakentrin from Sheep and Hog Pituitary Glands*

Several differences in the properties of the two pure hormones have been observed:

The isoelectric point of sheep hormone is 4.6 and that of hog hormone, 7.45.

The molecular weight of the sheep hormone (40,000) is considerably smaller than that of the hog hormone (100,000).

The metakentrins also differ significantly in their carbohydrate and tryptophan contents. The sheep protein contains 4.5 per cent of carbohydrate and one per cent tryptophan, while the hog protein contains only two per cent carbohydrate and 3.8 per cent tryptophan using the orcinol and glyoxalic acid methods, respectively (Li, Simpson, and Evans, 1942). Since the molecular ratio of hexose to hexosamine (Gurin, 1942) is almost one, the carbohydrate is probably composed of mannose and hexosamine in equimolar proportions.

The immunological properties of hog and sheep metakentrins have been studied by Chow (1942). The hormone from hog glands can stimulate the production of antibodies in the sera of rabbits. The presence of the antibody can be demonstrated by the precipitin as well as by the complement fixation reactions. The anti-hog metakentrin rabbit serum will react specifically with its homologous antigen, but it will not react with hormones from the posterior, intermediate, or even other hormones from the anterior pituitary of the hog. Homogeneous

metakentrin (sheep) prepared by Li likewise did not react with the anti-hog metakentrin rabbit serum. Therefore metakentrin from the hog pituitary glands is not only species specific but "lobe specific" and "hormone specific."

3. *The Purification of Thylakentrin*

No pure thylakentrin has yet been isolated from the pituitary gland of any animal. Fraenkel-Conrat, Simpson, and Evans (1940) reported a scheme of purification of thylakentrin of sheep origin and stated that "a preliminary electrophoresis experiment has shown follicle-stimulating hormone to consist mainly of one component." This does not mean that they have obtained an electrophoretically homogeneous thylakentrin, since they did not indicate that (1) their preparation was examined electrophoretically at different pHs, or that (2) the main component had the hormonal activity. Although their preparations were highly active in stimulating follicles, they still contained metakentrin. Similarly, highly purified thylakentrin has been prepared by Fevold (1941). His preparation also contained contaminating metakentrin.

Greep, van Dyke, and Chow (1942) obtained preparations of thylakentrin (hog) which were free from metakentrin. The latter hormone was not detected even when 25 times the minimum effective dose (62.5 micrograms of N) of their preparation was given to rats over a prolonged period. Estimation of this contaminating hormone (metakentrin) made by both the prostate method of Greep, van Dyke, and Chow (1941) and by the immunological method indicated the presence of less than 0.5 per cent and 0.04 per cent of metakentrin respectively. Tests for other hormones such as the lactogenic, chromatophorotropic, posterior, and adrenotrophic hormones gave uniformly negative results. Therefore, their preparation of thylakentrin may be considered biologically pure in the sense that no other hormones have been found by the biological assays to be present in the thylakentrin extract. Electrophoretic and ultracentrifugal analyses (Chow, 1943) showed that their biologically pure preparations were electrically inhomogeneous and ultracentrifugally polydisperse. From the determination of electrical mobilities of the biologically active fraction obtained after electrophoresis at several pHs, the isoelectric point of the hormone was estimated to be about 4.8.

4. *Comparison of Chemical and Physical Properties of Metakentrin and Thylakentrin*

Differences in the chemical and physical properties of metakentrin and thylakentrin from different species of animals may be summarized as follows:

a) *Solubility.* The difference in the solubility of metakentrin and thylakentrin has been utilized with success in separating the two hormones. Since metakentrin (hog) is neither soluble in one-third saturated ammonium sulfate solution at pH = 7.5 nor is metakentrin (sheep) soluble in 40 per cent alcohol, it may be classified as a globulin. On the other hand thylakentrin is soluble in a solution of half saturated ammonium sulfate and in 55 per cent alcohol, and it may be classified as an albumin, in the conventional sense of the word.

b) *Reactivity Toward Enzymes.* The action of proteolytic enzymes on the two pituitary gonadotrophic hormones has been studied by several groups of investigators. Chen and van Dyke (1939) digested the hypophysial extracts of sheep and horse pituitaries with a commercial preparation of trypsin. They found that the tryptic digestion destroyed most but not all the metakentrin and left the thylakentrin unimpaired. Similar results were independently reported by McShan and Meyer (1938), although they did not recognize the destructive effects of crystalline trypsin on thylakentrin. The differential rate of destruction of thylakentrin and metakentrin by trypsin was not confirmed by Abramowitz and Hisaw (1939). Chow, Greep, and van Dyke (1939) re-examined carefully the effects of digestion by proteolytic enzymes on the gonad-stimulating activities of the anterior pituitary extracts by comparing the action of digested and incubated control extracts in hypophysectomized immature male and female rats. They found that the percentage of protein digested was often of decisive importance in determining the destruction or survival of the hormones. Thus, if the tryptic digestion of the crude hog pituitary extract which contained metakentrin and thylakentrin had proceeded to a moderate extent (less than 48%), follicle growth was stimulated but the luteinizing action was absent. If digestion was carried to 61–75% both activities were abolished. It seemed reasonable to them that the discrepancy between the results of Abramowitz and Hisaw and those of other authors could be explained on the basis of differences in the degree of digestion. Later McShan and Meyer (1940) also determined the degree of digestion in conjunction with biological activity. Their results confirmed the importance of degree of digestion in determining the survival or destruction of the lutenizing hormone.

c) *Behavior Towards Picrolonic, Flavianic, or Picric Acids.* Fevold (1939) asserted that picrolonic, flavianic, or picric acids can precipitate both thylakentrin and metakentrin (sheep) completely but that "whereas the follicle-stimulating hormone is inactivated, the luteinizing hormone retains its physiological activity. Furthermore, the inactivation of the follicle-stimulating hormone is reversible and the activity is regenerated when the acid is removed." The difference in the reaction of the follicle stimulating

and luteinizing hormones and the reagents used indicated to Fevold that the prosthetic groups responsible for follicle stimulation and luteinization must be different. However, these results were not confirmed by Jensen and Tolksdorf (1940) or by Bischoff (1940). The precipitation of the proteins by these acids depends to a large extent on the pH of the solutions as well as on the concentration of the acids and proteins (Bischoff, 1940). Furthermore, the formation of the insoluble compounds involves not only formation of difficultly soluble salts of the acids but also induces some secondary alterations of the protein molecules. It is therefore easy to provide reasons for the failure to reproduce Fevold's results.

d) The Effect of the Route of Administration. Another difference in the two gonadotrophic hormones is shown by intraperitoneal injection. Fevold (1939) found that metakentrin (sheep) was fully active when given by this route as when injected subcutaneously while the thylakentrin was completely ineffective when injected into the peritoneal cavity. However, this difference in the effectiveness of the two gonadotrophins when given by these two routes requires further investigation.

e) The Immunological Response. Finally it may be recalled that the anti-serum of rabbits immunized against hog metakentrin does not react with thylakentrin preparations from the same species of animal. This immunological specificity is a direct reflection of a chemical difference between these two substances.

5. *The Inactivation of Hormone by Chemical Reagents*

In order to gain some insight into the chemical structure for the biological activity of the gonadotrophic hormones, attempts have been made to inactivate them with chemical reagents.

a) Acetylation by Ketene. Li, Simpson, and Evans (1939) treated a pituitary extract containing both thylakentrin and metakentrin with ketene at pH 5.0. They found that acetylation for five minutes significantly decreased the potency of metakentrin. In contrast with this, reduction of the activity of thylakentrin by ketene did not occur in five minutes but did occur in thirty minutes of acetylation. From these results they concluded that the physiological activity of both hormones was dependent on the free amino groups. They assumed that during acetylation under their experimental conditions (30 minutes at pH = 5.0) ketene did not react with the hydroxyl groups in the carbohydrate part of the molecule because Neuberger (1938) found ketene unable to acetylate the hydroxyl group in the carbohydrate resulting from the hydrolysis in the egg albumin as long as 18 hours at pH = 6.0. They further assumed that the phenolic groups were not attacked because Stern and White (1937–38) showed that at least 45 minutes were necessary to cause appreciable acetyla-

tion of the phenolic groups of insulin. These assumptions of course are not necessarily valid because the rate of acetylation of hydroxyl groups of carbohydrates or phenols may vary with different proteins.

b) The Action of Reducing Agents. Maxwell and Bischoff (1935) undertook chemical studies on the effects of reducing agents on impure pituitary gonadotrophic hormones. They found no detectable loss of activity upon exposure of crude hormones to nascent hydrogen, sulfur dioxide, hydrogen sulfide, hydrogen cyanide, and ferrous sulfate. Fraenkel-Conrat, Simpson, and Evans (1939) reduced unfractionated gonadotrophic extracts with cysteine at pH = 7.7 for two days and found that the biological activities were reduced 95 per cent. They concluded that disulfide linkages, important for the biological activities, were reduced by cysteine. McShan and Meyer (1940) confirmed the inactivation of gonadotrophic hormones by reduction with cysteine. Bischoff (1940) reopened the question of the reduction of the gonadotrophic hormones. While he agreed that the biological activities could be reduced on exposure to cysteine for a period of 24 hours, he disagreed with the hypothesis that disulfide linkages are an essential part of gonadotrophic hormones.

c) The Action of Formalin. Wallen-Lawrence (1934) reported the selective destruction of the luteinizing hormone by formalin. This destruction was disputed by Maxwell and Bischoff (1935). They treated the gonadotrophic hormones with a variety of chemical reagents including formalin but failed to detect a selective destruction of luteinizing principle. It must be emphasized that all these results arising from formalin-treatment are not clearly significant because normal and not hypohysectomized rats were used.

6. *The Rôle of Carbohydrate in the Biological Activity of the Gonadotrophic Hormones*

McShan and Meyer (1938, 1939) observed that ptyalin or takadiastase destroyed the follicle-stimulating activity but not the luteinizing activity of hypophysial extracts. Since these enzymes were assumed to attack carbohydrates but not proteins, the logical conclusion they drew was that the carbohydrate plays an important rôle in the biological activity of thylakentrin but not of metakentrin. The carbohydrate is not separable from the protein by dialysis or by chemical fractionation and it is assumed that it is united by one or more chemical linkages, although it is possible that a non-dialyzable carbohydrate is present. Thus thylakentrin is probably a glycoprotein. The proof of such a hypothesis lies in the eventual isolation of the hormone in a chemically pure form. The importance of carbohydrate groups has been observed by Fleischer, Schwenk, and Meyer (1938) and Hartmann and Benz (1938). Evans and his

collaborators (1939) found that although the thylakentrin fraction of sheep pituitary extracts undoubtedly contained more carbohydrate than any of the other hormone-fractions (including metakentrin) the determination of the carbohydrate content of a pituitary fraction could not be taken as a measure of its follicle-stimulating potency. Therefore, they searched for a more specific chemical characteristic of this gonadotrophic hormone and found a high glucosamine together with a high carbohydrate content to be valuable guides in the purification of the follicle-stimulating hormone.

II. The Lactogenic Hormone

The anterior pituitary secretes a lactogenic hormone which is able to initiate lactation and also plays the essential rôle in bringing about corpus luteum function. Various names have been proposed for this hormone: prolactin (Riddle), galactin (Turner), and mammotropin (Lyons).

1. *The Isolation of Lactogenic Hormone*

Alkaline solvents effectively extract the lactogenic hormone from acetone-desiccated pituitary gland, but a very considerable amount of other hormones and extraneous tissue proteins are also extracted. The acid-acetone method of Lyons' (1937) is recommended as a convenient and reliable method to obtain a relatively pure preparation as the starting material for further purification. The foremost advantage of Lyons' method is that the crude prolactin it yields is relatively free from other pituitary proteins; the chief contaminant is the adrenotrophic hormone.

Using the material obtained by acid-acetone extraction, White and his collaborators (1937) employed two methods which yielded crystalline products: (1) an acetic acid-pyridine procedure which is essentially that described for the crystallization of insulin, and (2) precipitation from dilute acetone solutions. Unfortunately neither of these two methods has been sufficiently standardized so that the results can be reproduced in other laboratories. The chief difficulties of preparing crystalline lactogenic hormone appear to be (1) apparent denaturation of the protein during crystallization resulting in a decrease of biological potency, (2) the low yield of the crystalline hormone.

The crystalline protein was shown to be homogeneous according to the schlieren diagrams of electrophoretic as well as the ultracentrifugal analyses. However, solubility studies were lacking. In a later report (White, 1943) satisfactory electrophoretic results with scanning patterns of a 2 per cent solution of the crystalline lactogenic hormone were published.

Li, Lyons, and Evans (1940) reported a method of preparing a pure lactogenic hormone, essentially similar to that previously published by Lyons. Electrophoretic studies of their preparation were made using 0.5

and 1.0 per cent protein solutions. The pH range investigated was between 3.75–7.85 and the isoelectric point was found to be 5.7. The schlieren photographs showed one sharp boundary in all electrophoresis experiments. In comparing the mobilities obtained by White with those of their own, the authors concluded that "the difference in the mobilities of their (White) undenatured crystalline and our (Li, *et. al.*) non-crystalline hormone indicates that the two substances are not identical." However, such a conclusion can hardly be justified on the basis of a small difference in the mobilities in buffers of different ionic strengths.

A much more convincing proof of purity of their hormone preparations was shown by the results of the solubility measurements in different solvents (Li, Lyons, and Evans, 1940–1941). The solubility curves were very close to that of a pure substance in equilibrium with its saturated solution.

A high degree of purity of the hormone preparations was also indicated by the ultracentrifugal analysis, The rate of sedimentation was found to be: $S_{20} = 2.65 \times 10^{-13}$ and $D_{20} = 7.5 \times 10^{-7}$, from which a molecular weight value of about 32,000 was obtained. The molecular weight of the hormone was measured by Li using an osmotic pressure method and was found to be approximately 26,500. This value can be considered as in good agreement with that obtained by ultracentrifugation. Li, Lyons, and Evans (1941) also determined the amounts of cysteine, arginine, tyrosine, and tryptophan in the hormone. From these analytical figures the minimum molecular weight of the lactogenic hormone was estimated to be approximately 25,000.

2. *The Effect of Chemical Agents on the Biological Activity*

The biological activity of lactogenic hormone can be easily destroyed by peptic or tryptic enzymes (McShan and French, 1937; White, 1943). The destruction of physiological activity occurs while fragments of the hormone protein are still precipitable by trichloroacetic acid. These enzymatic experiments therefore indicate that the hormone is a protein. The treatment of the hormone (McShan and French, 1937) with reagents such as sodium nitrite, formaldehyde, and diazonium compounds which react with amino as well as other groups results in a loss of the biological activity. Acetylation with ketene (Li, Simpson, and Evans, 1939) for five minutes also destroys the physiological activity completely. It is believed that during this short period of acetylation only amino groups are attacked and these are therefore essential for the biological activity. Another reagent which is commonly used to react with the amino group of proteins is phenylisocyanate. The phenyl ureido derivative of the lactogenic hormone (Bottomley and Folley, 1940) obtained by treatment with phenylisocyanate at 0° at pH 8.0, has only about 10 per cent of the biological

activity of untreated hormone. Although any one of the four reagents mentioned above may react with other groups in the protein molecule besides the amino group, the evidence taken as a whole more strongly suggests the essentiality of the amino group for the biological activity.

Li and his collaborators (1941) also studied the reaction between iodine and lactogenic hormone in a phosphate buffer of pH = 7.0. They found that the introduction of iodine in the phenolic group of the tyrosine molecules also resulted in a complete inactivation of the hormone. Moreover the introduction of iodine shifted the isoelectric point as determined electrophoretically from 5.7 to 4.7.

Fraenkel-Conrat, Simpson, and Evans (1942) studied the effect of reducing agents on the activity of the lactogenic hormone. They found that when a large excess of cysteine (200 fold) was employed to reduce the disulfide linkage in the protein, the biological activity was diminished. Thioglycolic acid, a more powerful reducing agent, also caused the destruction of the biological activity.

Lactogenic hormone can be inactivated by a number of chemical reagents, such as those which block the amino groups, or iodine which substitutes in the tyrosine ring, or cysteine which reduces disulfide linkages. Hence it is reasonable to assume that the biological activity of the hormone is dependent on several functional groups.

3. *Lactogenic Hormone from Different Species of Animals*

The lactogenic hormone from both beef and sheep pituitaries has been prepared by Li and his collaborators (1941 and 1942) in a homogeneous form. They showed that these two hormones have identical electrical mobilities over a wide pH range (2 to 10) and they both have an isoelectric point of 5.7. Bischoff and Lyons (1939) found that their purified lactogenic preparations of the pituitaries from oxen and from sheep were antigenically indistinguishable by the usual immunological tests. In other words lactogenic hormone is not species specific. In spite of the immunological and electrical identities, Li, Lyons, and Evans (1941) noted that sheep lactogenic hormone is more soluble than the beef hormone in acid solution. Furthermore they claimed that if solid sheep hormone were added to a saturated solution of the ox hormone, solution occurred. These results therefore indicate that the lactogenic hormone from the two species of animals are chemically different. They also found a significant difference in the tyrosine content (beef, 5.73 per cent; sheep, 4.53 per cent), but the same content of tryptophan (Li, Lyons, and Evans, 1940).

III. Adrenocorticotrophic Hormone

A recent addition to the list of pure hormones is the adrenocorticotrophic hormone, which selectively stimulates the adrenal cortex. This hormone

was isolated from sheep pituitary glands in a homogeneous form by Li, Simpson, and Evans (1942, 1943). Their preparations possessed constant solubility, and were homogeneous according to both electrophoretic and ultracentrifugal analyses. Some of the physical properties found are: isoelectric point = about 4.7; $S_{20} = 2.1 \times 10^{-13}$; $D_{20} = 10.5 \times 10^{-7}$; molecular weight = 20,000. It contains very little tryptophan.

More recently, Sayers, White, and Long (1943) also developed a method to purify the adrenotrophic hormone from hog pituitary glands, using acetone and ammonium sulfate as precipitating agents. Their purified preparation was free from lactogenic, growth-promoting, gonadotrophic, and thyrotrophic hormones. However, it did contain a small amount of pressor and antidiuretic principles. Its physical properties are surprisingly similar to those of adrenotrophic hormone from sheep: isoelectric point = 4.7 − 4.8; $S_{20} = 2.04 - 2.11 \times 10^{-13}$; and molecular weight = about 20,000.

A few points of interest in regard to the hormone proteins isolated from sheep and hog glands may be noted.

(1) Unlike other pituitary hormones (metakentrin and lactogenic hormone from sheep), adrenotrophic hormone lost its biological activity on treatment with trichloroacetic acid. In spite of its relative lability toward this acid the adrenotrophic hormone was resistant to treatment with heat and hydrochloric acid. For example, when the hormone solution in 0.1 *N* HCl was heated at 100° for 60 minutes, the activity was not lost. On the other hand, the activity was completely destroyed when the hormone solution in 0.1 *N* NaOH was heated at 100° for thirty minutes.

(2) It was pointed out earlier in the review that metakentrins from hog and sheep possess different physical (isoelectric points, molecular weights), chemical (tryptophan), and immunological properties. On the other hand lactogenic hormones from beef and sheep glands have apparently identical physical properties (isoelectric point and molecular weight), and they are also indistinguishable by ordinary immunological reactions. However, they have *distinctly* different solubility characteristics. Like lactogenic hormones, the adrenotrophic hormones isolated from sheep or hog glands have very similar if not identical properties. These properties include biological activity, nitrogen content, isoelectric point and sedimentation constant. The isoelectric point of the pure hormones is considerably different from pH = 6.6 − 6.8 formerly assumed to be correct by Lyons (1937).

(3) In the procedures of both Li and White, the adrenotrophic hormones were treated for several hours with concentrated ammonium hydroxide (one volume of concentrated ammonium hydroxide solution to two volumes of protein solution). The exposure of the hormone preparation to such a

concentrated alkali resulted in the destruction of much but not all the pressor principle, and probably in alteration of the hormone protein itself.

Since the manuscript was sent to the press, Li and Evans reported (Science 1944, *99*, 183) that they have isolated a growth hormone preparation, which is substantially free of other biologically active pituitary contaminants and homogeneous according to electrophoretic analysis. The isoelectric point is at pH = 6.85.

IV. Growth Hormone

The final proof of the existence of an anterior pituitary growth hormone lies in the isolation of a separate chemical substance which must not only promote growth but must be devoid of other activities. In the discussion which follows we shall assume that there is a definite and separate growth hormone.

The Purification of the Anterior Pituitary Growth Hormone

Beef pituitary glands are rich in growth promoting hormone. An efficient initial extraction usually involves the use of an inorganic base such as calcium oxide, barium hydroxide, or sodium hydroxide to bring the pH of the solvent to 8 or even higher. The turbid filtrate which contains the desired hormone can then be clarified by the addition of salts.

For the preparation of relatively pure growth hormone the methods published from Evans's laboratory in California are recommended, although they vary from one publication to another. The alkaline extract was used as their starting material and subsequently fractionated with ammonium sulfate. Like others (Teel, 1929; Fevold, Lee, Hisaw, and Cohn, 1940) they classified the growth hormone as a globulin, insoluble in water but soluble in salt solution. The common contaminants in the growth hormone preparations were thyrotrophic, lactogenic, and gonadotrophic hormones. To eliminate the activities of these specific contaminants Fraenkel-Conrat, Meamber, Simpson, and Evans (1940) treated a concentrated solution (5.7%) of the growth hormone preparations with "double the amount of cysteine." This method could be used apparently only in the early stages of the purification, because "with more potent globulin fractions, difficulties were often encountered, leading to loss of growth activity generally associated with precipitation of as much as 90 per cent of total protein in a form which could not be redissolved." Although the mechanism of the reaction between cysteine and the growth hormone is not apparent, the cysteine treatment seems to serve a dual purpose: (1) cysteine reduces gonadotrophin and other hormones thus rendering them inactive; however, it is not clear that this serves any useful purpose in purification although it may have advantages in biological assay; (2) cysteine precipi-

tates the lactogenic hormone under the precise experimental conditions given above.

The cysteine-treated growth hormone does not stimulate gonads or thyroids. It precipitates "isoelectrically" from pH 6.2 to 7.1, differing in that respect from untreated fractions which precipitate over a wider range from pH 5.6 to 7.0. Such preparations are almost free from carbohydrate (0.25% sugar and less than 0.90% glucosamine).

More recently Marx, Simpson, and Evans (1943) reported a modified procedure for the purification of the growth hormone. The procedure, briefly described, consists of five steps: desiccation of gland tissue with acetone, extraction with calcium hydroxide, precipitation with ammonium sulfate, treatment with cysteine, and further fractionation by pH variation. Electrophoretic analyses of their purified preparations showed two components, one of which represents 60 to 70 per cent of the total, although no mention was made as to whether the activity lies with the major or minor component. Their preparations contained very little gonadotrophic activities, less than 0.3 per cent of the thyrotrophic hormone, less than 0.5 per cent of the lactogenic hormone, and less than 0.5 per cent of the adrenocorticotrophic hormone.

Since the manuscript was sent to the press, Li and Evans reported (*Science* 1944, **99**, 183) that they have isolated a growth hormone preparation, which is substantially free of other biologically active pituitary contaminants and homogeneous according to electrophoretic analysis. The isoelectric point is at pH = 6.85.

V. The Posterior Lobe Hormones

Extracts of the posterior hypophysis produce several striking biological effects. These are attributed to (1) the oxytocic or uterine-stimulating principle, (2) the vasopressor principle probably identical with (3) the antidiuretic principle. Following the discovery of these effects there has been considerable controversy with regard to the number of chemical substances responsible for these biological effects. Abel (1923) and his collaborators (1930) maintained that the actual hormone is a unitary substance containing all three activities. On the other hand Dudley (1923) showed that partial separation of oxytocic and pressor activities could be effected. In 1928, Kamm and his collaborators reported a method of achieving partial separation of these biological activities so that one was substantially free from the other, although both fractions still contained antidiuretic activity. Furthermore, the separation was accomplished without loss of any of the total activities present in the original extract. This view of multiple hormones was further strengthened by confirmatory experiments by other methods devised in Stehle's laboratory (1933, 1935).

Even then Abel did not withdraw his contention that the true hormone is one substance but did admit that two active and separable principles could be extracted from the gland by appropriately drastic means.

1. *Purification and Separation of Oxytocic and Pressor Principles*

In general, dilute acetic acid has been preferred for the initial extraction of the gland because it is a good solvent and the pH of the resulting solution lies close to the pH of maximum stability (pH 3.0). The principles involved in the purification of the hormones depend on the solubility of the hormones in water or aqueous solvents and their relative insolubility in organic solvents and in concentrated salt solutions. The hormones are soluble to some extent in the lower aliphatic alcohols, particularly if small amounts of water are present. In partially purified preparations the oxytocic principle is more soluble than the pressor principle in certain organic solvents. This difference in solubility has been utilized for the separation of the hormones. Various organic acids and metal salts have been used with some success for the purification and separation of the hormones. Chromatographic separations have also yielded encouraging results. Recently Potts and Gallagher (1942) used chromatographic absorption on artificial zeolite and achieved purification and separation of two principles.

The details and scheme of three methods of fractionation are given in Table II from the paper published by Irving and du Vigneaud (1943). The initial step in all three methods involves a relatively drastic treatment of the extract. For example, according to Kamm's method the acetone desiccated powder is extracted with hot 0.25% acetic acid, and subsequently treated with glacial acetic acid; in the procedure of Stehle and Fraser, the desiccated gland is likewise treated with hot acid; in Irving, Dyer, and du Vigneaud's procedure the extract is acidified and boiled for ten minutes.

2. *Chemical and Physical Characteristics of the Separate Pressor and Oxytocic Fractions*

Although the two hormones have been separated and highly purified, neither one of them has been crystallized nor has their purity been adequately tested with any one of the physical methods. Both hormones are rapidly destroyed by trypsin but pepsin has practically no effect on them. Other enzymes, such as prolinase and arginase (Gulland and Macrae, 1933), exert no effect on the oxytocic hormone but tyrosinase (Freudenberg, Weiss, and Biller, 1935) causes complete inactivation of this principle. This observation leads one to believe that the phenolic group in tyrosine is essential to the biological activity of the oxytocic hormone. Polypeptidases of different kinds have been allowed to react with the extracts, but the

results did not provide enough information to elucidate the specific chemical grouping resonsible for the biological activity of the hormones.

The amino acid content of purified preparations has been investigated. From the analytical data the minimum molecular weight of the oxytocic and vasopressor principles can be estimated. The following values were obtained by Potts and Gallagher (1942): for the oxytocic principle, cysteine 18.3%, tyrosine 14.2%, arginine less than 0.8%; for the pressor principle, cysteine 19.0%, tyrosine 11.9%, arginine 12.3%. A strikingly low content of arginine in oxytocic principle in contrast with a significant amount of this amino acid in the pressor substance is worthy of note. This difference, as pointed out by the authors, may account for the fact that the pressor principle is a more basic ampholyte than the oxytocic preparation. The cysteine and tyrosine values of the oxytocic principle are in good agreement with a minimum molecular weight of 1300 assuming but one molecule of each amino acid. The pressor hormone gives a similar value although the agreement is not as good. The authors are aware that their products are not crystalline but believe that these figures on the molecular weight are of the correct order of magnitude.

3. *Effects of Various Chemical Reagents*

The posterior lobe hormones are destroyed upon exposure to alkali (1 *N*) for a few hours at room temperature. More vigorous treatment with alkali is accompanied by the liberation of hydrogen sulfide. Kamm and his colleagues (1928, 1937) found that reagents such as sulfite, thiosulfite inactivate the hormone. Other reagents like nitrous acid, nitric acid, hydrogen peroxide, and bromine likewise destroy the biological activity. These reagents as the authors realized are so drastic one refrains from discussing the structural imp ications of the results.

Since the hormones have an unusually high sulfur content it is interesting to examine the effects of reducing agents on the biological activities. Gulland and Randall (1935) found that the hormone was inactivated by reduction and the activity may in some cases be completely restored by oxidation. The degree of inactivation depended largely on the reducing conditions used and the extent of reduction. By using a series of oxidation-reduction dyes the authors found the oxytocic principle to contain a redox system having a potential of $E_o' = -0.025$-volts at pH 6.0. Attempts to elucidate the redox system have not been successful.

The most interesting information regarding the effect of reduction on the biological activity was obtained by Sealock and du Vigneaud (1935) using cysteine. They found that reduction of the hormones with this reagent did not affect the biological activity. However, if the reduced

material is benzylated or methylated the resulting compound becomes inactive. The authors concluded that the active principle contained a disulfide linkage and that a sulfhydryl group or a potential sulfhydryl is essential to the activity of these hormones. Gulland and Randall (1936) questioned the validity of these conclusions. They claimed that the behavior of the hormones towards reduction and benzylation can be explained on the basis of a number of oxidation-reduction systems, the reduced form of which may be susceptible to benzylation with accompanying loss of activity. Such reduced groups might be —OH or —NH—.

Cysteine is generally considered a specific reagent for the reduction of disulfide groups; in reply to the criticism of Gulland and Randall, du Vigneaud (1938) stated, "it would be necessary to visualize some grouping in the hormones which would be capable of being reduced by cysteine with a neutral pH at room temperature, and which, only after reduction, would be benzylated under the conditions used."

4. *Electrophoretic Studies of the Separate Hormone*

Kamm and his collaborators were led to conclude from their purification studies that both hormones appear to be basic in character and named their isolated products alpha and beta hypophamine, to signify that they may be amines derived from the hypophysis. Electrodialysis studies (Freeman, Gulland, and Randall, 1935) showed that the oxytocic principle can migrate toward the cathode, but not toward the anode. They concluded that the oxytocic principle is either a base or was absorbed on basic material. The electrophoretic studies were further extended by Cohn, Irving, and du Vigneaud (1941). They found that during electrophoresis neither hormone was able to enter a compartment where the pH was maintained at 12. Accordingly they studied the migration of the hormones over a wide range of pH in a special apparatus designed for this purpose as well as in the Tiselius apparatus. The direction and the distance of the migration of the hormone after suitable periods of electrophoresis was determined by assay and the net mobility was calculated. From the mobility and the pH curve they found that both principles are definitely amphoteric, and the isoelectric points are pH 10.8 in buffer of ionic strength = 0.02 for the pressor hormone, and pH 8.5 for the oxytocic principle. In none of these experiments have the authors followed the mobility of the hormone preparation during electrolysis with any optical device, because the concentration of the hormone used was not sufficiently great. Hence, the mobility data are not of much significance.

5. *The Isolation of a Protein from the Posterior Lobe*

As stated earlier Abel and his colleagues believed that the pharmacological activities of the posterior lobe were due to a single, large "mother

molecule." Doubts on the correctness of the view were cast by the experiments of Dudley and Kamm which showed that separate oxytocic and pressor principles could be obtained by suitable fractionation procedures. Even then Abel maintained that the treatments used for separation were sufficiently drastic to cleave the mother molecule into small active fractions but he was unable to isolate the hypothetical mother molecule. He based his convictions chiefly on his ability to obtain purified preparations in which the ratio of the pressor, oxytocic, and melanosome-dispersing activities was the same as that found in the untreated glands. When it was shown that the melanosome-dispersing activity was not a property of the posterior lobe the basis for Abel's argument became less secure. MacArthur (1931) obtained an active fraction from the posterior lobe which may have been the hypothetical mother molecule. His preparation contained both pressor and oxytocic activities and had an isoelectric point at pH = 5.0. The homogeneity of his preparation was not examined.

In 1940, Rosenfeld compared the behavior of purified pressor and oxytocic preparations with that of an untreated press juice from the posterior lobe. He found that the activities in the pressor and oxytocic preparations did not sediment appreciably even after almost six hours of centrifugation at 61,000 r.p.m., whereas both activities in the press juice sedimented rapidly and approximately at the same rate of centrifugation. It was concluded that in the press juice resided a single large molecule whereas in the purified oxytocic and pressor preparations the hormones have a very much lower molecular weight. The molecular weights were estimated to be about 20,000–30,000 and of the order of 600, respectively.

van Dyke, Chow, Greep, and Rothen (1942) isolated a protein from the extracts of ox posterior pituitary glands, which was found to possess constant solubility in 0.5 *M* acetate buffer pH = 3.90 and 6.5 per cent sodium chloride. Analysis of the protein in the Tiselius cell was made and the patterns, photographed by the method of Longsworth, showed that the protein was electrically homogeneous and no second component could be found. The isoelectric point obtained from the mobility and pH curve was about 4.8. In the ultracentrifuge the substance appeared to be a single homogeneous protein with a molecular weight of about 30,000.

One mg. of the protein was found to contain 17 U.S.P. units, whereas the most highly purified preparation of Potts and Gallagher contained 700 units of oxytocic activities per mg. solid. Although the separated substances have much higher activity (40–25 times) they likewise have smaller molecular weight by about 23 times. If we compare the activity on the basis of millimoles instead of milligrams the difference is not striking.

In order to present evidence that the biological activities are characteristics of the protein, van Dyke and his colleagues demonstrated the constancy of the biological activities of various fractions obtained:

(1) after electrophoretic migration of the protein,
(2) after centrifugation of a solution of the protein,
(3) in solubility tests.

All these fractions were assayed for both oxytocic and pressor activities on the basis of milligrams of nitrogen. In all cases they found that these activities remained constant. It was concluded that the hormone is either the protein or is very closely associated with it.

It appears, therefore, possible that the pure protein isolated by van Dyke and his associates is the "mother molecule" with prosthetic groups responsible for the oxytocic and pressor activities. If this be so, treatment of the pure protein with hot acetic acid or by chromatographic absorption in a manner similar to the procedures used for the separation of the hormones should cleave the prosthetic groups from the protein. Experiments of this nature should be performed before final judgment of the correctness of the "unitarian" hypothesis can be given.

The homogeneous protein hormone was found to be easily digested by crystalline trypsin or crystalline chymotrypsin with accompanying loss of the biological activities, whereas crystalline pepsin although digesting the protein caused little loss of the biological activities. Elementary analysis of the protein showed that it contains a high percentage of sulfur (4.89 per cent sulfur which is present almost entirely in the form of cystine). Anson's (1941) nitroprusside qualitative test for free sulfhydryl groups was applied, the protein being denatured by guanidine hydrochloride. This test was negative for as much as 2 mg. of the posterior lobe hormone but was positive for 0.085 mg. of egg albumin. It is therefore conc'uded that the molecule contains no cysteine. When the protein was treated either with cysteine or thioglycolic acid at pH 7.5 the biological activity was found to be abolished completely. Pressor and oxytocic activities were affected to an equal extent. This finding is particularly interesting in view of the fact that the biological activity of the preparations used by du Vigneaud and his collaborators was not abolished merely by reduction with cysteine.

D. Hormones Not of Pituitary Origin

Two gonadotrophic hormones are found to be associated with pregnancy and are not secreted by the pars glandularis of the pituitary body. They are chorionic gonadotrophin and pregnant mare serum.

I. Chorionic Gonadotrophin

Soon after Aschheim and Zondek (1927) reported that the urine of pregnant women contains "anterior pituitary hormone," many attempts have been made to isolate the hormone and study its chemical nature. The hormone can be adsorbed on charcoal or norite and eluted with 90 per

cent phenol. It also can be adsorbed on benzoic acid and the latter is freed from the hormone by solution in alcohol or acetone. The hormone also can be precipitated by ammonium sulfate, alcohol, uranyl acetate, tannic acid, etc. The adsorption method is preferred since such a product is less toxic than that obtained by the precipitation with inorganic salts or alcohol. Elden (1933) prepared the hormone by adsorbing it on kaolin and charcoal, from which the hormone may be extracted by sodium hydroxide. Although it is possible to obtain dilute solutions which possess physiological activities but fail to give positive biuret or millon tests, it would be erroneous to conclude from these tests made with dilute solutions that the hormone is not protein-like in nature, since the sensitivity of the biological assay far exceeds that of the chemical tests.

1. *The Isolation of Homogeneous Chorionic Gonadotrophin*

Using the hormone preparation obtained by the adsorption with benzoic acid as their starting material, Gurin, Bachman, and Wilson (1939) developed two simple processes further to purify the gonadotrophin. The first method consisted of a single extraction with 30 per cent aqueous acetone, and the second method involved an extraction with 50 per cent ethyl alcohol at pH 6.0 and re-extraction of this derived product with the same solvent at pH 4.8.

The homogeneity of their preparation was examined in a Tiselius apparatus. The schlieren pattern obtained after electrolysis in a phosphate buffer showed only a single migrating band. The hormone was found to be isoelectric at pH = 3.2–3.3 by cataphoresis. Ultracentrifugal analysis showed the preparation to be essentially monodisperse. The observations of the positions of the boundary in the cell were made by Lamm's scale method. The sedimentation rate was found to be $S_{20} = 4.3 \times 10^{-13}$ and the molecular weight 60,000–80,000.

No doubt, Gurin and his collaborators have obtained the most highly purified hormone preparation. Since no solubility tests have been made and no schlieren scanning patterns are available, their preparations may still contain a small amount of impurity.

2. *Chemical Studies on Chorionic Gonadotrophin*

Chorionic gonadotrophin is inactivated by trypsin but not by pepsin, (Reiss and Haurowitz, 1929; Wiesner and Marshall, 1931, and others). Therefore, it is protein or polypeptide in chemical nature. The gonadotrophin contains both polypeptide and carbohydrate and is therefore a glycoprotein. The molar ratio of galactose and hexosamine (2:1) suggests the possibility that the carbohydrate is built up of hexosamine digalactose units (Gurin, 1942). Since the carbohydrate component of the blood

group A substance has been identified to be composed also of galactose and hexosamine unit (Boyd, 1943), it seems reasonable that the hormone may react with the sera of rabbits immunized against blood group A substance. However, it was shown experimentally by Gurin (1942) that there was no demonstrable reaction between the gonadotrophin and the anti-serum. Hence, the authors concluded that the carbohydrate of the hormone molecule bears no serological relationship with the polysaccharide of blood group A substance.

That the union between the carbohydrate and the protein is a firm one is indicated by the fact that it is not possible to separate the carbohydrate from the protein either by dialysis or by heating with dilute alkali. Nor has it been possible to separate them by shaking aqueous solution of the hormone with chloroform at pH range of 5.0 to 8.0. It is interesting to note that a glycoprotein of molecular weight around 60,000–80,000 can be secreted by the normal human kidney (Gurin, Bachman, and Wilson, 1940).

Bischoff and Long (1936) allowed the gonadotrophin to react with a variety of chemical reagents. They found that the hormone can be inactivated by acetylation with acetic anhydride, benzoylation, reaction with H_2O_2, etc. Li and his collaborators (1939) reported that ketene inactivated the physiological activity of the pituitary and mare serum gonadotrophins more rapidly than that of human chorionic gonadotrophin. These results suggested to them that the free amino groups are essential for the biological activity of the pituitary as well as the mare serum hormones but not so essential to that of chorionic gonadotrophin. Such a conclusion was drawn merely on the basis of a difference in the biological activity in relation to time of acetylation by ketene. It would be more convincing if they had shown from analytical data that the acetylation of only amino groups by ketene affects the biological activities of pituitary and mare serum gonadotrophin but not of human chorionic gonadotrophin. Bischoff (1941) treated both pregnant mare serum and chorionic gonadotrophins with formaldehyde and found that both hormones were inactivated by this treatment (1 to 3 hrs.). He disagreed with the workers in Evans's laboratory on the essentiality of the free amino groups in the hormones. The activity of chorionic gonadotrophin is also completely destroyed at pH 8.5 by iodine, but it is stable to the same reagent at pH 3.5.

It has long been known that the inactivation of highly purified hormone from the urine of pregnant women can take place particularly in aqueous solutions even without detectable loss or rupture of the hormone molecule. Results of some experiments also suggest that the inactivation is the result of the spontaneous oxidation of the hormone. Bowman (1941) studied the oxidation-reduction properties of chorionic gonadotrophin. He titrated

the hormone potentiometrically with iodine and potassium ferricyanide. From the titration curves he obtained $E_0' = 0.354$ volt at 38°C. at pH 5.9. The oxidation depended on a single electron change. A few points of interest were noted. In aqueous solution the hormone undergoes slow spontaneous oxidation which can be prevented by the addition of certain reductants. On the other hand the oxidation is not greatly accelerated by the addition of strong oxidants unless the temperature is increased and phosphate buffer is present. In the presence of starch the oxidation-reduction reaction is altered. Although the hormone can be inactivated by oxidants the inactivated material can be only partially "restored" (20%) by reducing agents. These results therefore suggest that the redox system of the chorionic gonadotrophin is not one of a thermodynamically reversible system. The electron change is believed due to a bound metal iron or a derivative of tyrosine.

II. Pregnant Mare Serum Gonadotrophin

Gonadotrophic hormone has been found in the serum of the pregnant mare in considerable quantities during a limited part of the gestation period, but it is not secreted in significant amounts in the urine. Evans and his collaborators (1936) and Hellbaum (1937) reported the separation of mare serum into follicle-stimulating and luteinizing fractions. However, these findings were not confirmed by Cole, Pencharz, and Goss (1940) who concluded that the "gonadotrophic activity of the mare serum is dependent not upon a mixture of follicle-stimulating or luteinizing hormones but upon a single gonadotrophin."

1. *Isolation of the Gonadotrophin*

Pregnant mare serum is first concentrated as a precipitate following the addition of acetone to 70 per cent by the procedure of Cartland and Nelson (1937). This precipitate is dissolved in 50 per cent acetone pH 6 to 7. From this solution the hormone can be precipitated by the adjustment of pH to 5.5–4.5 (Goss and Cole, 1940). Li, Evans, and Wonder (1940) found that the hormone isolated with essentially the method described above showed only one sharp schlieren band during electrophoretic analyses. The pH range examined was from 1.6 to 7.5. From the mobility curve they estimated the isoelectric point of the hormone to lie at pH 2.60–2.65. They found that the mobility of the protein at 1 pH varied with the potential gradient used. This phenomenon, they believed, may be explained by the Wien effect for electrolyte solutions. Before the gonadotrophin from the pregnant mare serum can be admitted into the family of pure hormones, critical solubility tests and ultracentrifugal analyses must be performed. From the results of chemical analysis, Li and his collaborators estimated

the minimum molecular weight of the protein to be 30,000, on the assumption of the presence of two molecules of tryptophan and six molecules of tyrosine. This value must be considered as tentative until more reliable data are obtained with pure hormone by either the osmotic pressure or ultracentrifugal method.

2. *Inactivation of the Hormone by Various Chemical Reagents*

Inactivation experiments have been performed by numerous authors. Evans and Hauschild (1942) found that the hormone was inactivated rapidly by ptyalin and takadiastase but slowly by emulsin. They concluded that the carbohydrate was essential for the biological activity. Proteolytic enzymes likewise inactivated the hormone. Unlike the gonadotrophic hormones from the anterior pituitary there was no preferential destruction of the luteinization principle in the mare serum by trypsin.

Li and his collaborators (1940) found that the hormone could be inactivated by acetylation by ketene for thirty minutes. This result, therefore, suggested the essentiality of free amino groups for the biological activity of the hormone. Their finding was in accordance with the observation of Cartland and Nelson (1937) that 80 per cent of the biological activity was destroyed by exposing the hormone to a 4 per cent formaldehyde solution for three hours at pH 8. However, it is questionable whether formaldehyde reacts only with a free amino group under the above stated conditions as the authors inferred. Evans, Nelson, and Cartland (1942) treated the hormone with nitrous acid at 0°C. for thirty minutes and phenylisocyanate at 0°C. at pH 8 and found that the biological activity was likewise destroyed. These facts again indicated the importance of the primary amino groups of the hormone for the biological activity. It would be expected that the amino group of the lysine molecule would be covered by phenylisocyanate. However, the presence of lysine in pregnant mare serum has not been demonstrated.

Bischoff (1941) studied the inactivation of mare serum hormone with formaldehyde and found that an exposure of mare serum hormone to formaldehyde for one quarter of an hour resulted in almost no loss of the activity whereas a longer exposure (1 to 3 hrs.) produced progressive partial inactivation. He concluded that there was no indication that free amino groups are essential to mare serum activity. Bischoff (1942) also studied the effect of nitrous acid on the activity of mare serum hormone and found that it could be progressively inactivated by exposure to nitrite in acetic acid mixture. He pointed out that inactivation by nitrous acid was largely dependent on concentration, temperature, and time, and concluded "the results for the mare serum hormone neither prove nor disprove the essentiality of the amino groups in mare serum hormone activity."

Although such a conclusion may be correct, it cannot be drawn from the results of a reaction as complicated as that of proteins and formaldehyde or nitrous acid. At present data are lacking to show that in the reaction between the hormone and ketene or phenylisocyanate, only the amino group but not phenolic and other groups in the protein are affected. Nevertheless, the conclusion that amino groups are essential to the biological activity, is considered reasonable on the basis of the available data.

Fleischer, Schwenk, and Meyer (1938) expressed the view that pregnant mare serum does not contain any cystine although they did not give any data. Evans, Nelson, and Cartland (1942) obtained a positive nitroprusside reaction with a highly active pregnant mare serum hormone, only after treatment with NaCN, indicating the presence of a disulfide linkage in the molecule. It is, therefore, of interest to examine the relationship between the disulfide linkage and activity of gonadotrophin. Fraenkel-Conrat, Simpson, and Evans (1939) studied the effect of cysteine on the pregnant mare serum hormone. Cysteine was permitted to act on the hormone solution in a phosphate buffer of pH 7.7. for two days at 23°C., the ratio of cysteine to protein being 40:1. During the reaction the solutions were kept in tightly stoppered flasks with little air space to minimize oxidation. They found that cysteine did not inactivate the gonadotrophic preparations from pregnant mare serum. They concluded that the hormone "either contains no such bonds (disulfide bonds) or is equally potent in the thiol form." Later the same group of investigators (1940) allowed cysteine to react with 1.0 mg. of gonadotrophin per cc. instead of 0.1 mg. per cc. Under otherwise identical conditions they found that the hormone could be inactivated when present in higher concentrations. They attributed the inactivation to the presence of a disulfide linkage of intermediate activity. When the hormone is allowed to react for a longer period of time or in the presence of 40 per cent urea which is known to produce denaturation and to render the disulfide linkage more reactive, inactivation did occur. From these experiments they concluded that a disulfide linkage exists in the hormone.

Evans, Nelson, and Cartland (1942) again investigated the action of cysteine and other reducing compounds on mare serum gonadotrophin. They treated pregnant mare serum with sodium cyanide at pH 8.0. This mixture was allowed to stand for the desired period of time. The pH was then adjusted to 6.0 and the excess of cyanide was removed with iodine which was in turn removed with thiosulfate. The results showed that cyanide produced within thirty minutes some inactivation which was more evident after sixty minutes of exposure. The effect of other reducing agents on pregnant mare serum was also investigated. It was found that the hormone was not destroyed upon exposure to hydrogen sulfide up to one

hour. Neither was the hormone inactivated by nascent hydrogen, glutathione, or ascorbic acid.

E. Summary

In the past decade rapid advances in the isolation of protein hormones have been made possible because of the knowledge that has been gained from the study of other fields of protein chemistry and because of the new physical tools (Tiselius apparatus and ultracentrifuge) which are now more easily available. In this review we have limited our discussion only to those protein preparations whose purity has been examined either by electrophoretic or ultracentrifugal analyses or by solubility tests. It is the consensus of opinion among protein chemists that the solubility test for purity is probably the most reliable. There are instances where a protein has been shown to be homogeneous according to electrophoretic or ultracentrifugal analysis but inhomogeneous according to solubility tests, whereas the reverse appears not to be true. Only a limited number of preparations of protein hormones have been found to be able to comply with all these three tests. They are metakentrins from hog and sheep, lactogenic hormones from oxen and sheep, posterior lobe hormone from oxen, and adrenotrophic hormones from sheep and hogs. The isolation of a chemically pure protein with the biological activities of metakentrin and of biologically pure thylakentrin proves that there are two chemically distinct gonadotrophic hormones in crude pituitary extracts. Since the solubility tests of chorionic and pregnant mare serum gonadotrophins are still lacking, they cannot as yet be regarded as pure.

A pure protein possessing three biological activities of the posterior lobe hormone has been isolated in a homogeneous form, but to understand its exact relationship with the separated hormones of low molecular weight requires further experiments. If these small molecules are the prosthetic groups, they should be separable from the protein molecules by treatment with acid or by chromatographic adsorption method. Such studies will also be of great value in understanding the linkages in protein molecules.

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Soybean Protein in Human Nutrition

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I. INTRODUCTION

Probably no single proteinaceous material is attracting as much attention from so wide a group of scientists as is the soybean. Industrially soybean protein is utilized in adhesives, sizes for paper and textiles, and cold water paints in substantial quantities, and the possibilities of employing it in the production of synthetic fibers, plastics, insecticidal sprays, and emulsifying agents are being thoroughly investigated. The amount of soybean protein consumed in such uses is, however, relatively insignificant in comparison with that produced in the United States.

Approximately 97% of the available soybean protein is utilized for feed and food purposes. Nutritional studies on soybean oil meal have firmly established the excellent nutritive quality of the protein for animal feeding, and it is in animal feed that the major portion of the available soybean protein is utilized. The information gained by biochemists from experiments with soybean protein in the diets of such experimental animals as the rat, the chick, the dog, and the pig has served, along with the knowledge and experience gained in the Orient during 40 centuries of the use of the soybean, as a background for the development and acceptance of its use in the fortification of the human diet. Developments in this field are of special interest at this time: first, because soybean protein is readily available in large quantities and can serve as a supplement for limited and dwindling supplies of animal proteins; and secondly, because it provides the United States with a protein food of high biological quality which can be

produced and distributed at a cost low enough to meet the protein requirements of families with low incomes and for world-wide distribution on a competitive economic basis.

It is impossible to cover completely the history of the development of soybean cultivation and processing in the United States or of the chemical and technological advances which have made that development possible within the space allotted to this article. We can outline, however, the high points in that history as it pertains to the use of soybean proteins for human food, particularly in the present war emergency.

II. History of the Soybean

The first written record of the soybean appeared in "Materia Medica," written by Emperor Shennung in 2838 B. C.,[1] where it was described along with many other plants of China. As firmly as the history of the development of man in the western world is related to "bread and meat (animal protein)" the history in the eastern world is linked with millet, rice, and soybeans (vegetable protein). It is known, through legend, that the soybean has supplied, in the form of soya-milk, soya-cheese "tofu," and baked foods, the substantial part of the protein in the diet of the Chinese for at least 4000 years.

The soybean was introduced into Europe in 1712 by Kaempfer[1] and into the United States in 1804. Additional varieties of soybeans were brought into the United States in 1854, 1900, and 1905, but it was not until 1915 that they were grown in quantities sufficient for commercial utilization. In 1916, 30,000 bushels were processed for oil. The general shortage of fats and oils during the first world war gave a temporary, but rather short lived, stimulus to the production of soybeans for extraction purposes. In 1922, however, about 27,000 bushels of soybeans were crushed for oil, and the increasing demands for soybean oil have since that time given rise to the phenomenal growth of the domestic soybean industry. The protein chemist should keep in mind the fact that up to the present time, at least, soybean protein has been essentially a by-product of the oil and fat industry and that indirectly increased demand and use of soybean oil have made available soybean proteins in the form of soybean oil meal and flours with low fat content for animal feed, human food, and industrial purposes. It has been estimated that in 1944 over 160 million bushels of soybeans will be crushed for oil. Most of the beans being produced today are yellow-seeded varieties bred and selected by agronomists for their high content of oil, although there are over 2000 distinct and 100 well known varieties grown in this country.

[1] Dies, E. J. (1942) Soybeans, Gold from the Soil. The Macmillan Company, New York.

III. Economics of Production and Processing

The soybean is a leguminous plant and is consequently ideally adapted to the economy of the American farm. Because such plants have the ability, through their symbiotic relationship with nitrogen-fixing bacteria, to fix atmospheric nitrogen, they fit excellently into crop rotation plans designed to conserve soil fertility and provide for the most economic use of expensive nitrogen fertilizers. They are grown as a field crop, and adequate mechanical equipment has been developed for planting, cultivation, harvesting, and threshing; in 1939 it was calculated for the State of Illinois[1] that soybeans with an average farm price of 66 cents per bushel and an average yield of 28 bushels per acre were more profitable to produce than wheat yielding 23 bushels per acre at a farm price of 78 cents per bushel.

A key to the economics of processing is provided in the cost of the graded soybean as broken down in terms of the value of the resulting products. Thus, one bushel of Illini yellow soybeans, weighing 60 pounds, at $1.712[2] will yield 8.8 pounds of crude oil worth about 11.75 cents per pound, or $1.034, and 49.6 pounds of oil meal worth about 1.737 cents per pound, or 86.1 cents, or a total of $1.895. In terms of flour for human consumption, the gross processing margins have to be considerably larger to compensate for more careful selection of the soybeans and increased processing costs. The wholesale value of flour low in fat during the same month represented by the above figures for meal and oil was 4.25 cents per pound, and for flour rich in fat 5.9 cents per pound.

In terms of the unit cost of soybean protein for human consumption with average protein values of 40% for flour rich in fat and 50% for flour with low fat content, values of 14.25 cents and 8.5 cents per pound can be calculated. If we compare these values with the cost of protein in other proteinaceous human foods, as has been done in Table I, some idea can be gained as to the possible significance of the soybean in the economics of human nutrition. In all fairness, it should be pointed out that many of the foods listed in Table I have value as foods independent of the proteins present therein. However, this is also true of soybean flours which carry, in addition to the protein, readily digestible carbohydrates, fats, lecithin, and substantial quantities of the vitamin B complex as well as essential minerals.

IV. Composition of the Soybean

The results of hundreds of analyses of soybeans have been summarized by Bailey, Capen, and LeClerc (1935), and their results are given in Table II. From the point of view of nutrition it should be pointed out

[2] All values are calculated as of September, 1942, for the Chicago wholesale market.

that the ash of the soybean is decidedly alkaline in nature. Bailey, Capen, and LeClerc (1935) give the following percentages for the mineral content of soybeans, on an air-dried basis: K 1.913, Na 0.343, Ca 0.210, Mg 0.223, P 0.592, S 0.406, and Cl 0.024. Morrison (1936) reported that soybeans contain about 0.02% of iron, and Sherman, Elvehjem, and Hart (1934) found that 80% of the iron in soybeans is available to the animal organism.

TABLE I

Cost of Soybean Protein in Comparison with Milk, Meat, Egg, and Fish Protein[3]

Product	Cost per lb.	Protein	Approximate cost of protein per lb.
	dollars	*per cent*	*dollars*
Soybean flour (low in fat)	0.0425	50.0	0.085
" " (rich " ")	0.0590	40.0	0.143
Skim milk (dried)	0.1450	35.4	0.410
Whole " "	0.4500	26.9	1.67
American cheese	0.270	24.4	1.11
Salmon (canned, commercial, red)	0.320	20.8	1.54
Beef, lean (utility grade)	0.175	21.3	0.822
Pork (*extra* lean trimmings)	0.180	16.6	0.922
Mutton	0.120	19.8	0.606
Eggs (dried)	1.10	46.5	2.38

TABLE II

Chemical Composition of Soybeans

	Minimum	Maximum	Average
	per cent	*per cent*	*per cent*
Moisture	5.02	9.42	8.0
Ash	3.30	6.35	4.6
Fat	13.50	24.20	18.0
Fiber	2.84	6.27	3.5
Protein	29.60	50.30	40.0
Pentosan	3.77	5.45	4.4
Sugars	5.65	9.46	7.0
Starch-like substances by diastase	4.65	8.97	5.6

Reports from industrial laboratories[3] indicate that soybeans contain substantial quantities of thiamine (274 to 446 I.U. per 100 g.), niacin (4.77 to 8.99 mg. per 100 g.), and riboflavin (300 to 400 μ per 100 g.). Burkholder (1943) gave data on the B complex vitamins for mature soybeans that

[3] Sprague, P. E. (1940) Edible Soy Flour. Soy Flour Association, Chicago, Illinois.

substantiate these claims fairly well. The values, in micrograms per g. of dry weight, found for six varieties were as follows: thiamine 9.0, niacin 20.0, riboflavin 2.3, pyridoxin 6.4, biotin 0.61, pantothenic acid 12.0.

V. The Soybean in the Wartime Economy of Nations

By use of the average value of 40.0% of protein in soybeans, given in Table II, and the estimated 1943 production of 216 million bushels, it can be calculated that there will be produced in the United States, during the 1943 crop year, over 5 billion pounds of soybean proteins, an amount roughly equivalent to the total 5,867,000,000 pounds of protein[4] produced by the nation in 1942 in the form of beef, veal, lamb, mutton, pork, edible packing house by-products, eggs, milk, chicken, turkey, beans, and green peas. Only about 12% of the total soybean protein produced in 1943 will be made available for human food but the potentialities of a crop which can supply such a huge total weight of vitally essential protein within one crop season of from 3 to 4 months on relatively few acres (for the economy of a nation at war) are apparent, and the United States should make adequate use of these potentialities.

Repeated warnings have been given of the possibility of a critical shortage in food supplies if the United States attempt to fulfil the demands of our allies and the populations of occupied territories in addition to those of our own armed forces and domestic population. Critical shortages of protein may indeed occur if consideration is confined solely to those animal foods to which we have become accustomed, such as meat, milk, and eggs, all of which are relatively expensive in terms of national resources and labor. The excellent dietary properties of the proteins of these foods are well known and they are such desirable foods that people will continue to buy them just as long as they can afford to pay the price; hence the market for them is assured. However, can the American taxpayer afford to pay the price required in order to meet all wartime demands for protein with such foods? Since the correct answer to this question is obviously a matter of conjecture, there would appear to be only one prudent course of action for the nation to follow. That course involves the production, distribution, and utilization of a large volume of vegetable protein of known biological quality and low cost. With the soybean as one source of this protein, adequate nutritional levels of high quality protein can be assured even to the groups with low incomes at home and the homeless orphans of the wars in Europe and Asia.

That Germany and Japan are using the soybean as a primary weapon

[4] Jones, D. B., Morse, W. J., and Pollock, R. C. (1942) The Nation's Protein Supply. Food and Nutrition Board, National Research Council.

in war is well known. The greatest weakness in the German food economy, from the military standpoint, was the relative lack of essential protein foods. To overcome this the Germans developed a high quality soybean flour called Edelsoja. This flour is merely the "full-fat" flour processed today in the United States. It is used extensively in the German army field kitchens, and it has been stated that the rapid advances of the mechanized German army in Poland, France, and Russia were made possible through the use of this highly concentrated food of low bulk. It is also being used extensively in Germany to make up for shortages of animal proteins.

Manchuria has long been a large producer of soybeans, and Japan shrewdly counted on the recently acquired Manchurian Empire for this protein in order to provide food for her armies and to guarantee against shortages at home that might be induced by blockade tactics. It might safely be predicted that as long as Japan holds Manchuria she will be well supplied with this vitally important protein, for in 1939 Manchuria alone produced 149,435,000 bushels of soybeans,[1] or approximately 3½ billion pounds of soybean protein. We have every reason to believe that this production has been materially increased. Even though it might have remained at this figure, on the basis of an average daily protein requirement of 70 g. per person, we calculate that Japan has from this source alone enough protein to take care of the annual requirements of about 63 million people. The Japanese Islands themselves produce about 15 million[1] bushels of soybeans annually (360 million pounds of protein) in addition to those produced in Manchuria. This, on the same basis as the above, would account for the annual protein requirements of approximately 6½ million more people.

Although our primary interest here is in protein, it should not be forgotten that fats and oils are especially critical items during wartime and that the soybean supplies, in addition to all the protein mentioned, tremendous quantities of edible fats and oils both to ourselves and our enemies.

VI. Processing Soybeans for Human Food

In the manufacture of soybean flour, grits, and flakes for human consumption, considerably greater care must be exercised than in processing soybeans for oil and soybean oil meal. It is also necessary to introduce additional procedures. Raw, uncooked soybeans, except for the green vegetable varieties, have a very bitter taste, and this must be removed during the processing of products for human consumption. With flours of low fat content, the processes for removing the oils must be carefully supervised, and milling, screening, and air flotation methods of separation must be rigorously controlled if suitable uniform products are to be obtained.

Detailed information on the operations of the expeller and on extraction methods for removing the oil from the beans has been given by Markley and Goss (1942), and it should suffice to state here merely that with the former the oil is expelled from the beans by a machine that macerates and presses them simultaneously, resulting in the production of considerable heat. With the extraction process, flaked or rolled beans are treated with a suitable solvent such as hexane, which removes the oil. When the expeller cake is to be utilized in soybean oil meal for animal feed, no special attempt is made to control the heat involved in simultaneously macerating and pressing the beans. The high temperatures are conducive to more complete expulsion of the oil than can be obtained if cooling devices are employed to protect the expeller cake from caramelization by heat, as must be done in the manufacture of flour. Thus, soybean flour obtained from

TABLE III

Average Percentage Composition of Various Types of Soybean Flours[a]

	"Full-fat"	Low or medium fat (expeller)	Low or fat-free (solvent extracted)
	per cent	*per cent*	*per cent*
Moisture	5.5	5.0	7.0
Protein	40.5	50.0	53.0
Fiber	2.5	2.5	2.0
Ash	5.0	5.5	6.0
Fat	22.0	7.0	0.75
N. F. E.* (non-starchy) material	24.5	30.0	31.25

* = (*N-free extract*).

the expeller contains about 5 to 8% of the natural fats and oils of the soybean, an amount slightly greater than usually found in soybean oil meal. With the extraction process, efficient removal of practically all of the fats and oils can be obtained without heating to the extent of affecting adversely the color or odor of the extracted flakes. Thus there are, depending upon the basic processes employed, three distinct types of soybean flour; namely, (1) an essentially fat-free product, (2) a variety with low or medium fat content (expeller), and (3) the "full-fat" type. The average percentage compositions for these three products are given in Table III.

Soybean grits or flakes of essentially the same composition of any one of the three types of flours listed in Table III may be made, since the primary differences between the manufacture of such products and of the flour are in the methods of cracking, rolling, and grinding of the beans, in the expeller cake, or in the flakes, and in screening.

The processes employed by various companies for removing the bitter principle or principles of the bean differ. However, all companies make use of the fact that a large part of the bitter material can be either hydrolyzed or steam-distilled from the beans, or both. This bitter taste is commonly described as "beany," but its actual chemical nature has not been satisfactorily established, although a review of the available literature[5] on the subject indicates that it may be derivatives of *flavone* (2-phenyl-1,4-benzopyrone; 2-phenylchromone), yellow pigments that occur as saponin glycosides. Horvath (1939a) contends that the substances are derivatives of methyl n-nonyl ketone.

In the manufacture of soybean flour, special care must be exercised to prevent contamination with dirt. Thus, soybeans are carefully selected, dehulled, and in some instances washed prior to processing. Dehulling decreases the crude fiber content of the resulting products. It is also necessary to take special precautions to keep all machinery and conveyors from becoming infected with thermophilic and mesophylic rope spore bacteria, since the flours are commonly used in canned foods and bakery products in which such organisms may cause spoilage.

VII. Soybean Proteins

Our knowledge concerning the nature of soybean proteins as they occur in the bean itself, or in the native state, is very meager. Osborne and Campbell (1898) proposed the name glycinin for one of the globulin types of protein that they obtained from soybeans. They reported that this globulin made up 80 to 90% of the total crude protein contained in the beans. They also isolated a more soluble globulin resembling phaseolin and an albumin-like protein which they named legumelin as well as small amounts of proteose.

Jones and Csonka (1932) obtained five protein fractions from soybeans by fractional precipitation with varying concentrations of ammonium sulfate. That fraction which was precipitated from 10% salt solution by ammonium sulfate at 55% saturation and did not coagulate at boiling temperatures was called glycinin, since its properties agreed with those given by Osborne and Campbell for glycinin.

O'Brien (1936) reports the ultimate analysis of glycinin as follows:

	per cent
Carbon	52.12
Hydrogen	6.93
Nitrogen	7.53
Sulfur	0.75
Oxygen	26.30

[5] Cornell, G. N. (1942) Literature Search on The Debittering of Soybeans. Rpt. to Soy Flour Association (mimeographed).

Muramatsu (1920) showed that most of the protein in soybeans can be extracted with water and divided the water extract on the basis as follows: globulins 84.25%, albumins 5.36%, proteose and non-protein nitrogen 10.39%.

Horvath (1939b) has reported that some of the soybean proteins are bound to the phosphatides. In particular, he has pointed out that the arginine of glycinin is attached in a salt-like combination with cephalin. Tillmans and Phillippi (1929) have claimed that the protein is combined with a carbohydrate fraction. These contentions are not substantiated by any great volume of experimental evidence and need further investigation.

Csonka, Murphy, and Jones (1926) found pH 4.7 to be the isolectric point of glycinin. Later Hartman and Cheng (1936) reported a value of pH 5.02. These values are considerably higher than that of 4.1 established by Smith and Circle (1938) by precipitation methods for the mixed proteins of soybean oil meal, but the latter value is in absolute agreement with the findings of Watts (1937) on mixtures of soybean proteins obtained from extracts of beans by electrophoretic methods. Tado Koro and Yoshimura (1928) reported that the isoelectric point of glycinin was the highest of all of the protein fractions present in the bean and that of legumelin the lowest. Thus it would seem that the isoelectric point of the meal and mixed proteins is controlled by those protein fractions other than glycinin.

The solubility of the proteins in soybean flour may be a definite factor governing its use in many food preparations. In the utilization of soybean protein for some technical purposes it is especially important. For the preparation of certain of these products, low temperature solvent extracted flakes are preferable to materials obtained by the expeller process in which considerable heat denaturation is involved, since the proteins therein are lighter in color, more soluble, and can be utilized in a more diverse manner. The extent to which this holds true depends to a considerable degree on the nature of the solvent employed and the temperature of extraction. From the standpoint of the nutritional value of the protein, heat treatment, as a rule, produces a desirable result. From the standpoint of what might be loosely termed functional qualities or those properties that serve definite physical functions in prepared foods, however, this may not be true. For example, Watts (1937) found that flakes extracted in solvents at low temperature without heat denaturation possessed whipping properties which would allow such products to be utilized in baking in a manner somewhat similar to that of egg white. Watts and Ulrich (1939) subsequently showed that the specific substance responsible for this whipping property was not related to the globulin fraction, glycinin, but could be concentrated in a nitrogenous fraction containing 32% of protein. The whipping properties of this fraction were heat-labile, and thus it is reasonable to assume that the

whipping substance may be related to some other protein fraction of the bean the properties of which are altered by heat denaturation.

The work of Smith, Circle, and Brother (1938) and Smith and Circle (1938) on the peptization of soybean proteins by neutral salt solutions, acids, and bases provides a fairly comprehensive picture of the available knowledge on the solubility of soybean protein. They found that distilled water was a better dispersing agent than neutral salts in concentrations up to 2 N, and that at very low concentrations salts greatly inhibited the dispersion of the protein. Salts with divalent cations exerted in very dilute solutions a much stronger inhibiting action than those with univalent cations. They found also a rather high percentage of dispersion at a critical pH of about 1.5 as well as at pH 7.0 and above.

Most of the purified industrial proteins are separated from soybean oil meal by alkaline extraction methods.[6] The nutritional value of these materials has usually been adversely affected by treatment with strong alkali solutions. This is mentioned here for two reasons. First, there have been cases in which biologists used such products in feeding tests to compare the nutritional value of soybean proteins with that of other proteins; and secondly, to point out that if purified proteins are to be separated from soybeans for food purposes new procedures in which very slightly alkaline, neutral, or acid dispersion methods are used will probably have to be developed. In order to work out such methods, the data compiled by Smith and Circle may prove to be of great value (Fig. 1).

Woodruff, Chambers, and Klaas (1938) isolated a protein from commercially extracted soybean flakes and finely ground, fat-free beans with water, followed by acetic acid precipitation at pH 5.0, which yielded a tasteless, odorless, white powder. On the basis of tests made with this product on various foods, its use was suggested to increase the protein content. This protein material was, however, relatively insoluble after isolation and did not have the water-absorbing or other characteristic properties of soybean flour. It does not foam or whip, and for this reason the belief is expressed that the whipping properties observed by Watts are due to non-protein constituents of soybean flour. It would seem, however, that Woodruff's method would have left most of the albuminous portion of the soybean protein present in the filtrate or centrifugate, and thus the experimental evidence presented on this latter point would appear to be open to question.

VIII. Amino Acid Content of Soybean Protein

It has frequently been pointed out that soybean proteins have a remarkable empirical similarity to milk proteins. The amino acid contents of

[6] O'Brien, W. J. (1936) Soybean Proteins, pp. 254–260. Proc. Second Dearborn Conference of Agr. Ind. and Sci., Dearborn, Mich.

soybean glycinin and milk casein are directly compared in Table IV (taken from a report by Hayward, 1939) from which it is apparent that, although these two proteins may be basically similar in the number and kinds of acids present, there are several decided dissimilarities. For instance, the percentage given for valine, one of the nutritionally essential amino acids in glycinin, is so low as to indicate that this amino acid may well be a limiting factor in its nutritional value, whereas the percentage of valine in casein is exceeded by that found in only a few proteins.

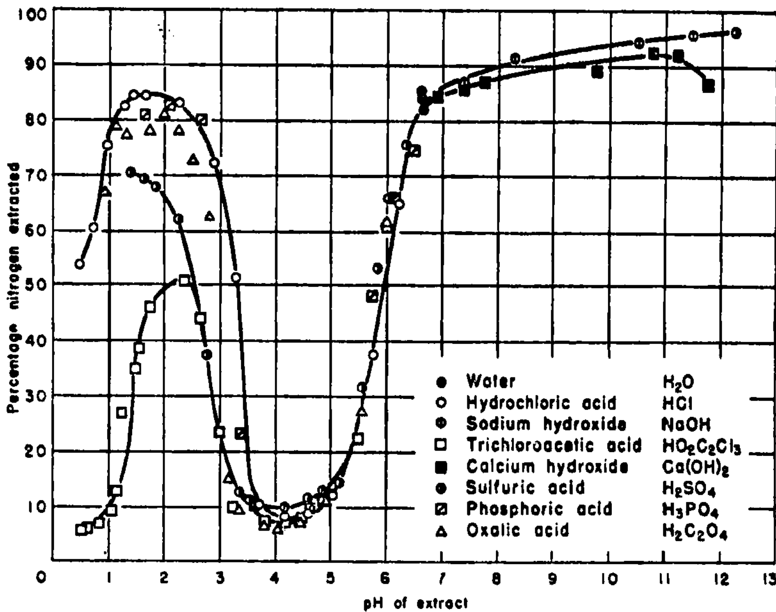

Fig. 1.

Percentage of total nitrogen extracted from oil-free soybean meal by various acids and bases
(Smith and Circle (1938). *Ind. Eng. Chem.* **30**, 1414–18.)

Unfortunately Table IV provides those interested primarily in the nutritional values of proteins with only a part of the story.

Glycinin is but one of the protein fractions of the soybean, and although we do not have adequate information on the amino acid composition of the other protein fractions, they cannot be excluded from the nutritional picture. Block and Bolling (1943) gave values for the ten essential amino acids in soybean protein as follows: lysine 4.8%, isoleucine 4.0, tryptophan 1.6, threonine 4.0, valine 4.5, methionine 2.0, histidine 1.8, leucine 7.7, arginine 5.3, and phenylalanine 5.7%. From an analysis of whole soybean protein, Sasaki (1935) reported values of 4.12% for alanine and 2.56% for

valine, and Mashino and Nishimura (1927) gave values of 7.3% for histidine, 6.08% for lysine, and 15.55% for arginine. There is considerable incongruity between all of these results and the data reported in Table IV.

Likewise, milk casein is only one of the proteins of milk, and the 2.29% of cystine in lactalbumin and lactoglobulin tends to make up for the obvious deficiency of this acid shown in Table IV and to supply additional and probably significant quantities of arginine, histidine, methionine, lysine, threonine, tryptophan, valine, and leucine.

Values that have been given for the lysine of soybean protein show considerable variation. The value of 2.71 given for glycinin in Table IV is considerably lower than values that are often found in the literature.

TABLE IV

Amino Acid Content of Glycinin As Compared to Casein

Amino acid	Soybean glycinin	Milk casein
	per cent	*per cent*
Glycine	0.87	0.45
Valine	0.68	7.20
Leucine	8.45	10.50
Proline	3.78	6.70
Phenylalanine	3.86	3.20
Aspartic acid	3.89	1.40
Glutamic acid	19.46	15.55
Tyrosine	1.86	4.50
	4.55	
Arginine	5.12	3.81
Histidine	1.39	2.50
Lysine	2.71	5.95
Tryptophan	1.94–2.84	1.50
Cystine	0.74–1.45	0.25
Methionine	1.84	3.25–3.53

The principal cause of the variations encountered with the values for this acid appear to be due to the methods employed in its determination rather than to its distribution in the various protein fractions of the bean. Jones and Waterman (1921) obtained a value of 9.06% for lysine by the Van Slyke method but were only able to obtain a value of 2.71% by the isolation method of Kossel and Kutcher. Felix (1920) called attention to the high values obtained for lysine by the Van Slyke method as compared with those found by the method of isolation of Kossel and Kutcher. By fractionation of the sulfuric acid hydrolyzate of glycinin he found the maximum value for lysine to be 5.77% in contrast to 3.34% which he obtained in the form of the picrate by isolation. Mashino and Nishimura (1927), using a somewhat

similar procedure, reported the lysine content of soybean protein to be 6.08%.

It should be pointed out that Csonka and Jones (1933) have shown considerable variation in the amino acid content of different varieties of soybeans, and such findings may presage the development of varieties of soybeans possessing even higher quantities of the essential amino acids.

IX. Digestibility of Soybean Proteins

The popularity and demand for soybeans and soybean oil meal for use in animal feeds can be traced in part to the relatively high percentage of total digestible nutrients. In soybeans, 86.2% of the total dry matter present has been found to be digestible in feeding trials with farm animals.

TABLE V

Digestibility of Soybeans in Farm Animals Compared to Other Common Foods

Food stuff	Protein		Fat		Fiber		N-free extract	
	Average composition	Digestion coefficient	Average composition	Digestion coefficient	Average composition	Digestion coefficient	Average composition	Digestion coefficient
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Soybeans	39.9	89	17.2	88	4.5	37	26.3	67
Soybean oil meal	44.3	85	5.7	86	5.6	68	30.3	98
Skim milk	3.7	94	3.7	97			5.0	98
Whole "	3.5	94	3.7	97			4.9	98
Oat meal	16.3	90	5.9	96	2.8	80	64.1	98
Wheat flour	15.1	92	2.5	87	0.8	50	69.1	99
Beans (field)	21.0	87	0.7	83	4.0	58	56.7	91
Peas "	23.8	85	1.2	62	6.2	87	56.2	93
Rice (grain)	8.3	76	1.8	76	8.8	10	64.7	91
Corn (dent)	9.7	76	4.0	91	2.3	57	71.1	94

Since the figures obtained in animal feeding experiments for digestibility of various foods would have about the same relative values for man, the digestibility figures given by Morrison (1936) in the case of farm animals for various common foods are given here in Table V.

In comparing the digestibility of soybean proteins with the proteins of cow's milk, Adolph and Wang (1934) found that cow's milk was more rapidly hydrolyzed by trypsin but less rapidly hydrolyzed by pepsin. Slightly more milk protein than soybean protein was digested by both of these enzymes. Ten day digestion trials on rats gave values for both proteins that were in very close agreement. The soybean proteins were found to be 84.9% digestible as compared to 86.6% for milk proteins. Piau (1930), on the other hand, reports a coefficient of digestibility of soybean proteins of 96% in rats.

London, Shokhor, Gagina, Kolotilova, Kutok, Markaryan, and Popel (1932) reported that 88% as much protein from soybeans as from meat proteins was utilized by humans. Tso and Chu (1931) found that 80% of the nitrogen of soybean milk was absorbed by infants as compared to 95% of the nitrogen in cow's milk. In nitrogen balance studies on human subjects, Cheng, Li, and Lau (1941) found coefficients of digestibility for protein from soybean curd above 97%, for mixtures of soybean and pork proteins of 96%, and for mixtures of egg and soybean proteins of 94%. Mendel and Fine (1912) found that 85% of the protein in soybean flour was utilized by humans, and Lyman and Bowers (1918) reported a coefficient of digestibility for man of 91%.

It should be pointed out here, even at the risk of being didactic, that a coefficient of digestibility does not necessarily indicate the percentage of an ingested nutrient that is retained in the body proper (blood stream and other organs). More specifically a coefficient of digestibility for a protein may give a false and misleading picture as to the *status quo* on the availability of the protein derivatives. On the other hand, a biological or nutritive value arrived at by comparing some factor such as the growth-promoting value of a given protein to a similar value for some known biologically efficient protein source, such as milk, does give an index of the over-all completeness of a protein as to availability and retention by the human body of the essential amino acids.

X. Nutritional Values of Soybean Proteins

Osborne and Mendel (1917a), Vestal and Shrewsbury (1933), Shrewsbury, Vestal, and Hauge (1932), and Hayward, Steenbock, and Bohstedt (1936) all found that raw soybeans when fed to rats as the sole or principal source of protein in an otherwise complete ration did not support appreciable growth. Much better growth resulted when they fed soybeans that had previously been cooked. Vestal and Shrewsbury (1933), Shrewsbury, Vestal, and Hauge (1932), and Robinson (1930) reported similar results with pigs. Mitchell and Villegas (1923), Mitchell and Smuts (1932), and McCollum, Simmonds, and Parsons (1921) reported experimental evidence in support of the fact that raw soybean protein has low nutritive value. Mitchell and Smuts (1932) and Shrewsbury and Bratzler (1933) reported that raw or uncooked soybean protein was definitely deficient in cystine.

As has been previously pointed out, the amount of heating or cooking involved in the preparation of soybean flours may vary considerably. Some cooking is involved in all types of flours, however. With "full-fat" flour considerable cooking is usually involved in the debittering process. With expeller flour of low fat content additional heating or cooking is obtained in the expeller, and in flour low or practically free of fat consider-

able cooking usually results from the use of steam to remove the solvents from the extracted flakes.

Since soybean flour is not usually consumed as flour but in cooked foods, it is not essential that the protein be completely cooked, and since overcooking may eventually impair nutritional values, it is usually not heated to an extent necessary to obtain an optimum nutritional value. In some instances when food processors are using the flour as an ingredient to perform certain physical functions, complete cooking may also be undesirable. The manufacturer of soybean flour, therefore, attempts, as a rule, to supply a line of flours that have received limited heat treatments, depending upon the type of product in which they are to be used.

Osborne and Mendel (1917b) showed that solvents did not increase the nutritive value of soybean proteins, and Hayward (1937) has recently reviewed all the information sustaining this early observation that the nutritional value of soybean protein is not increased by solvents as it is with heat denaturation.

The actual chemistry involved in the increase in the nutritional value of soybean protein under the influence of heat has not been definitely established, although considerable interesting information having a direct bearing on this subject has been developed.

Julian (1943) has recently reviewed the subject of heat denaturation of proteins. In this review, he calls attention to the intriguing and vexing problem posed by the improvement in the nutritive value of soybean oil meal by heating. He points out that soybean protein is among the very few proteins whose nutritional value may be improved by heating; he cites the works of Morgan (1931) and Morgan and Kern (1934) who showed that the biological values of heated or toasted cereal proteins, wheat gluten, casein, and raw beef tended to be lower than when they were not heated.

Julian outlines the measurable physical and chemical changes that may result from heat denaturation and interprets some of these effects on the basis of the prevailing views on protein structure. Finally, on the basis of the observation that heating oil-free soybean meal does not increase its biological values in the same manner as it does oil-containing meal he introduces the possibility that increased nutritional values in soybean oil meal may not be due solely to heat denaturation but to a chemical reaction between the proteins and other components of the meal under the influence of heat.

Johnson, Steenbock, and Parsons (1937) observed that the digestibility of soybeans was practically the same whether they were raw or cooked, although more nitrogen was retained by the animal from heated beans. Likewise, although 61 to 62% of the amount of sulfur ingested in both raw and cooked beans was absorbed, it was found that 25% was retained by

the animal from the heated beans and 11% from raw beans. From these observations, it was concluded that raw beans contained cystine in a form which could not be utilized by the body although it was absorbed.

Hayward, Steenbock, and Bohstedt (1936) in the light of results obtained by them on metabolism trials on raw and cooked soybeans in which the results for digestibility and biological values of the protein were corrected for endogenous nitrogen and in supplementation studies suggested that heat denaturation causes an increase in the availability of the methionine-cystine fraction of the protein molecule. Julian (1943) has pointed out that this effect could be accounted for quite readily on the basis of prevailing hypotheses on the unfolding, under the influence of heat, of globular compact protein molecules, exposing in that process, among others, SH or S—S groupings that normally face the interior of the molecule. Hayward and Hafner (1941) have recently studied the supplementary effect of both cystine and methionine for both raw and cooked soybean proteins in feeding studies with chicks and rats. They found that the proteins of raw soybeans were effectively supplemented by 0.3% cystine and even more so by 0.3% methionine. A combination of both of these amino acids produced no additional improvement in protein quality. Autoclaving the soybeans increased the nutritional value of their proteins to about the same degree as cystine and methionine supplementation, but additions of cystine and methionine to the cooked beans stimulated an even greater response in the test animals. Thus, raw soybeans supplemented with a combination of 0.3% cystine and 0.3% methionine contained protein that was not utilized as efficiently as the protein from the autoclaved beans supplemented with either amino acid separately. It should be pointed out that these feeding trials were conducted at a level of 10% protein and that, while studies at this level are helpful in revealing border line amino acid deficiencies, the rate of growth at this level is not great enough to allow results to be taken as a fair criterion for judging the over-all nutritional completeness of a protein.

The effectiveness of cystine and methionine in supplementing raw soybean proteins in these studies was attributed to the relief of an apparent deficiency of available cystine made more acute by the *in vivo* conversion of a large part of the methionine in the diet to cystine when the animal organism tends to overcome this deficiency. This nearly depleted the diets of available methionine necessary for building specific body proteins. The addition of cystine directly relieved the cystine deficiency, thereby releasing the available dietary methionine for the specific function of growth. In this manner cystine appeared to function in place of methionine. Methionine by virtue of its ability to serve either as cystine or methionine in the body proved more effective than cystine as a supplement.

It was not believed that the amount of cystine and methionine made available by heating could account entirely for the response noted in experimental animals. Hayward and Hafner (1941) have advanced the postulation that soybeans may contain homocystine in a form unavailable for growth, and that heat denaturation makes this amino acid available. This acid, according to the mechanism outlined for the metabolism of methionine by Brand, Cahill, and Block (1935), could after reduction to homocysteine be converted to methionine by *in vivo* methylation in the presence of soybean choline. Homocystine has not been identified in soybean proteins by analytical methods.

Evidence obtained in recent studies[7] on the effect of dry and moist heat on the nutritional value of soybean protein points strongly to the possibility that heat itself is merely an incidental factor in increasing nutritional efficiency, just as it is in a multitude of chemical reactions which take place

TABLE VI

Comparative Growth-Promoting Values of Soybean Proteins and Skim Milk Proteins When Used As Supplements to the Proteins of Wheat Flour

Source of protein supplement	95 parts wheat flour, 5 parts supplement		90 parts wheat flour, 10 parts supplement		85 parts wheat flour, 15 parts supplement	
	Gain in 42 days	Gain per g. protein eaten	Gain in 42 days	Gain per g. protein eaten	Gain in 42 days	Gain per g. protein eaten
	g.	g.	g.	g.	g.	g.
Soybean flour	39	1.38	75	2.16	93	2.27
Skim milk powder	49	1.15	77	1.86	85	2.06

more rapidly at elevated temperatures, and is not necessarily associated with heat denaturation in the true sense of the term.

Jones (1943) has shown that the growth-promoting value of the proteins of soybean flour, when fed as the sole source of protein (at a 9.1% level) in the diet, is inferior to the protein of casein or skim milk powder, but superior to the protein in patent wheat flour and whole wheat flour. At a 15% level the growth-promoting value of soybean protein increases about 60%, although it is still not equivalent to casein or skim milk powder. However, in mixtures with wheat flour at a 9.1% protein level, 5, 10, and 15% of soybean proteins gave results equivalent to or better than the same amounts of skim milk proteins. The results of these studies are reported in Table VI.

In discussing the increasing gains in weight obtained with increasing proportions of the oil-seed flours added to patent wheat flour, in rat-feeding

[7] Private communication of Dr. Percy L. Julian, Director of Research, The Glidden Co., Chicago.

studies, Jones and Divine (1942) have pointed out that these flours probably furnish one or more of the nutritionally essential amino acids not present in sufficient amounts in wheat flour proteins. They state, "There can be little doubt that one of these amino acids is lysine," and call attention to the deficiencies of patent flour in this connection and to soybean glycinin as a valuable source of supplementary lysine for use therewith.

Johns and Finks (1921) and Kon and Markuza (1931), in rat-feeding studies with baked breads containing varying percentages of soybean flour, have clearly demonstrated the supplemental value of soybean proteins for the proteins of wheat flour.

Possibly the greatest source of accumulated evidence relative to the biological value of soybean protein as compared to animal protein is found in the literature on poultry feeding. These data are not directly interpretable in terms of human requirements owing to the greater requirements in chickens than in mammalian animals for arginine, methionine, and glycine, as indicated by Briggs, Mills, Elvehjem, and Hart (1942), but it does not suggest that any great distinction can be drawn between the biological values of soybean and meat proteins. For example, Philips, Carr, and Kennard (1920) found that soybean meal was somewhat better than meat scraps as a protein supplement for 6 day-old chicks through a period of 26 weeks. Irwin and Kemster (1942) reported recently that a ration containing 25% soybean oil meal as a sole protein concentrate produced heavier chicks at 10 weeks than a control ration containing 5% dried buttermilk and 10% meat scraps. Bird and Groschke (1942) obtained practically as good growth with growing chicks up to 9 weeks of age with a diet containing soybean oil meal as the sole source of protein as with a diet containing 4% of fish meal in addition. Wilgus, Norris, and Heuser (1935) rated the protein of soybean meal as 88% as good as that of dried skim milk, while Heiman, Carver, and Cook (1939) gave it a rating of only 55% on the same basis. However, Draper and Rhian (1942), using the same procedure as Heiman, Carver, and Cook (1939), later rated soybean oil meal protein with a value of 85%. Almquist and coworkers (1942) from feeding studies with week-old chicks found that deficiencies with rations containing soybean oil meal as the sole source of protein are in all likelihood traceable to deficiencies of other nutrients than protein.

Van Landingham, Clark, and Schneider (1942) found that chicks utilized the nitrogen of soybean meal as well as that of dried whole egg and better than menhaden fish meal.

Synthesis of blood proteins results from the use of dietary protein. Measurements on the rate of blood protein regeneration in protein depleted animals on various diets can be used therefore as one index to the biological

quality of the proteins contained therein. For such studies the protein reserves of the test animals are reduced prior to the test period by either plasmapheresis or maintenance on low biological incomplete protein diets. McNaught, Scott, Woods, and Whipple (1936) reported that blood plasma regeneration in plasmapheresis depleted dogs on soybean protein diets compared favorably with that in dogs on a meat protein diet. Very recently Cannon (1944)[8] has found that plasma regeneration in protein starved (carrot diet) mature rats was nearly as good with animals on a soybean protein diet as with animals on a dried beef protein diet.

Mackay (1940), in clinical studies on 225 infant out-patients, compared the efficiency of a 50:50 mixture of dried whole milk and soybean flour with straight dried whole milk. On the basis of weight gains, total morbidity, and bone calcification, the soybean flour-dried whole milk mixture gave as satisfactory results as did the straight milk diet. The hemoglobin levels were good but about 2% lower on the average than those obtained with the whole dried milk diet. Rittinger, Dumbo, and Torrey (1935), Stearns (1933), and Hill and Stuart (1929) have used soybean milk successfully in infant-feeding studies.

XI. Use of Soybean Flour and Grits in Human Food

By far the major portion of the soybean protein available for human food is in the form of soybean flour or grits. These materials can at first be most effectively employed in the protein fortification of foods commonly used. Although soybean milk and soybean cheese are accepted as staple foods in the Orient, the acceptance of such foods by the American public, without a long expensive induction or introductory period, is doubtful. There is no time during a war emergency for the development of new taste and eating habits. Thus, to obtain the greatest immediate nutritional efficiency with the soybean products available, their use in bread, rolls, macaroni, hot and cold cereals, dry soup mixtures, and with meat in prepared loaves, scrapple, and sausages is being encouraged first by the War Food Administration, and the development of characteristic soybean foods will then be sponsored only as items possessing promising possibilities are developed. Consumer acceptance of fortification with soybean protein will depend to a large degree on the skill with which soybean products are employed in foods. Experiments have shown that soybean flour and grits can be used effectively in the products mentioned above, but their employment therein in quantities which definitely decrease edible quality or with-

[8] Private communication of Dr. Paul R. Cannon, Department of Pathology, University of Chicago.

out proper seasoning will produce an unfavorable reaction to soybeans. Careful education of the general public and food processors in ways and means of using soybean flour and grits will be required to avoid such reactions.

Food processors should remember that the flavor of protein food, good or bad, depends as a rule on the non-protein substances present. The flavoring substances of meat are a small part of the meat. Thus, making soybean proteins acceptable to the public will depend in a large part upon two factors; namely, (1) the production of soybean products free from bitter objectionable flavors, and (2) the development of suitable technique for flavoring and blending soybean products with other foods.

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Nucleoproteins

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I. Foreword

The year 1944 marks the hundredth anniversary of the birth of Friedrich Miescher in Basel. The isolation by Miescher of nucleic acid and of protamine from fish sperm is part of the classic history of biological science. It is impossible to read the account of this work (1) without a feeling of admiration for the resourcefulness and skill of the experimenter, for the care which he lavished over the isolation of his preparations, and for the imaginative insight with which he regarded the properties and biological rôles of the substances which he had discovered (1a). Had Miescher only isolated the nuclear materials and contented himself with their bare description his fame would have been enduring, but he went beyond this. Miescher was a biologist, or perhaps a biological chemist in the best sense of the hybrid term, which is to say he was interested in dynamic phenomena. Nucleic acid and protamine were to him a manifestation of biological development in which these components played no static rôle. The patient and tedious study of the development of the salmon gonads attested to this belief. This attitude extended also to the purely chemical characterization of the nuclear components, for Miescher drew the profound observation from the behavior of mixtures of nucleic acid, protamine, and salt that these components formed a dynamic system. The ions of sodium chloride distribute themselves in a polyvalent nucleic acid-protamine mixture whereby protamine chloride and sodium nucleate are formed and subsequently separated, and the distribution and separation vary according to the salt concentration. It is fitting to quote Miescher's own words (1, p. 71) "Wir haben also einen partiellen Austausch von Säuren und Basen zwischen Chlornatrium und Nucleo-Protamin; derselbe ist gebunden an bestimmte Grenzwerthe der NaCl-Concentration. Da das Nuclein . . . eine mehrbasische Säure ist, so werden mehrere neue Verbindungen neben einander entstehen können, welche Nuclein, Natrium, Protamin in verschiedenem Verhältnissen und Combinationen enthalten." This concept of a dynamic equilibrium among the nuclear components has perhaps wider implications than even Miescher envisaged, and in the peculiar properties of these substances and in their mutual interaction lie many sources for the phenomena of cellular development and the living process.

II. Nucleic Acids

Inasmuch as the inclusion of nucleic acid constitutes the distinguishing characteristic of the nucleoproteins, setting the latter apart from other types of conjugated proteins, it is desirable to give in brief detail certain of the more important features of this class of substances.

1. *The Basic Structure*

The nucleic acids which have been most completely studied, *e.g.*, from yeast and from the calf thymus gland, and which may be generally taken as models for this class of substances, are each composed of phosphoric acid, one kind of sugar, and four qualitatively different but quantitatively equivalent amounts of nitrogenous bases. When yeast nucleic acid is subjected to the action of dilute alkali, four separate components may be readily isolated. Each component contains one molecule each of sugar, of phosphoric acid, and of nitrogenous base (2, 3). The only distinction between the four components lies in the nature of the base. Four such components have not been isolated from thymus nucleic acid but there is good evidence, to be discussed later, that these components exist similarly within this acid as well. The trebly-constituted component, which forms the fundamental unit of all known nucleic acids, is referred to as a *nucleotide* and may be represented as follows:

(1) nitrogenous base-sugar-phosphoric acid.

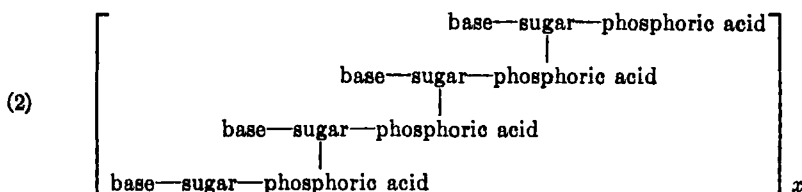
The phosphoric acid occupies a terminal position and is bound in ester linkage, for it can be readily hydrolyzed from the nucleotide by the action of purified monophosphatase or nucleotidase. The residue, which is the nitrogenous base-sugar combination, is referred to as a *nucleoside* (2). The base in turn must occupy the other terminal position in the nucleotide for it can be hydrolyzed from the nucleotide by acids, leaving the sugar-phosphate ester residue (2). Under the latter circumstance, or when the nucleoside is hydrolyzed by purified enzyme preparations called nucleosidases, the sugar exhibits reducing properties and mutarotation, thus indicating a glycosidic binding between base and sugar (2). All this evidence has been chiefly obtained on yeast nucleic acid from which the individual nucleotides are readily isolated. In the case of thymus nucleic acid, evidence for the order of the constituents within the nucleotides has been obtained through isolation of nucleosides from enzymatic digests of the nucleic acid (4, 5), and from the action of mineral acids which cause the splitting of certain of the bases from the nucleic acid leaving a residue richer in the sugar-phosphate esters (thyminic acid) (6, 7, 8).

2. *The Tetranucleotide Structure*

The nucleotides each form disodium salts below a pH of 10 (2). If the nucleic acid were composed of a free mixture of the four different nucleotides, the acidimetric titration of the former should reveal the presence of eight ionizable hydrogen atoms within the dissociation range of phosphoric acid. However, Miescher originally observed that the nucleic acid isolated from sperm was a tetrabasic acid (1), and the most reliable data at the

present time strongly indicate that both yeast and thymus nucleic acids consume four equivalents each of alkali per unit of four nucleotides before a pH of about 10 is reached (9, 10, 11, 12). Furthermore, the isolated sodium salt of thymus nucleic acid contains equivalent amounts of sodium and phosphorus atoms (12). It would appear therefore that four of the eight phosphate hydrogens of the group of four different nucleotides are bound in some fashion. The isolation from hydrolyzates of nucleic acid of nucleosides in which the sugar is doubly esterified with phosphoric acid at two different carbon atoms (13) provided one of the earliest clues to the solution of this problem, for it appeared probable that the extra phosphate on the nucleotide must have had its source in an adjacent nucleotide. The controlled action of phosphatases from different sources on nucleic acid was found to yield nucleotides without further splitting of the latter into inorganic phosphate, and in the process 3 to 4 alkaline equivalents per unit of four nucleotides were found to be set free (8, 14, 15, 16). The available evidence points strongly to the possibility that the four nucleotides are therefore bound together through phosphate ester linkages between adjacent nucleotides. Further evidence to this effect has been obtained by the hydrolysis of yeast nucleic acid into four different nucleotides by crystalline ribonuclease (16a). Such a tetranucleotide structure may be represented as follows:

(2)



3. *The Polymeric Structure*

This structure, first proposed by Levene (2), appears to be the most probable for yeast and for thymus nucleic acids, although other types of structure and possible formulations cannot be entirely excluded (10, 17). The tetranucleotide represented in structure *2* will yield five ionizable hydrogen atoms within the dissociation range of phosphoric acid but it has been mentioned above that the best available data show the presence of only four such atoms per tetranucleotide in nucleic acid. This discrepancy can be removed by a consideration of the physical state of both kinds of nucleic acid. The molecular weight of the sodium salt of thymus nucleic acid may vary in different preparations from 5×10^5 (18, 19, 20) to about 1×10^6 (18). The molecular weight of yeast nucleic acid is

about 2×10^4 (21, 22), but that of the yeast-type of nucleic acid isolated from the tobacco mosaic virus nucleoprotein may reach a value of about 3×10^5 (23). It should be mentioned that Miescher first noted that the nucleic acid which he isolated from sperm, since shown to be of the thymus type, was of high molecular weight for it failed to dialyze through parchment (1). In any case, it is evident that the free nucleic acids generally exist in the form of substances of high molecular weight, and what is more probable than that the tetranucleotide structure is repeated many times within molecules of such high weights? The linkage between the tetranucleotide in such molecules might well involve the fifth hydrogen atom of the individual tetranucleotide, and so as more and more tetranucleotides polymerize to form the large molecule, the basicity of the latter approaches more and more to the observed value of four per tetranucleotide unit. The reverse of this has been noted in the case of the yeast nucleic acid, for by treating the latter with the specific and crystalline preparation of ribonucleo-depolymerase, the fifth ionizable phosphate hydrogen makes its appearance (11), and simultaneously the translational diffusion constant of the nucleic acid increases (22). Thus, with increasing depolymerization of the acid, the basicity tends to increase to the value of five, characteristic of the single tetranucleotide. In the case of the thymus nucleic acid, the fundamental tetranucleotide unit has actually been isolated after treatment of the intact nucleic acid with a controlled and specific enzyme preparation (24).

The nucleic acids are thus highly polymerized structures. Since the molecular weight of the tetranucleotide is about 1400, the number of these units, *i.e.*, the value of x in formula *2*, is about 500–1000 for thymus and about 15 for yeast nucleic acid. If these polymers are arranged in linear fashion, the terminal nucleotide should have two free phosphate hydrogens, and the terminal tetranucleotide of which it is a part will have five such hydrogens. In such large polymers, the titration of this extra hydrogen would hardly be noticeable. In thymus nucleic acid, a linear structure appears to be plausible from the X-ray photographs of films of this substance (25). When the photograph is taken with the beam perpendicular to the direction of stretch and parallel to the surface, a strongly-marked period of 3.3 A along the "fibre" axis is noted. Astbury and Bell interpreted this finding as being due to a succession of flat nucleotides standing out perpendicularly to the long axis of the molecule to form an optically negative structure, the successive nucleotides separated from each other by the 3.3 A distance. If in such a structure, the phosphate in the terminal nucleotide of each tetranucleotide may be conceived as being bound in ester linkage with the sugar of a terminal nucleotide in an adjacent tetra-

nucleotide, the resulting molecule is a linear succession of nucleotide units which is in concordance with the scheme of Astbury and Bell. The vertebrae of such a column would be the phosphate ester linkages. Whether the molecule of yeast nucleic acid is arranged in this linear fashion cannot be stated definitely at the present time, but the tetrabasicity of this acid before and the pentabasicity after depolymerization, per tetranucleotide, renders quite plausible the assumption that the adjacent tetranucleotides are combined with phosphate ester linkages in the manner, if not the ultimate molecular shape, of the thymus nucleic acid.

As in the case of polymers in general, the degree of polymerization may vary according to the method of preparation. The highest polymer of thymus nucleic acid, or at least that material with the highest sedimentation rate in the ultracentrifuge, is that prepared by the Hammarsten method, and the lowest that prepared by the Feulgen-Levene technique (26). The former preparation is obtained by salt extraction in cold, neutral solutions and possesses a fibrous structure, whereas the latter preparation which is obtained through the use of boiling alkali is granular in appearance. All indications strongly suggest (12, 27) that the latter is a degradation product of the former, and it is therefore not unexpected that the former preparation yields 4 (12), the latter apparently 5 (2) ionizable phosphate hydrogens per tetranucleotide unit. The degree of polymerization of the yeast-type of nucleic acid isolated from the tobacco mosaic virus nucleoprotein also varies according to the method of preparation whereby the molecular weight of different preparations may range from 3×10^5 to 15×10^3 (23).

4. *Components of Nucleic Acids*

The nature of the sugar in the nucleic acids has been definitely established only in the cases of yeast and of thymus nucleic acid. The sugar in the former acid is *d*-ribose, in the latter acid it is *d*-2-desoxyribose. Yeast nucleic acid thus gives characteristic pentose reactions, *i.e.*, the Bial test and evolution of furfural in acid distillation. Small amounts of *l*-lyxose found in commercial samples of yeast nucleic acid might have been derived from carbohydrate impurities (27a). Nucleic acids from many sources, such as the wheat embryo (28), the pancreas β-nucleoprotein (29), the chicken embryo β-nucleoprotein (30), or the tobacco mosaic virus nucleoprotein (31), all give the reaction for pentose, and yield the same kind and proportion of bases as yeast nucleic acid. Such acids are referred to as yeast-type or pentosenucleic acids, sometimes less accurately as ribosenucleic acids, without commitment as to whether they are identical with yeast nucleic acid. The *d*-2-desoxyribose of the thymus nucleic acid gives specific color reactions with the Feulgen

(32) and with the Dische (33, 34) reagents, and thus in practice those nucleic acids isolated from such sources as the pancreas (29), liver (35), or spleen (2) are referred to as thymus-type or desoxypentosenucleic acids, less accurately as desoxyribosenucleic acids, without commitment as to whether they are identical with thymus nucleic acid. It is important to bear these generalizations in mind, and to recall in subsequent discussions that such a general differentiation between the two types of nucleic acid, except in the specific cases of yeast and thymus nucleic acid, is based upon qualitative tests for certain groups and configurations in the component sugars—after a period of hydrolysis of the nucleic acids—and not upon the absolute identification of either sugar.

The four bases in each tetranucleotide are different for each nucleotide, and the isolation of four so differently-constituted nucleotides in equivalent amounts from yeast nucleic acid was the first indication of the latter's tetranucleotide structure. Although four such different nucleotides have not yet been isolated from thymus nucleic acid because of the latter's relative instability, the isolation after complete hydrolysis of four different individual bases in equivalent amounts from this acid, together with other evidence cited above, makes the tetranucleotide structure also highly probable. In yeast nucleic acid the bases comprise the purines adenine and guanine, and the pyrimidines cytosine and uracil. Thymus nucleic acid also possesses adenine, guanine, and cytosine, but thymine is present in place of uracil. The tetranucleotide structure, which contains these bases, of the pentcsenucleic acid of the wheat embryo (28) and of the chicken embryo (30) was found to be identical or nearly identical with that of the yeast nucleic acid. All of these acids contained two purine and two pyrimidine nucleotides in equivalent amounts. The four bases found in thymus nucleic acid were also isolated from the desoxypentosenucleic acids of the spleen (2), and liver (2). On the basis of the present evidence, it seems probable that the yeast-type pentosenucleic acids and the thymus-type desoxypentosenucleic acids represent the major forms in which nucleic acid is found in living tissues. Nevertheless, the possibility that other forms may exist, even if in quite minor degree, cannot be excluded. This possibility is emphasized by the isolation of nucleotides involving thiomethylpentose from yeast (36) and of nucleic acid from the pancreas which appears to consist of a pentose-containing pentanucleotide structure (29). With this reservation in mind, we may however examine the fine-structure of yeast and of thymus nucleic acid insofar as it is known, to illustrate the specific diversity, apart from the composition, of the two general representatives of this class of substances.

5. *The Linkages between Components*

The attachment of the sugar in both acids appears to occur at N_9 in the purines and at N_3 in the pyrimidines (10). The ring structure of both the ribose and the desoxyribose components is of the furanose, rather than of the pyranose type (37, 38, 39, 40). In the yeast nucleic acid, the phosphoric acid is esterified in both the purine and pyrimidine nucleotides at C_3 of the ribose (41, 42). The position of the phosphoric acid in thymus nucleic acid nucleotides is not precisely known at the present time but it may be either at C_3 or C_5 (10).

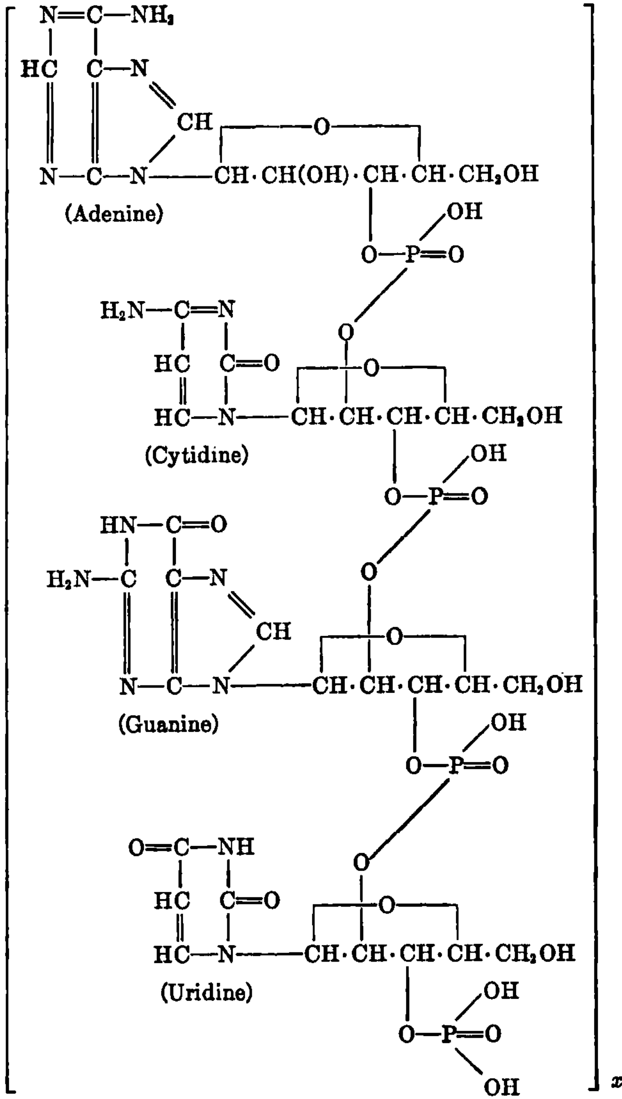
The manner of linkage of the individual nucleotides in either yeast or thymus nucleic acid has long been a source of controversy (see 10). The isolation of doubly esterified pyrimidine nucleosides with phosphoric acid has lent weight to the formulation of nucleic acids in which the linkage between the individual nucleotides is a sugar-phosphate ester. Each sugar, save possibly one, in the nucleotides of the fully polymerized nucleic acids is doubly esterified with phosphoric acid. Mixtures of diesterases and monoesterases readily dephosphorylate nucleic acid (10). The skeletal formula *2* given above represents such a diester structure if it is assumed that the fifth ionizable phosphate group is concerned with the polymerization of the tetranucleotides. The ester linkage between phosphate and ribose in the individual nucleotides of yeast nucleic acid concerns C_3 of the sugar. The ester linkage between adjacent nucleotides in this acid is assumed to involve C_2 of the pentose in view of the lability of esters at this carbon atom (37). There are only two free hydroxyl groups in the desoxyribose of thymus nucleic acid, namely at C_3 and C_5, and one of these is used in the intra-, the other in the internucleotide structure (37, 43). No choice between the two linkages can be made at the present time.

The order of the nucleotides in either of the two kinds of nucleic acid is not definitely known, but the isolation of the diphosphoric acid esters of pyrimidine nucleosides from thymus nucleic acid renders it quite probable that in this acid at least the purine and pyrimidine nucleotides occupy alternate positions within the tetranucleotide.

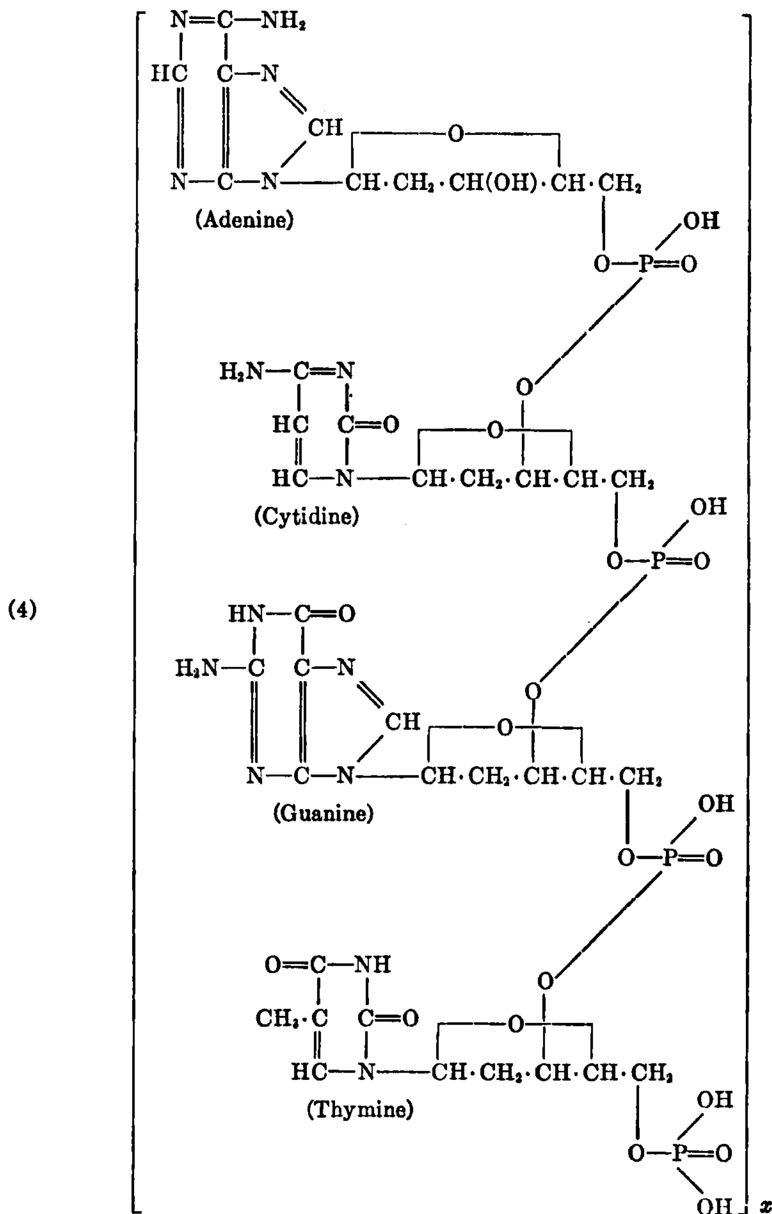
On the basis of the foregoing evidence, the tetranucleotide structure of yeast and of thymus nucleic acid may be tentatively represented as follows, in which the order of the nucleotides in both acids and the phosphate ester location in the thymus acid are arbitrarily selected, and the bases are formulated in the keto or lactam form:

Yeast Nucleic Acid

(3)



Thymus Nucleic Acid



6. *Molecular Size and Shape*

The structural formulae of yeast and of thymus nucleic acid reveal the differences in composition and configuration but they do not explain the

considerable differences in the physical properties of the two acids. Yeast nucleic acid is labile to the action of cold alkali, and the four nucleotides are readily separated by means of this reagent (28, 30). No such extensive hydrolysis apparently occurs as a result of the action of alkali on thymus nucleic acid. However, certain intramolecular rearrangements take place in the native form of the latter leading to the formation of gels in aqueous medium (12). Yeast nucleic acid is fairly stable to the action of acids, but thymus nucleic acid readily loses its purine bases in the presence of cold acid (7, 8), leaving as residue thyminic acid.

The native form of the sodium salt of thymus nucleic acid appears in the form of negatively-birefringent fibers (25). The aqueous solutions of this material show intense streaming birefringence (18, 27) and structural viscosity (27). The intensity of these properties, which depends upon the asymmetric shape of the particles, varies according to the method of preparation of the nucleate. The method of Hammarsten which leads to the most polymerized form of the nucleate also yields products which are highly birefringent and viscous in solution, whereas the Feulgen-Levene method yields products low in both molecular weight and the intensity of these properties (26, 27). However, even in the Hammarsten procedure, small and often inadvertent changes in the method lead to successive preparations the physical properties of which are rarely identical. The molecular weight and dimensions also appear to be functions of the particular preparation of thymus nucleate studied. Measurements of these quantities for so highly asymmetric a substance are beset by many uncertainties. The high degree of particle interaction leads to many anomalous findings. For example, the sedimentation constant of the thymus nucleate is a function of the concentration, increasing with increasing dilution (44, 19), and the curves for the measurements of diffusion are quite asymmetric. The values for the molecular weight as given in the literature invariably refer to finite concentrations of the material and hence are likely to be valid only for the particular concentration chosen and for the conditions selected. If such values are found for conditions removed from the ideal, they nevertheless possess a cogency for the interpretation of the physiological state, which, it is hardly necessary to point out, is also removed from the ideal. With the necessary reservations in mind, the data of several investigators for various nucleic acids are given in Table I.

The molecular weights and dimensions of the nucleic acids given in Table I are to be viewed more as orders of magnitude than as true values. Nevertheless, the data reveal that the thymus nucleate preparations are substances of high particle weight and considerable molecular asymmetry. To a lesser degree this is also true of the freshly-prepared nucleic acid removed from the tobacco mosaic virus nucleoprotein which is the first

example of a pentosenucleic acid found to show streaming birefringence. Like the thymus nucleate, this substance also shows particle interaction; in both cases the sedimentation constant increases with dilution (23). An explanation, in part, for some of the variation between the values for sodium thymus nucleate in Table I may be derived from the various values which have been reported for the partial specific volume of this substance, namely

TABLE I

Molecular Weight and Shape of the Nucleic Acids

(Thymus Nucleate Prepared by Method of Hammarsten)

Substance	Reference	Molecular Weight	a/b[1]	Length of Particle	Width of Particle
		× 1000		A	A
Sodium thymus nucleate	(44)	200[2]	—	—	—
" " "	(18)	500–1000[3]	300[3]	—	—
" " "	(25)	500[4]	—	—	16.2
" " "	(19)[8]	580[2]	400[2]	5200	13
" " "	"	430[2]	170[2]	2720	16
" " "	"	450[2]	200[2]	3000	15
" " "	[9]	1200[2]	—	—	—
" " "	(45, 46)	1235[3]	200[7]	4580	22
Thymus nucleic acid	(19)	4.8[2]	3[2]	42	14
Yeast nucleic acid	(21)	17[5]	—	—	—
Pentosenucleic acid from tobacco mosaic virus nucleoprotein	(23)	200[2,6]	32[2]	700	—
	"	290[2,6]	61[2]	—	—

[1] Ratio of long to short axes of the particle as ellipsoid of revolution.
[2] Results from ultracentrifugation.
[3] Results from streaming birefringence.
[4] Results from X radiation.
[5] Results from diffusion.
[6] Results on fresh material; on standing, the material breaks down to particles of lower weight and asymmetry.
[7] Results from viscosity.
[8] Thymus nucleic acid is rapidly depolymerized when the pH of its solutions is brought to 2.6 or 11.6 (20). The effect is reversed when the acid or alkaline solution is neutralized, but the reversed material is very inhomogeneous.
[9] Personal communication from H. Kahler.

0.55 (19), 0.62 (27), and 0.66 (46). Such variations are considerable, and since the molecular weights calculated are sensitive to this value, it might be expected that these weights would vary greatly. Apart from this, the entire problem of the size and shape of such anisotropic molecules is at the present time in a somewhat uncertain position, for only by a series of approximations can this problem be handled both technically and mathematically.

The lability of these substances is revealed by the variations between individual preparations and the ready, spontaneous breakdown of the virus nucleic acid into particles of lower weight and molecular asymmetry (23). The rotational diffusion constant for a particular preparation of thymus nucleate appeared to be in the neighborhood of 180 sec^{-1} (47). Since the latter constant varies roughly as the inverse cube of the particle length, and since the intensity of the properties of streaming birefringence and structural viscosity is largely dependent on the magnitude of this constant, small changes in particle length brought about by changes in the preparation of the material will be reflected in considerable changes in these physical properties (47). Free thymus nucleic acid has a relatively low molecular weight (19) reflecting the considerable instability of the substance in this form. It is interesting to note that the cross sectional diameters calculated in Table I are very nearly the same regardless of the length of the particle, which suggests that the molecules are polymerized end to end, and that the particles of lower weight result from cutting the long columns into smaller fragments of the same cross section (19).

It must be emphasized that the data reported in Table I were obtained on nucleic acid preparations free of protein, and the question inevitably arises as to whether the highly polymerized, asymmetric forms are not artefacts, products of the method of preparation. To some extent this question may be answered when the dimensions calculated for the nucleic acids are matched against the dimensions of the nucleoproteins from which they were derived.

7. *The Nucleases*

At neutral pH, the action of certain tissue extracts results in the loss of the specific physical properties of the nucleic acids, *e.g.*, the birefringence and structural viscosity of the thymus-type, (27, 48, 49) and the acid-insolubility of the yeast-type (50, 51). No nucleotides are apparently released under the conditions employed (27, 51) or dialyzable products formed, and the effect of the extracts has been interpreted (52) as being due to a depolymerization of the respective nucleic acids by specific enzymes in these extracts. The pentosenucleo-depolymerase, which has been isolated in the crystalline form (22) is relatively heat-stable and exerts no enzymatic activity on thymus nucleic acid (22, 53). Both in this form and in a crude intestinal preparation it does however attack both yeast nucleic acid and the nucleic acid isolated from the tobacco mosaic virus nucleoprotein (54, 55). The desoxypentosenucleo-depolymerase has not yet been isolated, but it appears to be heat-labile (27). The available evidence indicates that each type of nucleic acid requires a separate enzyme for its depolymerization. Moreover, such an enzymatic depolymerization is pre-

liminary to the further degradation of the nucleic acids to the nucleosides, phosphoric acid, and ultimately to the bases and sugar by the nucleotidases and the nucleosidases (26).

8. *The Absorption of Ultraviolet Light*

Similar physical changes in the polymerization of the two types of nucleic acid may also be achieved by the prolonged irradiation of their aqueous solution with ultraviolet light (56, 57). Fig. 1 illustrates the effect of such radiation on the structural viscosity of aqueous solution of the sodium

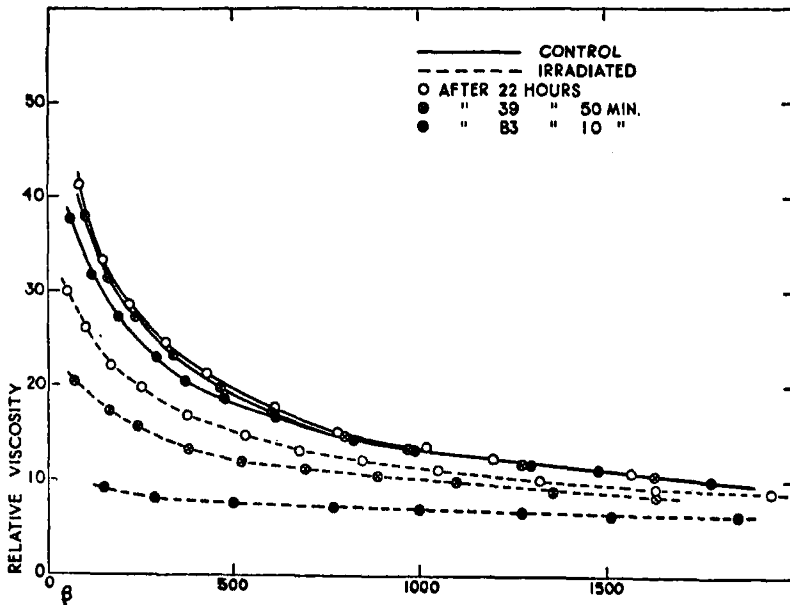


Fig. 1. The effect of ultraviolet irradiation on the structural viscosity of 0.5 per cent aqueous solutions of sodium thymus nucleate. Abscissa is function of the velocity gradient. From Hollaender, A., Greenstein, J. P., and Jenrette, W. V., *J. Nat. Cancer Inst.* **2**, 23 (1941).

salt of thymus nucleic acid. Simultaneous with a change in this property is a parallel decrease in the intensity of the streaming birefringence. No inorganic phosphorus or ammonia is split off from the material during the irradiation, and the absorption spectrum curve at the end of an 83 hour run is entirely congruent with that of the control, thus indicating that no primary decomposition of the thymus nucleate has occurred under the conditions employed. A similar decrease in the proportion of acid-insoluble yeast nucleic acid and without appearance of phosphorus or ammonia also accompanies the irradiation of solutions of this material. The possible

relation of these findings to the production of mutations in organisms induced by ultraviolet radiation will be discussed later.

The purine and pyrimidine components of the nucleic acids strongly absorb ultraviolet radiation with a maximum absorption at about 2600 A (58). For this reason, the nucleic acids also absorb radiation with a maxi-

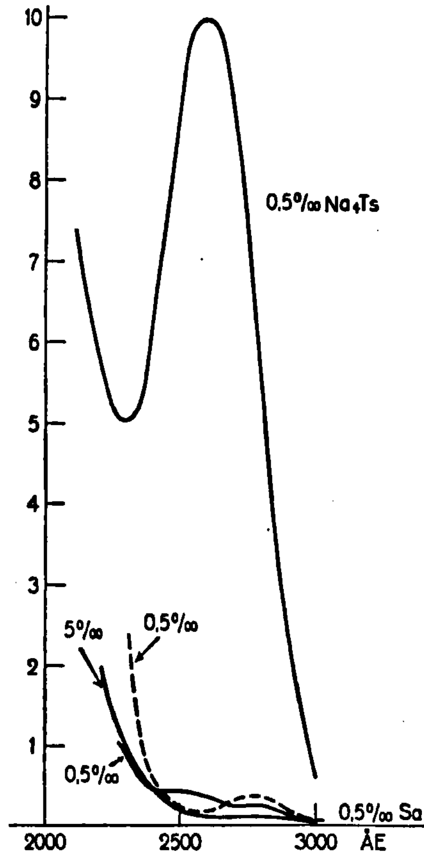


Fig. 2. The ultraviolet absorption curves of 0.5 per cent sodium thymus nucleate, 0.5 per cent serum albumin (dotted line), 0.5 per cent histone sulfate, and 5 per cent protamine sulfate. From Caspersson, T., *Fortschr. d. Zool.* **2**, 270 (1937).

mum at this wave length (59, 60), and indeed the intensity of this absorption may be used as a quantitative measure of the amount of these substances in nucleoproteins *in vitro* or in the cell. The absorption curves of thymus and of yeast nucleic acids are practically identical. Fig. 2 illustrates the absorption curve of nucleic acid, and for comparison the curves of certain proteins are included (61). It is clear that the maximum

absorption of the former is very much greater than that of the latter. Moreover the nucleic acid maximum occurs at 2600 A, the protein maximum at 2700–2900 A. The former maximum is due to the purine and pyrimidine components, the protein maximum to the content of tyrosine, phenylalanine, and tryptophan.

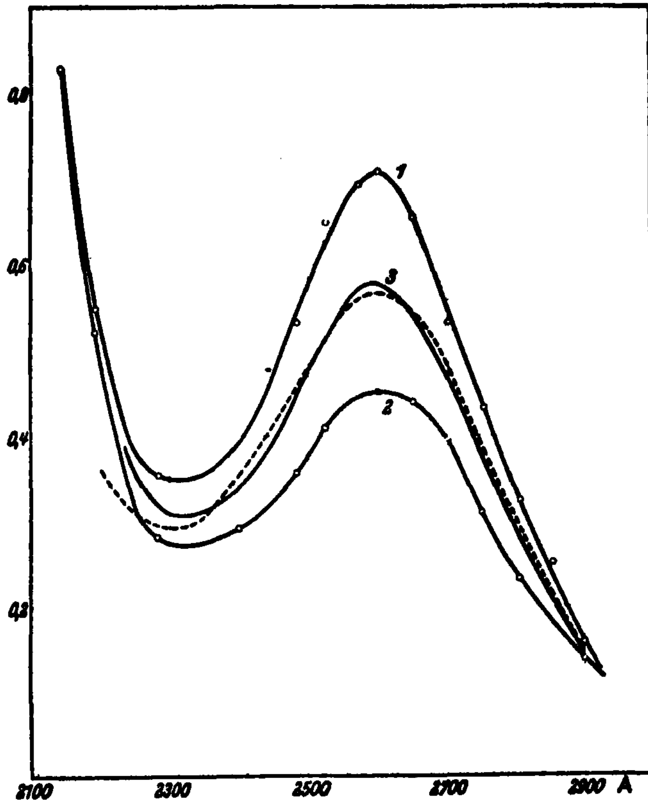


Fig. 3. Absorption spectra in the ultraviolet of a stretched film of sodium thymus nucleate. Curve 1 represents perpendicular direction, Curve 2 represents parallel direction of the incident beam, Curve 3 represents the arithmetic mean of 1 and 2. The dotted curve represents the absorption of unpolarized light. From Caspersson, T., *Chromosoma* **1**, 605 (1940).

The molecular asymmetry of sodium thymus nucleate has been hitherto revealed chiefly by measurements of viscosity at varying external pressure, of streaming birefringence, and by the combination of the methods of diffusion and ultracentrifugation. The probable orientation of the groups in the nucleate indicate that this substance should also show the phenomenon of dichroism. Thus if the pyrimidine rings which absorb ultraviolet light strongly at 2600 A were definitely arranged in space, the

absorption by these rings of polarized ultraviolet light vibrating parallel to the plane of the rings should be different from that of the polarized beam perpendicular to this plane. This phenomenon has actually been demonstrated for stretched films of the thymus nucleate by Caspersson (62). The absorption curves under these conditions are depicted in Fig. 3.

For complete orientation, the ratio of the peaks of the curves at 2600 A for the perpendicular and for the parallel incidence of the polarized beam, should be 2:1. Actually it appears to be 1.5–1.6:1. The reason for the discrepancy is not altogether clear, but it may be due either to incomplete stretching of the film or to incomplete orientation of all the absorbing groups in the molecule. Measurements with similar films of yeast nucleic acid revealed little if any dichroism (62) and hence as independently observed by other criteria, very little orientation.

III. The Interaction of Nucleic Acids with Proteins, Amino Acids, and Salts

1. *Relation of Nucleic Acid to Certain Nucleoproteins*

The nucleic acids of the thymus and of the tobacco virus nucleoprotein may be isolated free of protein and in this state appear to be highly polymerized and to possess a quite asymmetric molecular shape. The particle lengths of the free nucleates may be considered to be in the neighborhood of 4500 A and 700 A respectively (Table I); the particle diameter appears to be about 15–20 A for both. As a result of the asymmetric shape, these nucleates readily show intense streaming birefringence in aqueous solution. The nucleic acids are naturally associated with protein, the thymus nucleate with histone, and the virus nucleate with a more complex protein, in a binding the characteristics of which will be discussed fully in a later section. What relation do these elongated, polymerized forms of the isolated nucleates bear to the nucleoprotein molecules from which they are derived? This question is decidedly pertinent, for although the birefringent virus nucleic acid is derived from a strongly birefringent nucleoprotein, the strongly birefringent thymus nucleate is derived from a nucleoprotein (nucleohistone) which under ordinary conditions shows relatively little birefringence. Furthermore, preparations of thymus-type nucleates which show intense double refraction of flow in solution may be removed from nucleoproteins which appear to be nearly globular in shape and to lack any birefringent properties whatever. How then can the elongated forms of the isolated nucleates be fitted to the protein molecules with which they are naturally associated?

The length of the tobacco mosaic virus nucleoprotein at neutral pH is about 6000–7000 A and its width is about 150 A (63, 64, 23). There is about 5% of nucleic acid in this protein (65), and on the basis of a molecular

weight of this acid of 3×10^5 (23) and of the virus nucleoprotein of about 50×10^6 (64) there are at least eight molecules of the nucleic acid per molecule of virus nucleoprotein. Since the relative dimensions of the nucleic acid and the protein do not permit the former to lie within the width of the latter, it is likely that the acid particles are bound individually along the length of the virus protein molecule. There is no problem here of fitting the asymmetric nucleate within the framework of the molecule of the asymmetric nucleoprotein.

On the other hand, a similar attempt to fit the highly asymmetric thymus nucleate within the relatively less asymmetric thymus nucleohistone offers many difficulties. There is about 50 per cent nucleic acid within this nucleoprotein of molecular weight $2\text{–}2.3 \times 10^6$ (66, 67, 68). Since the molecular weight of the nucleate is about 1×10^6 (Table I), there is one molecule of the nucleate per molecule of nucleohistone. The ratio of long to short axes, a/b, of the nucleohistone is about 36 (68). From the equation:

$$b = \left[\frac{4MV}{\pi N\, a/b}\right]^{1/3}$$

the average particle length may be calculated to be 1548 A and the diameter 43 A. In a nucleoprotein of these dimensions the nucleic acid with the dimensions given above, namely 4500×16, can lie neither lengthwise nor crosswise. Too great weight should not be attached to the absolute value for any of the dimensions given, when the assumptions made in the derivation of the equations from which these values were obtained are considered. Nevertheless, on any basis there is a considerable discrepancy between the dimensions of the isolated nucleate and those of the nucleoprotein from which the former was derived. When this is taken into consideration, together with the observation that intensely birefringent preparations of thymus-type nucleic acid may be obtained from non-birefringent nucleoproteins, the question naturally arises as to whether the elongated forms of the nucleates described in Table I may not be largely artefacts, products of the method of preparation, and not necessarily representative of the condition of the nucleates within the intact nucleoprotein. In a sense, the problem of the possibly artificial elongated form of the thymus or thymus-type nucleic acid observed in isolated preparations may be partially resolved by studies of this acid within the intact cell. Schmidt (69) observed the negative birefringence of nuclear constituents within fixed tissue preparations and ascribed this property to the presence of thymus-type nucleic acid. By means of the measurement of the difference in absorption in different directions of polarized ultraviolet light (dichroism), Caspersson observed that the thymus-type of nucleic acid within the sperm of certain

species was strongly oriented (62). There is always the rather teleological argument based upon the presence in tissues of enzymes which catalytically effect a reduction in the birefringence and viscosity and hence the degree of asymmetry of the nucleate substrates. Whether the asymmetry of the free nucleic acid particles *in vivo* ever attains the degree found for preparations of the nucleates *in vitro* cannot however be answered at the present time.

The actual degree of asymmetry of the free thymus-type nucleate is on the whole a rather academic matter. The important fact is that when these nucleates are combined with protein there appears to be a distinct loss in the properties which depend upon this asymmetric shape. The problem raised by these observations comes close to involving the very nature of this important class of conjugated proteins, for it is evident that some profound interaction between the protein and its prosthetic group must occur. The protein must be able to accommodate these asymmetric particles spatially, to contain them within a form the limits of which are set by the interaction of the two substances, and in certain instances, this combination, whatever its nature, must be reversible.

2. *Complexes of Nucleic Acid and Protein*

It was first recognized by Miescher that when protamine was mixed with nucleic acid in neutral solution an insoluble salt of the components precipitated (1), and indeed this fact was utilized to show the presence of free nucleic acid in the sperm head. The protamines are not true proteins but in reality are simple, extended polypeptides (70) which, because of their high content of basic amino acids, have a very alkaline isoelectric point. The pH at which the precipitation occurs is well to the acid side of this point. The more complex proteins, such as egg or serum albumin, also form insoluble salts with nucleic acid when the components are mixed in a medium the pH of which is acid to the isoelectric point of the protein (12, 71, 72), and the composition of such precipitates, *e.g.*, the ratios of protein to nucleic acid, is dependent upon the original ratios as well as upon the absolute amounts of the components in the solutions, the pH of the medium, and the concentration of salt present. The isolated complexes are generally soluble in relatively high concentrations of neutral salts whereby a metathetical reaction takes place. The finding that, depending upon the conditions chosen, an entire series of different protein-nucleic acid complexes may be formed, is an event of profound cytochemical significance, for it provides a foundation for the dynamics, among other things, of the mitotic nucleus. The interaction between protein and nucleic acid may be expected to be modified at each instant by the available components, inorganic and presumably organic, of the living cell. Since the

complexes formed between protein and nucleic acid are dispersed by neutral electrolytes, the nature of the bond in such complexes must be largely electrostatic. These findings will be discussed in greater detail below when the forces between the components of the native nucleoproteins are considered, but it may be briefly stated at this point that the available evidence in the case of the latter class of substances indicates conditions essentially similar to those of the synthetic complexes formed of mixtures of the pure protein and nucleic acid.

A striking practical use of this phenomenon is in the purification of the "fermentation enzyme" as the yeast nucleate salt (73a). The nucleic acid is removed in a subsequent step by combination with protamine.

No precipitate is formed when protein and nucleic acid are mixed at a pH alkaline to the isoelectric point of the former, for the alkaline salts of the complex are soluble in water. In order to obtain evidence of the interaction of the components under this condition, sensitive methods which measure the specific properties of nucleic acid, particularly thymus nucleic acid, in homogeneous media, have had to be employed.

3. *Viscosity and Streaming Birefringence of Mixtures of Thymus Nucleate with Proteins, Salts, and Tissue Extracts*

Aqueous solutions of the sodium salt of thymus nucleic acid are highly viscous and show intense negative double refraction of flow. It has been stated above (p. 221) that when these solutions are treated with certain tissue extracts these properties progressively diminish. The rate at which these properties diminish under comparable conditions, is a measure of the enzymatic activity (depolymerase) of the tissue (73). With sufficient time of incubation, the viscosity of the mixture approaches that of the extract. Since the external pressure is kept constant under these conditions, and since the viscosity of the nucleate is a function of the external pressure (structural viscosity), the drop in viscosity and in birefringence is interpreted as being due to a decrease in the asymmetry of the nucleate.

When solutions of purified proteins, or the sera of certain species of animals, are added to solutions of sodium thymus nucleate there also occurs a decrease in the viscosity and streaming birefringence of the nucleate. But in contrast with the progressive effect exhibited by preparations containing the enzyme activity, the initial drop in these specific physical properties, which occurs instantly on mixing protein with nucleate, remains constant over a long period of time (27). This difference is well illustrated by Curves I and IV in Fig. 4 which refer respectively to the action of equal concentrations (protein) of rabbit and of horse serum on the relative viscosity of a 0.5 per cent solution of sodium thymus nucleate at 30°C. and 16 cm. water pressure (27). The rabbit serum contains the depoly-

merase, the horse serum does not. (The only animals so far investigated which contain the thymus nucleo-depolymerase not only in the serum but also in the milk are the rodents, 74.) Substantially the same sort of curves as IV in Fig. 4 were obtained with solutions of crystalline egg and serum albumin, and in all cases the extent of the drop in the viscosity and streaming birefringence of the nucleate in the mixture was proportional to the concentration of protein present. The viscosity and birefringence refer of course to the nucleate since the contribution of the protein is negligible in comparison. Inert and purified proteins, and, as will be shown later, amino acids and salts, have the property of reducing the molecular asymmetry of the thymus nucleate to a definite level which depends not only on the concentration but on the nature of the added substance. Curve I in Fig. 4 represents therefore the combined effects of (a) the inert proteins (and salts) of the rabbit serum in instantaneously reducing the viscosity; and (b) of the more progressive, subsequent decrease in this property by the enzyme. The horse serum (Curve IV) exhibits only part (a) of the effect. The heat-lability of the enzyme in the rabbit serum is illustrated by Curve II in Fig. 4, for after a short period of heating at 65°C. part (b) of the total effect practically vanishes. It is possible therefore to speak of a relatively non-specific depolymerization of the thymus nucleate induced instantly on mixing with inert protein, amino acid, or salt, and a specific depolymerization brought about by an enzyme, the effect of which is the sum of that induced (1) by the protein nature of the enzyme and any concomitant inert proteins and salts and (2) by the specific and progressive action of the enzyme (27). The difference in this kind of depolymerization is illustrated particularly clearly in the observed effect of crystalline ribonucleo-depolymerase on sodium thymus nucleate (53). Addition of this enzyme to solutions of the latter produce an instantaneous drop in viscosity which is proportional to the concentration of enzyme protein added, but there is no enzymatic depolymerization, for the value of the viscosity observed at every level of the protein concentration remains absolutely constant from the time of mixing to the end of a period of many hours.

The presence of a heat-labile depolymerase in dog and rabbit sera for thymus-type nucleates has recently been confirmed by Avery, *et al.* (74a). These authors further observed that the enzyme could be inhibited by fluoride.

The effect of the inert protein on the physical state of the nucleate is in turn dependent upon the native state of the former. When this native configuration is altered, as by heating at 65°C. for about 15 hours, the capacity of the proteins to effect the changes in the nucleate very sharply diminishes (Curves III and VI in Fig. 4).

Solutions of denatured protein have very little effect on the physical

properties of thymus nucleate in contrast with solutions of the same proteins at the same concentration in the native state. In order to give a curve identical with that of VI in Fig. 4, a solution of crystalline horse serum albumin had to be preheated at 65°C. not for 15 hours which sufficed for horse serum, but for about 60 hours, before mixing with the nucleate (27). As far as this property is concerned, the complex of proteins in the

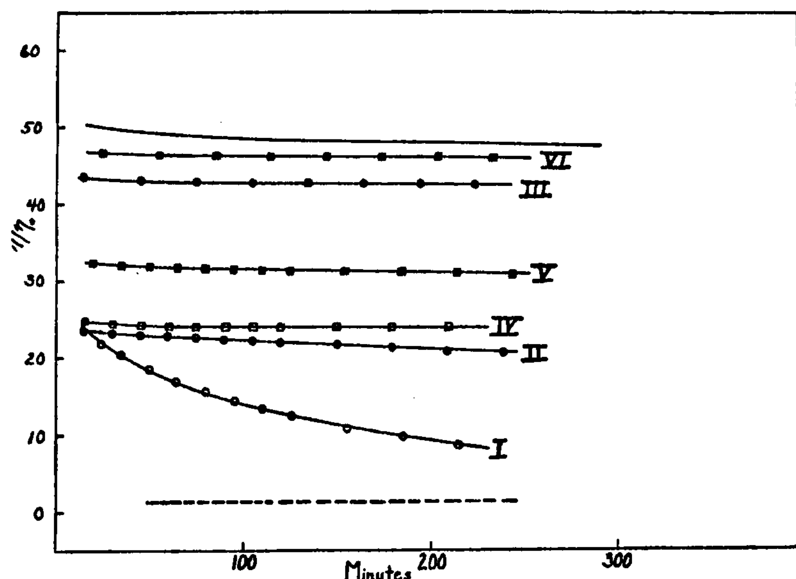


Fig. 4. The effect of adding equal volumes of horse and of rabbit serum (both containing 1.23 mg. protein N per cc.) to solutions of 1.0 per cent sodium thymus nucleate. Ordinate relative viscosity, abscissa time of incubation at 30°C. External pressure of 16.0 cm. water. Serum preheated at 65°C. for designated periods of time before mixing with nucleate solutions.

Curves: I, normal rabbit serum, II, rabbit serum preheated 5 hours, III, rabbit serum preheated 15 hours, IV, normal horse serum, V, horse serum preheated 5 hours, VI, horse serum preheated 15 hours.

Upper continuous curve represents 0.5 per cent solution of nucleate in water. Lower dotted curve represents sera diluted with equal volume of water. The effect of heating on these latter controls was to raise the viscosity so slightly as to be imperceptible on the graph. From Greenstein, J. P., and Jenrette, W. V., *Cold Spring Harbor Symposia Quant. Biol.* 9, 236 (1941).

serum is more readily denatured than the purified individual member of the complex. The theory of the denaturation of proteins is outside the scope of this review but it may be succintly stated that this process is accompanied by a loss in the unique configuration of the native protein molecule without the breaking of primary linkages (75).

The fact that denatured proteins fail to interact at least visibly with the

nucleate represents the converse of what happens when it is desired to isolate nucleic acid from protein in the nucleoproteins—the latter are denatured and the components then separate. The denaturation may be effected among other ways by heat (23), or by the use of neutral salts (76, 77). The reduction of the properties of structural viscosity and birefringence of the nucleate by salt or by protein is reversible. Thus when a highly viscous solution of thymus nucleate is mixed with protein or salt, the viscosity and birefringence of the former are reduced, but subsequent removal of the protein by denaturation or salt precipitation or of the salt by dialysis, restores these properties to a considerable degree (27). The liver nucleoprotein is globular in shape, and its solutions do not exhibit double refraction of flow nor structural viscosity. When it is denatured however, the nucleic acid split from it is highly viscous and doubly refracting (35). It is possible to speculate, therefore, on the rapid formation and breakdown *in vivo* of nucleoproteins induced by alternating and partly reversible native and denatured states of the tissue proteins concerned.

That inorganic, neutral salts would reduce the viscosity of sodium thymus nucleate was first observed by Hammarsten (12) who ascribed the effect to a diminution in the hydration of the particles of this substance. Later, when the shape of these particles was better apprehended, Wissler (46) and Greenstein and Jenrette (27) demonstrated that the intensity of the streaming birefringence of solutions of the nucleate was diminished by addition of salt, indicating that the effect of the latter was principally upon the degree of asymmetry of the particles. The latter authors (27) further showed that not only the viscosity but also the structural character of the viscosity function was reduced, and that the magnitude of the effect upon both the structural viscosity and the streaming birefringence was proportional to both the concentration and the nature of the salt employed. Thus at equivalent concentrations, the effect of the halides with a common cation was increasingly greater from chloride through bromide to iodide. The most effective cation was found to be the guanidinium ion and the striking parallelism of the effect of salts of this ion on the physical properties of the nucleate and on the denaturation of proteins is worthy of emphasis (27). In this connection it might be pointed out that the guanidinium ion is very wide-spread in nature, and as part of the amino acid arginine is present in nearly all proteins and particularly in the arginine-rich protamines and histones of certain cell nuclei. Amino acids produce effects upon the specific physical properties of the thymus nucleate in much the same way as inorganic salts, and in this case too the magnitude of the effect varies according to the concentration as well as the nature of the amino acid employed.

The conclusion of Edsall and Mehl (78) in respect to the parallel effect of the same reagents on the viscosity and on the streaming birefringence of myosin has been confirmed by the reviewer in the case of the thymus nucleate. It is indeed curious that the highly asymmetric protein and nucleic acid should behave so similarly toward the same inorganic and organic reagents.

In Table II, data on the effect of various substances on the viscosity of thymus nucleate at a fixed external pressure are collected. The measurements were performed on the same sample of nucleate under identical external conditions (27).

The most striking data in Table II refer to the relatively large effect which the proteins exert in the native state upon the specific properties of the thymus nucleate, an effect far exceeding that induced by simple salts. When largely denatured, the proteins exert an effect little different from that produced by an equivalent amount of glycine. Among the salts, those which contain the guanidinium cation or the iodide anion are particularly effective, and indeed the most effective salt found in reducing the specific properties of thymus nucleate is guanidine hydroiodide (27). The viscosity of the nucleate under the conditions described in Table II is a little over 50 in value, that of the various substances tested is little more than 1 in value; hence the changes observed in the mixtures can be measured with some degree of ease and accuracy.

The value for the viscosity of a nucleate-protein mixture, for any ratio of nucleate to protein, is between that of the protein and nucleate considered separately. In mixtures where the ratio of nucleate to protein is low, *e.g.*, 0.1 to 2.8 per cent respectively, addition of 8 *M* guanidine hydrochloride results in a marked increase in the viscosity of the mixture (which is still less than that of the nucleate alone), for the effect of the salt on the protein predominates over that on the nucleate. On the other hand, in mixtures where the ratio of nucleate to protein is higher than the above, *e.g.*, 0.5 to 2.8 per cent respectively, addition of the guanidine salt results in a decrease in the viscosity of the mixture, for in this case the effect of the salt on the nucleate predominates over that on the protein. Apparently in such mixtures each component acts as a separate entity, and the net effect is close to the algebraic sum of each effect considered separately.

The effect of all the agents described in Table II is produced without any measurable disintegration of the thymus nucleate molecule. No ammonia or inorganic phosphorus is evident in the mixtures. Furthermore, no products are formed which are capable of dialyzing through a cellophane membrane. The decrease in the structural viscosity and birefringence is a reflection of a corresponding decrease in the molecular asymmetry of the thymus nucleate. The loss in asymmetry can in turn be produced by a depolymerization of the thymus nucleate into smaller, more symmetrical particles similar to the interpretation given by Edsall and Mehl (78) for the effect of denaturing salts on myosin. This explanation has been hitherto adopted for the case of the thymus nucleate. However, it must be realized that the smaller particles need not necessarily remain as such but may subsequently combine into larger, presumably still somewhat symmetrical aggregates. To some extent this occurs to nucleate treated with acid or alkali (20).

TABLE II

The Effect of Various Substances on the Relative Viscosity of Solutions of Sodium Thymus Nucleate

Viscosity readings at 16 cm. H_2O external pressure and 30°C. Final concentration of nucleate 0.5%. Readings taken 15 minutes after mixing remained practically constant over a 4 hour period (27).

Mixtures (Equal volumes of reactants)	Relative Viscosity	Intensity of Streaming Birefringence
1 per cent Sodium thymus nucleate + H_2O	51.23	++++
" " " " + 2×10^{-5} *M* Guanidine hydrochloride	43.36	+++
" " " " + 2×10^{-3} *M* Guanidine hydrochloride	24.06	++
" " " " + 2×10^{-1} *M* Guanidine hydrochloride	18.12	+
" " " " + 2 *M* Guanidine hydrochloride	16.34	+
" " " " + 4 *M* Guanidine hydrochloride	14.83	+
" " " " + 8 *M* Guanidine hydrochloride	10.67	−
" " " " + 4 *M* Urea	20.32	+
" " " " + 8 *M* Urea	17.22	+
" " " " + 5×10^{-1} *M* NaCl	31.89	++
" " " " + 1 *M* NaCl	20.60	+
" " " " + 4 *M* NaCl	18.96	+
" " " " + 1 *M* NaBr	19.32	+
" " " " + 4 *M* NaBr	18.00	+
" " " " + 1 *M* NaI	16.22	+
" " " " + 4 *M* NaI	13.99	+
" " " " + 5×10^{-1} *M* Arginine hydrochloride	20.00	+
" " " " + 5×10^{-1} *M* Lysine hydrochloride	24.10	+
" " " " + 2×10^{-4} *M* Glycine	43.65	+++
" " " " + 5×10^{-1} *M* Glycine	37.22	++
" " " " + 5×10^{-2} *M* Histidine	31.38	++
" " " " + 1.6% egg albumin (app. 4×10^{-4} *M*)[1]	13.21	+
" " " " + 1.6% horse serum albumin (app. 2×10^{-4} *M*)[1]	21.15	+
" " " " + 1.6% horse serum albumin preheated for 60 hrs. at 65°C.	47.62	+++
" " " " + horse serum (1.6% protein)	22.05	+
" " " " + horse serum (1.6% protein)[2] preheated for 5 hrs. at 65°C.	32.65	++
" " " " + horse serum (1.6% protein)[2] preheated for 15 hrs. at 65°C.	47.48	+++

[1] The molecular weights of egg albumin and serum albumin are assumed to be 40,000 and 80,000 respectively.

[2] The viscosity of the serum itself increased from 1.18 to 1.23 after 5 hours heating, and to 1.42 after 15 hours heating; the change in the protein viscosity is thus negligible compared with that of the nucleate.

4. *Osmotic Pressure of Mixtures of Thymus Nucleate with Proteins*

Further evidence for the interaction between proteins and thymus nucleate as well as some implications of the state of aggregation of the latter in such mixture have been obtained by measurements of the osmotic pressure. In the absence of interaction between nucleate and protein, the osmotic pressure of the mixture should of course be additive. However, the nucleate in the presence of protein proved to be practically osmotically ineffective (79). The data are given in Table III.

TABLE III

Colloid Osmotic Pressure of Mixtures of Sodium Thymus Nucleate with Protein[1,2] (79)

Temperature 23°C. ± 0.5

Mixture	Protein: nucleate ratio	Colloid osmotic pressure
	Milligrams per milligram	*Millimeters H_2O*
Undiluted horse serum (5.6 per cent protein)	—	281 ± 3
2.0 cc. serum + 0.5 cc. H_2O	—	218 ± 4
2.0 cc. 0.9 per cent NaCl + 0.5 cc. 1 per cent nucleate	—	24 ± 1
2.0 cc. serum + 0.5 cc. 1 per cent nucleate	22.4:1	214 ± 4
2.0 cc. serum + 1.0 cc. H_2O	—	181 ± 3
2.0 cc. 0.9 per cent NaCl + 1.0 cc. 1 per cent nucleate	—	46 ± 2
2.0 cc. serum + 1.0 cc. 1 per cent nucleate	11.2:1	185 ± 4
2.0 cc. serum + 2.0 cc. H_2O	—	144 ± 3
2.0 cc. 0.9 per cent NaCl + 2.0 cc. 1 per cent nucleate	—	71 ± 1
2.0 cc. serum + 2.0 cc. 1 per cent nucleate	5.6:1	145 ± 5
2.0 cc. serum + 4.0 cc. H_2O	—	103 ± 2
2.0 cc. 0.9 per cent NaCl + 4.0 cc. 1 per cent nucleate	—	91 ± 2
2.0 cc. serum + 4.0 cc. 1 per cent nucleate	2.8:1	111 ± 5
2.0 cc. serum + 8.0 cc. H_2O	—	56 ± 1
2.0 cc. 0.9 per cent NaCl + 8.0 cc. 1 per cent nucleate	—	121 ± 2
2.0 cc. serum + 8.0 cc. 1 per cent nucleate	1.4:1	85 ± 2
Undiluted horse serum albumin (4.0 per cent)	—	172 ± 4
2.0 cc. serum albumin + 1.0 cc. H_2O	—	114 ± 2
2.0 cc. 0.9 per cent NaCl + 1.0 cc. 1 per cent nucleate	—	46 ± 2
2.0 cc. serum albumin + 1.0 cc. 1 per cent nucleate	8.0:1	117 ± 3
2.0 cc. serum albumin + 2.0 cc. H_2O	—	86 ± 5
2.0 cc. 0.9 per cent NaCl + 2.0 cc. 1 per cent nucleate	—	71 ± 1
2.0 cc. serum albumin + 2.0 cc. 1 per cent nucleate	4.0:1	86 ± 2
2.0 cc. serum albumin + 4.0 cc. H_2O	—	63 ± 2
2.0 cc. 0.9 per cent NaCl + 4.0 cc. 1 per cent nucleate	—	91 ± 2
2.0 cc. serum albumin + 4.0 cc. 1 per cent nucleate	2.0:1	69 ± 3

[1] The outside solutions were made up in the same salt concentrations as the inside solutions before the latter were tested.

[2] All readings were made after 60 minutes of equilibration in the osmometer.

Solutions of protein and of thymus nucleate each exert a definite osmotic pressure, but mixtures of the two, except when the ratio of nucleate to protein is very high, exert an osmotic pressure which is practically the same as that of the protein alone. Since there is no evidence of passage of any product of the nucleate through the membrane, this substance simply loses its osmotic activity, as well as its extreme asymmetric shape, when it is mixed with protein. The osmotic pressure exerted by sodium thymus nucleate in aqueous sodium chloride solution is that due to the unequal distribution of the salt across the membrane, and indeed Hammarsten showed that an equilibrium of this kind can be partially explained on the basis of the classic Donnan distribution (12). The position of such an equilibrium will in turn be dependent upon the degree of dissociation of the non-diffusible sodium thymus nucleate. If the latter is totally dissociated, then at any one concentration of nucleate and sodium chloride, the osmotic pressure will have a maximum value. If the thymus nucleate is totally undissociated, the concentration of salt is the same on both sides of the membrane, and the osmotic pressure will be that due to the non-ionic nucleate; since the latter has a very high molecular weight, the osmotic pressure will be at a minimum value. It is possible therefore to interpret the loss in osmotic activity of thymus nucleate in the presence of protein as due to a conversion of the former into an undissociated or nearly undissociated form. The term "undissociated" does not imply that the phosphate groups in the nucleate are not fully ionized when protein is present; it means that the sodium ions of the thymus nucleate are so closely associated with the relatively very large, polyvalent nucleate ion that they are osmotically ineffective. The electrical forces of attraction may be expected to be considerable in the neighborhood of the large polyvalent ions, and indeed Hammarsten (12) and Kern (80) respectively demonstrated that thymus nucleate and the polyacrylic acids under certain conditions could render smaller ions osmotically ineffective. Hammarsten actually pictured the sodium ions under these conditions as "hiding" within the framework of the giant thymonucleate ion. This phenomenon, often referred to as the Hammarsten effect, has been described by Kern as an "osmotic buffering," in analogy with the familiar buffering of weak acids and salts.

A further exemplification of these ideas may be drawn from the data of Stenhagen and Teorell (81) who studied the electrophoretic behavior of a mixture of serum albumin and thymus nucleate in the weight ratio of about 1.6:1. At neutral pH, part of the nucleate migrated with the protein at close to the mobility of the latter, part migrated independently with a mobility close to that of pure nucleate. At about this weight ratio (Table III), the osmotic pressure of the mixture was intermediate in value between that of the components taken separately, *e.g.*, part of the nucleate was unaffected; at higher ratios of protein to nucleate the osmotic pressure of the nucleate disappeared. For complete interaction between the protein and nucleate,

the former must be in excess. The results of Stenhagen and Teorell may be tentatively interpreted on the basis that part of the total nucleate was unaffected by the protein; this part migrated independently with the mobility of the pure component in buffer alone. That part which was affected by the protein migrated with the latter at very close to the mobility shown by the pure protein in buffer alone. The fraction of the nucleate which migrated with the protein must have borne a net electrical charge either close to zero or close to that of the protein. In either case, there must be a marked decrease in the degree of dissociation of the nucleate in the presence of protein and this is in general agreement with the explanation advanced for the results of the osmotic pressure measurements of such mixtures described.

Electrophoretic patterns of mixtures of yeast nucleic acid and egg albumin reveal that under certain conditions a reversibly-dissociable complex between these components can occur (82). The formation of such a complex is favored by low concentrations of salt and by a pH of the medium lower than that of the isoelectric point of the protein. This finding is in agreement with observations made by v. Przylecki and Grynberg (72) and by Hammarsten and Hammarsten (71) on the factors involved in the precipitation of such complexes (see above). The dissolution of such complexes at high pH values is illustrated in the separation of tuberculin protein from nucleic acid by subsequent electrophoresis (83).

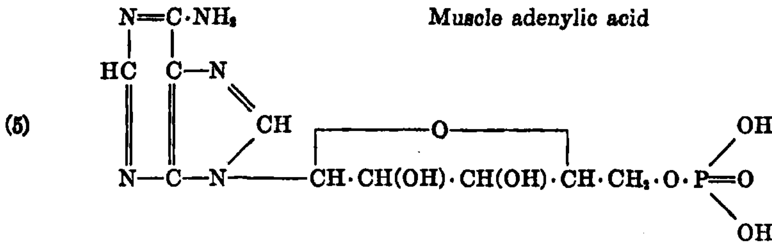
While the physical properties of thymus nucleate are greatly affected by the presence of native proteins, what happens to the protein in the mixtures? There is little evidence available to answer this question, although the data of the osmotic pressure measurements (Table III) indicate that the osmotic properties of the protein are unaffected, and the electrophoresis data reveal that the mobility of the protein, and hence its charge distribution, is relatively little affected. When the protein is specifically related to a nucleotide, as in the case of myosin and adenosinetriphosphate, definite physical changes in the protein molecule become evident as a result of the interaction (see below), but this may be a special case. When the protein is of an unspecific character it is very probably unaffected by the interaction with nucleate. The protein in this case can have only one native configuration; the nucleic acid is the labile component. The denaturation studies (Table II) indicate that the structure of the protein is probably more important than its constitution. If the number and kind of charged groups were the sole governing factors in the interaction of protein and nucleic acid then the native and denatured forms of the same protein should produce identical effects. It seems more probable that it is the distribution of these charged groups which is of primary importance. Denaturation of a protein changes the distribution of these groups, perhaps by unfolding (84). In the native protein a situation exists, at least in the present instance, in which the whole is greater than the sum of its parts.

In view of the evidence presented above, it is possible to hypothesize on the original problem which opened the section, namely, that concerned with the liberation from nucleoproteins of asymmetric nucleic acid the dimen-

sions of which were greater than those of the nucleoprotein. The nucleic acid presumably exists in this combination as aggregates of partially depolymerized particles which resume their highly asymmetric character when the protein is denatured and the nucleic acid is liberated.

IV. Free Nucleotides and Quasi-Nucleotideproteins

It is a matter of considerable biological interest that the nucleotides found in the nucleic acids have their counterparts rather wide-spread in nature. Indeed, the discovery of the nucleotide, inosinic acid by Liebig in 1847 (85) antedated the discovery of the nucleic acids, although the structure of the former substance was not determined until 1895 (86). Inosinic acid contains hypoxanthine, *d*-ribose, and phosphoric acid and is the deaminated form of muscle adenylic acid (87, 88, 88a).

(5) 

In this substance, in contrast with the nucleotide present in yeast nucleic acid, the phosphoric acid is esterified at the fifth carbon atom of the sugar (2). Closely related to muscle adenylic acid is adenosine-triphosphate (89, 90) in which adenylic acid is combined with two more molecules of phosphoric acid to form a structure the details of which are not yet settled. Adenylic acid and adenosine-triphosphate participate in the phosphate exchange during muscular activity and yeast fermentation (90, 91, 92).

The identification of the purified muscle protein, myosin, with the enzyme system which dephosphorylates adenosine-triphosphate to the diphosphate has been advanced by several investigators (93, 94, 95, 96, 97), and is discussed in more detail by Bailey elsewhere in this volume (p. 289). This enzymatic process, apparently induced by the contractile elements of muscle, liberates the energy for the physical changes in length of these elements. There is in muscle tissue therefore the case of a specific nucleotide which by interaction with a specific protein results in a catalytic and reversible change in both nucleotide and protein. That the nucleotides may also be associated with specific proteins in yeast during fermentation appears to be probable (98). The rôle of the adenine nucleotides as prosthetic groups of protein enzymes in fermentation reactions in animal tissues is illustrated by the work of the Cori group (98a, 98b, *cf.* also 98c).

Conjugated proteins with quasi-nucleotide prosthetic groups are found among the catalytic oxidation-reduction systems. The first representative of this class of substances to be isolated was the yellow enzyme (99, 100). The prosthetic group, riboflavin phosphoric acid, could be removed from the protein and subsequently reconstituted by simple combination of the two components (100). Riboflavin phosphate linked with adenylic acid forms the prosthetic group of specific proteins which comprise the enzyme systems, *d*-amino acid oxidase (101) and xanthine oxidase (102). In the oxidized form, the alloxazine prosthetic group is yellow in color; when reduced.

it is colorless. The reduction takes place by the addition of hydrogen to the alloxazine ring. Under physiological conditions, this hydrogen is contributed by reduced pyridine nucleotides which are the prosthetic groups of specific proteins (103). At the present time, two pyridine nucleotides are recognized, *e.g.*, di- and triphosphopyridine nucleotides. The essential oxidation-reduction component in these structures is nicotinic acid amide (103).

The diphosphopyridine nucleotide has the same structure as the above triphosphoderivative except that the former has one less phosphate residue. Both nucleotides are the prosthetic groups of specific proteins, and the catalytic effects observed, as in the case of the alloxazine nucleotide proteins, are dependent upon the conjugate as a whole (103). The nicotinic acid amide component of the conjugated proteins takes up hydrogen from certain substrates, thereby oxidizing the latter, and then transfers the hydrogen to the alloxazine proteins, which in turn, in the case of aerobic organisms, are oxidized by the cytochrome-cytochrome-oxidase system. The alloxazine and pyridine nucleotide proteins are thus readily reversible oxidation-reduction systems.

The conjugated proteins briefly considered in this section are not nucleoproteins in the sense in which the latter have been defined in this review, for the prosthetic groups of the proteins in the former case are not polymerized tetranucleotides but simple nucleotides or quasi-nucleotides. Nevertheless, they illustrate the range of biological activity which substances of a basic nucleotide structure may possess. Finally, it may be briefly mentioned that the alloxazine and pyridine components of these nucleotide prosthetic groups are dietary essentials for the higher animals, and are classed among the vitamins of the B group, the former under the name of riboflavin and the latter under the name of niacin.

V. The Simpler Proteins in Natural Association with Nucleic Acid

There have been considered up to now the general characteristics of the nucleic acids and related substances and the interaction of the former *in vitro* with proteins. In the present and following sections the emphasis will be laid on the characterization of the protein component of the naturally-occurring nucleoproteins for which data are available. There is a great diversity in size and composition among these protein components, ranging from relatively simple polypeptide structures with few amino acids contained therein to quite complex molecules of high particle weight and diversified amino acid composition.

1. *Protamines*

The protamines have been isolated from the sperm and ripe testicles of fish in which they exist in the form of salts with the thymus-type of nucleic acid (1, 104, 105). The characteristics of the protamines include (a) a relatively low molecular weight, ranging in the thousands (70, 44, 106, 107); (b) a highly basic reaction due to the inclusion of a predominating proportion of one or more of the basic amino acids, arginine, histidine, and lysine (70); (c) an extended structure in which the amino acids are united by peptide bonds (106); (d) the capacity to combine not only with nucleic

acid, but with certain of the higher, acidic proteins as well, in order to form relatively insoluble complexes (70, 108, 109); and (e) the property of splitting certain proteins into smaller fragments (110).

In different fish, the predominating basic amino acids are present in different amounts, and Kossel (70) based a method of classification upon this fact. In one group of protamines, arginine is the only kind of basic amino acid present, in another protamine group arginine plus either histidine or lysine are present, and in the third group all three basic amino acids are present. Arginine is the only basic amino acid present in all classes of the protamines. Indeed it is the most ubiquitous amino acid in nature for no member of the class of proteins, whether in animals or plants, is lacking in it. Only in the protamines, however, is it found in such high proportions. The group in arginine which confers upon the amino acid its strongly basic character is derived from guanidine. The guanidinium group in arginine has a dissociation constant at pH 12.5, and hence under physiological conditions this group will always be positively charged (111). In the protamines this group, like those of histidine (imidazole) and of lysine (ϵ-amino), is free and uncombined. The linkages between all of the amino acids is of a peptide nature because they are all dissolved through the action of mild hydrolytic agents and of enzymes, whereby amino (or imino) and carboxyl groups are liberated in equivalent amounts. The combination of the protamines with acidic higher proteins is, like their combination with nucleic acid, of a salt-like nature; the practical value of the former complex is illustrated in contemporary medical practice by the use of protamine-insulin in the treatment of diabetes. Fibrinogen may be removed from serum by adding protamine to the latter (111a).

Besides the presence of one or more of the basic amino acids, the protamines are composed of the monoamino-monocarboxylic acids; cystine, methionine, tryptophan, and dicarboxylic acids are lacking, and in only one member of the protamine group has the presence of tyrosine been claimed (70). Of the protamines, clupeine from herring, and salmine from salmon sperm, have been most completely studied. The amino acid composition of these substances is quite simple, for on complete hydrolysis there appear to be two molecules of arginine for every one molecule of mono-amino-monocarboxylic acid. No basic amino acid other than arginine is present. But far more important than the evidence for a relatively simple composition of these materials is the concept, due to Kossel, that the constituent amino acids within the protamine molecule are not arranged at random but possess a definite sequence along the polypeptide chain (70). The importance of this concept cannot be over-estimated, for it suggests that even in these primitive forms of protein molecules, a certain molecular pattern is followed. Kossel pictured the clupeine molecule to be arranged

in something like the following, in which A represents arginine and M represents any monoamino acid:

(6) AAMAAMAAM, etc.

Kossel's brilliant concept was based on relatively little experimental evidence and it remained for the investigations of Waldschmidt-Leitz and of Felix to rest it on firmer ground. Waldschmidt-Leitz deduced the structure of clupeine from the action of specific enzymes (106). Clupeine contains 15 amino acids of which 10 are arginine; of the 5 remaining acids one is proline, and there are at least one each of alanine, serine, and valine. Protaminase splits off two terminal arginine residues, yielding clupeine. Activated trypsin then attacks the latter, yielding two dipeptides and three tripeptides, the constitution of which was ascertained by the specific action of dipeptidase on the dipeptides, and of aminopolypeptidase and carboxypeptidase on the tripeptides. The results indicated that the two dipeptides had the structures AM and MA, and that the three tripeptides had the structures AMA, AMA, and APA, where A refers to arginine, M to monoamino acid, and P to proline residues respectively. Knowing the constitution of the split products, that of the original molecule may be inferred.

Clupeine (according to Waldschmidt-Leitz, 106)

(7) MAAMAAMAAPAAMAA

(where the position of proline, although known to be somewhere in the middle of the chain, is arbitrarily selected). Partial confirmation of the above structure has come from the isolation of arginyl-arginine (*i.e.*, AA) from incomplete hydrolyzates of clupeine by Felix (112). The molecular weight of clupeine so depicted is about 2000. The titrimetric studies on clupeine by Linderstrom-Lang yield a value for the molecular weight of about 4000 (107). It is possible therefore that two such chains as in formula 7 exist in the clupeine molecule. The enzymatic studies of Waldschmidt-Leitz suggested that the peptide linkages which are attacked by trypsin involve arginine. This was the first suggestion of the point of attack by this enzyme on proteins. The recent work of Bergmann and his collaborators has definitely decided this point for they have found that crystalline trypsin exerts a maximum effect on synthetic peptides involving arginine (113). The basic idea of a regular sequence of the amino acid pattern formulated above for clupeine has been greatly extended for the cases of the more complex proteins, such as egg albumin and hemoglobin, by Bergmann and Niemann (114). The latter proposed the concept of a periodicity or regular occurrence of certain amino residues along the protein chain, and suggested that such occurrence constituted a fundamental aspect of protein structure. Further consideration of this attractive concept is

outside the scope of the present review (compare Astbury, 115, and Chibnall, 116, for extended discussion from somewhat different points of view).

Serum proteins are split into smaller, well-defined fragments by the action of clupeine (110). This reaction may be attributed in great part to the content of this protamine in arginine, for the free amino acid (monohydrochloride) and guanidine salts also can produce this effect. Guanidine salts and arginine likewise are powerful agents in reducing the structural viscosity and streaming birefringence of myosin, and this has been interpreted by Edsall and Mehl (78) as a disaggregation of the highly asymmetric protein into smaller, more symmetrical particles. The profound effect of guanidine salts in disaggregating the virus proteins into smaller particles (76, 77) and in liberating sulfhydryl groups in many kinds of proteins (117, 118, 119) has recently been emphasized. The effect of such salts on the physical properties of nucleic acid has been described above (Table II). The reason for the remarkable properties of the guanidinium ion is not obvious at the present time. It may be pointed out however that this ion is capable of existing in several resonating structures and that those substitutions in this ion which reduce the possibilities of resonance also reduce its capacity to produce the denaturing effects noted (120, 121). The fact that such alterations in molecular aggregation may occur when protamine or related salts are mixed with higher proteins makes these interactions a matter of considerable significance for biological systems. Where these components exist together, an appreciable lability in molecular size among the higher protein components may be expected.

The naturally-occurring defatted complex of protamine and nucleic acid in the sperm head can be dissolved by either mineral acid or by sodium chloride solutions (1, pp. 66, 74) and a metathetical rearrangement takes place with separation of the components. The effect of the mineral acid may be likened to the displacement of a weak acid in a salt by a stronger. When the complex is treated with NaCl it swells and goes into solution and on treatment with much water the protamine largely precipitates while the nucleic acid to some extent remains in solution. The ionic exchanges under these conditions were first described by Miescher whose words on this subject have been quoted in the Foreword. So far no substances of a protamine nature have been found in sources other than fish sperm. In cells of more highly organized tissues, the protein in association with nucleic acid appears to be of a more complex structure and diversified amino acid composition, although of course the presence of protamine or protamine-like substances cannot be excluded simply because they have not yet been found.

2. *The Histones*

The histones form a class of proteins which in point of complexity, stand between the simple protamines, on the one hand, and the higher proteins, on the other. The histones are larger than the protamines in size and contain a greater assortment of amino acids. However, they resemble the

protamines in containing a high proportion of the basic amino acids, in particular arginine (70). It is not, however, the absolute amount of arginine in the histones which is necessarily characteristic of this class of proteins, for many other kinds of protein have as high a content of arginine (edestin for one, 116). It is rather the relative proportion of the total basic amino acids to the total dicarboxylic amino acids which is large, and it is this factor which confers upon the histones their marked basic character. In general, the histones may be divided at the present time into two classes, of which one is found in salt-like combination with nucleic acid, and the other in some as yet unknown combination with the heme pigments. The best known representative of the latter class of the conjugated histones is hemoglobin.

The nucleohistones, salts of histone and the thymus-type of nucleic acid, were first observed in the avian erythrocyte by Kossel (70) who was also responsible for much of the later study of this class of proteins. Similar preparations were later isolated from the ripe sperm of certain animals (70) and from the thymus gland (122, 123, 124). The material obtained from the latter tissue has been most extensively studied and may indeed serve as a model for this class of substances. The nucleohistones may be extracted from the tissues with either water or moderately-concentrated salt solutions (about 0.1 M) (122) and may be precipitated from the extract by addition of acid to a pH of 3–4 or as the calcium salt by addition of calcium chloride (12). These precipitates may be redissolved in moderately-concentrated salt solutions (123). When sodium chloride is added up to saturation of these solutions, the latter become increasingly viscous (66, 123) as the sodium thymus nucleate separates from the protein; the latter precipitates from solution when the solution is finally saturated with salt. The compounds of nucleic acid with protamines and with histones are thus equally separable through the agency of neutral electrolyte, metathesis takes place, and the nature of the salt linkage in both compounds is probably identical. On a weight basis, both compounds are made up in approximately equal measure of histone or protamine and nucleic acid (66, 105). The thymus nucleohistone has an acid isoelectric point which is revealed by measurements of the minimum solubility and by mobility to be near pH 4 (70, 67).

The analytical data on the thymus histone itself are incomplete, barely more than half the molecule being accounted for in terms of amino acids. Table IV lists the composition of thymus histone; the values given are minimal (70, 125).

The calf thymus nucleohistone possesses a molecular weight of about 2×10^6, and the shape of the molecule is asymmetric (68). The axial

ratio, *i.e.*, the ratio of long to short axis, is, from viscosity measurements about 36:1. As might be expected from such an anisotropic material there is considerable particle interaction, and the sedimentation constant increases with dilution (68). Half of the nucleohistone molecule is composed of nucleic acid, and the problem of fitting the much more asymmetric nucleic acid particles within the nucleohistone has been considered in detail above.

New methods of obtaining nucleohistones have been nearly simultaneously announced by Mirsky (105) and by Bensley (126). The technique depends upon extraction of the tissue with about 1 *M* sodium chloride, followed by precipitation of the nucleohistone in fibrous form by dilution with water. The recognition that the products obtained are rich in nucleic

TABLE IV

Amino Acid Composition of Thymus Histone (70, 125)

Amino Acids	Per Cent
Glycine	0.5
Alanine	3.5
Leucine fraction	11.8
Proline	1.5
Phenylalanine	2.2
Tyrosine	5.2
Tryptophan	1.1
Histidine	1.5
Arginine	15.5
Lysine	7.7
Glutamic Acid	3.7
(Sulfur)*	(0.62)*

* Dubious.

acid is quite recent. Nucleic acid of the thymus-type forms 31–60 per cent of the products obtained, and the absence of tryptophan from every preparation studied is reported (105). The relatively mild methods employed for the extraction and separation of these nucleoproteins are worthy of emphasis.

The transformation of the complex muscle proteins of the spawning salmon into the simple protamines of the ripe testis, first noted by Miescher, probably proceeds by way of the intermediate histone stages which are found in the unripe testis (70). Such a transformation, described in general terms for the nuclear development of animal cells by Kossel (70), has received a certain measure of support and amplification in recent studies which will be discussed below.

VI. The More Complex Proteins in Natural Association with Nucleic Acid

In contrast with the salts of nucleic acid with protamine or with histone, the binding of nucleic acid with more complex proteins in the nucleoproteins is of a firmer nature and is associated with the native configuration of the protein. High inorganic salt concentrations suffice to separate the components of the former type; to separate the components of the latter type denaturation resulting in loss of the specific configuration of the protein is necessary. The amino acids within all protein molecules are bound by strong peptide bonds to form long, folded, polypeptide chains. The chains in turn are bound to each other by relatively weak forces which nevertheless may be considered to be responsible for the unique native configuration of the protein molecule. The action of denaturing agents such as urea and the guanidine salts upon the native protein may be assumed to involve the dissolution of the labile linkages between the peptide chains leading to an unfolding and disorientation of the protein. In certain proteins such a reaction involves a disaggregation of the native protein in addition to the disorientation; in conjugated proteins, liberation of the prosthetic group from the protein would be simultaneously effected. The type of nucleoprotein considered in the present section involves protein components of a complex nature, containing a wide assortment of amino acids including cystine and methionine. Many nucleoproteins of this kind have been isolated but only the proteins of liver and the virus nucleoproteins have been characterized to any large extent.

1. *Liver Nucleoprotein*

A nucleoprotein fraction of mammalian liver has been isolated from several species by an isoelectric precipitation in the presence of salt (35). After two successive precipitations and washings, the ratio of N:P became constant. The proteins so isolated were more or less globular in shape. They contained about 15–20 per cent fat of which 30–40 per cent consisted of phospholipid. The protein made up about 5 per cent of the total protein content of the liver. From the proteins so isolated, a nucleic acid was separated by treatment with alkali which appeared to be similar to thymus nucleic acid, for besides giving the specific desoxypentose reactions it conferred upon its aqueous solutions the properties of structural viscosity and intense streaming birefringence. Thus from a nucleoprotein essentially globular in shape, demonstrating no asymmetric shape factors whatever, a component was isolated which was highly asymmetric. The nucleic acid was about 5% of the total weight of the nucleoprotein. The liver nucleoproteins so isolated appear to consist of a nucleo-lipoprotein complex. The presence of desoxyribosenucleic acid in such complexes strongly suggests

the nuclear origin of the latter. Similar complexes of lipoid with nucleoprotein of the ribose type however have been isolated by Claude from a variety of animal tissue (127), and these appear to stem from the cytoplasm. The vaccinia virus (128), the virus of equine encephalomyelitis and of the rabbit papilloma (129) and the chicken tumor virus (127, 130, 131) appear likewise to be complexes of nucleo-lipoproteins. In the case of the liver nucleoproteins, part of the fat is removable with cold ether, the remainder is removable only after boiling with absolute alcohol.

The analysis of the various preparations for certain elements and amino acids reveal that these constituents are, with one exception, very nearly identical for several species. The exception is concerned with the content

TABLE V

Analytical Values for Fat-Free Liver Nucleoprotein Preparations (35, 132)

Source	N	Amide N	P	Total S	Free SH in native Protein as Cysteine	Free SH in denatured Protein as Cysteine	Cystine-Cysteine	Methionine	Tyrosine	Tryptophan
	%	%	%	%	%	%	%	%	%	%
Rabbit	15.6	1.0	0.8	1.0	1.3	1.3	1.3	3.0	3.8	1.3
"	"	"	"	1.2	1.4	1.4	"	3.1	3.9	"
"	"	0.9	"	"	"	"	1.4	2.9	4.0	1.5
"	15.7	1.0	"	1.1	1.2	1.2	1.5	3.1	"	"
Calf	15.6	"	"	"	0.7	0.7	1.4	3.2	3.9	1.4
"	15.7	"	"	"	"	"	"	3.1	"	1.5
Cow	15.8	"	0.9	1.2	0.6	0.6	1.5	"	3.7	1.7
Rat	15.6	0.9	"	"	0.2	0.2	1.3	3.0	3.8	1.5
"	15.7	1.0	0.8	"	"	"	1.4	3.1	"	"
Transplanted Rat Hepatoma	15.5	0.9	0.7	1.1	"	"	"	2.9	3.6	"

of titratable sulfhydryl groups. The analytical data are given in Tables V and VI (35, 132).

The nucleoproteins of the livers of the various species are differentiated on the basis of their content of free sulfhydryl groups (Table V). Evidently all of the sulfhydryl groups exist in the free state in the native protein for no additional groups are revealed by denaturation. The rabbit liver nucleoprotein has the highest content of free sulfhydryl groups which when estimated as cysteine is equal to the value found by the Baernstein procedure for cystine-cysteine (Table V). Evidently the latter quantity refers in the rabbit-preparation only to cysteine, no cystine being present. The content of free sulfhydryl groups is relatively low in the rat liver nucleoprotein, and this value calculated as cysteine is only a fraction of the total

cystine-cysteine content. The latter quantity is thus presumably composed of about three times as much cystine as cysteine. The greater content of the rabbit liver nucleoprotein in sulfhydryl over that of the rat liver nucleoprotein is consistent with other findings in respect to these and other tissues. Thus the glutathione content of the liver (133, 134), and of the erythrocytes (133) of the rabbit is considerably higher than that of the rat. Furthermore, the total sulfhydryl content of aqueous extracts of the liver of the former species is also higher than that of the latter (135). It is of interest, therefore, that as far as the analyses depicted in Tables V–VI go, the only species differences found chemically resides in the content of free sulfhydryl groups. The comparison of the nucleoprotein of

TABLE VI

Proportion of Fat in the Liver Nucleoprotein Preparations (35, 132)

Source	Cold ether extract	Hot alcohol-acetone-ether extract	Fat from hot alcohol-acetone-ether extract	
			N	P
	%	%	%	%
Rabbit	4.4	10.1	0.7	1.6
"	4.5	12.5	.6	1.6
"	4.0	9.6	.7	1.9
"	3.9	12.5	.7	2.2
Calf	5.1	13.1	.7	1.9
"	16.3	21.5	.6	1.5
Cow	5.8	15.3	.8	2.2
Rat	5.5	14.7	.8	2.1
"	5.3	15.0	.8	2.2
Transplanted Rat Hepatoma	—	15.0	.8	2.0

normal rat liver and of the transplanted rat liver cell tumor reveals no essential chemical differences as far as the analyses given in Tables V–VI inform us. This similarity stands in considerable contrast with the enormous differences observed between the activity of many catalytic systems studied in the liver and in the hepatoma (136). It would nevertheless appear that the chemical constitution of the liver nuclear protein complex is to some measure preserved after the malignant transformation of the tissue. It is also difficult to avoid the impression that these nuclear complexes in the same tissue of different species of animals, are, on the whole, constituted rather similarly.

The idea stated in the preceding sentence is consistent with the data obtained by other investigators. Bailey (137) studied the myosins of various species and observed that the values for nitrogen, sulfur, amide N,

cystine, methionine, tyrosine, and tryptophan in each of the preparations of myosin of the rabbit, dog, ox, chicken, fish, and lobster were practically identical from one species to another. No data are available on the sulfhydryl groups of myosin other than that of the rabbit (117). It would be of interest to see whether this protein in the various species could be differentiated on the basis of the sulfhydryl group content in the manner employed for the liver nucleoproteins. The reaction of myosin with a specific nucleotide (p. 237) brings the analogy to the nucleoproteins considered even closer. Block (138) studied the amino acid composition of the neuroproteins of several species and observed that the nitrogen, histidine, lysine, arginine, cystine, tyrosine, and tryptophan values for the brain proteins of the human, monkey, cow, sheep, rat, and guinea pig were very nearly the same from one species to another. Similar results have recently been obtained (139) by the amino acid analyses of several animal tissue proteins from different species.

2. *The Particulate Components of Tissues*

A further analogy along the general line of the essential similarity of related tissue components may be observed in the comparison of the composition of the protoplasmic complexes isolated by centrifugation from the tissues of several species. Claude first demonstrated that a fraction of the chick embryo could be isolated, the physical properties of which were similar to those of the infectious fraction of the chicken tumor (127). Subsequent studies by Pollard (130) and by Claude (127) revealed that uniform complexes of lipoid with nucleoprotein of the ribose type could be isolated from a number of different animal tissues. Claude separated the nucleic acid from the complexes and estimated that the former represented about 16 per cent of the total weight of the complex. This author stressed the probable cytoplasmic origin of such materials (140). The analytical data of Claude and of Pollard are given in Table VII.

It may be emphasized that the technique of isolation in each of the cases cited was identical for the preparation in each group, *i.e.*, an acid isoelectric precipitation of the liver nucleoproteins, precipitation by dilution of the salt solutions of the myosin, an acid precipitation of the brain proteins, and centrifugation at known speeds for the cytoplasmic tissue particles. It would appear that identical methods of isolation of related tissues lead to products which at the present time appear to be quite similar in composition. However, the possibility always exists that further, more intensive analyses may reveal profound differences as well as similarities between such products. After all, only one member of the group of apparently similar complexes isolated by Claude possesses specific infective power. The particles isolated by Claude and by Pollard are not nucleoproteins in

a homogeneous sense, but rather are complexes of cytoplasmic components in probably native form.

3. *The Virus Nucleoproteins*

The essential property of this remarkable class of substances lies in their capacity for reproduction in favorable, living environments at the expense of their hosts. The term virus has been principally confined to agents residing in multicellular organisms; when free-living individual cells such as the bacteria are the hosts, these agents are referred to as bacteriophage. In nearly all cases the specific biological property of infectiousness, or obligate parasitism, has been found to be associated with certain nucleoproteins of high particle weight which bear little or no resemblance to the

TABLE VII

Composition of Particulate Components of Normal and Tumor Cells (127, 130)

Source	N	P	Total Lipoids	N on fat-free basis	P on fat-free basis
	%	%	%	%	%
(Claude)					
Chick embryo	8.22	2.10	51.0	13.80	1.21
Chicken tumor I	8.60	1.54	36.5	12.74	1.16
Mouse embryo	8.54	2.07	46.0	14.30	1.37
Mouse sarcoma 180	8.00	1.52	49.1	14.51	1.21
Mouse sarcoma 1549	9.26	1.88	42.4	14.90	1.23
(Pollard)					
Chicken tumor	—	—	40	12.4–13.5	1.0
Mellanby tumor	—	—	—	12.7	1.1
Tumor 7	—	—	—	13.3	1.0

normal nucleoproteins of the host, either chemically or serologically, and which themselves vary considerably from virus to virus in chemical composition, particle size and shape, and molecular stability. The only common ground, apart from the property of infectiveness, which the viruses and bacteriophage possess, is the fact that they are all nucleoproteins, or at least proteins associated with nucleic acid. However, the type of nucleic acid varies in the different viruses; the plant virus nucleoproteins contain a pentosenucleic acid as do the chicken tumor agent and the virus of equine encephalomyelitis. On the other hand the vaccinia, the rabbit papilloma, and probably the psitticosis viruses are associated with a nucleic acid of the desoxypentose type. Only a few of the virus nucleoproteins have received any extended chemical characterization, and these have been almost entirely the plant viruses which are available in relatively large

amounts. There will be given in this section therefore only a concise outline of the chemical and physical properties of those virus nucleoproteins which have been investigated, deferring until later some discussion of their distribution, significance, and occurrence. For the more detailed description of these substances reference may be made to the book of Bawden (65, 2nd edition) and to recent reviews by Stanley (141, 142, 143), Lauffer (64, 144, 145), and Smadel and Hoagland (128).

The chemical and physical characteristics of the plant virus nucleoproteins have been elucidated chiefly by the English workers Bawden, Pirie, and Bernal, and by the American group headed by Stanley. These proteins may be recovered from the infectious juices of various affected plants by means of salt or alcohol precipitation or by high speed centrifugation. The products so obtained are infectious, but their relation to the viruses as they are produced in the infected plant is not as yet fully clear. Except in possibly one case, that of the tomato bushy stunt virus, evidence of molecular individuality and homogeneity is largely lacking. A sensitive and rigorous test for homogeneity would be to show that each particle of a virus was equally infectious but such a test has not yet been developed. Nevertheless, the possession of specific biological activity by these virus nucleoproteins has greatly facilitated their critical examination to an extent impossible as yet for nucleoproteins apparently lacking this property.

Many of the virus nucleoproteins have been obtained in characteristic crystalline or paracrystalline forms. These and the virus preparations obtained in relatively pure but amorphous form all contain nucleic acid of the pentose type. The nucleic acid in the different viruses ranges from 5–40 per cent of the total nucleoprotein, and may be separated from the protein by various denaturation procedures, all of which employed to date result in loss of the infectious property of the viruses. The binding force of the nucleic acid to the protein varies in intensity among the different viruses; in some the nucleic acid is relatively tightly bound and strong denaturing methods must be employed for its separation, whereas in other viruses it is sometimes difficult to prepare the intact nucleoprotein because the nucleic acid is so readily split off. Bawden and Pirie (77) have recently paid considerable attention to the effect of various salts and neutral organic compounds which effect a denaturation of various virus nucleoproteins with separation of protein and nucleic acid and have demonstrated the range of susceptibility of the viruses to these reagents. Certain nucleoproteins required stronger denaturing salts than others, but in every case tested at least one neutral substance could be found which would effect the separation of protein and nucleic acid. This point will be emphasized again; it is mentioned at the present time to suggest that in these more complex representatives of the nucleoproteins as in the simpler types, the

linkage between protein and nucleic acid is probably of a salt-like character, but unlike the types where a more primitive kind of protein is present, the linkage is conditioned and modified by the native structure of the complex protein. The treatment of the virus nucleoprotein by neutral salts such as the detergents or guanidine hydrochloride or by neutral organic compounds as urea, frequently results in a disaggregation of the nucleoproteins into smaller particles accompanied by a loss in the anisotropic properties (*i.e.*, reduction in asymmetry) (76, 146), by the liberation of the nucleic acid component, and by the loss in infectivity by the virus (77). An analogous disaggregation of the anisotropic protein, myosin, by many of the same agents, has been observed (78, 147). In the case of the tobacco mosaic virus nucleoprotein (76, 148), as in the case of myosin (117), the addition of these neutral agents also causes the liberation of sulfhydryl and presumably other groups (149), not previously observed in the native, unaffected protein. Such effects mentioned above are not restricted to the rod-shaped protein molecules but may be exhibited by the more globular proteins as well, when treated with these agents (150, 151).

Other methods of removing the nucleic acid from the protein component of the virus nucleoproteins include heating (65, 23), high pressure (152), and treatment with acids and bases (65). The use of a brief period of heating of solutions of the tobacco mosaic virus nucleoprotein resulted in obtaining the nucleic acid in a highly polymerized and asymmetric form (23) (see Table I and discussion on pages 219–220). This nucleic acid breaks down spontaneously into smaller, less asymmetric particles, and the latter, on subsequent treatment with alkali yield in turn still smaller particles. Attempts to remove the nucleic acid from the intact tobacco mosaic virus nucleoprotein, or to affect it therein, by treatment of the nucleoprotein with purified nucleases known to attack the free nucleic acid gave negative results (54, 55). The latter results are probably due in part to the spatial configurations of enzyme and of nucleoprotein relative to each other. The problem of accomodating spatially the asymmetric nucleic acid within the asymmetric nucleoprotein has been considered above (p. 225).

a. *Absorption of Ultraviolet Light.* Although all of the plant viruses studied yield nucleic acids of the yeast or pentose type, the question as to whether all these acids are identical either with each other or with yeast nucleic acid itself has not yet been answered. On the basis of certain differences in the physical properties of the uridylic acid isolated from the nucleic acid of the tobacco mosaic virus from those of the corresponding nucleotide isolated from yeast nucleic acid Loring (31) suggested that the two acids were not identical but isomeric. The content of nucleic acid in the individual virus nucleoprotein preparations has been estimated by (a) the proportion of phosphorus in the latter, (b) actual isolation procedures,

and (c) the relative maximum absorption of ultraviolet light at 2600 A by known solutions of the viruses (65, 153). The agreement by all three methods is quite close, and among other things indicates that the phosphorus content of the purified preparations, as well as their carbohydrate content, is due exclusively to nucleic acid. Fig. 5 represents a comparison of the ultraviolet absorption curves of the viruses of the tobacco mosaic, the latent mosaic, and the tobacco ring spot (153). The relatively higher absorption by the latter nucleoprotein over that of the mosaic virus (at 2600 A) is consistent with the higher content of the ring spot virus in

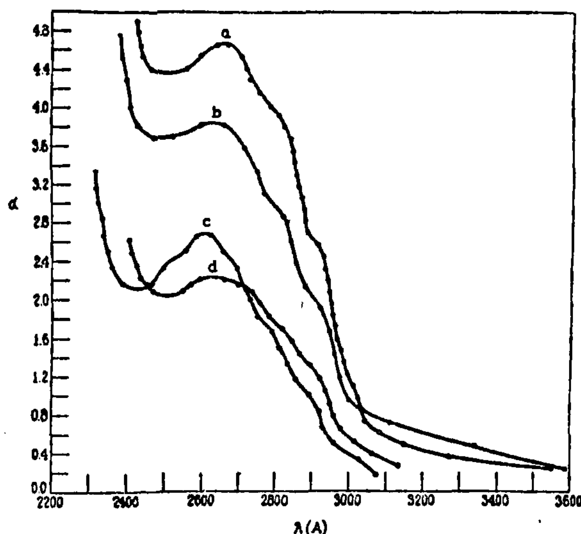


Fig. 5. Absorption spectra in the ultraviolet of a, tobacco mosaic virus prepared by ammonium sulfate precipitation; b, latent mosaic virus; c, tobacco ring spot virus (α divided by 10); d, tobacco mosaic virus. Preparations other than for curve a, obtained by centrifugation. From Lavin, G. I., Loring, H. S., and Stanley, W. M., *J. Biol. Chem.* **130**, 259 (1939).

nucleic acid (40 per cent) than that of the mosaic virus (5 per cent). A smaller absorption at about 2800 A is due to the protein component. When the nucleic acids were separated from the proteins in these viruses and subsequently mixed in the original proportions, the shape of the ultraviolet absorption curves and the relative positions of their maxima and minima, were similar to those of the original, intact virus nucleoproteins.

The viruses may be inactivated by prolonged ultraviolet radiation; in the case of the bushy stunt virus such inactivation can occur without loss of the crystalline form. The relative efficiency of inactivation by monochromatic ultraviolet radiation appears to attain at least one considerable maximum at a wave length of 2600 A which is characteristic of

the maximum absorption by the nucleic acid component (58). This is an interesting finding, (compare Fig. 2, pp. 222–225), for it suggests two things, (a) a method whereby a biologically active material may be determined to have nucleic acid associated therewith, and (b) a possible explanation for the effects of ultraviolet radiation on the mutation of living organisms.

b. *Size and Shape of Plant Viruses.* The first plant virus to be obtained in relatively pure form was the tobacco mosaic nucleoprotein. The streaming birefringence of the infectious juices of plants affected by this virus, noted earlier (154), has been shown to be due to the highly anisotropic nature of the virus nucleoprotein. Solutions of the latter exhibit besides streaming birefringence, structural viscosity, and electrical birefringence (155). From the infectious juices, the nucleoprotein is obtained as long, needle-shaped particles. Concentrated solutions of this protein if left undisturbed, settle into two layers, the lower of which is more concentrated and shows the phenomenon of spontaneous birefringence (*i.e.*, liquid crystallinity). The upper layer is birefringent only on stirring (65). The particles of this nucleoprotein are thus composed of paracrystals of highly asymmetric shape and relatively high weight. The potato virus X nucleoprotein in solution also shows the phenomenon of liquid crystallinity but the isolated preparations are amorphous (65). At least two other plant viruses have been obtained in crystalline condition, namely the tobacco necrosis (65), and the tomato bushy stunt (65, 156). The latter nucleoprotein satisfies many of the criteria for chemical homogeneity; in contrast with the tobacco plant viruses it appears to be globular rather than rod-shaped (156, 157, 158).

Determination of the size and shape of the plant virus nucleoproteins is beset by many difficulties for the methods of isolation of such proteins often lead to variations in their physical properties, and the methods of determination and calculation, such as centrifugation and diffusion, while valid for more or less spherical particles, may only be employed with some ambiguous approximations for the case of the highly elongated viruses. Particle interaction among the latter leads to many difficulties in interpretation (discussion of thymus nucleate on pages 218–221), and indeed it has been suggested that if contemporary methods of calculating the physical properties of the highly anisotropic viruses are carried to their logical extent, the diffusion constant would be zero and the molecular weight infinite (159, 160). This is a somewhat pessimistic view. The use of approximations is not unjustified as long as the limitations of the latter are understood. The sedimentation constants of the tobacco mosaic virus are indeed a function of the concentration of the latter; Lauffer has employed an extrapolation function to infinite dilution which appears to be valid and to yield results differing relatively little from those earlier ob-

tained on solutions at very low, finite, concentrations (157). The difficulties encountered with the elongated nucleoproteins are not met with in the case of the more globular bushy stunt virus. The data on the isolation procedures, elemental composition, and physical properties of the plant virus nucleoproteins are collected in Table VIII.

TABLE VIII

Isolation, Composition, and Properties of Some Plant Virus Nucleoproteins

Virus	Conc. in[1] Infected Sap	Host	Isolation	Form	Nucleic[2] Acid Content	Sedimentation Constant	Axial[3] Ratio	Average Particle Diameter
	g. per liter				*per cent*	*Svedbergs*		*A*
Tobacco mosaic (65, 161, 157, 64)	2[11]	Tobacco	$(NH_4)_2SO_4$ Centrifugation	Paracrystalline needles	5	193[5]	40	150
Ribgrass (162)	0.4	"	Centrifugation	"	"	187[5]	"	150
Cucumber 4 (65, 163)	0.3	Cucumber	Centrifugation	"	"	187[5]	"	146
Cucumber 3 (65, 163)	0.3	"	Centrifugation	"	"	187[5]	"	146
Alfalfa mosaic (164, 165)	0.2	Tobacco	Centrifugation	Amorphous	15	74[6]	(Spherical)[1]	165
Potato X (65)	0.1	"	$(NH_4)_2SO_4$ Centrifugation	"[4]	5	130[7]	(43)[1]	(98)[1]
Tobacco necrosis 1 (65, 166)	0.04	"	$(NH_4)_2SO_4$	Crystalline plates	15	112[8]	(Spherical)[1]	200
Tobacco ringspot (65)	0.012	"	Centrifugation	Amorphous	40	115[9]	(Spherical)[1]	190
Tomato bushy stunt (65, 158, 157, 167, 168)	0.05	Tomato	$(NH_4)_2SO_4$ Centrifugation	Isotropic dodecahedra	15	132[10]	(Spherical)[1]	260

[1] Approximate.
[2] Based on phosphorus content.
[3] Ratio of long to short axes of extended particles; the average length of the particles may be estimated by multiplying this value by the average particle diameter (last column).
[4] Shows strong streaming birefringence in solution.
[5] Corresponds to molecular weight of approximately 40 $\times$ 10[6]; from diffusion measurements (169) the weight is 59 $\times$ 10[6] for the tobacco mosaic virus.
[6] Corresponds to molecular weight of 2 $\times$ 10[6].
[7] Corresponds to molecular weight of 26 $\times$ 10[6].
[8] Corresponds to molecular weight of 6 $\times$ 10[6].
[9] Corresponds to molecular weight of 3 $\times$ 10[6].
[10] Corresponds to molecular weight of 7 $\times$ 10[6]; from diffusion measurements (170) the weight has been calculated to be 10 $\times$ 10[6].
[11] 1.3 in tomato and 0.15 in spinach.

The diameters of the particles given in Table VIII have been largely determined from ultracentrifugal, diffusion, or viscosity measurements, but where they have been checked by X-ray analysis (65, 171) or by electron microscopy (172, 166) fairly good concordance has been achieved. The X-ray patterns of many of the oriented virus preparations show definite

regularities in their structure. For the tobacco mosaic virus there is a spacing of about 2 A along the particle length; the particle itself appears to be composed of piles of sub-units of dimensions 11 × 11 × 11 A. The intra-molecular patterns of the cucumber viruses 3 and 4 which are strains of the tobacco mosaic virus are quite similar to the pattern of the latter. The X-ray photographs of the potato X virus suggests that this material is composed of sub-units essentially similar to those of the tobacco mosaic virus but that their arrangement is different. The pattern of the true crystals of the tomato bushy stunt virus shows the fixed regularity of the crystal lattice; it is of the same type as that of the other viruses although readily distinguished from them. The X-ray methods, although they permit an estimation of the width of the various particles, do not allow such an estimation for their length. Electron microscopy has revealed particles of varying lengths of certain of the elongated viruses, albeit identical diameter for each case, and in view of the known tendency of these particles to aggregate end to end (65, 173), this observation is not surprising. The axial ratios for such aggregated particles will thus be considerably higher than the values given in Table VIII. The similarity of this behavior to that of thymus nucleate (Table I and pp. 218–221) comes to mind. Like the values described for the latter substance, those given in Table VIII for the size and shape factors of the viruses should be viewed more as orders of magnitude. Whether the anisotropic virus particles in the infectious plant juices are already aggregated, and whether such particles are truly representative of their state within the plant leaf, form questions which cannot be entirely answered at the present time. Actually, of course, strongly birefringent inclusions in plants infected with the asymmetric viruses have frequently been observed (65), but whether these oriented inclusions represent pure virus particles or whether they include the virus in an oriented shell of non-virus plant material, also form questions at present largely unanswerable.

c. *Composition of Plant Viruses and the Problem of Variants.* The characterization of the viruses in terms of their amino acid composition has been attempted only in the case of the tobacco virus nucleoprotein and certain of its related strains. The data for the composition of the former are given in Table IX (143).

The content of cysteine in the hydrolyzed protein is equivalent to that observed in the intact, denatured protein, and accounts for all of the sulfur in the latter. Iodine added to the native nucleoprotein reacts with the sulfhydryl groups which would otherwise be revealed on denaturation, and a virus protein is thus formed in which the sulfhydryl groups are converted presumably to the disulfide form (174). This was the first example of a biologically active, chemically-altered virus protein. This altered virus

was found to be fully as infectious as the original material and the virus collected from plants infected with the former had the normal complement of sulfhydryl groups (174). Substitution of organic radicals on amino and phenolic groups of the nucleoprotein resulted in the formation of derivatives which were infectious, and, as in the case of the iodine-oxidized virus, the virus collected from the infected plants was indistinguishable from the original, unsubstituted nucleoprotein (175) and the outward aspects of the disease were unchanged. It would appear that the sulfhydryl groups and a considerable proportion of the amino and phenolic

TABLE IX

Composition of Tobacco Mosaic Virus Nucleoprotein (143)

Component	Per Cent
Nucleic acid (Ribose type)	5.8
Alanine	2.4
Amide N as ammonia	1.9
Arginine	9.0
Aspartic Acid	2.6
Cysteine	0.7
Glutamic Acid	5.3
Glycine	0
Histidine	0
Leucine	6.1
Lysine	0
Methionine	0
Phenylalanine	6.0
Proline	4.6
Serine	6.4
Threonine	5.3
Tryptophan	4.5
Tyrosine	3.9
Valine	3.9
Sulfur	0.2

groups of the virus molecule may be altered without interfering with the reproduction of the virus.

The specific infectious activity however of certain such chemically-altered derivatives while equal to that of the unaltered virus in one type of plant was found to be considerably less than that of the latter in another, related plant (175). This finding provided the first evidence of a known modification in the structure of an individual virus which leads to a change in the infectivity of the virus. Such a change in the virus, in the strict sense, is not a mutation for there is no evidence at the present time to show that these particular chemically-modified nucleoproteins are reproduced as

such. The attenuation of the virulence of viruses by such means as the passage through foreign hosts has been known since the classic work of Jenner and of Pasteur and this technique is employed at the present time in the case of the yellow fever virus. These classic techniques are inconvenient and uncontrollable; the chemical methods evolved by Stanley (143) and the radiation methods of Hollaender (176) offer perhaps more hope for the future practical control of virus-caused infections.

Under living conditions, the presumably spontaneous mutability of the tobacco mosaic virus has long been known, and several strains have been distinguished (65, 142). Each strain causes a different yet related disease and is characterized by a virus nucleoprotein, the physical features of which resemble closely those of every other member of this group (142, 148). Thus the crystalline form and the sedimentation constant of several members of the group are nearly identical. However, chemical analysis and immunological studies have revealed striking differences between the strains, and in each case, as might be expected, the differences revealed by analysis are paralleled by differences in the serological reactions (142). All of the nucleoproteins of the various strains have the same phosphorus content, which implies that the nucleic acid content is the same in each. Qualitatively, the nucleic acid of each strain of the virus is of the pentose type. Analyses of the content of certain of the amino acids are given in Table X for strains of the tobacco mosaic virus and of the closely related cucumber viruses (143, 148, 162, 177).

TABLE X

Amino Acids and Phosphorus in Nucleoproteins of Distinctive Strains of the Tobacco Mosaic Virus and of the Related Cucumber Viruses (143, 148, 162, 177, 178)

Virus	Tyrosine	Tryptophan	Phenylalanine	Phosphorus	Arginine	Histidine	Sulfur
	%	%	%	%	%	%	%
Tobacco mosaic	3.8	4.5	6.0	0.56	9.2	0	0.2[1]
Yellow aucuba	3.9	4.2	6.3	0.52	10.0	0	—
Green aucuba	3.9	4.2	6.1	0.54	10.0	0	—
Holmes' ribgrass	6.4	3.5	4.3	0.53	9.2	0.55	0.64[2]
Holmes' masked	3.9	4.3	6.1	0.54	9.0	0	—
J14D1	3.8	4.4	6.1	0.55	9.2	0	—
Cucumber 4	3.8	1.4	10.2	0.54	8.7	0	—
Cucumber 3	4.0	1.5	10.0	0.56	8.8	0	—

[1] Entirely accounted for as 0.68 per cent cysteine (148).

[2] Entirely accounted for in terms of 0.68 per cent cysteine (same as tobacco mosaic virus) and 1.9 per cent methionine (148).

The protein components of strains of the tobacco mosaic virus may

differ in their amino acid composition. Equally as interesting however are the points of similarity; although the ribgrass strain has three times the sulfur content of the mosaic, the cysteine content of the two strains is identical. The non-cysteine sulfur in the ribgrass strain is accounted for as methionine (148). None of the other strains listed in Table X contain the latter amino acid, nor, for that matter, histidine. It seems doubtful that the variants described in Table X could have their origin in the alteration of the amino acid composition of the intact parent virus. It seems more likely that the synthesis of the nucleoprotein under altered conditions within the host leads to the production of the new strain. Such variants however are reproducible and hence probably represent true mutant forms.

d. *Animal Viruses.* Far more complex than that of the plant viruses is the chemical composition of the viruses of vaccinia (128), of the chicken sarcoma (127, 130) (see Table VII), and of the equinine encephalomyelitis, and rabbit papilloma (129). These viruses have all been isolated by ultracentrifugation of extracts of the affected tissues; they are complexes of lipoid, nucleic acid, and protein. The nucleic acid of the first- and last-mentioned virus is of the desoxypentose type and forms about 4–5 and 6.8 per cent of the total molecule respectively, that of the chicken and horse is of the pentose type and forms about 10 and 4 per cent of the total molecule respectively. The vaccinia virus apparently contains, in addition to the components mentioned, copper, flavin, and several enzyme systems. Whether these latter components represent impurities adsorbed from the protoplasmic matrix during the isolation process is not clear at the present time. Apart from the presence of these components, the very large size of the vaccinia virus and its osmotic properties cause it to resemble on the whole a somewhat incomplete small organism. The sedimentation constant of the purified preparations of this virus reaches the high value of 4910 Svedberg units. The chicken sarcoma virus, which represents the first example of a cell-free, transmissable agent for an animal tumor, possesses a sedimentation constant of 550 Svedberg units, and in this respect, as well as in certain elementary composition, resembles the normal nucleoprotein fraction of the chick embryo. The sedimentation constant of the virus for equinine encephalomyelitis appears to be 253 Svedberg units; that of the rabbit papilloma, 278. All four of the virus complexes are thus of considerably higher particle weight than that of any of the plant nucleoproteins considered. New viruses recently isolated, such as the virus of influenza (178a, b, c) and of poliomyelitis (178d) appear to be nucleoproteins; the former virus is associated with a desoxyribose-type nucleic acid.

e. *Bacteriophage.* Related types of reproducible parasitic proteins are the bacteriophages. Northrop's preparations of the staphylococcus phage

gave a nitrogen value of 14.1–14.6 per cent and phosphorus from 4.6–5.0 per cent (179); the molecular weight of the purified preparations appeared to be quite high but its value varied in direct fashion with the concentration taken, indicating progressive dissociation with dilution. The presence of nucleic acid (type unknown) in the phage was inferred from the absorption curve in the ultraviolet of solutions of the latter material; the considerable maximum in the region of 2600 A was considered to be due to the nucleic acid component. The phage could be inactivated by prolonged ultraviolet radiation with a maximum efficiency at the wave length mentioned. These factors in addition to the very high phosphorus values make it likely that nucleic acid is present, but of course do not constitute incontrovertible evidence. If all the phosphorus found belonged to nucleic acid, the latter would constitute about half of the phage nucleoprotein. No data are as yet available of the amino acid composition of these or of the proteins described in the previous paragraph.

A preparation of active coliphage purified by Kalmanson and Bronfenbrenner (180), while obviously of protein nature, gave on analysis for phosphorus a value of 0.07 per cent. This low value led the authors to doubt the possibility that their preparation was a nucleoprotein and they tentatively ascribed the presence of the phosphorus to adventitious impurity. If the phosphorus was actually nucleic acid phosphorus, the nucleic acid would amount to 0.8 per cent. The small amount of material available and the low proportion of possible nucleic acid might well have made, respectively, chemical analysis and direct measurement of absorption in the ultraviolet region of the spectrum impracticable (p. 250). However, it would have been interesting in this case, to have observed the wave length dependence of irradiation on the inactivation of the phage in the ultraviolet, for a specific inactivation at 2600 A would suggest the presence of nucleic acid in the preparation.

VII. Nucleoproteins—General Considerations

1. *Definition*

The simplest and perhaps best available definition of a nucleoprotein would be to designate as such any protein with which nucleic acid is associated. The manner of association may refer either to primary (nonpolar) or to salt-like (polar) linkages between protein and nucleic acid. Nucleoprotein salts may be considered as arising largely from electrostatic forces of attraction between the positively-charged groups (ε-amino, guanidine, and imidazole) on the protein and the negatively-charged groups (phosphate) on the nucleic acid. The problem of the nature of the linkage between protein and prosthetic group arises here as in the case of other types of conjugated proteins. The weight of present evidence favors the view that the nucleoproteins are probably salts of oppositely-charged components profoundly modified by the presence of each. When the protein component is of a relatively simple protamine or histone nature, the

nucleic acid can be separated by the addition of neutral salt such as sodium chloride, and it is difficult to see how the linkage between the nucleic acid and protein can be other than salt-like. When the protein component is of a more complex nature, separation of the two components by metathesis is not so readily achieved. Here a profound intramolecular rearrangement and disorientation of the protein, and possibly of the nucleic acid as well, by denaturing processes is a necessary condition for such separation. When the denaturation is effected by the addition of neutral salts such as guanidine hydrochloride or of neutral organic compounds such as urea, whereby no primary linkages are presumably opened, it is difficult to avoid the impression that here too the binding forces between protein and nucleic acid are of a salt-like character with the added provision that the stability of such polar linkages is dependent upon the native configuration of both protein and nucleic acid and upon their mutual orientation. Striking examples of such separation by denaturation appear to occur in anisotropic nucleoprotein molecules, as in certain of the paracrystalline viruses, where the denaturation effects not only the separation of protein and nucleic acid but affects the size, shape, and infectivity of the molecule as a whole as well.

Neutral denaturing agents affect nucleic acid as well as proteins, and in neither case do we know the precise intramolecular changes which are involved. We can, however, follow in many cases the effects which these changes produce by analytical, physical, and physiological techniques. With the introduction of sufficient concentration of the agent to bring it within the critical range, guanidine hydrochloride or urea denatures protein or nucleic acid almost instantly. It would be indeed difficult to conceive of primary, nonpolar bonds being dissolved by a neutral substance such as urea. In cases therefore where nucleic acid is separated from protein by denaturation of the nucleoprotein with effective neutral salts or neutral organic compounds, the linkage between the two components may be assumed to be of a salt-like character.

Various denaturing agents differ in their effectiveness, *i.e.*, guanidine hydrochloride is more effective on either protein or nucleic acid than urea at equal molar concentrations, and likewise some proteins are more susceptible to denaturation than others. Thus, the tobacco mosaic virus nucleoprotein is readily denatured and the nucleic acid component separated by either urea or sodium dodecylsulfate, but the components of the bushy stunt virus nucleoprotein are separated only by the latter, stronger denaturing agent. The use of alkali for the separation of the nucleoprotein components has been advanced as evidence for the presence of a nonpolar bond between them (181, 182), but it is difficult in this case to distinguish among the following possibilities: (a) that the alkali actually splits a non-

polar bond, presumably an ester linkage by hydrolysis; (b) that by the denaturation induced by the alkali the consequent internal rearrangement and disorientation of protein and nucleic acid leads to separation of the salt in a manner essentially similar to that induced by neutral substances; and (c) that the alkali by converting the protein into an anion produces by the electrostatic repulsion of the similarly-charged nucleic acid anion a separation of the two components. The use of effective, neutral salts or compounds might enable one to decide among these alternatives.

The use of more selective agents such as the nucleases which are specific for nucleic acid give results which are inconclusive (54, 55). In the case of the tobacco mosaic virus nucleoprotein only an indefinite complex between enzyme and nucleoprotein is formed in which the virus is reversibly inactivated without loss of nucleic acid. Failure of the enzyme to affect the nucleic acid part of so anisotropic a nucleoprotein might be explained on steric grounds, *i.e.*, the shape of the extended nucleoprotein virus makes the nucleic acid component inaccessible to the enzyme. Negative evidence of this sort little assists in the elucidation of the nature of the linkages between nucleic acid and protein, but it does suggest that the nucleic acid found with protein within crystals or paracrystals of certain virus nucleoproteins is not just an indefinite mixture with the protein but is bound to the latter by salt-like linkages whose stability and orientation are conditioned by the integrity and molecular shape of the nucleoprotein as a whole. The same suggestion may be employed to explain the failure in certain instances to separate the components of isolated nucleoproteins by electrophoresis. Moreover, under certain conditions, designated mixtures of protein and nucleic acid migrate together in an electric field.

The combination of nucleic acid with protein has been considered above to be largely due to valence forces of a polar, electrostatic (coulombic) character, modified by the structural properties of each component. On the other hand, when the relatively large mass of the nucleic acid is taken into consideration, it is necessary to bear in mind also the possibility of van der Waals forces in the interaction between protein and nucleic acid. The affinity between the highly charged anion of the nucleate and the protein is not necessarily a special phenomenon except in the exclusively oriented cases involving the more complex proteins. The affinity of certain anions of high weight for proteins has been known for many years, *i.e.*, picrate, tannate, flavianate, etc., and in practical affairs has been utilized in such industrial arts as dyeing. The increase in such affinity for any one anion with decreasing pH on the acid side of the isoelectric point of the protein was demonstrated by Jacques Loéb (183) and this phenomenon holds true likewise in the case where the anion is a nucleate (71). It remained however for the work of Steinhardt and Harris (184, 185) to place the relative degrees of anionic affinity for protein on a firm basis. When a series of acids of equal strength is added to a given protein, each acid will combine with the latter to an extent which is determined as a first approximation by the molecular weight of the acid and by its valence type, and secondarily by the shape or configuration of the anion. As the pH is increased both the actual affinity and the differences

in affinity between various anions progressively diminish until on the alkaline side of the isoelectric point of the protein no anion is at all apparently combined. The high affinity of certain dye anions (orange II) for proteins has been interpreted as being due in part to the highly aggregated nature of the former (185).

The combination of nucleic acid with protein is therefore not at all a unique phenomenon for all that has been stated above for the cases of the smaller anions holds with augmented cogency, because of its large weight and polyvalence, for nucleic acid. Indeed, Osborne pointed out that the tendency of the alkaline molecule of edestin to crystallize in combination with hydrochloric acid might be taken as an illustration of an analogous behavior of the proteins and nucleic acid (186). The denaturation of edestin is accompanied by an alkaline shift in the reaction of the protein (187), an effect observed also with other proteins (120). It would be expected therefore that the affinity of any anion, nucleate or otherwise, would be reduced when the protein with which it is combined is denatured. To the disorientation of the protein which is the accompaniment of denaturation is thus added the alkaline shift of the dissociation constants of the free groups of the proteins (probably as a result of the disorientation).

2. *Methods of Isolation and Recognition*

The consideration of the intramolecular forces which govern the composition and integrity of the nucleoproteins possesses at once both an academic and a practical interest, for it not only permits a partial apprehension of the dynamic interchanges of the cellular components in living tissues but yields a logical basis for the limitations which must be understood in the isolation of this class of substances from the tissue matrix. The nucleoprotein is a readily-dissociable complex, and its integrity is governed among other things by the pH of its medium and by the concentration and nature of the salts present. In the mitotic cell it has long been known that nucleic acid is synthesized and degraded, and recent evidence suggests that there is likewise a protein cycle (188), similar to that once hypothesized by Kossel (70), which occurs in the mitotic nucleus. During certain phases of tissue growth as well as in certain adult secretory cells, a protein and a nucleic acid gradient (or at least that of the nucleate components) appears to exist in the cytoplasm (189, 190). In cells where the content and possibly type of nucleic acid and protein rise and ebb, the nucleoproteins must likely be of a relatively loosely-bound and transitory nature, and hence the conjugated proteins isolated therefrom will only be characteristic of the state of the tissue at the moment taken. In such compounds, however, the components will not necessarily be additive, because of the interaction between nucleic acid and protein which results in modifications of the properties of each. Such considerations offer a partial

explanation for the varying analytical data for the nucleoprotein preparations isolated by Osborne from the wheat embryo (186). On the other hand, in resting, adult cells, the nucleoproteins might be of a more permanent, if still possibly mixed character. The crystalline and paracrystalline viruses which in a sense are foreign to the cell and represent a special case, are apparently homogeneous insofar as they are largely free of components characteristic of the host, but crystallinity is not necessarily a *sine qua non* of purity and even here, the possibility of a mixture of biologically-differing components within the crystal is not excluded (65). It is impossible to determine at the present time what proportion of the nucleic acid in a tissue is free or bound, and since nucleic acid and protein interact to form a series of readily dissociable complexes which in turn may react further with protamine or other proteins, the nucleoprotein isolated from the tissue may rarely be a homogeneous, chemical individual. Such a situation need hardly act as a deterrent to the study of these substances when the very probable inhomogeneity of even the classic crystalline proteins is brought to mind (116, 191, 192). If the composition of a nucleoprotein is frequently a function of the method of preparation and the state of the tissue, the limitation imposed thereby is common to all amorphous preparations, and indeed is possessed by many representatives of the general class of proteins, both amorphous and crystalline. However, each method of isolation leads within narrow limits to preparations the properties of which are reproducible in successive preparations, and with this assurance the investigator in this field must for the present remain content.

The nucleoproteins have been isolated by one or a combination of the following methods: precipitation by dilute acids, by salts, and by centrifugation.

In 1882, Kossel (193) washed a number of ground animal tissues with dilute acid followed by alcohol and ether. The residue was represented as nucleoprotein and the distribution of the residual phosphorus closely followed the nucleus-cytoplasmic ratios of the intact tissues. This procedure with some modifications has at times been followed up to the present (194, 195, 196). It is obviously crude but it does permit a rough comparison between the nucleic acid phosphorus of widely-varying tissues and an approximate index of cellularity in tissues with many non-cellular elements, *i.e.*, tumors (196a). The possibility of including some lipoprotein phosphorus and phosphorylated esters of hyxroxyl-amino acids in the residual fraction is not entirely excluded. Lilienfeld (122) was the first to precipitate the nucleoprotein from neutral or slightly alkaline extracts of the thymus gland by the addition of dilute acid, and this technique with few modifications has been employed extensively since with a number of other kinds of tissue (see Samuely, 197; and Rollett, 198, for review of the older literature) (35, 104, 199). The method of acid precipitation

carefully performed leads to products which are reproducible in properties. On repeated precipitation the product must yield constant nitrogen to phosphorus ratios. Since the isoelectric point of most nucleoproteins lies within the pH range of 3–5, this procedure is essentially an isoelectric precipitation. The products however are not necessarily homogeneous.

Some of the nucleoproteins, particularly certain plant viruses, are not readily precipitable by dilute acid but can be removed from the tissue extracts by fractionation with added ammonium or sodium sulfate (180, 65, 179). The concentration of sulfate necessary to crystallize some of the virus nucleoproteins appears to be dependent upon the state of purity of the preparation (65). Extraction of certain nucleoproteins in strong salt solutions and their precipitation by subsequent dilution, which appears to be a promising method, has been described (105) (p. 243).

Methods of isolating proteins by acid and by salt precipitation are nothing new in protein chemistry, and indeed the use of the former agent dates back to the time of Mulder (200), the discoverer of this class of substances. The question always arises in these chemical methods of fractionation whether the products obtained are truly representative of their condition in the living tissue. For this reason, the technique of separating the nucleoproteins by differential centrifugation has been widely used. Because the nucleoproteins are generally of high particle weight they are readily separated by this method from the remainder of the tissue. The separation of a nucleoprotein complex by differential centrifugation was first accomplished in the case of the vaccinia virus (201) although the chemical nature of the virus was unknown at the time. This general method has since been used for the isolation of a number of different kinds of nucleoproteins from various sources, including as examples the plant viruses (65, 161), the chicken tumor virus (127, 130, 131), the chick embryo nucleoprotein (127), the virus of equinine encephalomyelitis and of the rabbit papilloma (202), and the particulate components of the protoplasm of various tissues (140, 203). In the case of the tobacco mosaic virus nucleoprotein, the product isolated by chemical fractionation is nearly identical with that obtained by the centrifuge. Where the nucleoprotein has specific biological properties as in the case of the viruses or of the bacteriophage, the fractionation by use of salt or of the centrifuge is the more readily accomplished. It is small wonder that the most successful attempts to obtain preparations of a satisfactory degree of homogeneity have been made with the viruses. Where the nucleoproteins are apparently lacking in biological specifity their separation is somewhat more difficult. Claude (140, 203) has studied this problem in detail and has to date made the most satisfactory contribution to the quite intricate separation of the various nucleoprotein complexes from the cytoplasm and from the nucleus

of various tissues. The essence of Claude's technique is to employ various rates of speed of the centrifuge and various periods of time during the centrifugation, all in a definite sequence of events.

Other methods of preparation, of more limited value, involve in certain instances the precipitation of the nucleoproteins by means of calcium chloride (123) and by means of various hydrophilic colloids (204). The former method has been applied to nucleohistones, and the latter to the crystallization of already purified preparations of various plant viruses.

The recognition and identification of the nucleoproteins depends upon specific tests for the nucleic acid component. The most unequivocal proof is of course the physical isolation of the nucleic acid from its combination with protein. This may be accomplished as described by any procedure which leads to denaturation of the protein molecule. The protein may be subsequently removed by salt (12, 123), by chloroform adsorption (205), or by tryptic digestion (206). The nucleic acid obtained, usually by alcohol or acid precipitation, is identified by its physical properties or organic structure or both. Thus the pentose nucleic acid of the tobacco mosaic virus nucleoprotein was identified by its insolubility in acid (65), by its nucleotide content (31), by its physical properties (23), and by its susceptibility to nuclease digestion (55). Similarly, the desoxypentose-nucleic acid of the liver nucleoprotein was identified by the streaming birefringence and structural viscosity of its aqueous solutions as well as by its content of purine bases (35). The nature of the nucleic acid of the so-called β-nucleoprotein of the pancreas, long in doubt, was cleared up by Jorpes (29) through painstaking analyses of its components.

Such positive identification has been possible only where sufficient quantities of the nucleoprotein are available for studies of protein and conjugate. When this is not the case, recourse is made to the use of specific tests for one or more of the nucleic acid components in the presence of protein after a brief period of hydrolysis. Such tests reveal the presence of the components, sugar, nitrogenous base, or phosphorus, but in no case are they specific for nucleic acid as such. The tests by which the presence of nucleic acid in nucleoproteins, short of its actual isolation, has been inferred, are specific only for certain of the components. This is undeniably an element of weakness in the characterization of nucleoproteins, which is illustrated in the case of those conjugated proteins which contain as their prosthetic group nucleotides involving pentose and phosphorus (yellow enzyme) or purine, pentose, and phosphorus (Coenzymes 1 and 2). Analysis of such conjugated proteins for only these components might lead to the inference that they were nucleoproteins, which is certainly not the case. Nevertheless, tests suitable for the identification of small quantities of the nucleoproteins may be useful when the necessary reservations are borne in mind. These tests are described below, and with each its limitations are set forth.

a. *Phosphorus.* After removal of free and presumably all the bound phospholipids, the residual phosphorus has been considered to belong to the nucleic acid component. This presupposes not only a complete removal of lipid phosphorus, but the absence of phosphate esters of the hydroxylated amino acids in the protein as well.

b. *Sugar.* Nucleic acids are divided at the present time into two categories, *e.g.*, those which contain pentose as the sugar component, and those which contain 2-desoxypentose. After mild hydrolysis of nucleoprotein the former sugar is recognized by the familiar orcinol reaction, the latter sugar by the Feulgen (32, 33) or by the Diesche (34) reactions. Certain types of phosphatides interfere with the Feulgen reaction (207) but they may be readily removed by the use of fat solvents. The orcinol reaction is common to most pentoses.

c. *Nitrogeneous Bases.* These components comprise purine and pyrimidine derivatives. For purposes of identification frequent use has been made of the fact that the pyrimidine ring structure which is common to all members of this class of substances strongly absorbs ultraviolet radiation with a sharp maximum of absorption in the close neighborhood of 2600 A (see p. 223). The presence of tyrosine and tryptophan radicals in the protein part of the nucleoprotein molecule results in absorption of radiation with a maximum at about 2800 A. Under comparable conditions the absorption by the nucleic acid is considerably greater than that by the protein and in nucleoproteins with a moderate or high ratio of nucleic acid to protein the absorption curve will clearly show the 2600 A maximum. The extinction coefficient at this maximum is proportional to the nucleic acid concentration and the nucleic acid-protein ratio in a relative sense can be estimated for various nucleoproteins. Where however this ratio is very small, the protein absorption will tend to mask the specific absorption by the nucleic acid and no maximum at 2600 A may be evident. A possible differentiation between nucleic acid and protein absorption may be accomplished on the basis of the sensitivity of the latter to changes in pH (208). The maximum absorption of free tyrosine in alkaline solution is at 2950 A and in acid solution at 2750 A (209). This shift also occurs in the absorption spectra of certain proteins (210, 211, 211a.).

The use of ultraviolet radiation as a means of inactivating certain biologically-active proteins may assist in their designation as nucleoproteins. In both the tobacco mosaic virus (212) and the bacteriophage (179) use of monochromatic radiation has revealed an inactivation at 2600 A which is the region of maximum absorption of nucleic acid. This method is admittedly indirect.

Micromethods for the estimation of purine nitrogen, involving a copper precipitation has been developed (213). The isolation and identification of the individual purine bases is quite difficult when protein is present.

It will be readily appreciated that tests a–c described above are not specific for nucleic acid but only for components of the latter. In certain cells, such as yeast, the amount of nucleic acid estimated from the degree of absorption of the ultraviolet radiation was found to be of the same order of magnitude as the amount of the acid actually isolated (214, 215). The position at the present time, however, permits us only to infer, often with a strong degree of probability, that when a protein preparation gives the above tests we are dealing with a nucleoprotein. Unless the nucleic acid is actually isolated, this nevertheless does not constitute proof.

VIII. Distribution of the Nucleoproteins—Cytochemical Evidence

1. *The Methods of Cytochemistry*

It has long been known from cytological observations that the nucleus exhibits a strong affinity for basic dyes (216), and the term "chromatin" was originally applied by Fleming (217) to the chromophilic material of the nucleus. The cytoplasm of certain cells, notably yeast and the basal regions of the pancreatic cells, also appeared to stain deeply with basic dyes. The fine-structure of the nucleus during mitosis revealed the presence of discrete units in which the chromatin was concentrated and which appeared to join and disjoin during separate phases of the mitotic cycle. These discrete units received the designation of chromosomes, and the work of modern genetics has identified these structures as the carriers of the hereditary characteristics of the cell. Since the number of these characteristics was far greater than the number of chromosomes, the latter structures were believed to be further divisible into still smaller units to each of which a single hereditary characteristic was ascribed. These units were referred to as genes. Pure nuclear material was first obtained from pus cells and from sperm heads by Miescher (1), and in the sperm, nucleoprotein comprised more than 90 per cent of the total. These and nucleoproteins isolated since from a wide variety of tissues all stained with basic dyes under the same conditions whereby chromatin in the fixed tissue preparations was stained. Indeed, on a quantitative basis, the greater the quantity of nucleic acid either in the fixed preparation on the slide or in the isolated nucleoprotein, the greater was the amount of basic dye bound (199). From the results of the classic staining methods, it appeared in general, that there was a rise and ebb in the mitotic cycle of basophilic material which was identified as nucleic acid. However, the results of such basic staining reactions were frequently obscured by the faculty of nuclear material to take on acidic stains, a faculty which was ascribed to the protein content of the nucleus, and there appeared to be therefore, similar to the case of nucleic acid, a rise and ebb of proteins in the nucleus.

The development of methods specific for desoxypentosenucleic acid by Feulgen permitted the differentiation of the chromatin on a chemical basis. This reaction of Feulgen was given only by nuclear material, whether of animals or plants, and never in the cytoplasm of any cell. When the basophilic staining reactions of the nucleus and of the cytoplasm of various cells were compared with the specific stain developed by Feulgen, it was revealed that not only was the cytoplasmic chromatin negative to the latter reaction but also part of the nuclear chromatin (218). In other words, there was in addition to the desoxypentosenucleic acid localized in certain regions of the nucleus and which reacted with both the Feulgen reagents and the basic dyes, material in both nucleus and cytoplasm which although Feulgen-negative reacted more or less strongly with basic dyes. This Feulgen-negative chromatin, evidently of acidic nature, was suspected of being of a pentosenucleic acid nature, but the presence of other acidic substances, such as mucoid or mucoproteins could not be excluded. The identification of the Feulgen-negative, basophilic chromatin of the cell with pentosenucleic acid was finally inferred from several lines of evidence, *e.g.*, (a) the observation of relatively large amounts of pentose in the egg (219) and in the pancreas (29); (b) the digestion of slide preparations of various tissues with specific nucleases for ribosenucleic acid whereby the original structure and stainability of both nuclear and cytoplasmic elements were altered (220, 221, 49, 222); (c) the high absorption at 2600 A of ultraviolet radiation in basophilic, Feulgen-negative regions of the cell; and (d) the isolation of pentosenucleic acid from the cytoplasm of certain cells including those free cells such as yeast and bacteria in which the presence of a nucleus in the accepted sense is dubious (223, 224, 225, 203, 226, 227). On the basis of the finding that the plastids contain a nucleoprotein of the ribose type (226), it has been suggested that the plant viruses may actually evolve in this region.

It is abundantly evident therefore that nucleic acid or nucleoprotein may occur in both nucleus and cytoplasm of cells, and in this respect the adjective "nucleic" is ambiguous and anachronistic. However, this adjective is used at present to describe a class of naturally-occurring substances without reference to their source. At the present time it is understood that nucleic acid of the desoxyribose type occurs only in the nucleus of cells, whereas nucleic acid of the ribose type may occur in both nucleus and cytoplasm. The gross characteristics of these two latter main structures of the cell have been chiefly elucidated by techniques which effect a separation of the two. The nuclei of cells generally possess a higher density than the cytoplasm, and the former may be obtained in a reasonable state of purity by centrifugal techniques (see Dounce, 228, for recent application and for review of the literature). Unfortunately, many of the isolation

procedures have involved the use of high acidities. Microdissection techniques for either whole nuclei or for nuclear chromosomal threads have been employed (229, 230), and Claude and Potter (203) have worked out a differential technique with the centrifuge whereby pure nuclear chromatin may be obtained. From the nuclei or nuclear fragments so isolated, the presence of desoxypentosenucleic acid has been definitely recognized (203, 228, 231). Indeed, by Claude's technique the nuclear chromatin material is morphologically as well as chemically indistinguishable from that observed in the intact nucleus. That the cytoplasmic nucleic acid is located in definite areas or within formed elements of the cytoplasm rather than diffusely spread appears probable from the work of Menke (232), Claude (140), Caspersson (215), and DuBuy and Woods (226). What role such cytoplasmic structures or their components play during cell division is not known at the present time.

The chemical isolation procedures and the dye-staining techniques (including the Feulgen), valuable though they are in the charting and partial identification of the nucleic acid components of nucleus and cytoplasm, do not and probably cannot reveal the finer topography of these components. For this purpose, the ultraviolet microscope with a resolving power roughly twice that of the ordinary microscope, with the aid of monochromatic radiation, is better capable of yielding information of the fine-structure of the elements containing nucleic acid, protein, or both. The specific absorption of nucleic acid and of protein may be distinguished on the bases given above (pp. 223, Fig. 2). With the aid of the quartz microscope and ultraviolet radiation, Caspersson and his collaborators have carried out a series of remarkable cytochemical investigations on various tissues. For convenience, these cytochemical studies together with related investigations by other authors will be discussed under the following headings, (a) the nucleoproteins of rapidly-growing and secretory cells, and (b) the nucleoprotein distribution in the nucleus.

2. *Rapidly-growing and Secretory Cells*

Very early in the study of chemical embryology it was observed that large quantities of purine bases were synthesized during the development of the eggs of a number of species (see Needham's classic, 233, for history). In the early stages a large content of the hexone bases was present, which with one exception, remained fairly constant until shortly before hatching. The exception, histidine, appeared to drop sharply as the purine content increased, thereby suggesting that the synthesis of the latter was at the expense of the former (234, 235, *cf.* 235a). Calvery (30) later succeeded in isolating relatively large amounts of pentose nucleotides and a desoxypentosenucleic acid from developing eggs which indicated that at least a

considerable proportion of the purine bases observed in the eggs by the earlier workers after hydrolytic procedures was originally bound within nucleic acids. The latter supposition received further support from the determinations of nucleic acid phosphorus in embryonic tissue which ran roughly parallel with the purine content (193, 194). In the later stages of embryonic development the hexone bases and the purine content decrease, until at birth both values are considerably reduced below those characteristic of the former state. The nitrogenous bases of the nucleic acids appear to be exclusively products of *in vivo* synthesis (235b).

The studies of Caspersson and coworkers revealed that by far the greater part of the increase in nucleic acid in the developing embryo or embryonic tissues was due to the accumulation in the *cytoplasm* of pentosenucleic acid. This observation appeared to hold true in the case of embryonic liver, kidney, intestine, and primitive erythrocytes (189), plants (214, 215), and insects (214). Wherever normally rapidly-dividing cells were compared with resting cells, whether of the developing embryo as compared with the adult, or of growing yeast cultures as compared with senescent ones, the absorption of ultraviolet light at 2600 A in the cytoplasm was invariably far greater in the former kind of cells than in the latter. However, not only was the maximum at 2600 A of a different magnitude for the embryonic and for the adult cell, but the shapes of the curves were distinctly different over the range of wave lengths studied (189, 190). Such differences in shape of the absorption curves Caspersson and Thorell (189) attributed to differences in the nature of the proteins in the embryonic and in the adult cell. The proteins in the former cell, containing a large proportion of the hexone bases and hence of the histone type, would be expected to yield a different type of absorption curve from that of the proteins of the adult cell, which are of a more complex, globulin type (189, 208). As an illustration of these findings, the absorption curves in the ultraviolet for the cytoplasm of embryonic and adult liver are given in Fig. 6. The protein absorption maximum for the lower curves (adult cells) occurs at a shorter wave length than that for the proteins in the upper curves (embryonic cells). According to Caspersson's observations, the longer-wave (beyond 2800 A), maxima are characteristic of histone-type proteins in which the hexone bases predominate, the shorter-wave maxima (below 2800 A) of the more complex, tyrosine and tryptophan-containing proteins with a lower proportion of hexone bases (208). Spectra of the former type, including intense absorption at 2600 A due to nucleic acid (here of the ribose-type) were observed in the cytoplasm of all embryonic cells studied; spectra of the latter type were found in adult cells (189). These observations are in accord with the classic Kossel hypothesis that the histone-type of protein represents an intermediate phase in the ontological development

of the proteins into later, more complex representatives of this class of substances (70, see p. 241). The upper curves in Fig. 6 may be compared with the curves for the viruses given in Fig. 5.

Malignant tumors, also, form a class of rapidly-dividing cells. Loss of differentiation occurs, and it might be expected that, functionally, a tumor

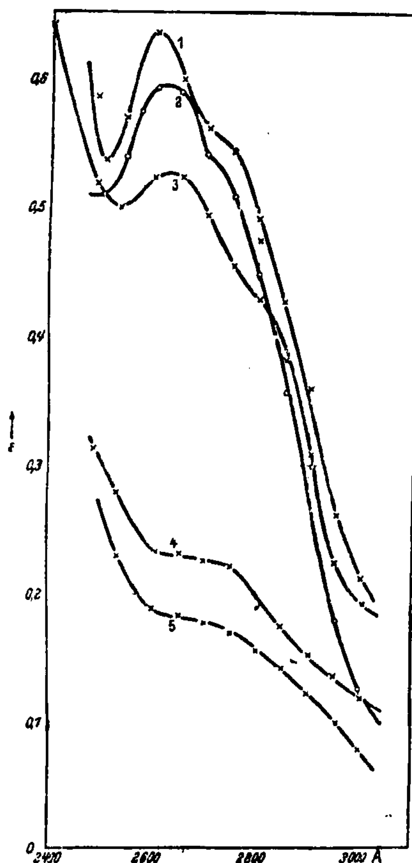


Fig. 6. Absorption spectra in the ultraviolet of cytoplasmic regions of the liver cells of: Curve 1, 3-day-old, Curves 2 and 3, 6-day-old chick embryo, Curves 4 and 5, newly-hatched chick. From Caspersson T., and Thorell, B., *Chromosoma* **2**, 132 (1941).

should resemble undifferentiated fetal tissue more than the normal adult tissue of its origin. Comparative studies on several enzyme systems in a series of homologous tissues of the rat, *i.e.*, normal adult liver, fetal liver, and hepatoma, have indeed revealed that the hepatoma resembled fetal liver far more than it did the adult liver (236). Two enzymes, however,

remain appreciably constant in activity (per gram of tissue) in fetal, adult, and neoplastic liver tissue—these are the depolymerases for thymus and for yeast nucleic acids. It would be of interest to observe the distribution of the comparable nucleic acids, as well as the proteins, in these tissues. Dounce (228) has demonstrated in the case of the desoxypentosenucleic acid a lower concentration in the isolated nuclei of the rat hepatoma than of the adult liver of this species; the corresponding figures are, approximately 20 and 40 per cent of the dry weight.

The nucleus in the embryonic cells appears to be rather low in either kind of nucleic acid, but the nucleolus on the other hand like the cytoplasm appears to be quite high in the ribose-type of nucleic acid (189). Similar in certain respects to the embryonic cell with regard to the general cytology and to the topography and quantitative distribution of nucleic acid are the somatochrome nerve cells (237, 238). In the latter cells, pentosenucleic acid appears to be highly concentrated in the basophilic Nissl-bodies of the cytoplasm, as well as in the nucleolus, whereas the nucleus itself is relatively low in any kind of nucleic acid. The finding of nucleoproteins of the ribose type localized in condensed areas of the cytoplasm of certain nerve cells is consistent with similar observations in other types of tissues. It should be pointed out however, that the smaller, caryochrome nerve cells contain much nuclear nucleic acid and little or no Nissl-bodies.

Not only embryonic cells in general, but actively-secreting, exocrine cells of certain mammalian tissues appear to be rich in cytoplasmic nucleic acid of the ribose-type and in protein of a histone nature (190). The secretory cells of the pancreas extrude large amounts of protein (pancreatic enzyme secretion) and hence the synthesis of protein in these as in other types of secretory as well as embryonic cells must proceed at a high rate. In the pancreas zymogen cells there is a gradient of pentosenucleic acid and type of protein, starting at the base of the cell with a high concentration of nucleic acid and histone type of protein and progressing with a diminishing content of nucleic acid and change in type of protein from histone to the more complex as the lumen is approached. Similar findings were observed in the case of the gastric mucosa and of the parotid gland (190). In general, it would therefore appear that in normal cells wherein a high rate of synthetic activity occurs, nucleic acid of the ribose type and protein of the histone type are found in relatively high concentration in the cytoplasm. A curious finding in regard to the *endocrine* cells of the pancreas consists in the failure to observe more than traces of nucleic acid in these cells (190), a finding consistent with earlier observations by Hammarsten, *et al.* (239). It is quite probable that the amount of insulin produced by the islet cells is almost negligible in comparison with the proteins produced by the various kinds of exocrine cells.

3. *Nuclear and Cytoplasmic Distribution*

Only in embryonic and secretory and in certain kinds of nerve cells does the cytoplasmic content of nucleic acid form a relatively large proportion of the total nucleic acid content of the cell. In most adult cells of the vertebrates, the greater proportion of nucleic acid is contained in the nucleus. The determination of the total nucleoprotein content of various tissues was first attempted by Kossel (193); the best recent data are due to Javallier and coworkers (194, 240, 241, 242). Of some interest is the finding of the latter workers that the nucleoprotein content of similar tissues in all classes of vertebrates studied was very nearly the same. The relative size of the nucleus to the cytoplasm varies considerably from tissue to tissue; quantitative estimates of the nucleus-cytoplasm ratio are beset by some difficulties however; see (243) for excellent review. An interesting question arises as to whether the nucleoprotein content of a cell invariably follows the order of the relative size of the nucleus to the cytoplasm. Since the nucleus contains desoxypentosenucleic acid whereas the cytoplasm contains only pentosenucleic acid, a supplemental question might well be concerned with the relative ratios of the activity of the enzymes specific for these two acids as a function on the one hand of the relative total nucleic acid content and on the other of the relative order of the nucleus-cytoplasmic ratio in various tissues. These questions are answered to some extent by the data collected in Table XI (52, 242).

On considering the series of mouse tissues from thymus to muscle, it is clear that with one exception, the content of nucleoprotein runs parallel with the order of the nucleus-cytoplasmic ratio, *i.e.*, the greater the latter, the greater the content of nucleoprotein. The significant exception is the pancreas; from the discussion above the cause for this exception is to be found in the very high content of nucleic acid in the cytoplasm. No particular correlation with these findings may be discerned in the absolute activity of either DRD (desoxyribosenucleo-depolymerase) or RD (ribonucleo-depolymerase) for the series of tissues, but the arbitrary *ratio* of these activities (last column in Table XI) is quite interesting. The order of the ratio of the activity of DRD to RD, with again a significant exception for the case of the pancreas, runs *inversely* with the total nucleoprotein content and the nucleus-cytoplasmic ratio. The exceptional position assumed by the pancreas is due to the fact that the ribonucleo-depolymerase activity for this tissue is extremely high; in fact, because of its high content of this enzyme the pancreas has been the source for the isolation of the crystalline enzyme preparations (22). Excluding consideration of the pancreas therefore, it would appear that with decreasing size of the nucleus in relation to the cytoplasm, the greater is the activity of the desoxyribose nuclease (exclusively nuclear function) relative to that of the ribose nuclease

(chiefly cytoplasmic function). The relationship is thus a curiously inverse one.

When homologous pairs of tissues are considered, *e.g.*, fetal and adult liver, regenerating and resting liver—which represent comparisons based on normal, controlled growth—and tumors and their normal tissue of origin which represent comparisons based on abnormal, uncontrolled growth, an interesting relation between the DRD and the RD activities for each pair

TABLE XI

Interrelation of the Nucleo-depolymerases, Nucleoprotein Content, and the Nucleus-Cytoplasmic Ratio

Tissue	Total Nucleo-protein Phosphorus (242)	Order of Nucleus Cytoplasm Ratios[1]	Activity of DRD (52)[2]	Activity of RD (52)[3]	Ratio of DRD:RD[4]
	mg. per 100 g. dry tissue				
Mouse thymus	1.30[5]	1	3.1	0.12	25
" pancreas	.64[5]	5	5.4	.78[7]	7[8]
" spleen	.39[6]	2	16.1	.28	61
" liver	.20[6]	3	14.3	.12	119
" kidney	.13[6]	4	10.2	.08	127
" muscle	.05[6]	6	12.0	.07	171

[1] Rough approximation from two-dimensional inspection; highest ratio = 1, lowest ratio = 6.

[2] DRD = desoxyribonucleo-depolymerase (sodium thymus nucleate as substrate). Activity based on viscosity measurements by method described (52). All tissues studied under identical conditions.

[3] RD = ribonucleo-depolymerase (sodium yeast nucleate as substrate.) Activity based on increase in acid-soluble phosphorus after two hour digestion during period of linearity. All tissues studied under identical conditions.

[4] Since activities of DRD and RD were obtained with different methods, the significance of these ratios lies chiefly in the comparisons between the tissues.

[5] Values given for horse tissues which are nearly the same for all vertebrates studied.

[6] Values given for rat tissues, ditto above.

[7] Probably still larger than value given.

[8] Probably still smaller than value given.

of tissues is revealed (52). When fetal tissue becomes adult, or when regenerating liver reaches the resting state, or when normal tissues become neoplastic, both DRD and RD activity, or presumably nuclear and cytoplasmic specific functions respectively, *are altered in the same direction.* It would appear that the metabolism of both kinds of nucleic acid during either normal or abnormal transformations of the tissues studied are distinctly interrelated.

It has been tacitly assumed that in the cells considered the high absorp-

tion of ultraviolet radiation at 2600 A is due to nucleic acid, and in the absence of the Feulgen reaction, specifically to pentose nucleic acid. It is well to recall that this absorption is due only to the pyrimidine ring. The fact that the pentose (29, 239) and phosphorus (244) content of these cells runs parallel with the degree of specific absorption of the radiation is however strongly suggestive that the latter indeed may refer to nucleic acid.

The absorption of ultraviolet radiation at 2600 A in striated muscle fibres is due nearly entirely not to nucleic acid but to the high content of free adenine nucleotides within the fibres. In resting muscle the nucleotides appear to be localized almost entirely in the isotropic elements, but in working muscle these substances wander into the contractile, anisotropic segments (245). It appears therefore that the energy for muscular work is supplied by the transfer of adenosine-triphosphate from the reservoir in the isotropic elements to the region of the phosphatase activity of the myosin of the anisotropic, contractile elements (p. 237). Whether this transfer takes place as a prelude or as a sequel to contraction is not at present clear.

4. *Nucleoprotein Distribution in the Nucleus*

The nuclei of plant and animal cells contain formed elements, the chromosomes (p. 266), the nucleoprotein nature of which is revealed by (a) staining reactions with dyes, (b) digestion by proteases and nucleases, and (c) specific absorption by ultraviolet radiation. At the start of the mitotic cycle (prophase) there is an accumulation of nucleic acid in the chromosome which reaches a high value in the metaphase and largely disappears at the telophase (59). This nucleic acid is of the desoxyribose-type for it yields a positive Feulgen reaction; in no place other than the chromosomes is this type of nucleic acid found. At the end of mitosis, small nuclear organelles are formed which are called nucleoli and moreover a residuum of nucleoprotein is observed at the site previously occupied by the metaphase chromosome. This residuum together with the nucleoli, in the resting nucleus, do not contain desoxypentose—but only pentosenucleic acid, similar in type to the nucleic acid of the cytoplasm (246, 247). It would appear therefore that although the greater part of the chromosome was composed of desoxypentosenucleic acid, a part is also composed of pentosenucleic acid. The particular regions on the chromosomes associated with hereditary characteristics, the genes, have been located at positions in which only nucleic acid of the desoxyribose-type is present. This genic region is referred to as euchromatin. The part of the chromosome which presumably persists into the resting state of the nucleus and which contains only nucleic acid of the ribose type has been presumed to be relatively genetically inert, and is referred to as heterochromatin. The rôle of heterochromatin and its relation to the euchromatin has been extensively studied by Caspersson and Schultz (246, 248) who advanced the suggestion that the heterochromatin is responsible for the synthesis of the desoxypentosenucleic acid of the euchromatin. This

suggestion was largely based on the remarkable finding that translocation of part of the heterochromatin to euchromatic regions results in an increase in the nucleic acid content of the latter in the immediate neighborhood of the translocation, together with an instability of the neighboring genes leading to somatic variegation. In any event, the heterochromatic regions of the chromosomes have far fewer genes, per unit length, than the euchromatic regions, and these genes possess very slight phenotypic effects (249).

Parallel with the nucleic acid cycle in the nucleus runs a protein cycle, which on a smaller scale reflects again the basic Kossel hypothesis (p. 241). In prophase, as the content of desoxypentosenucleic acid begins to increase, the relatively large amount of the complex protein present begins to decrease and alter into protein of the histone type; at metaphase the ratio of desoxypentosenucleic acid to protein is at the highest value, about 1:1,

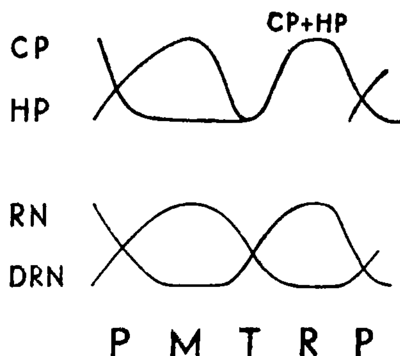


Fig. 7. Sketch of the changes during the mitotic and interkinetic phases of the nuclear elements. P, prophase; M, metaphase, T, telophase; R, resting; HP, histone-type protein; CP, complex-type protein; RN, pentose-nucleic acid; DRN, desoxypentosenucleic acid.

and the protein is exclusively of a histone nature. At telophase, the euchromatic elements of the chromosomes begin to lose the desoxypentosenucleic acid and simultaneously to alter in protein nature to the complex type, whereas the heterochromatic elements produce protein of the histone type. In the resting, interkinetic phase, the nucleus (nuclear sap) shows the presence of protein of the complex type, presumably largely products of the telophasic euchromatin, but the formed elements, namely the heterochromatin and the nucleoli contain only histone-type protein and pentosenucleic acid (208). The metaphase chromosome thus resembles the ripe sperm which is nearly wholly composed of equal quantities of protein of a primitive, basic nature and of desoxypentosenucleic acid; the resting nucleus resembles the unripe germinal cells. The nucleic acid-protein cycle during mitosis and the resting stage, based upon the work of Caspersson (208), is broadly illustrated in Figure 7.

The inverse relation between pentose- and desoxypentosenucleic acid had been pointed out by Brachet for the case of the developing embryo (219). The distribution of the complex and of the histone types of protein in the chromosome, revealed by the absorption curves in the ultraviolet has been independently inferred by enzymatic experiments. Pepsin exerts little or no effect on the chromosomal structure (49), which would be expected if the greater amount of protein present were of a simplified histone or histone-protamine nature. Some alteration occurs in the loci occupied by the more complex type of protein which might be expected to be affected by pepsin. On the other hand, trypsin, which attacks both types of protein, removes practically all the proteins of the chromosomes leaving little but nucleic acid behind (59). Treatment of the chromosome with purified nuclease resulted in loss of the nucleic acid as demonstrated by the absence of the typical staining reactions and of specific absorption in the ultraviolet, but the protein skeleton remained intact (49).

5. *The Pattern of the Chromosomes*

Nucleic acid is inextricably bound with protein synthesis. Since the chromosomes may be considered to be elongated, extensible nucleoprotein fibres, containing in certain stages much nucleic acid of the desoxyribose type, it has been frequently believed that the fibrous nature of the latter type of nucleic acid was responsible for the morphological pattern of the chromosomes. The spatial orientation of the chromosomes was supposed to be guided by the elongated, polymerized fibres of this nucleic acid. The latter, alone, shows a marked dichroism (Fig. 3). In ripe sperm, containing a high proportion of the acid, the latter also shows a strong dichroism, or a high degree of orientation (62). In the salivary gland chromosomes however, no such dichroism or orientation is observed, and it must be concluded that in these structures the desoxypentosenucleic acid is not anistropic, or at least not of such a magnitude as to be revealed by polarized radiation within the resolving power of the ultraviolet microscope. These findings are not at all surprising in the light of the known interactions between protein and thymus nucleate (Table II and pp. 225–237). The presence of a much greater quantity and of a more complex type of protein in the chromosome would be expected to reduce the anisotropic character of the nucleate. This does not imply that the desoxypentose type of nucleic acid is not partially responsible for the orientation of the chromosomal proteins, for as Caspersson has pointed out, a nucleic acid chain of 200 A, which dimension lies well under the resolving power of the microscope, could produce by lengthwise combination with protein a fibre with a molecular weight of many thousands (62). It seems to the reviewer that the problem of the morphological characteristics of the chromosome is to

be viewed in the light of the problem involving the combination of thymus nucleate with histone in thymusnucleohistone and with the globular proteins (see discussion on pp. 225–237). Nucleate which is highly asymmetric in the free state must become less so, either by previous depolymerization followed by aggregation (or perhaps by coiling) before combination with protein may take place. Denaturation of the latter, depending upon the extent, would then liberate nucleate with varying degrees of asymmetry. The nucleoproteins formed must be of a transitory nature and evidently involve the salt-like linkages postulated above. Nucleic acid in nuclei isolated from liver under neutral conditions has been observed to be almost entirely loosely-bound (228). The ease with which the nucleases attack the nucleic acid of the chromosomes suggests still further the loose binding between protein and the nucleic acid in these structures, a feature in marked contrast with the negative results obtained in trying to digest the nucleic acid away from the intact virus nucleoprotein. Finally, to the purely protein effect upon the asymmetry of the desoxypentosenucleate must be added the effect of the depolymerase, for the latter catalyst apparently occurs only in the chromosomes (49).

Study of the fine-structure of certain of the larger chromosomes revealed that the latter are discontinuous, whereby dark-staining bands containing nucleic acid and protein of the histone type alternate with light-staining bands containing protein of a complex type with little or no nucleic acid present. The genic elements are located in the former bands. In this connection it is of interest to note from Fig. 7 that during mitosis the content of histone and desoxypentosenucleic acid run parallel. The gene is thus associated with nucleoprotein of a desoxypentosenucleohistone type; whether it is identified with the latter cannot be definitely answered at the present time. Very rough estimates of the size of the gene suggest a molecule 1000 A long and 200 A wide (250). These dimensions are not far from those observed for the proteins. Somewhat indirect evidence for the nucleoprotein nature of the genes has been obtained by studies of the mutations produced by ultraviolet irradiation of the germinal material (176, 251). High efficiencies for mutation of many organisms have been observed in the neighborhood of the wave length of 2600 A at which nucleic acid absorbs very strongly. Fig. 8 illustrates such findings.

The possible fate of the nucleic acid under such conditions is illustrated in Fig. 1. The toxic effect of the radiation also reaches a maximum sensitivity at 2600 A, but the two processes of mutation and toxic action are not congruent at other wave lengths (176). Hollaender and Emmons point out that both euchromatin (genetic and containing desoxypentosenucleic acid) and heterochromatin (relatively genetically inert and containing pentosenucleic acid) would be affected by the radiation, the former

affect producing the mutative changes, the latter producing the cell division inhibition. Further indirect evidence bearing on the nucleoprotein nature of the gene has been drawn from the chemical nature of the viruses which are nucleoproteins and which, like the genes, possess the ability of reproduction and possibly of susceptibility to mutation by ultraviolet irradiation. In the chemical sense, the plant viruses being nearly all of a pentosenucleoprotein nature, are more comparable with the relatively genetically-inert heterochromatin than with the genic euchromatin.

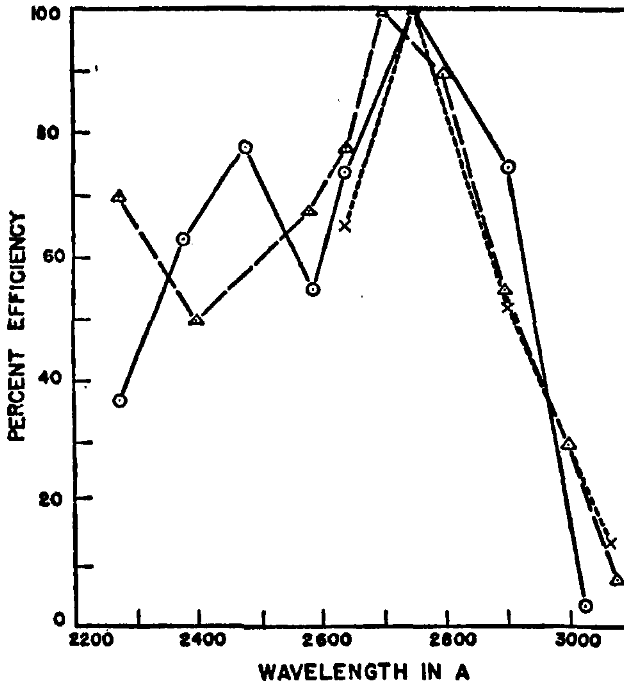


Fig. 8. Broken line: Relative absorption spectrum of sodium thymus nucleate taking absorption at 2600 A as 100 per cent. Solid line: Relative effectivity for mutation production in fungi. Dotted line: Relative effectivity of mutation production for liverwort spores (248) taking the effectivity at 2650 A as 100 per cent. From Hollaender, A., and Emmons, C. W., *Cold Spring Harbor Symposia Quant. Biol.* **9**, 179 (1941).

6. *Protein Synthesis*

It has been stated in the previous section that the synthesis of protein in the cytoplasm appears to be correlative with large amounts of the pentosenucleic acids. The latter owe their origin to the heterochromatic regions of the nucleus, and in this sense the nucleus is the focal point for protein synthesis. Indeed, as a general rule, those normal cells which are concerned with the most rapid protein production have proportionately the largest

nucleoli, whereas the converse holds in the case of those cells with little or no protein production (with the exception of certain nerve cells). The relationship between the nucleoli and heterochromatin is very close and when extra quantities of the latter appear, as in the case of a Y chromosome in the *drosophila* egg, the content of pentosenucleic acid and of protein in the nucleolus and cytoplasm increase (248). In many cases the increase in protein and in pentosenucleic acid occurs with a rather high concentration of these components near the nuclear membrane, as when the zymogen activity of the pancreas is stimulated by administration of pilocarpine (190). In such a case the rate of synthesis apparently exceeds the rate of diffusion of these materials. In yeast cells, the volutin-bodies which contain much pentosenucleohistone, and hence resemble heterochromatin or the nucleolus, dissolve when the yeast comes in contact with nutriment; as protein is synthesized the nucleohistone diffuses throughout the yeast cells (215). The general similarity in the functions of the formed bodies of the yeast and of the animal cells is of interest. It is probable therefore that the pentosenucleic acid synthesized in the heterochromatin combines with histone in the various cells and by diffusing into the cytoplasm stimulates or perhaps acts as a template by which the production of proteins is effected. If the pentosenucleohistone acts as a model upon which subsequent proteins are synthesized in its image, then a case analogous to that of the virus could be envisaged, but in this case as many models must be prepared as there are different kinds of proteins to be synthesized. Then again not all the proteins to be synthesized are necessarily nucleoproteins. Perhaps the stimulation exerted by the nucleoproteins originating in the heterochromatin on the synthesis of proteins is better envisaged by a mechanism analogous to that of antibody formation, whereby the nucleoproteins affecting each specifically a mass of protein precursors produce a series of proteins distinct in themselves, and although related to their instigators perhaps spatially, are not necessarily similar in chemical composition. In this sense, the heterochromatin and the viruses have very little in common for the latter only induce the synthesis of more of themselves in a form which is foreign to the host cell. The unidirectional growth of the virus nucleoproteins as contrasted with the cyclic, controlled development of chromatin nucleoproteins makes the multiplication of the former material resemble rather cancerous as contrasted with normal growth.

The agent which induces the transformation of unencapsulated R variants of Pneumococcus Type II into the fully encapsulated cells of Type III has by strong evidence appeared to be a thymus-type nucleic acid (74a). Only the highly polymerized and viscous form of the nucleic acid was active, for treatment with depolymerase from serum (p. 228) caused inactivation of the agent. The induced changes corresponded in type

specificity to the encapsulated cells from which the agent was isolated, and the newly acquired characters were readily reproduced in successive generations as also the agent itself in amounts far greater than the inciting dosage. Inheritable morphological, serological, and chemical alterations in a living organism are thus induced by a specific chemical substance, in this case a polymerized desoxypentose nucleic acid. It is possible to conceive the latter acting as the prosthetic group of one or more proteins of the host, the interaction between them leading to an alteration of the protein specificity and hence also in the direction of their morphological development. The implications of this important work are indeed wide-spread over many fields of biology.

In a recent study by ultraviolet microscopy of several human tumors (251a) it is suggested that "disturbances in the heterochromatic system seem to be a necessary prerequisite for the malignant growth." The findings revealed a considerable heterogeneity in cell type for any one tumor whereby "different parts of a carcinoma show extraordinarily different functional activity of the system of protein formation." The suggestive linking of the pentose nucleic acids of the heterochromatin to protein synthesis in tumors is similar to that postulated previously in the cases of embryonic and secretory cells. An enhanced nucleic acid concentration in the chromosomes of cancer cells has been reported (251b).

Whenever growth occurs within a living medium, nucleoproteins are found in high concentration, whether in the metaphase chromosome, the normal gland cell, the embryonic cell, the avian neoplasm, or the plant or animal lesion. Many different functions are involved in any one tissue and to our knowledge at the present time the forms in which nucleic acid appears are too limited to explain the possible diversity of function of the nucleoproteins. Thus all the plant viruses contain pentose nucleic acid, but the biological activity of each is distinct. Since the proteins possess a far greater capacity to exist in diverse forms, they might be considered to be responsible for the specificity of each type of nucleoprotein. If this is the case, then what is the function of the nucleic acid? The same question might be raised for all conjugated proteins, but in the case of proteins conjugated with oxidation—reduction systems such as the hemins, and alloxazine or pyridine nucleotides, the answer is more obvious. The latter prosthetic groups are responsible for the chemical effects produced, but the biological specificity is determined by the conjugated protein as a whole (103). Perhaps the specific properties of a nucleoprotein are conditioned by the molecule as a whole. It is certain in any event that the physical properties of a designated mixture of nucleic acid and protein are not additive, and in the interaction between the two some cause leading to the desired specificity of function or of morphology may be discerned.

In the consideration of the chromatin material of the nucleus little attention has ever been paid to the properties of the nuclear sap. Yet the chromosomes must be dependent to some degree at least upon the nature of their environment. Chalkley (252) has presented an interesting picture of a cyclic process involving the sulfhydryl groups of the nuclear proteins, in which these groups apparently increase in amount during reorganization of the nucleus after fission, persist during the prophase of the succeeding mitotic cycle, and disappear coincidentally with the breakdown of the nuclear membrane at metaphase. In *Amoeba proteus* these protein sulfhydryl groups are concentrated in regions nearest the chromatin. There must be some relation between the sulfhydryl and the mitotic cycles of the neighboring materials and indeed it has been observed that both nuclear growth and fission may be stimulated by addition of sulfhydryl compounds such as glutathione (253). The effect of such compounds on stimulating the activity of the intracellular proteases has long been known. On the other hand the inhibitory effect of such compounds on nuclease activity has been noted (254). In the balance among these effects, regulated fundamentally by the tension of available oxygen, may be discerned certain of the factors upon which the behavior of the neighboring chromatin depends (255, 256).

Addendum

Stedman and Stedman, *Nature* **152,** 503 (1943), have recently made some preliminary observations of a protein to which the name "chromosomin" is given, and which is presumed to be a major constituent of the chromosomes. *Cf.* Barber, H. N., and Callan, H. G., *Nature* **153,** 109 (1944).

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The Proteins of Skeletal Muscle

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I. INTRODUCTION

Several reviews on widely different aspects of muscle (Muralt, 1933; Weber, 1939; Fischer, 1936, 1941; Bernal, 1937; Kalckar, 1941; Brown, 1941; Engelhardt, 1942) have shown that the most diverse evidence is being collated and directed towards an explanation of muscle contraction in terms of the contractility and intra-molecular aggregation of polypeptide chains. Our present knowledge of muscle proteins is derived (1) from the 'colloid-chemical' studies which had their origin in the work of Kühne and have continued up to the present time in the researches of Weber, Edsall, Muralt, and Smith; (2) from the more purely enzymic studies either of well defined proteins, or more commonly, of ill-defined fractions, which have been used to elucidate the reactions of the glycolytic cycle; (3) from the direct study of muscle and of artificial myosin fibres by optical and X-ray methods; (4) from the more purely physiological studies. The most comprehensive data covering these various fields are obtained from studies of skeletal muscle, and for this reason little reference will be made to other muscle types.

Systematic research on muscle proteins began in the middle of last century when Kühne (1859) attempted to relate the clotting of muscle plasma with changes which occur during rigor. Most workers who fol-

lowed Kühne were perhaps influenced by his example in their attempts to refer the *in vitro* properties of muscle protein to the vital behavior of muscle itself. This early historical work, associated mainly with the names of Kühne, Danilewsky, von Fürth, and Halliburton, does not lie within the scope of the present review, but forms an interesting background for the study of modern work. Some of it has been concisely summarized by Howe (1924), Edsall (1930), and Smith (1930).

Striated muscle consists of fibres which are composed of numerous fibrils arranged roughly parallel to each other and to the fibre axis. In diameter, the fibrils are approximately 1μ and the fibres 10–100μ according to the type of muscle and to the species of animal. The fibrils are embedded in the sarcous medium and separated from each other by intervals of about 0.5μ. Optically, the fibres are characterized by striations running throughout their whole width, the isotropic I bands alternating with the anisotropic A bands. The length of these bands varies from species to species, but in the case of vertebrates is approximately 1.5μ. The difference of refractive index in the two bands makes the A layer appear dark in ordinary light and the I layer bright, while in polarized light the effect is reversed. Ramifying throughout and between the contractile cells is a framework of connective tissue fibres which are attached ultimately to the tendon. This tissue, consisting of collagen, elastin, and some vascular substance, is termed the extracellular protein, while the protein components of the sarcoplasm and fibril are intracellular. As will be seen later, the fibril is considered to consist of the globulin myosin in the form of a concentrated gel; the sarcoplasm contains a complex mixture of proteins, some globulin-like in character, others albuminous, these latter corresponding to the myogen of von Fürth (1895).

II. The Preparation of Muscle Proteins

When fresh muscle is minced and extracted with salt solutions (KCl or LiCl) of ionic strength 0.5 and pH 7–8, a viscous fluid is obtained which contains most of the intracellular protein. On progressive dilution of the sol at pH 7 there appears a flocculent precipitate of myosin which often exhibits a sheen when the solution is stirred. If instead of diluting, the extract is treated with increasing amounts of slightly alkaline $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, or NaCl, the first precipitate to appear is again the myosin fraction. The dilution procedure is undoubtedly superior for the initial separation of myosin from the remaining proteins and has generally been adopted in the methods described by Edsall (1930), Greenstein and Edsall (1940) and by Bailey (1937, 2; 1942, 1).

The non-myosin intracellular fraction is usually prepared from a different type of extract. Fresh muscle is minced, and glycolysis is allowed to

proceed in presence of isotonic NaCl solution for about 30 minutes, when the pH falls from about 7 to 6. The juice can then be extracted by squeezing and contains some 20% of the muscle N of which 70% is protein N (Weber and Meyer, 1933). The myosin-free juice, on dialysis to a specific conductivity of 10^{-4}, yields a precipitate which is largely soluble again in salt and was termed by Weber and Meyer "Globulin X." The residual myogen fraction on further dialysis remains soluble except for the precipitation of small amounts of denatured protein which result from a slow spontaneous denaturation of one or more of the myogen components. This insoluble protein is identical with the myogen-fibrin of von Fürth (1895). When a salt-free myogen sol is acidified to pH 3–4, denaturation is greatly accelerated (compare Bailey, 1942, 2), and on neutralizing, 85–95% of the original protein precipitates (Smith, 1937). The residual soluble protein has an isoelectric point lower than that of the original myogen, and between pH 2–7 slowly denatures and flocculates to give a product which, unlike the original myogen, is insoluble in dilute acid. To this fraction Smith gave the name "myoalbumin."

These experiments, following somewhat traditional lines of investigation, and effecting the separation of proteins into fractions which still consist of gross mixtures, are not, as might be supposed, of minor importance. The physical data thus derived led to the recognition of certain protein types which by comparison with the properties of muscle itself could be allocated to definite histological sites. Weber (1934, 2) has made a detailed survey of these data, and the more informative figures are listed in Table I.

Electrophoretic studies on the proteins, other than myosin, of the striated muscle of the rat, have been carried out by Mehl and Sexton (1942); and similar studies on rabbit muscle by Singher (1942). In both cases the electrophoretic studies indicated the presence of at least four components in addition to myosin. The principal component observed in Singher's studies appeared to correspond to Weber's myogen, but the results indicated that it was not electrophoretically homogeneous.

In recent years some progress has been made in the purification of proteins from the sarcoplasmic fraction. In 1939, Baranowski isolated two crystalline proteins from the myogen of rabbit muscle, one occurring in the form of hexagonal bipyramids (myogen A) and the other as thin transparent needles (myogen B). Myogen A was found to be homogeneous in the ultracentrifuge (Gralén, 1939), and gave a particle weight of 150,000 by sedimentation diffusion and of 136,000 by sedimentation equilibrium. Engelhardt (1942) has since found that myogen A possesses aldolase-zymohexase activity. Independently, Bailey (1939, 1; 1940) obtained a crystalline protein apparently identical with myogen B. The best preparation, obtained in the form of large rectangular needles, gave a tentative particle

weight of 150,000. Herbert, *et al.* (1940) observed similar crystals during the isolation of zymohexase. No physiological properties have as yet been assigned to myogen B, although it resembles in form and description the lactic dehydrogenase isolated by Straub (1940) from heart muscle. The zymohexase of Herbert, *et al.* (1940) also forms part of the myogen fraction and was isolated in a cataphoretically pure, though non-crystalline, condition. It comprises some 1% of the total muscle protein, and possesses an approximate particle weight of 100,000 and an I.P. of about 6.3; it contains no phosphorus or carbohydrate. The crystallization of zymohexase has since been effected by Warburg and Christian (1943).

TABLE I

Properties of Myosin, Globulin X, and Myogen

(After Weber, 1934, 2)

	Myosin	Globulin X	Myogen
Isoelectric point	5.4	5	6.3
Viscosity (1 per cent sol)	~10	1.15	1.04
Solubility in KCl, pH 6.8	0.23% at 0.3μ	0.15% at 0.005μ	Sol. in water
Values of μ at which precipitation begins in $(NH_4)_2SO_4$, pH 6.5–7.0	3.5	–	6.3*
Temperature of coagulation	No precipitation at 100°C., pH 7.1, 1.3 *M* KCl; 45°C., pH 6.3 1.3 *M* KCl	80°C., pH 7, 0.3 *M* KCl; 50°C., pH 6.5, 0.3 *M* KCl	58°C., *M*/110 buffer pH 8; 52°C., *M*/110 buffer pH 6
Particle weight (O.P.)	$\sim 10^6$	–	81,000
(Sedimentation)	–	160,000	90,000
Flow birefringence	+	–	–

* Progressive precipitation up to full saturation.

Recently, Green and Cori (1943) have described the crystallization of the muscle phosphorylase which catalyzes the reaction: glucose-1-phosphate $\rightleftharpoons$ polysaccharide + inorganic phosphate. The preparation involves extraction of ground rabbit muscle with water, dialysis of the extract and subsequent removal of an isoelectric precipitate at pH 5.8, and precipitation of the supernatant from this precipitate with 1.7 *M* ammonium sulfate at pH 6.8. The dissolved precipitate is dialyzed against a cysteine-glycerophosphate buffer at pH 6.8, and crystals form after most of the salt has dialyzed out. Rabbit muscle is estimated to contain 40 to 80 mg. of phosphorylase protein per 100 g. Cysteine greatly increases the solubility

of the enzyme in dilute salt solutions. A note by J. L. Oncley in the same paper reports the sedimentation constant S_{20} as near 13.7 Svedberg units; the diffusion constant D_{20} appears to lie between 3.2 and 3.8 $\times 10^{-7}$ giving an estimated molecular weight of 340,000 to 400,000.

Before the crystallization of this enzyme, phosphorylase had been obtained in a much more soluble but non-crystalline form (phosphorylase *b*), which was inactive without added adenylic acid, but showed as much as 90 per cent of the activity of the crystalline enzyme (phosphorylase *a*) when adenylic acid was added. A very careful study of these two forms of the enzyme has been made by Cori and Green (1943). They showed that muscle and other tissues contained an enzyme—called by them PR (prosthetic group removing) enzyme—which converts form *a* to form *b* by splitting off a prosthetic group which apparently contains adenylic acid and other unidentified groups. In the preparation of crystalline phosphorylase (*a*), the PR enzyme is removed in the isoelectric precipitate of the water extract at pH 5.9. If this precipitation is omitted, the enzyme is obtained chiefly in the amorphous form *b*. Crystalline trypsin, at pH 6.0–6.2, acts like the PR enzyme in converting form *a* to *b*, splitting off the prosthetic group with relatively little action on the rest of the molecule; this suggests that the group is bound by a peptide linkage to the rest of the enzyme molecule. The linkage is certainly a strong one; the prosthetic group is not removed by dialysis. The connection between phosphorylase *b* and added adenylic acid, on the other hand, is very loose; the adenylic acid can be rapidly dialyzed away again. Form *a* contains pentose—approximately 0.3 μg per mg. of protein—while *b* gives no measurable pentose reaction.

The activity of phosphorylase *a*, although high even when no adenylic acid is added, can be increased by about thirty per cent in the presence of added adenylic acid. The full activity of the enzyme is displayed only in the presence of cysteine (0.015 *M*) or other reducing agents; in the absence of cysteine the activity is only one fourth to one half as great. These facts are set forth by Cori, Cori, and Green (1943) in the course of a detailed study of the kinetics of phosphorylase action, into which we cannot enter here. The nature of the polysaccharide formed in the reaction, and the mechanism of glycogen formation, are considered by Cori and Cori (1943).

III. The Quantitative Determination of Proteins in Muscle

The partition of N between the various types of intracellular and extracellular protein is of great importance, although the results obtained by different investigators are inconsistent. Weber and Meyer (1933), using as extractant 0.6 *M* KCl buffered to pH 8–9, obtained excellent yields of

intracellular protein as judged by their evaluation of stroma protein, but the partition of N between myosin and non-myosin protein differs from the later analyses of Smith (1937). They determined myosin by diluting the intracellular extract to a salt molarity of 0.04, and it is probable, as Smith has indicated, that an error of about 10% arises from the myosin residuum which remains dispersed. The extractability of the intracellular fraction would appear to be determined by (1) pH, ionic strength, and ionic type of extractant; and (2) by adequacy of grinding. Smith (1934, 1; 1935, 1937) showed that 10 per cent NH_4Cl or 7 per cent LiCl were equally effective extractants and better than 10 per cent KCl-phosphate or 5 per cent $MgSO_4$, although all are adequate for the solution of the intracellular proteins after extraction. These latter can also be extracted in the form of soluble protein chloride by the use of $N/100$ HCl, and the protein N obtained in this way may be greater than that in the corresponding salt

TABLE II

Analyses of Muscles

(N as % total coagulable N)

Muscle	Author	Stroma	Myosin	Globulin X	Myogen
Rabbit (white muscle)	Meyer and Weber (1933)	17	39	22	22
Rabbit (red muscle)	Meyer and Weber (1933)	27	39	17	17
Rabbit (white muscle)	Smith (1937)	16	57	18	9
Haddock	Reay and Kuchel (1936)	3	67	—	—
Torpedo	Bailey (1939, 2)	11, 7, 12	71, 65, 67	—	—

extract, due to the presence in the stroma of unextracted intracellular protein, found by Smith to consist largely of myosin and denatured globulin X. His final analysis of muscle (1937) was derived (1) from the N partition of the LiCl extract, (2) by application of corrections for the residual solubility of myosin, (3) from the N partition of the unextracted intracellular fraction remaining with the stroma. The amount of stroma protein varies with age and with species; fish muscle may contain as little as 3% of the total protein N (Reay and Kuchel, 1936), while the myosin content is much greater than any figure reported for mammalian muscle. Some typical analyses are listed in Table II.

Muscle Proteins During Rigor and Fatigue

Deuticke (1930, 1932) and Hensay (1930), following the observations of Saxl (1907) found that the protein extracted by phosphate buffer, pH ∾ 7.2,

was greater in fresh rabbit and frog muscle than in fatigued or rigor muscle. Weber and Meyer (1933) in extending this work found that the decrease for muscles stored over a period of 24 hours resided largely in the myosin fraction, and to a less extent in the globulin X fraction. In contrast, Smith (1934, 1; 1937) using rabbit muscle and beef, observed only very small changes in the amount of protein extractable before and after storing at 0°C. for periods from 24 hours to 3 weeks. Various extractants, $MgSO_4$, NH_4Cl, and LiCl, were employed. Later (1938) he showed that rigor muscle, after exhaustive extraction with Deuticke's phosphate solution, yielded further intracellular protein on extraction with LiCl. Two explanations for Deuticke's results were proposed: (1) those parts of the fibre not destroyed by mincing are only partially accessible to the phosphate ion, and even less so on rigor, and that salts such as LiCl have a greater penetrative power; (2) that phosphate does not disperse the aggregates of myosin formed during rigor. Both Deuticke and Hensay conceded that their observed solubility changes were apparent only by the use of certain extractants. When Na acetate buffers were used, more protein dissolved from stored muscle than from fresh. More recently, Weber (1939) has claimed that the factor responsible for diminishing the solubility under his conditions is transmissible from fatigued muscle to fresh.

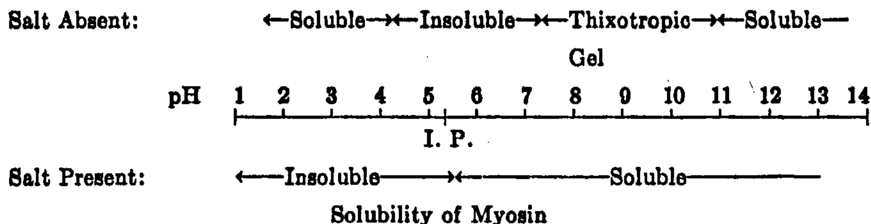
It would seem on the whole that the apparently conflicting results of different workers are resolvable by accepting Smith's explanation under the heading (2). The state of rigor or fatigue would thus involve a type of coagulation or aggregation of myosin *in situ* which prevents its dispersal in some types of salt solution. This form of coagulation has been adequately discussed by Mirsky (1937). It would lead to the supposition that in some states of rigor, coagulation has proceeded so far that dispersion cannot be effected by any solvent. Fish muscle, which yields a comparatively unstable myosin, is a case in point. Both Reay and Kuchel (1936) using Haddock and the author using Torpedo (unpublished) found large decreases in the amount of protein soluble either in LiCl or in $N/100$ HCl after the muscle had been stored.

IV. Properties of Myosin

In the fibril, myosin exists in the form of a concentrated gel, which in the A band at least consists of an organized structure of protein chains. It cannot be supposed that dispersed myosin exists in the form of single molecules in a monodisperse condition. The ultracentrifuge first indicated a polydispersity (Svedberg, 1930), and the electron-microscope photographs of Ardenne and Weber (1941) imply a pronounced tendency towards aggregation into long thin micellae. In considering the properties of dispersions of myosin, the micellar character must always be borne in mind.

1. *Solubility Characteristics*

The solubility of native myosin in absence and presence of salt is primarily a function of pH and is represented diagrammatically below.



Solubility of Myosin

Its solubility is comparable to that of a typical 'corpuscular' protein such as edestin; it is unique, however, in the way in which it swells in absence of salt in the pH range 7–9 to give extremely thixotropic gels of protein concentration 0.2–0.3% (Edsall, 1930). In polarized light, such gels give rise to pronounced pseudo photo-elastic effects (Muralt and Edsall, 1930, 1).

The solubility of myosin in dilute salt solution is of interest in relation to its state of dispersion within the fibril. In phosphate buffers, it is soluble at pH 7.4 in the range 0.25–0.3μ but its solubility increases more than a thousand fold between 0.2–0.4μ, a range which includes the ionic strength obtaining in muscle (Edsall, 1930). From a consideration of dispersion values in unbuffered KCl, Smith (1934, 2) concluded, however, that at pH 7.5—the approximate pH of resting muscle—not more than 5% of the fibrillar myosin could exist in the dispersed condition.

Most globulins on acidification in absence of salt (Bailey, 1942, 2) pass into solution, and after neutralization precipitate in the isoelectric zone to give a denatured product no longer soluble in salt. Myosin is unique in that it exhibits no gross change of solubility after exposure to pH values even lower than 1.

2. *Isoelectric Point (I.P.)*

The zone of minimal solubility in salt and water suggests an I.P. in the range pH 4.5–7. The isoelectric point as deduced from electrophoretic neutrality of myosin particles in KCl-buffer (0.05μ) lies near pH 5 (Weber, 1934, 2) but the point of minimal combination with H and OH ions was found by Salter (1926) and Edsall (1930) to occur at a much higher pH of 6.3. This latter value coincides with the pH of myosin suspensions washed many times with CO_2-free water. In spite of these findings, Hollwede and Weber (1938) showed by analytical means that myosin thus washed still contained cations which could be freed by reducing the pH to 5.4. It was thus established that both isoionic and isoelectric points lie in the pH range 5.0–5.4.

3. *Myosin and Adenosinetriphosphatase (ATP-ase)*

In 1939, Liubimova and Engelhardt made the simple yet none the less dramatic discovery that the enzyme associated with the breakdown of adenosinetriphosphate (ATP) was extracted by the methods normally employed for the preparation of myosin and was in fact always associated with the myosin itself. They tentatively suggested that the enzyme might constitute some part of the myosin molecule. In the years preceding this discovery it had been emphasized by some workers, and particularly by D. M. Needham (1938), that ATP breakdown is not only closely associated in point of time with the act of contraction, but is capable of supplying free energy to the contractile system. For this reason, the observations of the Moscow workers were seen to be of great significance. The ATP-ase activity of myosin was confirmed by several workers (Szent-Györgyi and Banga, 1941; Edsall and Singher, private communication), but experimental details have been reported only in the papers of D. M. Needham (1942), Bailey (1942, 1), and Kleinzeller (1942).

Properties of ATP-ase. Liubimova and Engelhardt studied the action of the enzyme on the Ca salt of ATP in bicarbonate, veronal, and borate buffers. They found the reaction to be optimal between pH 8.5–9 and to be greatly reduced by short exposure of the myosin to pH values below 6. Activity was measured in terms of Q_P, the volume of hypothetical gas (μl.) equivalent to the amount of P (in μg.) liberated by 1 mg. myosin in 1 hour. The Q_P values obtained, ranging from 400 to 1,000, were sufficiently low by comparison with other enzymes to advise caution in postulating an identity between myosin and ATP-ase. Extending these results, D. M. Needham (1942) used the Na salt of ATP in presence of added Ca and Mg and found that one PO_4 group was split rapidly and the second only slightly or not at all. The enzyme was inactive towards α-glycerophosphate and hexose diphosphate and was unable to transfer phosphate from ATP to fructose-6-phosphate. The abolition of SH groups by treatment with iodoacetate had no effect upon activity. At this time the effects of activation by divalent metals were obscure, and Bailey (1942, 1), continuing D. M. Needham's earlier work, showed that myosin after 3 precipitations was capable of splitting only one phosphate group, and that the reaction appeared to be activated by several divalent metals, and inhibited by heavy metals such as Cu and Hg. When, however, the myosin concentration was reduced to the point where no hydrolysis of ATP occurred in absence of added activator, it was found that the Ca ion was pre-eminent in effecting activation, Mn having a slight effect, and Mg none at all. Many of the conflicting results obtained by addition of metallic activators in the early experiments were due to the presence of a phosphatase capable of splitting adenosinediphosphate (ADP) to adenylic acid, a reaction activated by

the ions Mg and Mn. After three precipitations, myosin is generally freed from this impurity. Similar results were reported at this time by Liubimova and Pevsner (1941), who claimed that ATP-ase was actually inhibited by Mg. This latter observation, considered in conjunction with the activating effect of Ca, led DuBois, *et al.* (1943) to suggest that Mg anesthesia—a condition which is relieved by injection of Ca salts—might be explained by the replacement of Ca by Mg on the active ATP-ase surface of the muscle. In support of this thesis, they found that ATP tends to accumulate in the muscle of Mg-anesthetized animals. Greville and Lehmann (1943) have also shown that addition of Mg to myosin ATP-ase prevents the activation by the Ca ion—an antagonistic effect which, however, they are cautious to accept as an explanation for Mg anesthesia.

Under what appeared to be favorable conditions for the action of myosin ATP-ase (*e.g.*, pH 9, bicarbonate-carbonate buffer, 37°C., Ca activation) the Q_p values for rabbit myosin rarely exceeded 1,000, and other myosin types, particularly cardiac and amphibian, were only feebly active (Bailey, 1942, 1). Repeated solution and reprecipitation under carefully controlled conditions of pH and temperature produced only minor variations in the Q_p value, and fractionation procedures were quite ineffective in yielding any fraction of greater activity than the original. Cataphoretic experiments indicated an electrical homogeneity. Moreover, there appeared to be no obvious relation between the activities of myosin preparations possessing pronounced flow birefringence, and those which were non-refracting or only feebly so.

By the use of the Sørensen glycine buffers, however, it was found that a 2–4 fold enhancement of activity was obtainable, and other amino acids (alanine, leucine, serine, cysteine, glutamic and aspartic acids) exerted a similar effect. Lehmann and Pollak (1942) have observed that arginine and other α-amino acids likewise increase the velocity of phosphate transfer from ATP to creatine. In our case, the amino acid effect resides partly in the removal by co-ordination of heavy metals normally present in myosin preparations, partly in the increased heat stabilisation of the enzyme, and partly in some specific action of the dipole ion. This enhanced activity led to the supposition that carnosine or anserine might also exert an activating effect, for the function of these two peptides, peculiar to muscle and comprising some 0.5% of the total muscle substance, has never been elucidated. Their buffering range, effective from pH 6.3–9.8, suggests they may merely augment the buffering due to phosphate and phosphate esters. The Q_p value in carnosine buffer (3,300) was slightly less than that in glycine (3,600) at pH 9, temperature 37°C. The pH activity curves, however, in both glycine and carnosine at room temperature have some interesting features. When freshly prepared buffer-protein sols are employed,

the amount of splitting increases progressively up to and beyond pH 10, but sols which have stood for 24 hours show a marked diminution in their ability to cleave ATP at pH values more alkaline than 9.5 (unpublished experiments). The optimal pH at 37°C. (*i.e.*, 9–9.2) appears to be the point at which the increased rate of splitting is at last overtaken by an increased rate of inactivation as one proceeds from the neutral to more alkaline pH values.

The specificity of myosin ATP-ase is remarkable. It does not attack the simple P-esters (see above) nor the pyro-P grouping in Na pyrophosphate and K diphenylpyrophosphate (Bailey, 1942, 1). Accepting the Lohmann (1935) formula for ATP—a chain of three phosphate groups attached to the adenosine residue—it would seem that ATP-ase is either (1) an unspecific triphosphatase, or (2) that its triphosphatase activity is dependent upon the presence of the nucleoside residue. By its ability to attack sodium triphosphate (J. Needham, *et al.*, 1942) and inosinetriphosphate (ITP) (Kleinzeller, 1942), the enzyme was thought to be a triphosphatase under category (1). Using myosin concentrations lower than Needham, *et al.*, but still greatly in excess of those required to measure Q_p with ATP as substrate, the author has been unable to obtain any splitting of inorganic triphosphate. At the moment, then, it seems probable that ATP-ase attacks only nucleoside triphosphate. The modification of the adenosine residue to inosine (*i.e.*, substitution of OH for NH_2) does, however, appear to influence the rate of splitting. A recalculation of Kleinzeller's figures shows that the Q_p value at 37°C. in glycine buffer is 6,700 using ITP as against 2,330 with ATP; the highest Q_p value observed with ATP is 6,000. Just how important is the rôle of ITP in muscle we cannot assess, but as Kleinzeller has recognized, the known liberation of NH_3 during muscular contraction is linked directly with the deamination of adenine nucleotides. Moreover, ITP itself was isolated from frog muscle by Lohmann (1932).

In contrast to this specificity, the phosphatases present in liver and electrical tissue[1]—organs in which myosin is absent—are able to degrade ATP to adenylic acid (in the case of liver) and to adenosine (in the case of electrical tissue of *Raia clavata*). Although activation by the Ca ion was discernible, it was not of the characteristically rapid type observed with myosin, and on prolonged incubation, the Mg-activated systems showed most splitting (Bailey, 1942, 1).

None of the evidence either proves or refutes the idea that myosin and the enzyme are identical. While it is true that the enzyme has not been separated from the myosin it is equally true that conditions affecting

[1] This tissue arises embryologically from muscle rudiments (compare Bailey, 1939, 2).

activity have not been related to systematic changes affecting the myosin molecule as a whole. This difficulty is enhanced because there are no absolute criteria to distinguish 'native' from 'changed' or 'degraded' myosin. For example, unless care is taken in maintaining the pH of myosin above 7 it is possible to obtain preparations of feeble activity without any obvious change in the physical properties of the protein. A comparable enfeeblement is also induced on storage. Again, the enzyme character may be lost (1) by heat, (2) by acidification, (3) by dehydration with organic solvents, and (4) by depolymerizing reagents such as urea, but the irreversible coagulation of the protein is effected only by (1) in the pH range 5–7, and by (3). It is entirely reasonable to expect from the behavior of most other enzymes, that such drastic treatments will affect the enzyme grouping even when the gross solubility of the protein is surprisingly unchanged. Long thin molecules are always liable to undergo changes in their state of aggregation, and such changes will be reflected in the enzyme character only when certain groupings are involved. If ATP-ase is a universal component of muscular tissue, but independent of the myosin, we would expect its properties to be also independent of the *individual* characteristics of the accompanying myosin. This is not so. The peculiar instability of myosins from cardiac and mammalian smooth muscle,[2] *i.e.*, the facility with which they aggregate to give pseudo sols, runs parallel with their feebly enzymic character. Assuming that the enzyme is not eventually isolated unassociated with myosin, the evidence for identity must be massively built up from many types of investigation. At the moment, the highest Q_p values obtained for myosin are lower than for most purified enzymes (*e.g.*, lactic dehydrogenase, diaphorase, Q_{O_2} 60,000 and 180,000 resp.) but comparable with those of xanthine oxidase, uricase, and carboxylase. Such comparisons relate of course to the older conception of enzymes as catalysts present in small amount, but on the view that myosin and ATP-ase are identical, activity values are important only in relation to the capacity of the muscle to hydrolyse ATP. It will be realized how great is this hydrolytic ability from the fact that 100 g. muscle may contain 10–12 g. myosin.

4. *Birefringence and Viscosity Studies*

The difficulties attending the interpretation of results derived from the study of viscosity and double refraction of asymmetric molecules cannot be discussed here. Several reviews and papers may be cited: Muralt and Edsall (1930, 1 and 2); Edsall (1942); Fischer (1936, 1941); Mehl (1938); Robinson (1939); Edsall and Mehl (1940).

[2] Dr. J. W. Mehl in a private communication reports that mammalian smooth muscle, as distinct from invertebrate (see Mehl, 1940) does not respond to normal methods for the extraction of myosin. The protein which can be extracted under certain conditions is probably derived from extracellular tissue.

The total birefringence (TB) of rod-shaped particles arranged in an anisotropic manner is derived from their form birefringence (FB) and, when present, from their intrinsic birefringence (IB). If the refractive index of the medium surrounding the rods is changed, a point is reached at which TB is minimal (if IB is present), or zero (if it is not). Both forms of birefringence are found in muscle, as also in the artificial myosin fibres studied so intensively by Weber (1934, 1). From the figures quoted by Fischer (1941) it may be seen that the birefringence of muscle is due directly to the anisotropic arrangement of intrinsically birefringent particles in the fibril.

	TB	FB	IB
Myosin fibres	3.5	1.9	1.6×10^{-3}
Skeletal rat muscle	2.2	1.3	0.9

It should be noted, however, that the IB of a fibre depends upon the degree of orientation of the chains composing it (Barnes, 1933), and the value obtained for myosin fibres in the β-form will be much greater than for the oriented α-form, and greater therefore than that of the myosin particles in muscle itself, which gives a diffraction diagram of the α-type.

When brought into solution under carefully controlled conditions, myosin sols exhibit streaming birefringence (SB). In general terms, SB is ascribed primarily to the orientation of anisotropic particles, the asymmetry of which is inferred (in this case) from their anomalous viscosity and from the theory underlying the magnitude of the angle of isocline. It is due only secondarily to photo-elastic effects. The intensity of flow-birefringence is related to the concentration of particles, to their degree of optical anisotropy and of orientation, to their rigidity, to the depth of the solution through which the polarized light passes, and to other factors discussed below.

Defining a coefficient Z as follows:

$$Z = \frac{N_e - N_0}{G\eta_{sp} C}$$

where $N_e - N_0$ = observed double refraction
G = velocity gradient
η sp = specific viscosity
C = protein concentration

Edsall and Singher (private communication) found that Z was independent of pH between 6 and 8.5 and does not vary as the protein is purified by solution and re-precipitation; in the case of rabbit myosin its value at 20°C. lies between 4 and 9×10^{-7}. Edsall and Mehl (1940) showed that SB was diminished or destroyed by a variety of inorganic and organic compounds. KI, KSCN, $CaCl_2$, $MgCl_2$, arginine and guanidine salts were all effective at concentrations <0.3 *M*, and in most cases a concomitant decrease in specific viscosity occurred. Some of these reagents (guanidine,

urea) augment the number of titratable SH groups, which are present in native myosin to the extent of 0.4% as cysteine (Greenstein and Edsall, 1940). Others (glycine, arginine, NH_4^+) diminish them, while others still (Ca^{++}) are without effect. Although the loss of SB could thus be related to a decrease in specific viscosity, it could not be correlated with a systematic alteration of SH groups.

J. Needham, *et al.* (1942, and private communication) have extended the studies of Edsall and Mehl by utilizing the Couette viscosimeter for the simultaneous measurement of streaming birefringence and viscosity. Four variables were observed: (1) intensity of streaming birefringence; (2) angle of isocline; (3) anomalous viscosity, *i.e.*, variation of apparent viscosity with rate of shear; (4) limiting relative viscosity, *i.e.*, η/η_0 where η_0 is viscosity of water, and η the limiting value at which viscosity becomes independent of shear-rate as the latter increases. Birefringence was found to increase linearly with protein concentration, and was unchanged by the ionic species in the case of Li, Na, K, Rb, and Cs, all present as chloride at ionic strength 0.5—the concentration at which birefringence is maximal. The continuous fall of SB with further increase of ionic strength was accompanied by a rise of relative viscosity up to ionic strengths of 1.5–2, when viscosity too began to fall. It was found possible to restore SB in sols previously treated with Mg, NH_4, and Ca chlorides, or in those allowed to age, merely by incubating at 37°C., without producing any great change in the angle of isocline. A similar restoration was effected in certain cases by removal of the reagent which originally caused loss of birefringence.

The most interesting feature of this work, when considered in relation to the foregoing enzyme section, is the reduction of streaming birefringence on addition of salts of ATP. At concentrations as low as 0.004 *M*, ATP will reduce streaming birefringence by some 50% in a protein sol of 1–3% strength, and although flow anomaly is unaffected, the limiting relative viscosity falls by about 14%. After an interval of time (15′ to several hours), birefringence and viscosity return to their original values, and the effect may then be repeated. ITP acts in a similar way, but ADP and Na triphosphate have no effect. Once-precipitated myosin preparations do exhibit the ATP effect with ADP, due possibly to the myokinase impurity (Kalckar, 1942) which effects its conversion to ATP and adenylic acid.

It is not absolutely clear to what extent this remarkable action of ATP is related to the enzyme character, though the restoration of streaming birefringence does run roughly parallel to ATP breakdown; whether, in fact, ATP acts on the birefringence by virtue of, or independently of, its combination at a specific enzyme grouping, and whether a myosin inactivated by ageing, by heavy metals, or by complete absence of Ca activator would give a comparable effect. The above authors have recognized

(J. Needham, *et al.*, private communication) that the interpretation of birefringence changes, even when considered in relation to viscosity changes, is many-sided, and as yet no peremptory interpretation of the salt or ATP effect is available. The ultimate explanation of the latter may conceivably link with the observations of Engelhardt, *et al.* (1941), that enzymically active myosin fibres are more extensible on immersion in ATP solutions.

5. *Amino Acid Composition of Myosin*

Apart from the purely nutritional standpoint, the amino acid composition of myosin is of great importance because the ultimate mechanism of muscular contraction must be connected with the disposition of groups along the polypeptide chains. Published assays are derived mainly from the work of Bailey (1937, 1, 2) and Sharp (1939), but more recently, chromatogram values for the mono-amino acids have been obtained by Synge (see Astbury, 1942) and by Tristram, and for the hydroxy-amino acids by Rees. The analyses of Bailey were confined to the determination of total N, amide N, total S, cystine, methionine, tryptophan and tyrosine, but values were obtained for myosins from a number of different species, *viz.*, rabbit, dog, ox, chicken, fish (teleost), and lobster. At that time physico-chemical studies of myosins from various sources (Weber, 1934, 2; Edsall, 1930; Muralt and Edsall, 1930, 2; see also Mehl, 1940) had indicated the fundamental similarity of myosin with respect to flow-birefringence, viscosity, etc., but had revealed minor differences which might be due to (1) differences of chemical composition, or (2) differences in the state of aggregation. The seven analytical criteria studied were expected to reveal whether in the course of evolution a fundamental and invariable amino acid composition had been elaborated for the myosins of all species, or whether minor differences could be considered to uphold the general principles which have been the *leitmotiv* of Astbury's interpretation of X-ray and analytical data, *viz.*, that the mechanisms for the elaboration of protein pattern are few in comparison with the endless diversity of the proteins themselves, and that out of the superficial complexity of proteins which are members of any one group (*e.g.*, the keratin-myosin, the collagenous, etc.) may perhaps be sifted some fundamental, perhaps simple, skeletal plan. In a more generalized form, this point of view is reflected most consistently in hypotheses which from time to time have become the cynosures in protein research, the Svedberg hypothesis, the Wrinch theory, the Bergmann-Niemann frequency theory, and the Roche plasticity hypothesis. The collected data of Table III do indicate a constancy of composition in the mammalian and avian myosins, but with teleost and crustacean myosin, small variations outside the range of experimental error are apparent.

Thus, although we cannot maintain that all myosins are chemically

identical, the indications are that variations are not very large, a point which can be confirmed only by more extended analysis. The water-soluble proteins of muscle (myogen fraction) differ from myosin in composition, and subsequent analyses revealed a high degree of non-uniformity amongst various samples.

Some comment on the analyses of Table III is necessary. In the original paper (Bailey, 1937, 1, 2) attention was drawn to the serious decrease in cystine on continued hydrolysis, due most probably to destruction of SH groups shown to be present by Mirsky (1936), Todrick and Walker (1937), and Greenstein and Edsall (1940). A more reliable estimate of cystine-S is obtained by the difference of total S and methionine-S for the following reasons: unidentified forms of S in proteins have been reported from time to time (*e.g.*, Blumenthal and Clarke, 1935), and in particular that which is present in acid hydrolyzates and is oxidized to sulfate by bromine water.

TABLE III

Analyses of Muscle Proteins after Bailey (1937, 2)

Results expressed as % protein weight

	Myosins						Rabbit Myogen
	Rabbit	Dog	Ox	Chicken	Fish	Lobster	
Total N	16.7	16.6	16.6	16.6	16.6	16.6	16.6
Total S	1.10	1.06	—	1.12	1.21	1.21	1.29
Methionine-S	0.73	0.72	—	0.74	0.79	0.76	0.60
Cystine-S (see text)	0.37	0.34	—	0.38	0.42	0.45	0.69
Tyrosine	3.4	3.3	3.2	3.3	4.3	3.6	4.2
Tryptophan	0.8	0.8	0.8	0.8	0.95	0.85	1.5
Amide-N as % of total-N	7.20	7.17	—	7.00	6.66	6.75	5.53

This reaction is so reminiscent of the facile oxidation of the SH group of thiolhistidine that the presence of this amino acid in proteins (in addition to cystine and methionine) has been suspected. In unpublished experiments, however, the author found that the bromine-oxidizable S of edestin and wool hydrolyzates is also reducible to H_2S by nascent hydrogen, and is due to elementary S held firmly in solution by the amino acids. It is derived most probably from cystine breakdown (see Routh, 1938). The total N and all other values reported are obtained from myosin rigorously extracted with organic solvents to remove a loosely associated lipoid complex—present to the extent of 5–10%—which has led to discrepancies in the N values of other workers.

A more comprehensive analysis of rabbit myosin is given in Table IV. The dicarboxylic acids were isolated by the Foreman (1914) ethanol-lime method and the bases by Block's procedure; the mono-amino acids either

by fractional distillation of the ethyl esters or by chromatographic analysis, and the hydroxy-amino acids by a modification of the method of Nicolet and Shinn (1939). According to Chibnall's revised analyses of proteins (Chibnall, 1942; Bailey, Chibnall, *et al.*, 1943; Chibnall, *et al.*, 1943) obtained by the most exacting isolation procedures ever reported in protein chemistry, it is probable that glutamic acid and base figures are reasonably correct, and the aspartic acid somewhat low. The glycine figure by the Fischer method must be considered a minimal value, while the individual figures from chromatographic estimates represent merely an order of magnitude rather than very exact values. The chromatographic data, however, do complete the analysis to the point where a reasonably correct value for the average residue weight (ARW) can be derived, *i.e.*

$$\text{ARW} = \left(\frac{\Sigma x}{\Sigma y} - 18\right)$$

(where x = weight of amino acid, and y = number of g. residues/100 g. protein) = 115, a value agreeing exactly with that calculated from X-ray spacing and density measurements; the true value is probably somewhat lower since glycine is underestimated (see also Note 3, Table 5). The yield of identified N as a % of the total N amounts to 88%.

Minimal Molecular Weight. Significant values of the M.M.Wt. can be obtained only from integral constituents present in small amounts. The P content of 0.054% (Bailey, 1942, 1) and tryptophan, 0.82% (Bailey, 1937, 2) give the values 57,000 and 25,000 respectively. Cysteine determinations, 0.31% (Mirsky, 1936), 0.27% (Todrick and Walker, 1937), 0.42% (Greenstein and Edsall, 1940) lead to M.M.Wt. values of 39,000, 45,000, and 29,000 respectively. The scattering of these values is so great that no reliable estimate can be made. For the purpose of calculating numbers of residues, we shall adopt the arbitrary value of 100,000.

Acid-Base Equivalence and Acid-Base Binding

The sum of arginine, histidine, and lysine groups amounts to 121.7 mol./10^5 g.; the dicarboxylic acid minus amide to 132.3, leaving an excess of 10.6 acid groups. Whether the phenolic groups of tyrosine (18.8 mol.) are free to dissociate in the predicted pH zone is quite unknown. The correlation of analytical data with titration data shows some unresolved divergences.

In a private communication, Edsall has drawn attention to the fact that while titration data are reported in most cases as mol./g. of lipoid-free protein, the undried myosin which is titrated contains phosphatide. Some error is thus involved in the titration of extraneous P and of choline and ethanolamine.

Authors (titration data)	pH Range	Acid-Base Binding mol./10^5 g.	Analytical Figures
Gale and Singher*	6.3–1.8	174	Total base = 121.7
Hollwede and Weber (1938)	6.3–5.4	4.5	
Bailey (unpublished)	5.5–1.8	146	
Salter (1926)	6.3–1.8	150	
Perlmann (1941)†	—	150	
Gale and Singher*	6.2–8.3	34	Histidine = 11
" " "	6.2–10.4	96	Histidine + lysine = 81.5
Salter (1926)	6.3–12.2	130	Total non-amidized-COOH = 132.3

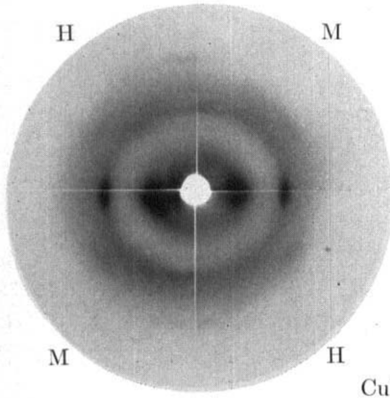
* Private communication.
† Metaphosphate binding.

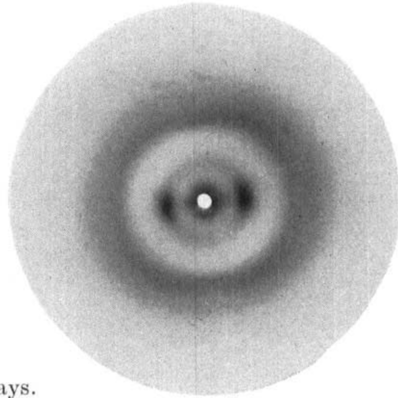
6. *The Interpretation of Analytical Data*

The interpretation of analytical data in terms of stoichiometric principles has recently been reviewed (Astbury, 1943) and will not be discussed here. Certain aspects of the amino acid composition of myosin, however, have led to a line of inquiry which suggests that some of the analytical and structural features of the myosin molecule are reflected in those of fibrinogen. Before these points of comparison can be appreciated, it is necessary to outline the primary facts which any theory of protein structure must embrace. X-ray evidence suggests that a characteristic type of folding may be common to proteins which in the analytical sense show wide variations amongst their amino acid constituents. Of this general principle, keratin and myosin are good examples. Any attempt to resolve the detailed plan of protein structure in terms of individual amino acids would seem rather hopeless; the more logical procedure has been adopted by Astbury (1942) in considering the possible arrangement of whole groups of amino acids, each group being defined by the character of its side-chain residue. The broadest classification of such residues naturally distinguishes between the polar and non-polar side-chains, but these are further subdivided into particular groups, *e.g.*, those with acidic, basic, and H-bonding properties, and those with paraffinic properties. It is supposed that for proteins which possess the same type of structure (as deduced from X-ray and physical properties) the broad structural pattern is defined by the arrangement of these whole groups of amino acids, and that only the detailed pattern is varied by altering the proportions of amino acids within the group—serine for threonine, aspartic acid for glutamic, glycine for alanine, etc.

This type of approach seems logical when we consider how dissimilar are keratin and myosin from the analytical standpoint, and how similar struc-

turally. Firstly, X-ray evidence has shown that myosin in the isolated, dried state consists for the most part of polypeptide chains in the regularly folded α-form, which gives rise on partial stretching to oriented α, and on complete stretching to oriented β. The latter form, when allowed to contract in water, again assumes the α-form (Astbury and Dickinson, 1935,

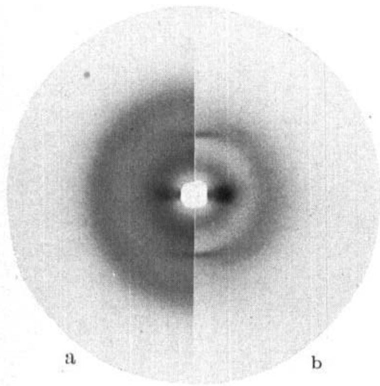




CuKα rays.

Fig. 1. X-ray comparison photograph of foot retractor muscle of *Mytilus edulis*. (a) living muscle, (b) dried muscle

Fig. 2. X-ray sector comparison photograph of α-horn (H) and α-myosin (M).



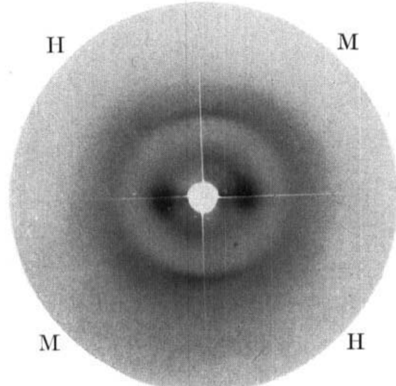


Fig. 3. X-ray sector comparison photograph of β-horn (H) and β-myosin (M).

Fig. 4. X-ray photograph of α-fibrinogen.

1, 2; 1940). This reversible α-β-transformation is completely characteristic of the keratins as the photographs in Figs. 2 and 3 demonstrate. But in addition to X-ray evidence, the more purely elastic and selective orientation properties of air dried myosin resemble those of keratin; not so much native keratin as the super-contracting form in which disulfide bonds have

been broken—a phenomenon not unexpected in view of the low cystine content of myosin and the characteristically high one of keratin.

The more detailed interpretation of the keratin-myosin pattern has been sought in terms of the α-model of Astbury (1941) which has been derived solely from considerations of density, elasticity, and crystallographic data. The particular postulate which Astbury (1942) makes with respect to the keratin-myosin group is that individual side-chains in the polypeptide chain are alternately polar and non-polar, whence it follows, by reference to the α-model, that the side-chain triads on one side of the chain are all polar, and those on the other, non-polar. This type of intramolecular aggregation predicts not only the long-range elasticity of myosin and keratin, but is also in keeping with the findings of Gordon, *et al.* (1941) that the basic amino acids are linked to all types of acids with non-polar side-chains. From such a postulate follows at once the provision that the polar amino acids must constitute about one half of the total residues, which indeed is the case in myosin and keratin.

In the case of fibrin, however, it was noticed that the analytical figures available in the literature were in many ways similar to those of myosin, and although the structural details as deduced by X-rays had not been studied in any great detail, it was suspected that here was a case in which both structural and analytical features were more alike than had been realized. It may be recalled that the physico-chemical properties of fibrinogen bear comparison with those of myosin. Like myosin, fibrinogen is an asymmetric molecule of high particle weight,[3] whose solutions show anomalous viscosity (Wöhlisch and Kiesgen, 1936) and flow birefringence (Boehm and Signer, 1932). Again like myosin, it has globulin-like properties and is loosely associated with lipoid, and gives rise during the enzymic clotting of plasma to doubly refracting threads of fibrin, which resemble Weber's myosin threads. Moreover, there exists a biological parallel in that Ca ions play an important part in the clotting of blood and in the activation of myosin-ATP-ase. In collaboration with Drs. Astbury and Rudall, the comparison was extended to an X-ray investigation of fibrinogen and fibrin (Bailey, *et al.*, 1943). The utmost difficulty was experienced in obtaining 'clean' X-ray photographs such as are readily obtained with myosin and keratin, but it became apparent that fibrinogen gives a photograph of the α-type (Fig. 4), which with some difficulty may be transformed to β. Most unexpectedly, and contrary to the results of Katz and de Rooy (1933), fibrin gave also a predominantly α-pattern, containing small amounts of poorly oriented β. The relation of these results to the clotting of blood cannot be discussed here, but they indicate that fibrinogen and

[3] Mr. G. S. Adair has kindly carried out O.P. measurements on fibrinogen dissolved in 0.333 M $(NH_4)_2HPO_4$, pH 8.5. Tentative values indicate a particle wt. of 500,000.

fibrin are members of the keratin-myosin group, although admittedly the α-β-transformation is as yet neither so complete nor so perfect as in the latter.

Parallel with the X-ray experiments, comparative analyses of rabbit myosin and human fibrin were commenced, in which most attention was paid to those acids hitherto unsatisfactorily determined in the case of fibrin, *viz.*, tyrosine, tryptophan, serine, threonine, the imino and mono-amino acids. Yields are reported in Table IV and converted in Table V to residue numbers/10^5 g. of protein. It will be seen that the really striking difference between myosin and fibrin is the high hydroxy-amino acid content of the latter,[4] and in this respect fibrin more resembles keratin than myosin. Approximate agreements are found, however, in histidine, arginine, lysine, total dicarboxylic acids, and cystine; the chromatogram values are not sufficiently accurate to justify a comparison. In addition, it may be noted that I.P., acid binding, density, and average residue weight do not differ widely.

For the present, it is unwise to lay too much stress on such individual similarities until we know just what is the significance of those amino acids which show the most obvious divergence. The main emphasis must be laid upon the comparative figures for amino acid groups, shown at the base of Table V. Here it is seen that fibrin, like myosin and keratin, conforms to Astbury's postulate that a protein exhibiting the α-β-transformation must contain about 50% of the total groups as polar groups. One other point is of considerable interest. The total charged groups of myosin and fibrin (*i.e.* base + free acid) comprise more than one quarter[5] of the total, and this high valence, considered also in conjunction with the great asymmetry of both molecules, is just the character which might be expected to play an important part in effecting changes of aggregation state. Such changes are unquestionably of fundamental importance in relation to the biological function of these two proteins, the contractility of myosin, and the enzymic transformation of soluble fibrinogen to insoluble fibrin. Moreover, at the physiological pH, all such groups may be assumed to be fully ionized with the exception of histidyl groups (of which in any case there are few), and the total charge on the protein as distinct from the net charge, will approach the maximal value.

The evidence as it stands would thus seem to indicate an analytical relationship between myosin and fibrin in the group distribution of amino

[4] The high threonine value is interesting in view of the isolation by Rose & coworkers of this amino acid from fibrin hydrolyzates.

[5] Comparing the figures derived for other proteins, it is found that two others, and these also globulins, have a comparably high valence: edestin, 26.9%; β-lactoglobulin, 26.7%. Compare ovalbumin, 19.6%; casein, 17.0%; insulin, 9.5%; zein, 3.2%.

TABLE IV

Analysis of Myosin and Fibrinogen-Fibrin

Yields in terms of wt./100 g. protein

	Rabbit Myosin	Method	Fibrinogen-Fibrin (C, cattle; H, human)	Method
Total N	16.6–16.8[3,4]		17.1[9] H	
Amide N	1.19[3]	Mild hydrolysis	1.33[9]	Mild hydrolysis
Total S	1.10[3]		0.97–1.10[10,11]	
Arginine	7.0[4]	Isolation	7.7[12,13] C	Isolation
Histidine	1.7[4]	"	2.5[13] C	"
Lysine	10.3[5]	"	10.1[13] C	"
Glutamic acid	22.1 } 31.0[4]	"	14.8* } 27.4[14] C	Isotopic
Aspartic acid	8.9 }	"	12.6 } C	"
Tyrosine	3.4[3]	Colorimetric	5.15[9] H	Colorimetric
Serine	3.9[8]	Oxidation	6.95[8] H	Oxidation
Threonine	4.95[8]	"	6.15[8] H	"
Cystine	1.4[3a]	See text	1.5[18]	Mercaptide
Methionine	3.4[3]	Volatile iodide	2.6[15]	Volatile iodide
Tryptophan	0.8[3]	Colorimetric	3.25[9] H	Colorimetric
Glycine	>1.9[4]	Fischer	5.7[14] C	Isotopic
Alanine	6.5[6]	Chromatogram	3.7[6] H	Chromatogram
Leucines	15.6[6]	"	11.7[6] H	"
Valine	2.6[6]	"	3.9[6] H	"
Proline	1.9[6]	"	3.85[6] H	"
Phenylalanine	4.3[6]	"	5.0[6] H	"
Total	100.65		107.15	
Identified N as % total N	87.8		94.1	
Isoelectric point	5.4[1]		5.2–5.6[16] H	
Acid binding	0.062 g. HCl/g.[2]		0.06 g. HCl/g.[17]	
Density	1.265[7]		1.269[7] H	

* Corrected for moisture and ash to N basis 17.1%.

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TABLE V

Analysis of Wool Keratin, Myosin, and Fibrin

g. Residues/10^5 g. of protein

	Wool keratin[1]	Myosin[2]	Fibrin[2]
Arginine	59.2	40.2	44.3
Histidine	4.5	11.0	16.1
Lysine	18.2	70.5	69.2
Glutamic acid	95.9	150.4	100.7
Aspartic acid	49.4	66.9	94.7
Tyrosine	25.7	18.8	28.2
Serine	98.1	37.2	66.3
Threonine	53.8	41.5	51.8
Cystine/2	98.9	11.7	12.5
Methionine	4.7	22.8	17.5
Tryptophan	8.8	3.9	16.0
Glycine	87.0	25.3	76.0
Alanine	46.4	73.0	41.6
Leucines	86.3	119.1	89.3
Valine	41.0	22.2	33.3
Proline	59.1	16.5	33.5
Phenylalanine	22.7	26.1	30.3
Amide-N	81.0	85.0	95.0
Average residue wt.[3]			
(a) X-ray and density	118	115	116
(b) From analysis	107	115	113
(c) From N distribution	112	111	112
Present probable value	112	114	114
Residues/10^5 g. protein	893	877	877
As % of total residues:			
Polar groups	45.3	49.8	53.8
Base + free acid groups	16.4	29.0	26.2
Hydroxyl groups	19.9	11.1	16.7
Amide groups	9.1	9.7	10.8

[1] After Astbury (1942).

[2] This paper, Table IV.

[3] Method for (a), see Astbury (1942); (b) calculated from the expression $\left(\frac{\Sigma x}{\Sigma y}\right) - 18$, where x = wt. of amino acid/100 g. of protein, and y = g. residues of amino acid/100 g. of protein; (c) derived by calculating the total g. residues of amino acids from a consideration of the amide-N and the N content of histidine, arginine, lysine, and tryptophan in relation to the total N content. On discussion with Dr. Astbury, it appears that the present X-ray estimates (a) are probably too high on account of the proteins in question not being exhaustively dehydrated when they were photographed: the question is now being submitted to further examination. Keratin presents the difficulty, too, that the available analyses afford no more than a composite picture of a variety of wools.

acids, and to some extent in the amounts of particular acids such as the basic ones. This, considered with the biological, physical, and X-ray aspects, indicates a biogenetic relationship which is worthy of further study.

V. Muscle Proteins in Relation to Structure and Contractility

There is no reason to suppose that the basic mechanism of muscular contraction is different for the various types of plain and striped muscle. Striations seem to have been developed as specialized devices for reducing the high chronaxie associated with smooth muscle. In its contractility and anisotropy, only the A band resembles the smooth muscle fibre, while the I band appears to be a more extensible structure of unknown function, which lengthens considerably in isometric contraction and less so in isotonic contraction (Buchtal, 1936). In view of the fact that some evidence exists (see below) for the localization of certain muscle metabolites in one band or the other, it may well be that a striated structure facilitates the supply of material to the actively contracting sites of the muscle.

The points of similarity between the properties of isolated myosin and the protein as studied in the A band prove that myosin really is the contractile element of muscle. These properties are: (1) the asymmetry of the myosin molecule as deduced from viscosity, streaming birefringence, X-ray diffraction, and electron optics; (2) the intrinsic birefringence and mechanical anisotropy of artificial myosin fibres, used by Weber (1934, 1) as a model for the fibril; (3) the fundamental similarities in the diffraction patterns of muscle, living *or* dried, with those of myosin films in various states of orientation. There has always existed a doubt as to whether the I band is composed of myosin chains in a less oriented condition, or of some other protein (see Weber, 1934, 2). The high myosin content found by Smith (1937) in mammalian muscle, and by Reay and Kuchel (1936) and Bailey (1939, 2) in fish, suggests the correctness of the former view. It would indeed be difficult to imagine that any other type of protein—apart from the stroma—could withstand the tension exerted by the A band on the I during contraction.

Accepting then that the fibril is wholly composed of myosin, it is of some interest to elucidate the size of the smallest structural units, the single micelles, and the manner in which these are arranged in the A and I band. Weber (1934, 1) first deduced the size of the single micelle as 55 mμ long and 5 mμ wide. The calculation was derived from the particle weight of myosin (10^6), from the partial volume (deduced from form birefringence), and from the fact that myosin fibres do not imbibe myogen molecules of estimated diameter 9.5 mμ. The long-spacing equatorial diffractions observed by Astbury (unpublished) and by Meyer and Picken (1937) in muscle itself indicate, as Schmitt (see Fischer, 1941) has noted, that the

unit may be much wider than Weber's 50 A. The great length of micellar aggregates, however, is very striking. Tentative calculations from studies of flow birefringence give the value 11,600 A for rabbit, and 28,000 A for snail myosin (Edsall, 1942). The electron microscope reveals that myosin aggregates obtained by fixing a very dilute sol with osmium are several thousand mμ long by 5–10 mμ wide (Ardenne and Weber, 1941). In Schmitt's view (see below) the single micelles should not be considered as isolated rodlets, but as units connected by anastomosing chains to form a syncytial lattice. Possible and probable modes of micellar aggregation have also engaged the attention of Ardenne and Weber (1941).

X-ray studies of resting muscle (Astbury, unpublished) indicate that the myosin chains within the micelle exist in the folded α-configuration. The drying of muscle or of isolated myosin under certain specified conditions merely involves a denaturation connected with the aggregation of the α-form, and does not produce any fundamental change in the diffraction pattern. This fact, quite generally unappreciated, is of profound importance, and provides a firm basis for inferring the structure of muscle from the elastic and micellar properties of isolated myosin. By the kindness of Dr. Astbury is shown on one and the same film (Fig. 1), hitherto unpublished, (a) the living foot retractor muscle of *Mytilus edulis*, and (b) the same dried. The latter is quite similar to the diffraction pattern of isolated, oriented α-myosin (Fig. 2), and the comparison brings out very convincingly how the essential configurational features of the myosin molecule remain the same, whether in living or in dead muscle, or in the isolated protein. The additional rings visible in (a) arise from the fluid contents of the muscle. It should be pointed out that during exposure to X rays, the living muscle was moved longitudinally to minimize the deleterious action of the beam, and was physiologically active at the end of the experiment.

For limited contractions of muscle there is no evidence from X rays of any extensive disorientation of the α-form. Astbury has shown (private communication) in a series of photographs of living muscle that the disorientation of the α-form is negligible at 10% contraction and very little even at 20% contraction; that, in fact, it is always less than that given by calculations based on micellar disorientation alone. The effect is very similar indeed to what is observed when α-keratin is caused to supercontract: in both cases the α-pattern suffers delayed disorientation but preserves its identity (Astbury and Woods, 1933, and unpublished data). This and other phenomena of a like nature that occur in the stretching and 'setting' of the keratin-myosin fibres indicate that the correct point of view is to consider the chain molecules as acting both in series and in parallel. In the process of supercontraction the less 'crystalline' parts contract first, and because these and the more 'crystalline' parts lie both

in series and in parallel, all that appears in modest contractions is a delayed disorientation of the latter. As for the actual kind of folding that is brought about in the supercontraction of keratin or the contraction of muscle, it seems that no new *regular* structure is generated, but we may visualize two possible processes. It may be that some chains are imperfectly folded, and the contractile mechanism induces greater regularity in this respect; or chains that are already folded fold still further on account of increased facilities for aggregation among the side-chains. In very large contractions the latter process must predominate, as, for example, in muscle such as the retractor of the holothurian, Thyone, which can shorten to $^1/_{12}$ of its original length. Here it is not possible in any sense to visualize contraction in terms of random micellar disorientation, which at the most could lead to contraction of the order of 100%.

The relation between contractility and the chemical reactions occurring in muscle is at present obscure. It has been supposed that they affect ultimately the ionization (and hence the length) of myosin chains either (1) by a gross change of pH (Meyer, 1929; von Muralt, 1933), (2) a localized change in the neighborhood of certain groups, (3) by esterification of some side-chain group which previously contributed to the total charge. Bernal (1938) has pointed out that it is, in fact, only necessary to invoke a different ionic environment in order to produce a change in length in hydrated fibre systems. Of these alternatives, (1) is quite improbable, but the rest have some bearing upon the present view that myosin contains an active ATP-ase grouping. In general, any localized reaction taking place at a group or grouping in a polypeptide chain will influence the configurational disposition of other groups, whether it results in the formation of a co-valent link, or merely, as is probable with ATP, in the formation of metastable links between enzyme group and reaction products. Such a reaction may be set in motion, as already suggested (Bailey, 1942, 1), by making available, via the stimulus, of a specific activator—in this case the Ca ion. In these terms we are reverting to the older concept of contraction, that chemical change is coincidental with, or just initial to, the act of contraction, but it is possible also to formulate theories by which ATP takes part in the relaxation process (Needham, *et al.*, 1941). On present evidence it is undesirable to explore alternative hypotheses, but it is interesting to find that Brown (1941), merely by considering the cycle of energy liberation in muscle, has proposed a theory rather similar to those derived from a consideration of myosin as ATP-ase. Into such theories must ultimately be fitted points of detail; why, for example, the adenine nucleotides are located in the isotropic regions in relaxed muscle, showing diffusion into the anisotropic on fatigue (Caspersson and Thorell, 1942), and why the mineral constituents (Ca and Mg) are localized in the refracting band

(Scott, 1932). Such facts are not only of general importance if ATP is to remain the key substance in relation to muscle contractility, but they may also illustrate, as mentioned above, the kind of difference which exists between skeletal and smooth muscle.

With few facts and much logic, Aristotle, in his 'De Motu Animalium' proposed with supreme confidence that the cause of animal locomotion lay in the ability of the 'spirit' to expand and contract. The contemporary reviewer, with many facts, less logic perhaps, and certainly with less confidence, can indicate merely the type of theory towards which several lines of investigation appear to converge. Even so, the gaps which separate fact from theory are obvious, and some speculation seems unavoidable. If this must be justified, it can at least be said that the most peripatetic hypothesis has often contributed something towards the ultimate truth.

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The following papers may be noted, which appeared too late for discussion in the review: H. M. Kalckar "Adenylpyrophosphatase and Myokinase," *J. Biol. Chem.* **153**, 355 (1944); M. Ziff and D. H. Moore "Electrophoresis, sedimentation, and adenosinetriphosphatase activity of myosin," *J. Biol. Chem.* **153**, 653 (1944). Determinations of amino acid content of fibrin (compare Tables IV and V of this article) are reported by E. Brand, B. Kassell, and L. J. Saidel, *J. Clin. Investigation* **23** (in press) (1944).—*Note added by editor, June 1944.*

* This reference has been available to the author only in the form of an extended summary prepared by J. T. Edsall.

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