ADVANCES IN ENZYMOLOGY

AND RELATED SUBJECTS OF BIOCHEMISTRY

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I. Introduction

During the past ten years there has been a great increase in the amount of work done on the purification of plant viruses and many confident statements, of varying degrees of validity, have been made about their physical and chemical properties. These statements have often been contradictory and, although many of the earlier contradictions have been resolved by further and more critical work, some of them still remain. It is to be expected that these also will soon be resolved, for the study of plant viruses involves no more intrinsic difficulties than the study of any other group of proteins. Under present circumstances, however, a critical review is bound to be colored by the reviewer's opinions; no claim is made that this review is impartial.

No virus preparation has yet been made that can be looked on as pure in an ideal sense. Such a preparation has been defined (89) as containing "particles identical in size and chemical composition and each carrying the

full unmodified activity of the starting material." With no plant virus preparation has it been possible to show that every constituent particle is infective. The physical and chemical properties of many virus preparations, however, probably resemble those of the actual infective agents closely. Although it is legitimate to be uncertain whether any preparation consists exclusively of infective particles, it would at this stage be unreasonable to doubt that the properties of these preparations have a bearing on those of the infective particles.

Preparations of many viruses besides those discussed in this review have been made; their properties have been described in a recent book (3), and the amount of work that has so far been done on each is so small that the time does not seem ripe for any further review of their properties. It may be said, however, that all purified preparations have consisted largely or exclusively of nucleoprotein. The viruses worked on are not, however, a random sample from the whole group of plant viruses, for only those that are stable for at least a few days *in vitro* have been purified, and it may well be that this initial selection by the research worker is the cause of the apparent chemical uniformity of the group. The diversity in the symptoms caused on susceptible plants by these viruses has little bearing on this question of initial selection by the research worker, for in no other field of pathology is there any close connection between the chemical composition of the infective agent and the type of symptom caused.

II. Tomato Bushy Stunt Virus

Of the viruses that have been studied by several different groups of workers, that causing tomato bushy stunt is the one upon whose properties there is now the greatest measure of unanimity. This is in part due to the fact that only one strain of the virus has been recognized, and this appears to exist in only one physical state.

The first purified preparations (7) were made from sap that had been heated to remove most of the normal leaf protein, greatly simplifying the preparation but leading to a product of reduced infectivity. Later methods of preparation (12, 109) do not entail this partial inactivation, and the product is as infective as the sap from which it came. This does not necessarily mean that it has suffered no inactivation because the other components in the sap tend to reduce infectivity. There is as yet no means of demonstrating that the preparations are in an "ideally pure" state. Preparations are generally made from the leaves of infected tomato plants, but *Datura stramonium* and *Nicotiana glutinosa* have also been used.

Virus occurs not only in the sap but also in the fibrous leaf residue and can be liberated by fine grinding or by enzymatic digestion (8).

1. *Physical Properties*

Preparations made simply by differential centrifugation appear to be slightly contaminated with normal leaf chromoprotein for they are described as pale green (84, 109); those made by precipitation with ammonium sulfate (12) are colorless, and there is no reason to suspect the presence in them of any material not closely related to the virus. The virus, like other nucleoproteins, shows an absorption maximum at 260 mμ (7, 109).

The precipitate that separates when an excess of $(NH_4)_2SO_4$, Na_2SO_4, $FeSO_4$, $ZnSO_4$, $MgSO_4$, $MnSO_4$ or $Na_2S_2O_3$ is added to a virus solution at room temperature (13) is amorphous. Slow precipitation, especially in the cold, by any of these agents leads to the separation of rhombic dodecahedra, which crystallize reliably over a wide *p*H range; crystals up to 1 mm. across are readily made. The insoluble complex that is formed with clupein sulfate likewise crystallizes in rhombic dodecadedra (7), but it is interesting to note that the complex with heparin crystallizes as isotropic prisms (26) and that with ribonuclease has not been crystallized (71). X-ray examination shows that the ammonium sulfate crystals have a body-centered cubic structure, good evidence that the particle is nearly spherical (20).

The virus is soluble in water and dilute salt solutions over the whole *p*H range in which it is stable. The solutions have the opalescence characteristic of macromolecules and do not show anisotropy of flow under the usual conditions of testing. The specific refractive increments at 23° C. for light of wave lengths 366, 436 and 546 mμ are 0.00178, 0.00170 and 0.00164, respectively (74). At 25° and *p*H 7.1, the diffusion constant is 1.15×10^{-7} (84); this value has been confirmed (75) and has also been shown to agree with the observed spreading of the boundary during centrifuge runs (57). This spreading can be used as a measure of the uniformity of the particle weights in a preparation; and it is clear (57) that the standard deviation from the mean is less than 2%.

Several independent measurements of the sedimentation constant have been made because of an error, due to temperature irregularity, in the first measurement (74). There is now general agreement (56, 63, 86) that the value $S^{20^\circ}_w$ is 132×10^{-13}. The value is but little affected by variations in the *p*H (74) or concentration (56) of the fluid that is being centrifuged, but the *p*H does affect the character of the pellet that separates (8).

Near the isoelectric point, *p*H 4.1 (74), the pellet is firm. It gets less compact as the *p*H moves away from this value. Under all conditions the pellet is isotropic and appears to be structureless.

Density measurements on solutions lead to a partial specific volume of 0.739 (74) or 0.724 (14) for the virus. It is not possible to move directly from this value to the density of the particles as they actually occur in solution, for the extent of hydration is unknown. Furthermore, there is no reason to think that it is unaffected by the nature and concentration of the other components of the solution. It is obvious that there is some hydration, for the most tightly packed centrifuge pellet contains about 70% of water and only about one-third of this could be held in the interstices between close-packed spheres. The crystal form, x-ray measurements, diffusion measurements and absence of anisotropy of flow all suggest that the particle is spherical, or nearly so, and hydrated; and a careful collation of these various lines of evidence leads to the conclusion (75) that there is 0.76 g. of water associated with each gram of dry virus in solution. These various measurements also lead to a figure for the "molecular weight" of the particle. The inadvisability of using the phrase "molecular weight" in connection with these large particles has already been stressed (89), the suggestion being made at the same time that the symbol Mh be used for a mass 1,000,000 times one-sixteenth of the mass of an oxygen atom. This convention has a special advantage in a case like this, for about 43% of the mass is made up of loosely associated water. The available evidence suggests that, in solution, the particle weighs about 18 Mh.

The diameter of the particle can be deduced from the sedimentation constant and diffusion constant or, more directly, from the x-ray measurements and from filtration data. The unit cell in a wet crystal made with ammonium sulfate is a cube of edge 39.4 mμ (20). After drying, it is 31.8 mμ. When tested on collodion membranes with a more or less uniform pore size, there is a sharp filtration end point when the average pore diameter is 40 mμ (102). This value agrees so well with the x-ray measurements that it is argued (75) that the assumptions made in earlier attempts to relate average pore diameter at the filtration end point to particle weight were in error and that with some particles the ratio of particle diameter to average pore diameter may be nearly unity.

2. Chemical Composition

The elementary analysis of purified preparations made by fractional precipitation with ammonium sulfate has given the following mean values

(7, 12, 109): carbon 48.5%; hydrogen, 7.7%; nitrogen, 16.1%; sulfur 0.6%; and phosphorus 1.5%. Even after prolonged dialysis at the isoelectric point, a residue is left that is not all metaphosphoric acid. It consists largely of calcium and sodium, but traces of twelve other metals have been found (14). The carbohydrate content of a preparation is a useful criterion of its purity. The value varies with the method of estimation used and with the standard of comparison; 6% is a usual value when glucose is used as standard in a method based on the color developed on heating with orcinol in strong sulfuric acid. Crude preparations, especially when made by differential centrifugation only, generally have higher carbohydrate contents than this (109). The contaminant is generally removed by prolonged dialysis followed by low-speed centrifugation at the isoelectric point. The carbohydrate and phosphorus are present largely, if not exclusively, in the form of nucleic acid; and material with the general properties of nucleic acid has been isolated from virus preparations disrupted by warm acetic acid, spreading agents or alkali (7, 109). This nucleic acid is of the ribose type, but the actual presence of ribose in it has not yet been demonstrated. Nothing has been published on the amino acid composition of the protein part of the virus.

3. Stability

There is no evidence that any modification can be brought about in the virus that does not lead to a loss of infectivity; many treatments, on the other hand, will make a preparation noninfective without affecting those chemical, physical or serological properties that have so far been studied. Within the *p*H range of 2 to 9, infectivity is unaffected during a few hours at room temperature; but if the *p*H or temperature is raised, a crystallizable and serologically active but noninfective product results (13). Similarly, suitable irradiation with ultraviolet light removes infectivity without affecting the other two qualities (7). The infectivity is reduced to about one-third by exposure, in neutral solution, to 10^5 ergs per sq. cm. at 254 mμ (67). The nature of the changes undergone by the virus during these treatments is not known; but some chemical agents, if used under suitable conditions, have a similar effect. Among these may be mentioned nitrite, formaldehyde and hydrogen peroxide (7) and also dimethyl sulfate, methyl iodide and iodoacetic acid (14).

The virus, in dialyzed and isoelectric solution, is inactivated and coagulated by freezing. It is coagulated by freezing more readily the more concentrated the solution (13). At neutrality, it is more stable towards freezing than it is at the isoelectric point; and it can also be protected from

inactivation by the presence of other solutes in the solution that is being frozen. Under the conditions in leaf sap, the virus is therefore stable towards the intensity of freezing normally used as a pretreatment of the leaves in carrying out a virus preparation. As with the other treatments, however, a duration of freezing that is insufficient to cause denaturation and loss of serological activity will cause loss of infectivity. Freezing is not therefore a wholly unobjectionable step in a preparation.

No enzymes are known to digest the virus nor has any enzymatic activity been claimed for it. Little work has been done on the products formed when the virus is destroyed chemically, although it is known (74) that exposure at *p*H values outside the stability range leads to the formation of particles with smaller sedimentation constants than the virus. Several protein-dissolving and denaturing agents (10, 11) inactivate the virus in neutral solution with approximately simultaneous disappearance of infectivity and serological activity. In general, these agents do not separate the protein part of this virus from the nucleic acid. The concentration needed to bring about inactivation varies greatly; the substances tested can be arranged in the following order of increasing potency: urea, guanidine, salicylate, phenol and dodecyl sulfate.

III. Tobacco Mosaic Virus and Related Virus Strains

A description of the physical and chemical properties of the group of viruses related to tobacco mosaic virus is complicated by two factors that do not appear to arise with bushy stunt: many virus strains exist and preparations, even when derived initially from a single lesion, generally contain a mixture of strains; furthermore the virus can exist in a series of different physical states.

Preparations are generally made from the leaves of infected tobacco plants. They have also been made from tomato leaves and roots and from the leaves of petunia, spinach and phlox. There is little or no virus in the vacuolar sap (77). Most of it is in the cytoplasm and comes out when the leaf cells are damaged by rough maceration, but part remains attached to the fiber and is liberated from it by fine grinding or enzymatic digestion (8). Many different methods have been used for the isolation of virus from infective sap, but no method is wholly satisfactory. It is probable that every preparation that has so far been made is significantly contaminated either by normal plant components and virus breakdown products or by trypsin that has been added to get rid of these. Essentially, the purification depends on the precipitation of the virus at its isoelectric point or by high concentrations of neutral salts, on the sedimentation

of the virus in strong gravitational fields and on the separation of normal leaf components from it by adsorption, heating or incubation with enzyme preparations. Changes, generally tending towards aggregation, go on during these processes of purification so that the physical properties of the purified material are not necessarily those of the main virus component of the original sap. But the infected leaf itself appears to contain virus particles of several different types, and the changes that go on during purification are at least comparable to those going on normally in the infected leaf. A quite unreal distinction is sometimes drawn between "chemical" and "physical" methods of isolation and purification, and it is confidently asserted that the latter are milder and less likely to alter the virus. The available evidence suggests, however, that any process that concentrates the virus or that removes the last traces of contaminant from it is likely to cause aggregation. The published data on the properties of virus preparations need rather careful scrutiny if any attempt is to be made to relate the results on different preparations, for these preparations are likely to have been aggregated to different degrees.

1. *Physical Properties*

True crystals, which apparently contain virus, are common in the cells of infected plants; but there is only one (44) report of the preparation of similar crystals *in vitro* and this is unconfirmed (3). In neutral solution, there is a smooth gradation from a mobile fluid to a stiff gel as the concentration is increased and no appearance of the separation of a solid phase in equilibrium with the solution. The precipitates that separate on the addition of agents which do not inactivate the virus are generally fibrous. The individual fibers are often fairly regular and pointed at each end; although originally described as crystals (106) they lack the full regularity of crystals and are now generally spoken of as paracrystals (19, 20). Material is precipitated in this state by many neutral salts (23) and by agents as diverse as: acids, clupein (5); dodecyl sulfate (104); salicylate (24); ribonuclease (71); guanidine, nicotine and arginine (11); heparin, hyaluronic acid and gum arabic (26). There is no reason to believe that the initial state of aggregation of the virus affects the character of this precipitation. The ammonium sulfate precipitate has no definite solubility, but the amount of virus remaining in the fluid depends on the intensity of centrifugation. It also depends on the method used in preparing the virus and on the weight of solid phase present (70). From the phase rule standpoint, therefore, the virus cannot be looked upon as a single homogeneous component. Consistent differences in precipitablity between

the virus strains have not been systematically looked for, but the clupein precipitates are more soluble in dilute salt solution when they are formed with aucuba mosaic and cucumber viruses 3 and 4 than with the other strains tested (6). X-ray measurements on masses of this paracrystalline material orientated by rolling show that there is, here, a two-dimensional regularity only (19) and also (20) that, as the isoelectric point is approached or as the ammonium sulfate concentration is increased, the distance between the particles from which the fibers are built up diminishes. This approximation is also evident from the increased tightness of packing when such materials are centrifuged in the course of a virus preparation.

The virus is soluble in neutral solution but precipitates on the addition of acid. In the presence of traces of salts, the *p*H of maximum precipitation with most of the strains is 3.2 to 3.4 (21, 72), but it is 4.8 with cucumber viruses 3 and 4 (6). In the absence of salt, the pH of maximum precipitation is increased by 0.5 to 1.0 units (5, 6). Dilute solutions are colorless and faintly opalescent. The ultraviolet absorption spectrum shows a maximum at 260 mμ, as would be expected with a nucleoprotein, and general absorption below 245 mμ (5, 65). The partial specific volume is 0.73 (5).

In all states of aggregation the virus has the properties so far described. Solutions also have an anomalous viscosity and show some anisotropy of flow (double refraction of flow or flow birefringence), but the extent to which they show these properties and others associated with the presence of anisodimensional particles varies with the state of aggregation. Most work has been done on largely aggregated material, which will be described first, although it appears to be a minor component of the total virus of the leaf. Preparations made from infective sap that has been heated before the fractionation, or preparations that have been incubated with trypsin, are in the fully aggregated state, a state approached by any preparation that has been kept for a few weeks, even in the cold, after purification. In general, therefore, preparations may be assumed to be partly or fully aggregated if special precautions have not been taken to avoid aggregation.

Crude infective sap may show slight anisotropy of flow (112). This is much stronger with purified preparations (5), for it is easily seen when a 0.2 g. per liter solution is allowed to flow from end to end of a narrow tube while being watched in polarized light. Simultaneous measurements, in a coaxial viscometer, of the viscosity and anisotropy of flow at different known rates of shear (94) show that, for rates of shear so low that maximum birefringence has not been attained, *i. e.*, where orientation is incomplete, the relative viscosity falls with increasing rate of shear. Quantitatively,

these results agree well with theoretical expectations based on x-ray measurements of virus dimensions; they have also been confirmed (66). On the basis of these measurements (93) and of measurements of the anomalous viscosity made in a capillary viscometer (30), the use of viscosity measurements (*e. g.*, 54) in making deductions about the shape and size of virus particles has been emphatically condemned, for the theory underlying these deductions assumes that there is no interaction between the particles, whereas the anomaly demonstrates that interaction exists. The interaction between particles is also shown by the ability of dilute solutions to resist small forces by elastic deformation only, *i. e.*, to show rigidity, and by their discontinuous flow in a capillary (34). Stronger solutions, *e. g.*, 1%, are highly thixotropic (32); and it is general experience that any pattern imposed on such a solution, for example by stirring, does not spontaneously disappear for many hours.

Viscosity and anisotropy of flow are greatly affected by the nature of the solution in which measurements are made. This is a matter of qualitative experience with those who have examined preparations at various stages in the course of the purification, but some quantitative data are also available. In the presence of methemoglobin, anisotropy of flow is reduced (66); and, in the presence of even $M/1000$ potassium chloride, the viscosity is markedly increased (34). The effect of substances that ultimately decompose the virus will be considered later (pages 21–22).

Dilute solutions of virus are not orientated by magnetic fields up to 5000 gauss, but they are orientated by alternating electric fields (20). This phenomenon has been examined at 60 cycles with various potential gradients and virus concentrations (55). Neutral solutions containing 1 to 2 g. per liter of virus show positive birefringence (*i. e.*, similar to that shown by virus orientated by flow) at all potential gradients; but, at *p*H 5 or with more concentrated solutions, low-potential gradients give a negative birefringence that may become positive as the potential is increased. The precise concentrations at which this transition occurs varies with the strain and age of the virus preparation used and presumably depends on its state of aggregation. The interpretation of these results is by no means clear, for what is measured is an average orientation value, whereas the individual particles are presumably changing their orientation in phase with the current. It is probable, however, that this technique will ultimately be of the greatest use in clarifying the still confused picture of virus aggregation.

As the concentration of a virus solution is increased, the intensity of interaction between the particles increases until, at a concentration that

depends on the *p*H temperature, state of aggregation and presence of other solutes, a new phase separates (5). It is only in the presence of a third component besides water and virus that the two phases can exist in equilibrium with each other (89). The new phase is at first in the form of spindle-shaped drops or tactoids, but these coalesce to form what is generally called "bottom layer." The latter is spontaneously birefringent and is an example of class RRD in Hermann's classification of liquid crystals (20). The surface of a tactoid is defined by forces analogous to surface tension but, since the component rod-shaped particles are arranged parallel to the long axis of the tactoid, this interfacial tension has different values in the two directions; the surface is not therefore spherical but, over most of its area, toroidal (20). The state of affairs at the pointed ends or cusps of the tactoid is not clear, but they may be occupied by particles of impurity. This supposition agrees well with experience in carrying through a virus purification, for when this can be carried no further by precipitation or differential centrifugation a layer of insoluble material will gradually form between the top and bottom layers of an equilibrium mixture, as would be expected to happen during the coalescence of the tactoids into bottom layer. Preliminary attempts to interpret the concentrations of the top and bottom layers when in equilibrium have been made (5, 20) in terms of the dimensions of the particles and the probable amount of free movement possible in the two types of liquid. The conclusions will be referred to later.

From an examination of tactoids with the polarizing microscope, it is clear that the orientation of the virus particles in them is substantially complete (20). The liquid crystal layer formed by their fusion can also be orientated, by flow, etc., and, if not shaken, it keeps for a considerable time any orientation that it may have been given. Ultimately, however, it turns to a three-dimensional mosaic of regions arranged at random to one another but in each of which all the particles lie approximately parallel. The average size of these regions of parallel orientation varies inversely with the virus concentration (5). They are clearly visible to the naked eye if a 5 or 6% solution is examined between polarizing screens. With increasing concentration, the liquid crystalline material becomes more rigid and its birefringence increases. When dried, however, there is a fall in the birefringence, and a series of cracks arranged in a zigzag or herringbone pattern appear. This is interpreted as evidence that the greater shrinkage takes place along a line parallel to the short axis of the rods owing to the loss of loosely held water from between them. During the last stages of the drying of a gel, the refractive index rises more (1.484 to 1.532) in

this direction than it does in the direction parallel to the long axis (1.490 to 1.536); the birefringence therefore falls from 0.006 to 0.004 (20).

Further optical evidence that the particles are either anisodimensional or anisotropic or both comes from the partial depolarization of polarized light that has been scattered by virus solutions (53). The simplest interpretation of all the optical properties of virus solutions is in terms of the orientation of anisotropic rods, but they would also be explicable if the rods were isotropic; in this case the anisotropy of flow should disappear in a medium with the same refractive index as the particles. The medium chosen should not inactivate the virus; and it should not be so anhydrous that the birefringence attributed to the virus from a study of partly dried gels would be reduced by further drying in the manner already described. These conditions have not been achieved for, although the anisotropy of flow disappears in mixtures of aniline and glycerol (53), the mixture used is nearly anhydrous and the stability of the virus in it is disputed (11). Furthermore, if the result were in fact due to the equalization of the refractive index of the medium to the single refractive index of the virus, anisotropy of flow should reappear if the refractive index of the medium is increased further; this has not been demonstrated.

It has long been known that tobacco mosaic virus will sediment on high-speed centrifugation (17). The pellet is liquid crystalline and has been to some extent freed from the lower molecular weight components of the system (5). Much effort has been expended on the measurement of the rate of sedimentation in centrifuges of the Svedberg type and in attempts to relate these rates to the weight of the particle. Two major difficulties are encountered in these attempts: In the first place the laws relating velocity of sedimentation to particle weight all assume that there is no interaction between the particles, but the anomalous viscosity of the virus shows that such interaction exists. A particle weight derived by proper correction from the sedimentation constant would be infinite (29). In the second place, the laws are derived from the motion of noninteracting spheres and, although they can be corrected so as to apply to a somewhat ellipsoidal shape, there is no adequate theoretical treatment of particles so anisodimensional as the extreme character of the orientation phenomena shows the virus to be. It is to be expected *a priori* (5), and has been observed in practice with the methyl celluloses and polystyrol resins, that increases in the length of a uniform rod will not significantly affect its sedimentation rate.

The ultracentrifugation of a fluid with anomalous viscosity and showing rigidity toward small shears (34) is more strictly comparable to the

expression of fluid from the interstices in a loose gel than to the independent movement of particles through the fluid (31). This picture offers a ready explanation of many phenomena, notably the increase in the rate of sedimentation (56) and in the water content of the pellet (5) as the initial virus content of the fluid that is being centrifuged is diminished, for the elastic compressibility of a gel becomes greater as its concentration, and therefore strength, becomes less. A few phenomena are, however, left unexplained. Among these is the existence in some preparations of double boundaries (114) indicative of components sedimenting at different rates. This could only appear if the particles were to some extent independent from one another, for it is hard to picture interpenetrating gels as being compressed at different rates.

The values found for the sedimentation constant have varied within the range 100 to 260 $\times 10^{-13}$ cm. sec.$^{-1}$ dyne^{-1}. This variation has been related to such factors as the age of the plant used as host, the duration of the infection, the time of year of harvesting, the strain of virus, the treatments to which the preparation was exposed during purification and the final conditions under which the centrifugation was carried out. In view of the unsatisfactory state of the theoretical interpretation of sedimentation constants with this virus and of the constant's dependence on so many factors, it would appear that all evidence (*e. g.*, 58) based on sedimentation constants and purporting to demonstrate the purity, homogeneity or general authenticity of a virus preparation is valueless. Centrifugal homogeneity in a fraction made by careful differential centrifugation no doubt does credit to the technical skill of a research worker. The argument that this homogeneity is evidence that the virus is necessarily monodisperse does less credit to his common sense.

Measurements of the diffusion constant of tobacco mosaic virus have been made; but these, again because of the anomalous viscosity, are likely to be misleading. The particle weight of the virus derived by making the conventional corrections from the diffusion constant is 0 (29). A fully aggregated product gave the observed value 4.5 $\times 10^{-9}$ cm.^{2} sec.$^{-1}$ (35), and a probably less fully aggregated one gave 3 $\times 10^{-8}$ (85); as would be expected if there were interaction between the particles, the rate of change of concentration across the boundary is not that characteristic of independent particles.

2. Size of the Virus Particle

Breadth Measurements.—X-ray measurements on a series of orientated preparations of three strains of tobacco mosaic virus with varying water

content show (20) that the rods lie in a hexagonal pattern and distribute themselves so as to fill the available space as evenly as possible. The distance between the centers of the rods varies with the water content from 100 to 15 mμ. The latter figure is therefore the most probable value for the diameter of the dry particle and, although first proposed nine years ago (15), has not so far been seriously disputed. Two other strains, cucumber viruses 3 and 4, give dry preparations with 14.6 mμ between the centers of the rods (20), and in Holmes rib-gross strain the distance is also 15 mμ (40). It is not easy to deduce from these figures the effective diameters of the particles as they exist in solution. Dry preparations are extremely hygroscopic and absorb 10–15% of water in a moist atmosphere; it is therefore unlikely that they do not hold at least this much water when in solution. The x-ray measurements on virus precipitated as fibers by the addition of acid or ammonium sulfate give distances of 18.2 and 17.3 mμ between the centers of the rods (20). These measurements were made on rigid orientated preparations in which it is likely that the rods were touching laterally and therefore that they had these diameters. If the increase is due to a layer of water surrounding a cylindrical particle 15 mμ in diameter, layers of water with cross-sectional areas 47% and 33% of that of the virus rod would be called for. Under these two conditions, therefore, each gram of virus would be associated with 0.35 or 0.24 g. of water. These amounts of water are probably smaller than those held by the particles in dilute salt solutions at neutrality, for it is usual to relate the separation of a protein from solution in visible form with a reduction in its water-holding power. The translucent mass left after the centrifugal separation of the water from an acid precipitate contains 50% of water (5). The pellets ultracentrifuged from neutral virus solutions have never had as low a water content as this (14). On the basis of these considerations, it is reasonable to suppose that the virus is normally associated with about its own weight of water; the diameter of the particle in solution would then be about 23 mμ. The same conclusion has been reached from a consideration (75) of the diffusion and sedimentation constants. It is argued (18, 20) that, if this water penetrates into the particles, there would be a swelling of the protein framework and that this would result in an increase in the short x-ray spacings due to internal structure across the particles. No such increase on wetting a dry preparation is in fact observed. The particles appear, therefore, to be substantially dry inside but to be surrounded by a layer of water several molecules thick. It is not known whether the thickness of this layer is not greatly affected by

minor changes in the medium, for no fully satisfactory theory has so far been proposed for the manner in which it is held on to the particle.

Length Measurements.—The properties considered so far are shown, to a greater or less extent, by materials at all stages of aggregation and are either relatively independent of the state of aggregation, or else partly or fully aggregated material only has hitherto been used for their study. The x-ray data and the physical changes that accompany aggregation give us reason to think that it proceeds linearly, that is to say, initially somewhat rod-shaped particles aggregate end to end. Some consideration of the evidence for this aggregation forms a useful preamble to any discussion of the length of the particle.

There is no dispute over the fact that virus preparations can exist in different states of aggregation. The extent to which any preparation has been separated from normal leaf components without undergoing at least partial aggregation is, however, in dispute. No property is known that is dependent on the length of the virus particle and that can be satisfactorily studied in sap as it is expressed from infected leaves, because the virus dilution is either too great or else the other components of the sap interfere with the observation. Considerable difficulties arise, therefore, in any direct and unequivocal demonstration of aggregation. Anisotropy of flow is a valuable guide. Unfortunately no measurements of the birefringence built up in unaggregated material at different known rates of shear have been published; but it is qualitatively obvious that the amount of light passing if a sample of sap is stirred when between polarizing screens is increased by centrifugal sedimentation or precipitation with ammonium sulfate (5, 9). This difference is apparently not shown when measurements are made on fluid streaming through a capillary tube (62, 73); but under these conditions of intense shear short rods are probably as completely orientated as long ones. No increase in birefringence should then be caused by aggregation, for the total amount of orientated material in any section of the tube has not been increased by aggregation. The treatments to which sap is exposed to bring about this change do, however, demonstrably affect other components besides the virus, for the centrifuge pellet or ammonium sulfate precipitate will not redissolve completely on suspending it in either water or its own supernatant. It can be argued, therefore, that the observed changes are due to the removal of this material rather than to the aggregation of the virus. A similar objection can be leveled against the demonstration (5) that purification reduces the filterability of the virus. Careful comparisons of the filtration end points of material at different stages of purification have not been made; but ma-

terial partially purified by centrifugation is known to pass a membrane of average pore diameter 190 mμ (73). Since the filtration end point of the virus in untreated infective sap is about 40 mμ (102), this establishes an upper limit only to the extent of the aggregation brought about by centifuging. Fully aggregated material will not pass a membrane of average pore diameter 450 mμ (5). It should at first sight be possible to get evidence from a comparison of the infectivities of preparations at different stages in the purification; but the apparent infectivity of a preparation is greatly affected by changes in the medium in which it is suspended (3); an aggregation, if accompanied by the removal of an inhibitor, might well leave the apparent infectivity unchanged. The interesting observation (103) that, weight for weight, the virus from recently infected plants has only one-fourth the infectivity of that from older plants is relevant in this connection.

There is no evidence that a preparation has yet been made in which all the particles have the same length, nor is there evidence that the lengths remain unaltered after the solution has been diluted or concentrated. Any length measurement, therefore, must be looked on as applying to the precise conditions obtaining during the measurement, and it cannot, without further evidence, be generalized to cover particles derived from the same preparation but exposed to different conditions. A wholly satisfactory demonstration of the average particle length in a solution containing 20 or 0.01 g. per liter does not necessarily tell us the average particle length in the original sap containing 2 g. per liter. Furthermore, since the properties used are not in general affected to an extent proportional to the length of the rod, some will attach disproportionate importance to those rods that are longer and others to those that are shorter than the mode for the preparation.

The following estimates of particle length have been made on aggregated material by methods that are, for the reasons already outlined, of doubtful validity:

430 mμ, from viscosity measurements taken in conjunction with the sedimentation constant (54).

532 mμ, from viscosity measurements taken in conjunction with the width measured by x-ray diffraction (75).

580 mμ, from the rotational diffusion coefficient taken in conjunction with a length-width ratio obtained from the viscosity (79).

1350 mμ, from viscosity extrapolated to zero shear taken in conjunction with x-ray measurements of the width (93).

1400 mμ, from the critical concentration for "bottom layer" formation on the assumption that at this concentration each rod has insufficient room to turn about a short axis, and using the x-ray value for the width (5).

1000 mμ, minimum, from a consideration of the sharpness of the x-ray diffraction lines due to reflections along the length of the rods; it is assumed that the sharpness depends on the number of times a fundamental unit is repeated inside each rod (20).

Three of these values depend on the x-ray determination of the width of the particle and this has been taken as 15 mμ; but, since all the measurements were made in solution, a hydrated value for the width, *e. g.*, 23 mμ, is probably more nearly correct. There are, however, so many other uncertainities in these measurements that it seems best simply to point out this correction and adhere to the published values.

Photographs of the virus have now been taken in several institutes with electron microscopes. They are in good agreement with the general picture that has been arrived at by the other techniques, but they have not as yet added to our unequivocal knowledge of the particle size. The subject has recently been reviewed (78) and therefore needs no detailed description here. Before use, the preparation must be dried, and drying is known (5) to cause inactivation of the virus as well as great loss in the ability to show anisotropy of flow. This presumably means that the virus has disaggregated, but it is not known whether the whole preparation has disaggregated to some extent or whether some particles retain their original size. Tobacco mosaic virus, like other proteins, is known (66) to form a strong surface film; the more dilute the solution the larger will be the proportion of the virus in this film, a significant factor in the making of an electron microscope specimen, for here a tiny drop of a 0.01 g. per 1. solution (or weaker) is allowed to evaporate on the mount. The specimen will therefore contain a mixture of particles dried from the bulk of the fluid and from the surface film. The photographs that have been published show a tendency for the particles to lie side by side and to touch rather than to overlap. This is most readily explicable on the assumption that the photographer tends to choose parts of the surface film when looking for a suitable field. There is no evidence whether this factor will lead to a selection of the longer or shorter particles in a preparation.

The resolution attainable in electron microscopy is of the order of 5 mμ. Particles about 15 mμ wide are not therefore suitable material for width measurements. The lengths have been given as 150 to 300 mμ (46), 140 and 190 mμ (80), and 280 mμ (110) by those who have made the photographs. The results have, however, been reinterpreted as showing, on the one hand (33), that the rods are built up of units 37 mμ long and are all integral multiples of this length, and, on the other hand (90), that there is a complete absence of regularity in the lengths of the rods. Such a difference of opinion is by no means unusual in the development of our

knowledge of this virus! It would, however, appear that our uncertainty of the effects of dilution, drying, selection by the photographer and even bombardment by the electron beam robs the controversy of some of the significance it would otherwise have had.

In summarizing this section on the length of the particle a few points may be repeated. There are several methods whereby a figure can be derived from reproducible measurements on solutions of known antecedents; here it is clear to what material the measurements refer, but the steps relating these measurements to particle length are uncertain. The electron microscope, on the other hand, can give a precise measurement of the length of the particles that are being photographed, but the relationship between these and the average of the particles as they occur in the original virus solution is uncertain. The lengths found range from 1400 mμ in preparations that have been fractionated by liquid crystal formation to 140 mμ in preparations that have been dried. There is no evidence that a particle at either of these extremes is still infective.

Bulk Measurements.—From measurements of the x-ray dosage needed to inactivate the virus and on the assumption that one x-ray quantum can, presumably by causing local ionization, inactivate one particle, the volume of the particle can be calculated. The number of ionizations produced by a given dose of radiation in a given volume is known. Different samples of virus give different values for this volume; and the inactivation is greater on the acid side of the isoelectric point (76). Extreme values are 4600 and 42,000 mμ^3. An independent measurement is 7500 mμ^3 (36). Two objections can be raised against the basic assumption underlying this method. On the one hand, ionizations tend to occur in groups of about three together in a volume smaller than that of the particle; it has therefore been suggested (67) that three quanta are normally responsible for one inactivation and that the true volumes are three times those given. Making this correction and using the value 23 mμ for the width of the particle in solution, lengths of 34 and 300 mμ are arrived at. On the other hand, there is also evidence that not all impacts of a quantum on a virus particle cause total inactivation. The frequency with which normal virus appears in cultures of the aucuba strains and vice versa is increased by x-raying (37), and weak irradiation with x-rays is stated (45) to activate the virus. These phenomena suggest that a proportion of the impacts has an irreversible effect falling short of inactivation, and there seems to be no reason to assume that the effect of all impacts is irreversible. The magnitude of this factor, which would lead to an increase in the volume deduced from the measurements, is uncertain.

3. *Chemical Properties*

Composition.—After considerable preliminary conflict, which is now of only historical interest, there is substantial unanimity that the first analyses (5, 15) to be carried out on material whose physical and chemical properties bore any relationship to those of the virus were approximately correct; the values now accepted lie within the ranges then given. Reasonable values are: carbon, 50%, hydrogen, 7%, nitrogen, 16.6% and phosphorus, 0.53%. Differences outside the experimental error have not been found in the percentages of these four elements in the different strains even when these are not closely related. The sulfur content, partly perhaps because the analytical methods that have been used are less reliable, is variable (5, 48, 95) in the range 0.2 to 0.64%. The smaller figure is usual with the strains generally handled. Cucumber viruses 3 and 4, which are only distantly related to tobacco mosaic virus, contain 0.84% sulfur (51). Preparations always leave a residue after combustion, but its composition has not been investigated.

There is no evidence for the presence of any component in the virus besides protein and nucleic acid. In the undenatured protein, 0.8% of the total nitrogen is present as amino nitrogen (42, 82) estimated by the Van Slyke method with nitrous acid and by the color given with ninhydrin (98). The undenatured protein contains no free —SH groups, but after denaturation of the normal strain an amount is produced, estimated by porphyrindene titration, that agrees closely with the total sulfur content (111).

The virus strains differ little if at all in the properties so far described in this review. By analogy with other proteins, it is to be expected that there should be greater differences in the proportions in which the constituent amino acids occur; this is in fact observed when the strains are not closely related. The figures for amino acid content must, however, be treated with some reserve because preparations large enough for a determination are generally made from bulk cultures and are therefore liable to contain mixtures of strains. Even a preparation derived recently from a single lesion is liable to be mixed, for new strains appear frequently in this group of viruses. The published figures for tobacco mosaic virus are given in Table I. The value which seems to be the best has been given, but references to any papers tending to confirm it are set out as well. All the figures are for the weight of amino acid that would arise from the hydrolysis of 100 g. of dry protein. In only four cases has the configuration been determined; aspartic acid, glutamic acid, leucine and tyrosine

all belong to the normal or *l*-series (100). Four strains of virus with a close serological relationship to tobacco mosaic virus contain the same percentage of tyrosine, tryptophan and phenylalanine (51), but there are significant differences with three less closely related strains. Holmes ribgrass virus contains 2.0% methionine and 0.55% histidine (49), amino acids that are absent from the normal virus, and contains 4.3, 3.5 and 6.4% of phenylalanine, tyrosine and tryptophan, respectively (52). The percentages of these three amino acids in cucumber viruses 3 and 4 are 10.1, 1.5 and 3.9, respectively.

TABLE I

AMINO ACID CONTENT OF TOBACCO MOSAIC VIRUS

Method employed	Amino acid	Content, %	Reference
Colorimetric only	Cystine or cysteine	0.7	38, 95
	Threonine	5.3	96
	Tryptophan	4.5	52, 96
Gravimetric only	Alanine	2.4	97
	Ammonia	1.9	97
	Aspartic acid	2.6	97, 100
	Glutamic acid	5.3	97, 100
	Leucine	6.1	97, 100
	Valine	3.9	97
Colorimetric and gravimetric	Arginine	9.0	96, 97
	Phenylalanine	6.0	52, 96, 97
	Proline	4.6	96, 97
	Serine	6.4	96, 97
	Tyrosine	3.9	52, 96, 97, 100
	Believed to be absent		**Reference**
	Glycine		96, 97
	Histidine		96, 97
	Hydroxyglutamic acid		97
	Hydroxyproline		97
	Lysine		96, 97
	Methionine		95

All the phosphorus in the virus is satisfactorily accounted for by the 5.5 to 6.0% of nucleic acid it contains. The usual color reactions show that the sugar in this nucleic acid is a pentose rather than a desoxypentose (5, 69); it is generally assumed to be ribose, but there is no evidence for this—there is, in fact, a suggestion to the contrary, for "uridylic acid" isolated from the virus nucleic acid has an anomalous solubility and optical rotation (69). Adenine, guanine and cytosine have been positively identified in nucleic acid hydrolyzates, and the presence of uracil as opposed to thymine is probable but less certain (69). It is clear, therefore, that the nucleic acid has affinities with yeast or ribonucleic acid but that the iden-

tification is incomplete and the possibility that other bases are present has by no means been excluded. The matter is worthy of further study because of the suggestion, unsupported by definite evidence, that differences in the nucleic acids are responsible for the differences between virus strains (87).

Crude preparations of virus nucleic acid do not diffuse through membranes which permit the passage of yeast nucleic acid made in the usual ways (5). Purified preparations have likewise larger particles, for they have lower osmotic pressures (28) and diffusion constants (68) and higher sedimentation constants (28). The nucleic acid used for these measurements was made by fission of the virus with cold alkali, strong acetic acid or heat denaturation. There is no information on the effect of these agents on the state of aggregation of a nucleic acid; and, although these treatments seem milder than those to which yeast is subjected in the course of a yeast nucleic acid preparation, it would be premature to assume that the nucleic acid has the same size and shape as it had in the virus before fission. Without this evidence the physical properties of the virus nucleic acid can shed only an uncertain light on the internal structure of the virus particle.

Stability.—In neutral or slightly acid solution, the virus is more resistant than most proteins to inactivation by heating; it is most stable at about *p*H 5. Short periods of heating at 65° C. rob it of infectivity without causing denaturation; but at higher temperatures, in the presence of electrolytes, there is coagulation, which proceeds more quickly the greater the virus dilution and which follows a first-order reaction course (61). Exposure to high pressure causes similar changes; first loss of infectivity and then coagulation with separation of nucleic acid. The coagulation is preceded by a disaggregation of the virus (60). The extent of the coagulation increases with the pressure and the time for which the virus is exposed to it; at *p*H 7 and 30° C. half is coagulated in 100 minutes at 7500 kg. per cm.^{2}

Exposure to ultraviolet light (107) or x-rays (5) reduces the infectivity without affecting those physical, chemical or serological properties which have been studied. As would be expected, those wave lengths in the ultraviolet that are most strongly absorbed by the virus are most effective in inactivating it (39).

The virus is unstable in alkaline solution at room temperature. With some samples, loss of infectivity is rapid at *p*H 8 (22) while others are relatively stable at *p*H 9 (11). It is possible that this discrepancy is due to the use of different virus strains. Material that has lost the greater

part of its infectivity is somewhat disaggregated but suffers no other marked physical change. More intense treatment with alkali splits off nucleic acid; and the protein is at the same time disaggregated into fragments with sedimentation constants, in alkaline solution, between 4 and 30×10^{-13} (50). Neutral solutions are unstable and quickly form aggregates; these are also formed when the breakdown products from virus strains with distinguishable amino acid composition are mixed (50). There is as yet no evidence to show whether the virus rod is built up from dissimilar protein pieces or by the replication of identical units.

There is an extensive range of substances which disintegrate the virus in neutral solution. Of these, urea has been the most extensively studied although, as has already been pointed out in a survey of the action of urea on proteins (10), it is by no means the most powerful. Ten effects of urea on the virus have been studied:

(*a*) reduction in viscosity,
(*b*) decrease in infectivity,
(*c*) decrease in serological activity,
(*d*) appearance of —SH groups,
(*e*) increase in diffusion constant,
(*f*) appearance of protein insoluble at neutrality in the absence of urea,
(*g*) appearance of material with a small sedimentation constant when in a suitable solvent,
(*h*) disappearance of anisotropy of flow,
(*i*) increase in the clarity of the solution and
(*j*) a rise in the osmotic pressure.

Kinetic measurements have not been made on all these changes; but they can be arranged in approximate order and some of them have been related together. There is an immediate reduction in viscosity on mixing virus with urea (30), followed by changes *bcd* proceeding at comparable rates (10, 64). Changes *efghi* go on after this and more or less together (10, 35, 64), *j* being the slowest of all (64). There is no evidence that there can be any great reduction in the average weight of the particles without a loss of infectivity (10, 64) although the contrary has been maintained on the basis of viscosity measurements. Change *a* can, however, be caused by lower concentrations of urea, *i. e.*, *M* instead of 4–6 *M*, than have been shown to affect any of the other properties. It is probable, therefore, that it is analogous to the reversible effects dilute urea is known to have on proteins (*cf.* 10) rather than to the deep-seated alterations involved in all the other changes. Material of reduced infectivity can be isolated cen-

trifugally (64) after partial inactivation, suggesting that change *b* precedes *f* and *g*. But because of the probable heterogeneity of the original virus, this is not certain. Changes *fgi* follow the course of a first-order reaction (59). Change *i* was the most fully investigated and its rate was found to depend on the concentration and nature of the electrolytes present and on the initial concentration of the virus. Changes *bchi* proceed most slowly at about 20° C. (10, 59), in agreement with general experience on the action of urea on proteins; the existence of this temperature minimum differentiates the action of urea from that of all similar substances which have been tested. No wholly convincing hypothesis to account for this anomaly has been proposed. The same changes proceed more rapidly as the concentration of urea or the *p*H is raised (10, 59). The osmotic pressure goes on rising after all the infectivity has disappeared and no further change is apparent in the other properties (64), finally corresponding to a molecular weight of 41,000. Nucleic acid is split off at an early stage; but the kinetics of the reaction have not been followed.

Guanidine (11, 64), salicylate (11, 24) and a range of substances including pyridine, phenol and benzoic acid (11) disintegrate the virus. They have been investigated in less detail than urea; all of them are well known as solvents for other proteins. The wetting and spreading agents, notably sodium dodecyl sulfate, also act in this way and at greater dilutions. The action proceeds more rapidly as the temperature and *p*H are raised, and can be followed by the loss of anisotropy of flow, by ultracentrifugal sedimentability and by the separation of nucleic acid from the protein (104). The resulting virus protein,* like that made by alkaline fission, is unstable and readily aggregates to an insoluble slimy mass. Such behavior is not unusual with the protein moiety of a conjugated protein. Dodecyl sulfate can also disintegrate the virus in acid solution (81).

Formation of Complexes.—There is no satisfactory evidence for the enzymatic hydrolysis of tobacco mosaic virus although claims that it is split by pepsin (105) and by an intestinal nucleophosphatase (99) have been made; both claims have been contradicted (27, 47). Trypsin (47), ribonuclease (71) and papain (5) combine with the virus, but the complexes, although of reduced infectivity, do not undergo the hydrolysis that generally follows enzyme–substrate combination. These complexes are dissociated

* I have always referred to infective products as "virus preparations" or, where the context does not demand so much caution, as viruses, and have condemned (89) the use of the phrase "virus protein" as either redundant or overprecise. It has the added disadvantage of leaving no suitable phrase to cover products such as this. The point of view is now gaining acceptance, for other workers (50) are now using the phrase "virus protein" in this, exact, sense for material free from nucleic acid and derived from a virus.

by dilution or by changes in *p*H or salt content; those with ribonuclease and papain are insoluble only so long as the salt concentration is low. There is a group of substances of unknown nature that reduce greatly the infectivity of the virus but that, like the substances already mentioned, can be removed with restoration of infectivity. Extracts from a number of insects have this effect. The component responsible is thermolabile and indiffusible through cellophane; it can be removed from the virus by separating the latter on a more porous membrane or by ultracentrifugation (25). Similarly, virus solutions made noninfective by the addition of milk or a number of bacterial and vegetable extracts can be reactivated by diffusing the inhibitors away through agar, for the virus has a lower mobility in this gel (41). The existence of these phenomena complicates the interpretation of the fact (103, 108) that partly purified virus from leaves infected for less than a week is less infective than that from leaves infected for three weeks, for the effect may be due to variation in the amount of such components in the leaves.

Virus preparations do not contain amylase, asparaginase, catalase, chlorophyllase, oxidases, peroxidase or urease (92), but protease (113) and glucosemonophosphatase (91) have been found. Details of this work are not yet available but, since both enzymes are known to occur in the sap of healthy plants, the possibility that the virus is coming out as a naturally occurring, but presumably dissociable, complex must be borne in mind. The existence of natural complexes can also be inferred from the presence of fibers or hexagonal crystals in many of the cells of an infected plant (16, 43). Their nature is not satisfactorily explained, but it is certain that they contain virus and that they are insoluble under conditions in which free virus would be soluble. Stable soluble complexes form when virus solutions are heated with serum proteins (4); these precipitate with antisera against either the virus or the serum protein but not with other antisera.

Chemical Modification of the Virus.—Nothing is known about the nature of the linkages that are affected by the substances considered in the last two sections. In this section a group of substances is considered which can bring about definable chemical changes in a protein. As usual, with actions involving proteins there is no evidence that the actions that have been followed chemically are the only ones taking place. This group is differentiated from the agents considered under the heading of stability largely because the members of it do not tend to disintegrate the virus, and from those described as complex formers only because they are of known composition. Both distinctions are clearly somewhat arbitrary.

Our knowledge is still too meager for any serious attempt at a classification of the changes brought about by agents of known composition, although some arrangement is already possible. There are three types of change: first, those that do not affect the infectivity; second, those removing infectivity without causing recognized changes in the other properties; and last, those causing a general modification of the virus. The first type is the most interesting and can be interpreted in three different ways: (*a*) The parts of the particle affected may not be essential for the process of infection; (*b*) the change may be of such a character that the plant can restore the *status quo* and then become infected; (*c*) there is the remote possibility that these agents attack preferentially those particles in the original mixture that were not infective at the beginning. Examples of agents causing changes of this first type are: acetyl chloride, benzenesulfonyl chloride, benzoyl chloride, carbobenzyloxy chloride, *p*-chlorobenzoyl chloride, iodine, ketene and phenylisocyanate. Clearly it is necessary to establish in each case that at least the greater part of the virus has undergone a chemical change before its continuing infectivity is significant.

Tobacco mosaic virus gives the nitroprusside reaction for —SH groups after it has been denatured but not when in the native state; after treatment with mild oxidizing agents preliminary treatment with cyanide is necessary before the —SH reaction can be elicited. On exposure to increasing concentrations of iodine (2), this reversible oxidation of the potential —SH groups is followed by an action that is not reversed by cyanide and later by a disappearance of the Millon reaction for tyrosine. It is only at this last intensity of treatment that there is a pronounced fall in the infectivity of the virus. All that is known of the nature of the action taking place in the intermediate zone where infectivity is unaffected is that the potential —SH is probably not being oxidized to —SO_3H, for the normal —SH content is found after hydrolysis with hydrogen iodide, and this treatment is not known to reduce the —SO_3H group.

Treatment with ketene or phenylisocyanate (101) first removes the free —NH_2 groups; later the phenol and indole groups are affected. During the first action there is little change in the infectivity, appearance or sedimentation constant of the virus, but, as would be expected when —NH_2 groups are replaced by acetyl or phenylureide, there is a change in electrophoretic mobility. The reaction with phenol and indole groups is associated with loss of infectivity. Mixtures of normal virus and virus modified in these ways are readily detected electrophoretically (82) and there is reason to think that, although it has not proved possible to cover all the —NH_2 groups without affecting infectivity, most of the —NH_2 of all the

particles has been covered rather than all the $—NH_2$ of some of them while others remained unaffected. Treatment with acetyl or benzoyl chlorides (1) likewise causes disappearance of the $—NH_2$ and reduction in the Millon reaction without loss in infectivity.

One of the most interesting phenomena so far observed with plant viruses has appeared in the study of the carbobenzyloxy, chlorobenzoyl, and benzenesulfonyl derivatives, for here an almost complete inactivation toward one host plant can occur after an intensity of action that has but little effect on the infectivity on another (83). With all three agents, *Nicotiana glutinosa* was infected more easily than *Phaseolus vulgaris*. Ketene produces a similar but less pronounced effect.

There is no evidence with any of these virus derivatives tending to show whether the derivative itself is infective or whether enzyme systems exist in the plant capable of splitting off the added group and reforming the original virus. Macerated leaves are not able to bring about this action (83), but it is well known in other fields that many enzyme systems are destroyed by maceration. So little is known at present of the mechanisms by which viruses multiply or by which proteins are built up and broken down in the cell that there is no basis for looking on one interpretation as more probable than the other. The modifications made so far have not affected the character of the infection produced in the host plant, and the virus recovered from plants infected with these modified viruses has had the normal properties (1, 82, 83); this is not at first sight surprising, but there is every reason to expect that it will prove possible so to modify the virus particle that the modification will prove transmissible.

After more intense treatment with the agents in the first group, the virus loses infectivity; this loss is not reversible or not readily reversible. Other agents, notably nitrite, hydrogen peroxide, formaldehyde and other aldehydes, iodoacetamide, iodoacetic acid, methyl iodide and dimethyl sulfate, have only been found to cause an inactivation of this second type; but we may legitimately doubt whether there is need in all these cases to assume the existence of a real distinction. It has not been clearly shown with these agents that the loss of infectivity and the recognizable chemical changes proceed *pari passu*. With the first group of substances, chemical changes have been looked for after an intensity of treatment that does not lead to loss of infectivity; but with the second group, chemical changes at this stage have not been looked for. Only the action with formaldehyde has been studied in any detail, and it has been found to be complex. After extensive inactivation at *p*H 7, there is a 60% fall in the $—NH_2$ value as measured by the Van Slyke (42, 98, 107) or ninhydrin (42) tech-

niques and a similar fall in the color with the Folin phenol reagent (42, 98). At *p*H 3, on the other hand, although there is loss of infectivity, these chemical changes are not detectable or hardly detectable (42). Under certain conditions it is stated that dialysis causes partial reactivation (98), but this has not been confirmed (42). Treatment with hydrogen peroxide at neutrality or with nitrite at *p*H 3 also reduces the $-NH_2$ value (98). These three agents cause no marked change in the sedimentation constants, for those found with inactivated materials have fallen within the normal range (114). The general appearance of an aggregated virus preparation is unaffected by these treatments. After inactivation with nitrite, no alteration has been detected (5) in the serological behavior of the virus. The action of some other aldehydes agrees qualitatively with that of formaldehyde (98); here also reactivation has been claimed. Details of the properties of these derivatives have not been published nor have they for the derivatives with iodoacetamide (2) or for iodoacetic acid, methyl iodide or dimethyl sulfate (14), all of which cause loss of infectivity without gross physical changes.

The third group of agents, those inactivating the virus with complete loss of all its characteristic properties, need not be discussed here because in these actions the virus is not clearly distinguished from other proteins. The salts of heavy metals and strong oxidizing agents, acids or alkalies belong to this group.

It will be clear from this survey that, although there is still some disagreement, there is now as large a body of accepted knowledge about the tobacco mosaic group of viruses as there is about any other protein with the exception of hemoglobin. The viruses have certain special advantages as materials for the study of the chemical and physical behavior of proteins, for they have an unusually large number of properties that are well adapted to the quantitative study of the changes that are being brought about.

In conclusion, it may be permissible to point out that no rigid distinction has been, or perhaps can be (88), drawn between those systems conveniently referred to as "living" and "nonliving," and that no observation has so far been made on plant viruses which has any bearing whatsoever on this metaphysical or semantic problem.

Bibliography

1. Agatov, P., *Biokhimiya*, **6**, 269 (1941).
2. Anson, M. L., and Stanley, W. M., *J. Gen. Physiol.*, **24**, 679 (1941).
3. Bawden, F. C., *Plant Viruses and Virus Diseases.* Chronica Botanica, Waltham, Mass., 1943.

4. Bawden, F. C., and Kleczkowski, A., *Brit. J. Exptl. Path.*, **22**, 208 (1941).
5. Bawden, F. C., and Pirie, N. W., *Proc. Roy. Soc. London*, **B123**, 274 (1937).
6. Bawden, F. C., and Pirie, N. W., *Brit. J. Exptl. Path.*, **18**, 275 (1937).
7. Bawden, F. C., and Pirie, N. W., *ibid.*, **19**, 251 (1938).
8. Bawden, F. C., and Pirie, N. W., *ibid.*, **25**, 68 (1944).
9. Bawden, F. C., and Pirie, N. W., *Nature*, **142**, 842 (1938).
10. Bawden, F. C., and Pirie, N. W., *Biochem. J.*, **34**, 1258 (1940).
11. Bawden, F. C., and Pirie, N. W., *ibid.*, **34**, 1278 (1940).
12. Bawden, F. C., and Pirie, N. W., *ibid.*, **37**, 66 (1943).
13. Bawden, F. C., and Pirie, N. W., *ibid.*, **37**, 70 (1943).
14. Bawden, F. C., and Pirie, N. W., *unpublished.*
15. Bawden, F. C., Pirie, N. W., Bernal, J. D., and Fankuchen, I., *Nature*, **138**, 1051 (1936).
16. Bawden, F. C., and Sheffield, F. M. L., *Ann. Applied Biol.*, **26**, 102 (1939).
17. Bechold, H., and Schlesinger, M., *Phytopath. Z.*, **6**, 627 (1933).
18. Bernal, J. D., *Proc. Roy. Soc. London*, **B125**, 299 (1938).
19. Bernal, J. D., and Fankuchen, I., *Nature*, **139**, 923 (1937).
20. Bernal, J. D., and Fankuchen, I., *J. Gen. Physiol.*, **25**, 111 (1941).
21. Best, R. J., *Australian J. Exptl. Biol. Med. Sci.*, **14**, 1 (1936).
22. Best, R. J., *ibid.*, **14**, 323 (1936).
23. Best, R. J., *ibid.*, **18**, 307 (1940).
24. Best, R. J., *Nature*, **145**, 627 (1940).
25. Black, L. M., *Phytopathology*, **29**, 321 (1939).
26. Cohen, S. S., *J. Biol. Chem.*, **144**, 352 (1942).
27. Cohen, S. S., and Stanley, W. M., *ibid.*, **142**, 863 (1942).
28. Cohen, S. S., and Stanley, W. M., *ibid.*, **144**, 589 (1942).
29. Frampton, V. L., *Science*, **90**, 305 (1939).
30. Frampton, V. L., *J. Biol. Chem.*, **129**, 233 (1939).
31. Frampton, V. L., *Phytopathology*, **29**, 495 (1939).
32. Frampton, V. L., *ibid.*, **30**, 666 (1940).
33. Frampton, V. L., *Science*, **95**, 232 (1942).
34. Frampton, V. L., *Arch. Biochem.*, **1**, 83 (1943).
35. Frampton, V. L., and Saum, A. M., *Science*, **89**, 84 (1939).
36. Gowen, J. W., *Proc. Natl. Acad. Sci. U. S.*, **26**, 8 (1940).
37. Gowen, J. W., *Cold Spring Harbor Symposium Quant. Biol.*, **9**, 187 (1941).
38. Hess, W. C., Sullivan, M. X., and Palmer, E. P., *Proc. Soc. Exptl. Biol. Med.*, **48**, 353 (1941).
39. Hollaender, A., and Duggar, B. M., *Proc. Natl. Acad. Sci. U. S.*, **22**, 19 (1936).
40. Holmes, F. O., *Phytopathology*, **31**, 1089 (1941).
41. Johnson, J., *ibid.*, **31**, 679 (1941).
42. Kassanis, B., and Kleczkowski, A., *Biochem. J.*, **38**, 20 (1944).
43. Kassanis, B., and Sheffield, F. M. L., *Ann. Applied Biol.*, **28**, 360 (1941).
44. Kausche, G. A., *Naturwissenschaften*, **27**, 77 (1939).
45. Kausche, G. A., and Stubbe, H., *ibid.*, **26**, 740 (1938).
46. Kausche, G. A., Pfankuch, E., and Ruska, H., *ibid.*, **27**, 292 (1939).
47. Kleczkowski, A., *Biochem. J.*, **38**, 160 (1944).
48. Knight, C. A., *J. Biol. Chem.*, **145**, 11 (1942).
49. Knight, C. A., *ibid.*, **147**, 663 (1943).

50. Knight, C. A., and Lauffer, M. A., *J. Biol. Chem.*, **144**, 411 (1942).
51. Knight, C. A., and Stanley, W. M., *ibid.*, **141**, 29 (1941).
52. Knight, C. A., and Stanley, W. M., *ibid.*, **141**, 39 (1941).
53. Lauffer, M. A., *J. Phys. Chem.*, **42**, 935 (1938).
54. Lauffer, M. A., *J. Biol. Chem.*, **126**, 443 (1938).
55. Lauffer, M. A., *J. Am. Chem. Soc.*, **61**, 2412 (1939).
56. Lauffer, M. A., *J. Phys. Chem.*, **44**, 1137 (1940).
57. Lauffer, M. A., *J. Biol. Chem.*, **143**, 99 (1942).
58. Lauffer, M. A., *ibid.*, **151**, 627 (1943).
59. Lauffer, M. A., *J. Am. Chem. Soc.*, **65**, 1793 (1943).
60. Lauffer, M. A., and Dow, R. B., *J. Biol. Chem.*, **140**, 509 (1941).
61. Lauffer, M. A., and Price, W. C., *ibid.*, **133**, 1 (1940).
62. Lauffer, M. A., and Stanley, W. M., *ibid.*, **123**, 507 (1938).
63. Lauffer, M. A., and Stanley, W. M., *ibid.*, **135**, 463 (1940).
64. Lauffer, M. A., and Stanley, W. M., *Arch. Biochem.*, **2**, 413 (1943).
65. Lavin, G. I., and Stanley, W. M., *J. Biol. Chem.*, **118**, 269 (1937).
66. Lawrence, A. S. C., Needham, J., and Shen, S.-C., *J. Gen. Physiol.*, **27**, 201 (1944).
67. Lea, D. E., and Smith, K. M., *Parasitology*, **32**, 405 (1940).
68. Loring, H. S., *J. Biol. Chem.*, **128**, lxi (1939).
69. Loring, H. S., *ibid.*, **130**, 251 (1939).
70. Loring, H. S., *J. Gen. Physiol.*, **23**, 719 (1940).
71. Loring, H. S., *ibid.*, **25**, 497 (1942).
72. Loring, H. S., and Stanley, W. M., *J. Biol. Chem.*, **117**, 733 (1937).
73. Loring, H. S., Lauffer, M. A., and Stanley, W. M., *Nature*, **142**, 841 (1938).
74. McFarlane, A. S., and Kekwick, R. A., *Biochem. J.*, **32**, 1607 (1938).
75. Markham, R., Smith, K. M., and Lea, D. E., *Parasitology*, **34**, 315 (1942).
76. Marshak, A., and Takahashi, W. N., *Proc. Natl. Acad. Sci. U. S.*, **28**, 211 (1942).
77. Martin, L. F., and McKinney, H. H., *Science*, **88**, 458 (1938).
78. Marton, L., *Ann. Rev. Biochem.*, **12**, 587 (1943).
79. Mehl, J. W., *Cold Spring Harbor Symposia Quant. Biol.*, **6**, 218 (1938).
80. Melchers, G., Schramm, G., Trurnit, H., and Friedrich-Freska, H., *Biol. Zentr.*, **60**, 524 (1940).
81. Miller, G. L., *J. Biol. Chem.*, **146**, 339 (1942).
82. Miller, G. L., and Stanley, W. M., *ibid.*, **141**, 905 (1941).
83. Miller, G. L., and Stanley, W. M., *ibid.*, **146**, 331 (1942).
84. Neurath, H., and Cooper, G. R., *ibid.*, **135**, 455 (1940).
85. Neurath, H., and Saum, A. M., *ibid.*, **126**, 435 (1938).
86. Ogston, A. G., *Biochem. J.*, **37**, 78 (1943).
87. Pfankuch, E., *Biochem. Z.*, **306**, 125 (1940).
88. Pirie, N. W., in Needham, J., and Green, D. E., *Perspectives in Biochemistry.* Macmillan, New York, and Cambridge Univ. Press, London, 1937, p. 11.
89. Pirie, N. W., *Biol. Rev. Cambridge Phil. Soc.*, **15**, 377 (1940).
90. Rawlins, T. E., *Science*, **96**, 425 (1942).
91. Rischkov, V. L., *Phytopathology*, **33**, 950 (1943).
92. Rischkov, V. L., and Sukov, K. S., *Compt. rend. acad. sci. U. R. S. S.*, **21**, 265 (1938).
93. Robinson, J. R., *Nature*, **143**, 923 (1939).

94. Robinson, J. R., *Proc. Roy. Soc. London*, **A170**, 519 (1939).
95. Ross, A. F., *J. Biol. Chem.*, **136**, 119 (1940).
96. Ross, A. F., *ibid.*, **138**, 741 (1941).
97. Ross, A. F., *ibid.*, **143**, 685 (1942).
98. Ross, A. F., and Stanley, W. M., *J. Gen. Physiol.*, **22**, 165 (1938).
99. Schramm, G., *Ber.*, **B74**, 532 (1941).
100. Schramm, G., and Müller, H., *Naturwissenschaften*, **28**, 223 (1940).
101. Schramm, G., and Müller, H., *Z. physiol. Chem.*, **266**, 43 (1940).
102. Smith, K. M., and MacClement, W. D., *Parasitology*, **33**, 320 (1941).
103. Spencer, E. L., *Science*, **94**, 96 (1941).
104. Sreenivasaya, M., and Pirie, N. W., *Biochem. J.*, **32**, 1707 (1938).
105. Stanley, W. M., *Phytopathology*, **24**, 1269 (1934).
106. Stanley, W. M., *ibid.*, **26**, 305 (1936).
107. Stanley, W. M., *Science*, **83**, 626 (1936).
108. Stanley, W. M., *J. Biol. Chem.*, **121**, 205 (1937).
109. Stanley, W. M., *ibid.*, **135**, 437 (1940).
110. Stanley, W. M., and Anderson, T. F., *ibid.*, **139**, 325 (1941).
111. Stanley, W. M., and Lauffer, M. A., *Science*, **89**, 345 (1939).
112. Takahashi, W. N., and Rawlins, T. E., *ibid.*, **77**, 26 (1933).
113. Vorobieva, M. N., *Compt. rend. acad. sci. U. R. S. S.*, **30**, 466 (1941).
114. Wyckoff, R. W. G., Biscoe, J., and Stanley, W. M., *J. Biol. Chem.*, **117**, 57 (1937).

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THE COAGULATION OF BLOOD

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CONTENTS

I. Introduction

"To invent without scruple a new principle to every new phenomenon, instead of adapting it to the old; to overload our hypothesis with a variety of this kind, are certain proofs that none of these principles is the just one, and that we only desire, by a number of falsehoods, to cover our ignorance of the truth."

These words from Hume's *Treatise of Human Nature* (Book II, Part I, Section III) could well serve as a motto for a discussion of the present state of our knowledge of the mechanism of blood coagulation. It is true, the number of theories of clotting does not greatly exceed that of the workers in the field; but there have been many workers in the field—the vast, and at that incomplete, compilations of the literature include 490 publications

up to 1905 (157), 902 papers between 1905 and 1928 (219), about 550 papers published in the following eleven years (221)—and the intuitive approach to the problems has, unfortunately, been only too prevalent in the subject of this discussion. Many weird reactions have been described and many unique findings reported; and the laws of chemistry often appear suspended for the duration of the experiments. Many commonly accepted terms seem, in the minds of some authors, freely exchangeable, so that what is usually referred to as thromboplastin (or thrombokinase) will sometimes be found called thrombin or even fibrinogen. Formulations in which heparin is said to "neutralize" cephalin, *i. e.*, one acid another, are also not infrequent.

The development of a scientific problem will, in general, be found to pass through four stages: (*1*) formulation; (*2*) oversimplification; (*3*) anarchy; (*4*) solution. In the field of blood coagulation we appear at present to be in the third stage: the confused terminology, the multiplicity of hypotheses, the feeling of discomfort experienced by anyone approaching the problem critically, would all seem to point to that. At the same time, considerable progress has been achieved in the purification of the various agents concerned with blood coagulation. When the chemical reactions brought about by the interaction of these substances can be studied in clearly defined systems, the first step toward a generally acceptable clotting mechanism will have been made.

In this report, no attempt will be made at a historical or comprehensive treatment of the problem of blood clotting. The primaey objective of the following discussion is the consideration of the chemistry of the controlling factors. This approach, whose prime virtue lies in its limitations, will of necessity exclude a large part of the physiology and pathology of blood coagulation. It is amost superfluous to add that no new clotting theory will be found in these pages; for, in the opinion of this author, more facts are needed rather than additions to our terminology or to the arsenal of expendable hypotheses.

II. The Controlling Factors in Blood Coagulation

1. Schematic Formulation

The conception of the coagulation of blood as an enzymatic process rests, in the main, on the fundamental researches of Alexander Schmidt. This formulation to which, at one time or another, much excellent work has been contributed by Denis, Buchanan, Hammarsten, Pekelharing, Morawitz, Bordet, Howell, and many others, has successfully weathered the attacks

by those who attempted to explain as specific a phenomenon as the clotting of blood by means of completely unspecific reactions. It is, however, obvious that the widespread acceptance of this useful working hypothesis is due not only to the persuasiveness of truth but also to the strength of the acclamation. The coagulation of blood or of plasma involves an extremely complicated system to which more factors may contribute than we are able to enumerate at present, and it may safely be stated that the problem of blood clotting will not be solved completely before the clotting of pure fibrinogen is fully understood. This, in turn, will have to await the clarification of the underlying catalytic system, studied with as highly purified components as can be obtained.

What is usually called the classical theory of blood coagulation may, in its simplest form, be formulated as follows:

$$\text{Prothrombin} \xrightarrow{Ca^{++},\ \text{activator}} \text{thrombin} \qquad (1)$$

$$\text{Fibrinogen} \xrightarrow{\text{thrombin}} \text{fibrin} \qquad (2)$$

In a preparatory step (first phase), the enzyme precursor prothrombin, present in circulating blood, is assumed to be converted to the active enzyme thrombin. Two factors are required for this conversion, *viz.*, ionized calcium and an activator (thromboplastin or thrombokinase) which is present in the formed elements of blood, especially in the blood platelets, in tissue cells and perhaps in plasma. In the coagulation step proper (second phase), the soluble plasma protein fibrinogen is, under the influence of thrombin, converted to the insoluble protein fibrin.

In this report we shall consider briefly the chemical evidence pertaining to the several factors postulated by this working hypothesis which, although it has not led to a solution of the problem, has demonstrated its serviceability throughout a long period. Much additional evidence which cannot be included here will be found in a number of monographs, reviews and progress reports (67, 108, 159, 173, 175, 201, 219–221). At least one recent review article written from a divergent point of view should also be cited (163).

2. *Calcium*

The role of the substances necessary for the conversion of prothrombin to thrombin, *i. e.*, calcium and the thromboplastic substances, is perhaps the most controversial and unsatisfactory phase of the current conceptions. The understanding of the specific calcium effect in blood or plasma is made difficult by the multiplicity of the proteins present which, in addi-

tion to some of the plasma lipides, may be expected to react with this cation. [Compare the recent review by Greenberg (94).] Much may be expected here from a study of the effect of calcium on pure prothrombin.

The necessity of calcium for the formation of thrombin in plasma was first shown to be probable by the discovery of the anticoagulant effect of oxalates (3) and more clearly demonstrated by the work of Pekelharing (167) and Hammarsten (99), which emphasized the specific action exerted by calcium salts. The other alkaline earth elements exert a much weaker effect in the following order of decreasing activity: strontium, barium (148).

In partially purified prothrombin concentrates, spontaneous conversion to thrombin in the absence of calcium has been observed repeatedly (150, 152, 154). It is, however, probable that this activation was due to the presence of contaminating substances, perhaps proteolytic enzymes.

While there exists impressive circumstantial evidence that calcium is, in a largely unexplained manner, possibly as a catalyst (108, 138), necessary for the first phase of blood coagulation, *i. e.*, the formation of thrombin under physiological conditions, the often repeated claims (*e. g.*, 61, 139, 186) of its essentiality for the second phase, *i. e.*, the action of thrombin, are completely unconvincing. [Compare, for instance, (218).] One gains the impression, as so often in the field discussed here, that studies carried out with impure reagents in, for the most part, inadequate experiments lead to the observation of a multiplicity of concurrent reactions, thus creating a syndrome whose disentanglement can come only from investigations of highly purified simpler systems.

The calcium factor in blood coagulation has been reviewed in detail by Ferguson (72).

3. The Activator System

It has long been known that tissue cells and blood platelets contain an agent which by its postulated activating effect on prothrombin is assumed to initiate the process of blood coagulation. This factor or, better, these factors, known by a multiplicity of names, have been described by their several rediscoverers as either thermolabile and soluble in water (52, 87, 97, 228), or thermostable and soluble in alcohol and ether (189, 190). The innumerable controversies between the adherents of these two groups were finally resolved by the recognition that both were right, with the reservation, however, that the lability to heat of the water-soluble factor is not pronounced in purified preparations and may be due to the presence of contaminating substances in the crude specimens. It will, for the time being, be of advantage to make a clear distinction between these two groups of

agents. The water-soluble factor will be discussed in the following section as the thromboplastic protein, the agent soluble in lipide solvents as the thromboplastic lipide.*

The Thromboplastic Protein.—The presence in tissue cells of water-soluble activators of prothrombin has, from the very beginning of our knowledge of blood coagulation, been demonstrated frequently. [Compare the reviews in (157) and (219).] It is significant that Wooldridge, one of the earliest and most effective workers in this field, already considered this agent as a phosphatide–protein complex (228), and that, as early as 1905, Morawitz (157a) recognized it as a general constituent of protoplasm. The distinction between this tissue activator and the prothrombin of plasma was, however, difficult in the beginning. This led to a large number of erroneous statements and untenable theories, although the early experiments of Fuld and Spiro (88) and of Morawitz (156) should have sufficed to disprove the assumed identity of these two agents.

It is at present impossible to decide whether the thromboplastic protein, *i. e.*, the activator extracted from tissue cells by means of aqueous solvents, is identical in different organs and different animal species. It is not unlikely that we are dealing with an entire family of conjugated proteins whose similarities and dissimilarities will have to be determined by detailed chemical and immunological studies. There exist indications of a species specificity as regards the thromboplastic effect of the tissue activators (87, 137, 172).

The isolation of a globulin fraction from normal human plasma with an activity resembling in certain, but not all, respects that of the thromboplastic factor has been reported by Patek and Taylor (166). This agent seems to be lacking in hemophilic blood. It is difficult to assign it at present its place in the current conceptions of blood clotting. On the other hand, the globulin fraction first isolated from rabbit plasma (165) and later found in other plasmas (1), which is sometimes designated "clotting globulin," appears to contain thrombin.

A clue to the possible function of the globulin fraction of Patek and Taylor (166) may be derived from later experiments of Tagnon *et al.* (129, 208, 209). A globulin fraction isolated from plasma, previously treated with chloroform, was found to have the following activities: it digested fibrinogen and fibrin, as well as other proteins; and it converted

* It would probably make for greater precision in nomenclature, if these factors were termed *thrombinoplastic*, since they are assumed to contribute to the formation of thrombin and not of the thrombus. But this review will attempt to avoid terminological innovations.

prothrombin to thrombin in the absence of calcium.* Comparable preparations from hemophilic plasma exhibited much lower enzymatic activity (210).

Water-soluble tissue activators are almost ubiquitous. Similar substances have been demonstrated in blood platelets and other blood cells [compare the comprehensive reviews of Morawitz (157) and Wöhlisch (219, 221)], in human milk (188, 204) and in saliva (90). Whether blood, apart from its formed elements, contains this activator, is not yet quite clear. A comparative study of the thromboplastic activity of the extracts of various organs of the hen showed the lungs to be the richest organ followed, in decreasing order, by striped muscle, heart, kidney, spleen, brain and liver (80). During the past few years the thromboplastic protein of lungs has been studied in great detail, and the following discussion will be limited to this substance, which may be considered as a representative of the water-soluble activators present in tissue cells.

It was Mills (153) who revived interest in the nature of the thromboplastic factor of lung tissue. He adduced evidence to confirm Wooldridge's conception of this substance as a lipide–protein complex (228) and showed that it had the properties of a globulin with an isoelectric point at *p*H 5 to 6. Mills (153), in his studies, emphasized the fact, repeatedly recognized (26, 29, 185, 202), that the activity of the thromboplastic protein greatly exceeds that of the thromboplastic lipides obtained by the use of organic solvents.

Most of the early preparations of the thromboplastic protein were contaminated with prothrombin. It has, however, later been possible to demonstrate the absence of prothrombin or thrombin from carefully prepared extracts of lungs (202) and brain (169).

The study of the chemical properties of the thromboplastic protein was carried out in greater detail in the writer's laboratory. In all experiments, beef lungs were used as the starting material. The preparation of the thromboplastic protein by *fractional salt precipitation* of saline extracts of beef lungs was investigated by Cohen and Chargaff (48). The active principle was prepared 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 *p*H 5.1 (50). This material was far less active than the preparations obtained by fractional high-speed centrifugation, which will be discussed presently. The concentration *in vacuo* of the frozen solutions of this substance, followed by washing with acetone, resulted in stable powders containing about 12% nitrogen, 0.8% phosphorus, 0.9% sulfur, and 18% of firmly held lipides that could be removed by extraction with alcohol–ether, but neither with chloroform nor with ether alone. 30 γ of this material clotted 0.1 ml. of rooster plasma in 3 to 6 minutes at 30° C.

* It is not yet possible to decide whether this enzyme preparation is related to the fibrinolysin investigated by Rosenmann in a number of studies (179–183).

The examination of these thromboplastic protein preparations in the Tiselius apparatus showed them to be 90 to 95% homogeneous with respect to their electrophoretic behavior (50). A small, faster moving component appeared to consist of nucleic acid. The study of the action of heparin on these compounds revealed an interesting reaction (39). The treatment of the thromboplastic protein with heparin resulted in the displacement of the lipide constituents by heparin and the formation of a heparin–protein complex with markedly anticoagulant properties.

The protein component, after the removal of the lipides, had no thromboplastic activity (48). On the other hand, it was found in immunization experiments that rabbit antibodies toward the intact lipoprotein gave precipitation with the lipide-free protein moiety, showing that the phosphatides were not essential for the capacity of the thromboplastic protein to combine with antibodies (48). The complex between the thromboplastic protein and its antibody was, interestingly enough, active in the promotion of clotting.

TABLE I

COMPOSITION AND PROPERTIES OF THROMBOPLASTIC PROTEIN PREPARATIONS (32)

Preparation No.	Centrifugal method*	Yield per kg. tissue, mg.	N, %	P, %	N/P	Thromboplastic activity.** γ	Phosphatase activity	
							Phosphatase units per mg.	Initial activity, A_{100}
1	U	470	7.4	1.6	10.2	...	...	...
2	M, U	440	7.8	1.5	11.5	0.003	1.6	2.8
3	S, M	535	7.6	1.6	10.5	0.008	1.6	4.5
4	M	480	7.8	1.6	10.8	0.003	2.2	3.8

* U = Air-driven vacuum ultracentrifuge; M = International Multispeed centrifuge; S = Sharples Laboratory Super-Centrifuge.

** Expressed as the smallest amount clotting 0.1 ml. of rooster plasma (normal clotting time about 90 minutes) within 30 minutes.

The lipides contained in the thromboplastic protein proved to be an extremely complex mixture (49). 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 (50). Considerably more potent and homogeneous preparations of the thromboplastic protein (with respect to both electrophoretic and ultracentrifugal properties) were obtained

by Chargaff, Moore and Bendich (34) by the *fractional ultracentrifugation* of beef-lung saline extracts. Equally potent preparations were later isolated by a simplified procedure in which a Sharples laboratory supercentrifuge and an International centrifuge with Multispeed attachment were employed (32). Data on a number of such preparations are summarized in Table I. It will be seen that these preparations exhibited, in addition to some phosphatase activity, an extremely high thromboplastic activity towards plasma. The action of this material on purified prothrombin will be discussed in Section IV (page 57); but it should be pointed out here that purified preparations of the thromboplastic protein proved remarkably stable to heating (32). The heat stability of crude thromboplastic protein preparations has been frequently noted (136, 187a). Crude brain extract appears to be somewhat more labile to heat than crude lung extract (173a). Additional data on the stability of crude preparations of the thromboplastic protein will be found in a paper by Marx and Dyckerhoff (144).

The great potency of the highly purified thromboplastic protein obviously speaks against the often repeated assumption that the activation of prothrombin is due to a completely unspecific cause, *viz.*, the contact with surfaces. The general barrenness of such explanations requires no extended comment. While it is true that many causes may produce the same effect, the conception of the existence in biological systems of an anarchic free-for-all has never yielded fruitful results. It may be of interest to present here the assay protocol for one highly active preparation, in order to illustrate the truly remarkable potency of these compounds, which in this case permitted the demonstration of 3×10^{-10} g. (32). The experiments were performed at 30.6° C. by mixing 0.1 ml. of fresh rooster plasma with 0.03 ml. of the solution of the indicated amounts of the thromboplastic protein in borate buffer of *p*H 8.5.

	γ of thromboplastic protein in experiment (32)							
	0.22	0.074	0.024	0.008	0.0027	0.0009	0.0003	0
Clotting time, min.	3	5	9	15	21	34	47	>90

When lung extracts are subjected to centrifugal fractionation, the highly potent thromboplastic protein is found to sediment at 20,000 r.p.m. (31,000 g). A coarsely particulate fraction may be separated at a much lower speed, 8000 r.p.m. (5000 g). It is noteworthy that this material had barely one-thousandth of the thromboplastic potency exhibited by the active fractions (32). This finding in itself should be sufficient to dispose of the notion of an unspecific contact effect as the cause of the thromboplastic action.

The thromboplastic protein preparations which are isolated by differential centrifugation rather than by fractional salt precipitation and undergo no chemical manipulation nor are in contact with organic solvents in the course of their purification, lose about one-half of their weight by exhaustive extraction with hot alcohol–ether (32). Between 40 and 45% of the material may be recovered as purified lipides whose composition may be tentatively estimated as follows (in per cent of total lipides): cholesterol, 19 (almost entirely in the free state); fat, 18; phosphatides, 63 ("lecithin" 26, "cephalin" 25, "sphingomyelin" 12). A small amount of acetal phosphatides (about 1.5%) is also present. The residue remaining from the extraction of the lipide portion consists in the main of proteins, some carbohydrates and a nucleic acid of the ribose nucleic acid type (about 1.8% in the intact complex).

Physical measurements have been carried out with several highly purified preparations of the thromboplastic protein (34). The partial specific volume was $V_{27} = 0.87$, the sedimentation constant $s_{20} = 330S$, the diffusion constant $D_{20} = 0.38 \times 10^{-7}$. This corresponds to a particle weight from rate of sedimentation of 167,000,000. 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 was found at 8.0 and 8.4 $\times 10^{-5}$ sq. cm. per v. per sec. at *p*H 7.5 and 8.6, respectively.

The evidence available at present seems to permit the classification of the thromboplastic protein from lungs as a lipoprotein of a very high particle weight and probably of cytoplasmic origin.* Both the protein and the lipide components appear necessary for the very considerable thromboplastic activity of this substance. No more than passing reference can be made here to recent experiments (31, 32) on the disintegration of this macroprotein by freezing in the presence of ether. A review on the chemistry of lipoproteins (25) may also be found of interest in this connection.

The Thromboplastic Lipide.—It has long been known that alcoholic or ethereal cell extracts contain a factor, called "zymoplastic substance" by its discoverer Schmidt (189, 190), which functions as a clotting activator. This agent was recognized as being associated with the phosphatides (14, 105, 227, 229) and, more particularly, with what then was called the cephalin fraction (105, 146, 147) of animal tissues. Plants, and even microorganisms, appear to contain similar substances (29).

* In view of the very high particle weight of the thromboplastic protein, it should be possible to ascertain its presence in plasma by studying the behavior of plasma following centrifugation at a very high speed. Indications of the presence of a sedimentable clotting factor in plasma have been reported briefly in the literature (141).

Later investigations of the thromboplastic lipide (81, 93, 214, 234, 235) confirmed their association with the crude cephalin fraction, but the significant finding was made (234) that the active agent could be extracted from the cephalin preparations by means of alcohol. A number of investigators later arrived at similar conclusions (26, 29, 42, 49, 81). During an investigation of the phosphatides isolated from horse-blood platelets it was furthermore observed that the fractionation of the active cephalin fraction by means of alcohol resulted in the accumulation of most of the activity in the alcoholic mother liquors (29). Essentially similar results were obtained in a study of the phosphatides contained in preparations of the thromboplastic protein from beef lungs (49). In view of the assumed role of the thrombocytes in the initiation of blood clotting, it is significant that one of the phosphatide fractions isolated from platelets (29) has proved the most potent thromboplastic lipide encountered in the laboratory of this author.

All these findings served to throw considerable doubt on the conception that the thromboplastic lipide was identical with the phosphatide traditionally termed cephalin, *viz.*, ethanolamine phosphoryl diglyceride; and with the discovery of the complex nature of brain cephalin (84, 85) and of other tissue phosphatides (41), the question of the identity of the active lipide once more became open.

The thromboplastic activity of tissue phosphatides was again investigated by Chargaff (26). Numerous phosphatide fractions obtained by a variety of methods from pig heart, beef heart and beef brain were examined for thromboplastic activity. The most potent preparations, especially those from pig heart, were very soluble in ethyl alcohol. Phosphatidyl serine from beef brain was completely inactive; brain cephalin itself (*i. e.*, the ethanolamine-containing phosphatide fraction) showed some activity. But the specimens from pig heart which exhibited the highest activity did not resemble cephalin. It is at present not possible to identify the thromboplastic lipide, if indeed we deal here with a lipide at all, with any of the known phosphatides. In view of the entirely different level of activity of the thromboplastic protein, as compared with even the most potent lipide specimens—the difference is at least 1000-fold—no conclusions as to the nature of the active fraction, which may be present in the lipide preparations in a very small concentration only, and the mechanism of its action appear justified. Not even the lipide nature of this compound can be affirmed. It must, moreover, be borne in mind that any substance that is susceptible of phosphorylation in the organism may occur, if only in traces, in the form of a phosphatide.

It should be mentioned that egg-yolk phosphatides, following their conversion to lysophosphatides by means of snake venom, have been found to lose their thromboplastic activity (33). A thromboplastically active fraction, easily soluble in most organic solvents, has been isolated from beef lungs (42). This material is stable to heat and oxidizing agents, but its chemical nature is unknown.

This is not the place to discuss the role of the *blood platelets* in blood coagulation. Their origin, properties and function have been reviewed in a very complete monograph by Tocantins (211) and, more recently, by Quick (173b). It is unfortunate that the difficulty of obtaining these fragile bodies in sufficient quantity has, for the time being, prevented attempts to isolate the thromboplastic protein by the high-speed centrifugation of platelet extracts. A chemical and immunological comparison between thromboplastic protein preparations from lungs and platelets of the same animal species should be of interest in view of the conception that the platelets originate from the cytoplasm of lung megakaryocytes (109). The isolation of a potent thromboplastic lipide fraction from the blood platelets of the horse has already been mentioned.

4. Prothrombin

Recent evidence (203, 216) appears to furnish a complete confirmation of the old conception (162) that the liver is intimately concerned with the production of prothrombin. The evidence, which is entirely convincing, is based on two main lines of experimentation: the effect of liver poisons and liver injury on the prothrombin level in plasma; and the influence exerted by vitamin K on the formation of prothrombin. It is probable that prothrombin is present in most body fluids; but the evidence as regards its distribution in tissues is unsatisfactory, since the contamination of the examined material with blood, and therefore with traces of blood prothrombin, is extremely difficult to prevent. Purified platelets apparently contain no prothrombin (65, 73).

Most prothrombin samples used until very recently were extremely impure globulin preparations from plasma. The experiments of Mellanby (149) led to the isolation of a somewhat purer material. The essential step consisted in the precipitation of a protein fraction from diluted oxalated plasma at *p*H 5.3, *i. e.*, near the point of lowest solubility of prothrombin, followed by the selective elution of prothrombin by means of a dilute solution of calcium bicarbonate.

The technique of Mellanby was later refined by Smith and collaborators (200), and this in turn led to the isolation by Seegers (193, 197, 199) of highly potent prothrombin preparations. The most active prothrombin specimens (197) were obtained by a procedure which involved the precipitation of a crude fraction from diluted plasma at *p*H 5.3 and the treatment of the solution of this precipitate with a suspension of magnesium hydroxide, followed by the elution of the adsorbate. The eluate was subjected to a

fractional precipitation with ammonium sulfate, when the activity was found to reside in the fraction precipitable between 50 and 65% saturation with ammonium sulfate. The final preparations contained 11,000 to 14,000 prothrombin units per mg. of tyrosine. One prothrombin unit is defined as that amount which, when completely converted into thrombin, will clot 1 ml. of fibrinogen solution in 15 seconds (217).

The analytical data available for less pure prothrombin preparations (193) showed them to be nondialyzable proteins containing about 4% of carbohydrate. The point of minimum solubility was near *p*H 4.9. The substance was completely inactivated by the heating to 60° C. of its aqueous solution or by the lowering of the *p*H below 4.8. A reaction above *p*H 10 also markedly inactivated the material.

In the procedure for the fractionation of human plasma developed by Cohn and collaborators (51, 70), the prothrombin is mainly found in one fraction (Fraction III-2) which consists of 75% of β-globulin, 10% of α-globulin, and 15% of γ-globulin. Purified prothrombin preparations from beef plasma, on the other hand, have an electrophoretic mobility in the Tiselius apparatus of 6.7×10^{-5} sq. cm. per v. per sec. at *p*H 7.0 and of 7.6 at *p*H 8.0 (196), which is considerably higher than the average value, *viz.*, 3.3, found for the β-globulin of human plasma at *p*H 7.8 (155).

The necessity of calcium for the conversion of prothrombin to thrombin by the thromboplastic factor has given rise to many explanations. Quick (171) has sought to show that prothrombin contained calcium in its molecule. Other workers (108, 138) assign a purely catalytic function to this cation. The final decision will have to await the analyses of pure prothrombin. It is possible that experiments with radioactive calcium could contribute to a solution of this problem.

A discussion of methods for the determination of the prothrombin level in blood will be found in several recent monographs (16, 173).

5. *Thrombin*

The progress in our knowledge of the chemistry of thrombin closely parallels the results obtained with prothrombin. This is natural, since the only test available for prothrombin is the conversion of the inactive precursor to thrombin. The methods previously employed for the preparation of crude thrombin (12, 104, 148, 150, 189, 190) will probably in the near future be completely superseded by procedures in which the highly purified prothrombin preparations, discussed in the preceding section, will be utilized. The ideal thrombin preparation, unrealized so far, would require a system consisting of physically and chemically homogeneous prothrombin of maximum potency and constant solubility, of calcium and of the pure thromboplastic protein (isolated from the same animal species as the particular prothrombin sample).

Attempts at the isolation of pure thrombin were more recently initiated by Smith and collaborators (195) and continued by Seegers (193, 198). A different and, it would seem, more laborious procedure was used by Milstone (154). Other recent methods (4) appear to yield thrombin of much lower potency.

From what little is known of the chemical properties of thrombin, it may be concluded that, like prothrombin, it is a nondialyzable carbohydrate-containing protein (193). The point of minimum solubility of thrombin is near *p*H 4.3, *i. e.*, lower than that of prothrombin. Thrombin is much more soluble than prothrombin, when compared at the respective points of lowest solubility of these two proteins. Saline solutions of thrombin are irreversibly inactivated by acid at *p*H 3.5 and by temperatures higher than 60° C. Marked inactivation also takes place in alkaline solution near *p*H 11. Solubility curves obtained with purified thrombin samples indicate the presence of two active components with different solubilities (198).

The stability of aqueous solutions of purified thrombin can be increased considerably by the addition of glycerol (154) or carbohydrates, especially glycosides (194). Prothrombin, on the other hand, is not stabilized as readily.

It has long been known that thrombin is rapidly inactivated in serum, but that the activity may be re-established by treatment with alkali or acid (189, 223). This inactive modification has been designated meta-thrombin (157b). A considerable amount of work has been carried out with respect to the mechanism of thrombin inactivation. It appears that the disappearance of thrombin is due to its combination with a fraction of the serum proteins (177) that resides in the albumin portion (136, 170). More will have to be said on this point later in connection with the discussion of the heparin complement and antithrombin (page 511).

The enzymatic nature of thrombin has been heatedly debated by adherents and opponents of the enzyme theory of blood clotting for many years. Many of these discussions degenerated to mere exercises in terminological dialectics. It is certain that the best evidence available at present supports the assumption of an enzymatic effect (221a).

6. *Fibrinogen and Fibrin*

The liver is usually considered as the site of formation of fibrinogen in the organism. The evidence is not entirely clear-cut; but it is certain that an appreciable decrease in plasma fibrinogen takes place when the liver is excluded from the circulation or injured by poison or disease.

Our present knowledge of the chemistry of fibrinogen suffers from all the

limitations and uncertainties to which our understanding of the chemistry of proteins is still subject. In addition, the investigation of fibrinogen is made difficult by the lack of methods for the preparation of this delicate protein in an electrolyte-free and unaltered state. Most analyses, therefore, have been carried out with fibrin, thus assuming what remains to be shown, namely, that the chemical changes involved in the conversion of fibrinogen to fibrin are too small to affect the chemical composition of the respective proteins noticeably. Laki (133) recently reported the crystallization of hog fibrinogen. A method permitting the isolation of crystalline fibrinogen in quantity would undoubtedly be of great importance.

Fibrinogen is easily changed by outside influences and is apparently sensitive to the removal of salts by dialysis (83). Because of the lack of reliable analyses it cannot be decided whether the products obtained by the coagulation of fibrinogen by heat or other action and those obtained under the influence of thrombin are identical. It certainly is misleading to designate all these substances as fibrin, a term which should be reserved for the coagulation product obtained with thrombin. In all other cases, the designation "coagulated fibrinogen" may be more appropriate.

The amino acid composition of cattle fibrin has been studied by Bergmann and Niemann (8). What appears to be the first comparative analysis of the amino acid distribution in genuine and in coagulated human fibrinogen was recently published by Brand *et al.* (15). The authors do not insist on the significance of the observed differences in the contents of cysteine, cystine, methionine and tryptophane. The conclusions that might be drawn from the analytical findings will be on safer ground when more analytical data on a variety of preparations become available. One feature which may in the future acquire importance is the comparatively high content in the hydroxyamino acids serine and threonine. The ideal experiment evidently will consist in a comparison between purified fibrinogen preparations and the fibrin samples obtained from them under the influence of highly purified thrombin.

The average fibrinogen content of normal human plasma is about 0.25% (70, 98). The protein is usually prepared from oxalated or citrated plasma that has been previously freed of prothrombin by treatment with calcium phosphate, barium sulfate or the hydroxides of aluminum or magnesium. The precipitating agent most commonly used is sodium chloride or ammonium sulfate. Recently, large amounts of fibrinogen from human plasma have become available as the result of the plasma fractionation project undertaken at Harvard Medical School (51) and have found numerous interesting applications in medicine in form of films, foams and plastics [compare (10, 76)].

The average electrophoretic mobility of fibrinogen in normal human plasma has been found at 2.1×10^{-5} sq. cm. per v. per sec. (155). It thus is intermediate between the β-

and γ-globulins. It is a highly asymmetric large molecule with dimensions of about 900 × 33 Å and an approximate particle weight of 500,000 (70). Its solutions are viscous and exhibit double refraction of flow. [Compare the detailed discussion of the physical chemistry of fibrinogen by Wöhlisch (221b).] In these properties fibrinogen is reminiscent of myosin. But it apparently does not share with the latter the important property of causing the breakdown of adenosine triphosphate, as has been found in unpublished experiments (233). The similarities between fibrinogen and myosin were recently discussed by Bailey (5); but, in spite of certain common features in the x-ray diffraction patterns of fibrous proteins (6), it would, as far as a clarification of the problem of blood clotting is concerned, appear advisable not to lay much stress on these resemblances.

The question of whether the association of fibrinogen with lipide material is a prerequisite for the coagulability of the protein by thrombin remains controversial. It was first raised in connection with observations made following the extraction of plasma with organic solvents (185, 230); but later experiments with purified fibrinogen (127, 225) failed to contribute much to our understanding of the problem.

The existence of a soluble precursor of fibrin, recently termed profibrin (2), which occupies a position intermediate between fibrinogen and fibrin, has been postulated by several investigators. It is quite conceivable that the chemical changes taking place along the fibrinogen molecule, which lead finally to its conversion to the insoluble fibrin, could, under proper circumstances, be arrested before the precipitation of fibrin takes place; but a decision regarding the existence and the properties of such an intermediary product will have to await further experimental work.

7. *Other Substances with Activating or Coagulating Properties*

In the following discussion it will be preferable to distinguish between substances having an activating effect on prothrombin and those that are themselves thrombin-like in action. The most interesting substance, as far as a thromboplastic effect is concerned, probably is trypsin. That blood, even in the presence of oxalate (100), could be clotted by this enzyme has long been known (56). The same effect was later demonstrated for crystalline trypsin (164). The action of trypsin was first thought to be a proteolytic one, exerted on fibrinogen (215); but this explanation did not remain undisputed (207), and Eagle and Harris (68) demonstrated that the action of trypsin was exerted on prothrombin and did not require the presence of calcium. Other workers (75) have found calcium and "cephalin" to enhance the thromboplastic potency of trypsin considerably.

It is at the present time not yet possible to give a reasonable explanation for the thromboplastic action of trypsin. The proposed analogy be-

tween trypsin and the thromboplastic protein (68), in which proteolytic enzyme activity is assigned to the latter, is made improbable by the apparent absence of a trypsin-like potency from highly purified thromboplastic protein preparations (32). Crude thromboplastic protein preparations also were found to lack proteolytic activity (144), even when tested with prothrombin as the substrate (62). Similarly, the comparison of the thromboplastic action of trypsin with that of a hypothetical weakly proteolytic "thromboplastic enzyme," which acts by making "cephalin" available for the activation of prothrombin (74, 75), is open to objection on at least two counts: (*a*) the much lower potency of the thromboplastic lipide as compared with the thromboplastic protein; and (*b*) the absence from the latter of proteolytic activity. Therefore, while trypsin could, in certain respects, be regarded as a model substance for the thromboplastic protein, it should be understood that the mechanism of its action may very well be entirely different. The isolation of a fibrinolytic enzyme fraction from chloroform-treated plasma (208), similar to trypsin in its action on prothrombin, has been noted before on page 35.

The clotting properties of various snake venoms have long been known (142, 143), although the manner in which this effect was produced remained a matter of conjecture. More recently, the presence in snake venoms of a thromboplastic agent has been demonstrated repeatedly. The effectiveness of the much investigated Russell viper venom (*Daboia*) (140) appears to be increased by the addition of various phosphatide mixtures (69, 101, 134, 141, 212). Other proteolytically active snake venoms, mostly of the *Bothrops* group, have been shown to bring about the conversion of prothrombin to thrombin (66). Some snake venoms (*Crotalus, Bothrops*) are, in addition, able to coagulate fibrinogen directly, *i. e.*, they appear to contain thrombin or a thrombin-like substance (66).

The direct clotting of fibrinogen is also brought about by preparations of the proteolytic enzyme, papain (68, 224). In this connection, experiments of Dyckerhoff and Gigante (59) are of interest which indicated that the proteolytic and the thrombin-like activities of papain preparations were due to separate components of the enzyme mixture. This is of importance in view of the many speculations to which the assumed relationship of fibrinogen coagulation to proteolysis has given rise.

An entirely different group of agents able to coagulate fibrinogen directly is represented by a number of simple organic substances (30, 37). These substances are (in the order of their activity): chloramine-T (sodium N-chloro-*p*-toluenesulfonamide), potassium 1,4-naphthoquinone-2-sulfonate, sodium 1,2-naphthoquinone-4-sulfonate, ninhydrin (1,2,3-indantrione hy-

drate) and, much less markedly, alloxan and salicylaldehyde. Neither the thromboplastic factor nor calcium was required in this reaction which proceeded with human or horse fibrinogen solutions free of prothrombin. The clotting times depended on the concentrations of both the fibrinogen and the clotting agent. A strict comparison between genuine fibrin and the fibrinogen coagulation products obtained with the aid of the synthetic clotting agents is difficult; but with fibrinogen solutions in a concentration of between 0.4 and 0.7%, the clotting agents, with the exception of chloramine-T (which formed a loose coagulum), produced firm, coherent, rapidly retracting clots. With ninhydrin and the α- and β-naphthoquinone sulfonates, the amounts of fibrinogen nitrogen recovered in the clots were of the same order of magnitude as found in the corresponding fibrin samples. Alloxan and chloramine-T appeared to attack the fibrinogen more energetically. A large number of carbonyl compounds, the oxidation products of inositol and ascorbic acid, and quinone derivatives related to vitamin K were inactive. Certain conclusions, based on these experimental findings, which may contribute to our understanding of the reactions involved in the conversion of fibrinogen to fibrin under physiological conditions, will be discussed on pages 57–59.

III. The Inhibition of Blood Coagulation

1. Schematic Formulation

A consideration of the factors controlling the coagulation of fibrinogen (compare pages 32–33, Section II) will show that a substance acting as a clotting inhibitor may do so by reacting with one (or more) of five agents: prothrombin, calcium, activator, thrombin and fibrinogen. In fact, one more possible place of interference could be added, *viz.*, fibrin. An agent able to destroy this insoluble protein could very actively interfere with the blood-clotting mechanism. The function, if any, of the fibrinolytic enzymes of the body within the physiological clotting process is, however, difficult to define, although the fibrinolysins undoubtedly are of great biological importance in enabling the organism to rid itself of the coagulation product.

The schematic formulation of anticoagulants would lead to the following tentative classification:

A. Interfering with prothrombin:

1. Dicumorol, 3,3′-methylenebis[4-hydroxycoumarin], active *in vivo* by depressing the plasma prothrombin level. The activity is perhaps

due to the interference with the formation of prothrombin in the organism.*

2. Heparin (in presence of the heparin complement), preventing the conversion to thrombin (17, 110). The effect of heparin on the first clotting phase has been denied by several workers [compare (64)]. The synthetic anticoagulants of the polysulfuric acid ester type, which will be mentioned later, probably have a similar effect.

3. Rare earth salts, *e. g.*, neodymium (58, 61, 96) praseodymium, lanthanum, cerium, samarium (213); probably active by inhibiting the formation of thrombin (60, 64).

B. Interfering with Ca^{++}:

1. Oxalates, citrates, fluorides.

C. Interfering with thromboplastic activator:

1. Heparin (39), compare the discussion in Section II (see page 37).

2. Protamines (23) and histones (36). This effect is probably due to the inactivation of the thromboplastic lipides. The action on the thromboplastic protein is not known, to the exception of a few data in (48).

D. Preventing the disintegration of blood platelets:

1. Heparin (11).

E. Interfering with thrombin:

1. Heparin (in presence of the heparin complement) (110, 116, 170, 231, 232). The synthetic polysulfuric acid esters probably act in a manner similar to heparin.

2. Lipide inhibitor (20, 21). The point of attack of this factor is not known exactly; but it is included here by analogy to the behavior of the synthetic cerebroside sulfuric acids (22).

3. Hirudin (91, 92).

4. Serum antithrombin [(221), p. 334]. This factor will be discussed together with the heparin complement to which it is possibly related.

5. Reducing agents, *e. g.*, cysteine (160), glutathione (131), sodium bisulfite (30).

* The discussion of this interesting subject, the development of which is due to the pioneering work of Schofield (191) and Roderick (178) and to the brilliant chemical investigations of Link and associates (18, 19, 205), lies outside the scope of an article that centers on the controlling factors of blood clotting normally present in the organism. A review article will be found in (174). The recent claim (57) that dicumarol interferes with the first clotting phase by reacting with the thromboplastic factor appears unconvincing.

F. Interfering with fibrinogen:

1. Fibrinogenolytic enzymes (fibrinogenase) (128, 158). The physiological and pathological functions of these enzymes and their relationship to the fibrinolysins are not yet clear.

2. Protamines. These substances have been claimed to act as specific precipitating agents for fibrinogen (161).

It ought to be understood that the formulation attempted here must remain provisional until more is known of the chemical properties and of the reactivities of the many factors which compose the phenomenon of blood coagulation. More or less unspecific effects (as, for example, inhibition by heavy metals, salt effects, the action of urea and of detergents, etc.) have not been included. Nor could the very important problem of the influence of active surfaces (*e. g.*, glass) and inert surfaces (*e. g.*, amber, collodion, paraffin, etc.) be considered here. Reference should be made to a brief treatment of this problem by Wöhlisch (221c).

The following discussion will be limited to a brief consideration of the anticoagulants known to occur in the mammalian organism.

2. Heparin

Heparin was discovered in 1916 by McLean (146) in the course of an investigation of liver phosphatides. Our present knowledge of the chemistry of this potent anticoagulant is in the main due to the efforts of Howell, of Charles and Scott and of Jorpes. A detailed monograph has been published by Jorpes (120).

The pioneering work of Howell [compare, for instance, (107)] on the purification of heparin was followed by the studies of Charles and Scott (43, 45, 192), whose procedure made possible the preparation of heparin in large quantity from beef liver and lung. Jorpes (119, 123, 124, 125) demonstrated the presence in purified heparin of large amounts of sulfuric acid in ester linkage and of glucosamine, and obtained indications of the presence of a uronic acid, thus establishing the relationship of heparin to mucoitin sulfuric acid.*

Most of the later work has been carried out with heparin preparations recovered from the crystalline barium salt of beef-lung heparin (46). Whether heparin samples isolated from various tissues of the same animal

* It should be mentioned that Howell (107) was the first to draw attention to the probable presence in heparin of uronic and sulfuric acids, and that the importance of the $-SO_3H$ group for the inhibition of blood clotting had been emphasized previously by several workers (55, 78).

species are identical and a chemical individual (47) or whether heparin is constituted of a series of different polysulfuric acid esters of mucoitin [(125), compare also (132)] cannot yet be decided. The evidence available at present would seem to favor the view that, even in the same animal species, there may occur several heparins of closely related structure but of different activity (121, 122). It is undoubtedly of great significance that the crystalline barium salts of heparin isolated from different animal species differ greatly in their anticoagulant potency, although their chemical composition appears fairly similar (118).

Masamune *et al.* (145) claimed to have identified the uronic acid of heparin with glucuronic acid; but, since this identification was achieved by extremely indirect means, it will have to be accepted with caution. The same workers made the significant observation that the amino group of the glucosamine constituent of heparin was neither free nor acetylated. This finding was later confirmed by Wolfrom and collaborators (226), who added a number of other interesting observations.

The composition (226) of the crystalline barium acid salt of heparin (prepared from Hoffmann-La Roche heparin) was found to be (in per cent, on an ash-free and dry basis): carbon, 20.19; hydrogen, 2.85; nitrogen (Dumas), 1.94; sulfur, 11.31; ash, 40.41; anhydrohexuronic acid, 17.9; barium, 23.78; anhydrohexosamine, 17.3 ± 1.2. The specific rotation was $[\alpha]_D^{25} = +47.5°$. The substance contained neither free amino groups nor N-acetyl. The ratio obtained was anhydrohexosamine:anhydrohexuronic acid:SO_3:Ba = 2.0:1.8:6.0:3.0. There were indications of the presence of another as yet unidentified constituent. The repeated recrystallization of the barium salt from warm, dilute acetic acid was found to bring about the almost complete destruction of its anticoagulant activity. The inactivation was accompanied by the appearance of free amino nitrogen.

Heparin appears, in general, to be rather easily inactivated. Treatment with nitrous acid, formaldehyde or acidic methyl alcohol (46), or prolonged drying of the barium salt at 100° C. (226) produce a destruction or a diminution of the anticoagulant potency, and tissue extracts are said to inactivate heparin by means of a specific enzyme (112).

Heparin is widely distributed in the animal body. The largest quantities are found in lung, liver and muscle; much smaller amounts are present in spleen, heart and blood, whereas the quantity found in serum is almost negligible (44). As regards the source of heparin in the body, the studies of Holmgren and Wilander (102, 126) have indicated that the metachromatic granules of the mast cells of Ehrlich contain heparin.

The incoagulability of the blood in peptone shock and in anaphylactic shock has long been known. Recently, the suggestion of Howell (106) that this phenomenon may be due to the presence of heparin in the blood was fully borne out by the isolation of heparin (as the crystalline barium salt) from the blood of dogs in anaphylactic shock (117).

The high content of sulfuric acid groups confers on heparin a strong

electronegative charge. This property, together with its high molecular weight, is probably responsible for certain reactions of heparin described in the literature, *viz.*, the displacement of the flocculation point of proteins (78, 176), the inhibition of the action of fumarase (82) and of trypsin (103), and the "neutralization" of the hemolytic complement (86). Salts between heparin and proteins or protamines have been studied in a number of cases (23, 35, 79, 113). In plasma, heparin appears to be largely bound to a portion of the albumin fraction (40), and this is of importance for the mechanism of its inhibiting effect.

Before the mechanism of the action of heparin is discussed, however, attention should be drawn to a number of synthetic anticoagulants which may be considered as heparin models. It is a remarkable fact that, even before the composition of heparin was known, the anticoagulant effect of sulfonic acid derivatives, *e. g.*, of germanin (206), had been noticed repeatedly. Demole and Reinert (55) described a polyanethole sulfonic acid ("Liquoid Roche") in 1930; and at about the same time the anticoagulant effect of a dye, Chicago blue, was recorded by Rous *et al.* (184). This substance and other related dyes, all sulfonic acid derivatives, were studied by Huggett and associates [compare (111)]. Later, a series of compounds that resembled heparin more closely was described, namely, sulfuric acid esters of high molecular substances of which the derivatives of polyvinyl alcohol (28), cellulose (9, 28) and chitin (9) exhibited the greatest potency. The activity, however, amounted to no more than about 6% of that of pure heparin. The relation of the constitution of synthetic anticoagulants to their toxicity was recently reinvestigated (130). The sulfuric acid esters of cellulose glycolic acid ether, $(C_6H_9O_4.OCH_2.COOH)_x$, and of other water-soluble cellulose derivatives were less toxic than cellulose sulfuric acid itself. Several polysaccharide sulfuric acid esters, isolated from algae, have likewise been found to exhibit some anticoagulant activity (9, 28, 71). The fact that the sulfuric acid esters of substances of low molecular weight are inactive (9, 28), and that the phosphoric acid ester of cellulose has only a small activity (28, 130), demonstrates that solely water-soluble sulfuric acid esters of high molecular weight may be expected to inhibit blood clotting to an appreciable degree.

3. *Mode of Action of Heparin, Heparin Complement, Antithrombin*

The important clinical applications that heparin has found in recent years, as in the prevention of postoperative thromboses, have, of course, served to stimulate interest in the mechanism of its action. Chargaff and Olson (35) studied the mode of action of heparin and several synthetic

anticoagulants in dogs. The experiments on the intravenous administration of these agents demonstrated that the action of both the natural and synthetic anticoagulants followed a similar course. While the maximum inhibiting effect was reached very rapidly and uniformly in all cases, the duration of the effect was entirely a matter of dosage. With large doses of heparin, the anticoagulant effect was demonstrable for about five hours. In the course of these experiments the interesting finding was made that the biological activity of circulating heparin could be entirely stopped by the intravenous injection of the protamine salmine. The application of salmine immediately before or after the intravenous injection of heparin prevented the anticoagulant effect completely. When the application of heparin was followed after some time by the injection of salmine, the prolonged clotting time produced by heparin was immediately brought back to the normal level. The reaction between heparin and protamines or other basic proteins was later frequently studied and employed [compare, *e. g.*, (23, 113, 115, 117)].

It has long been known that heparin, in contrast to its striking effect on blood, does not prevent the coagulation of a fibrinogen solution by purified thrombin (110). It is also unable to prevent the conversion of isolated prothrombin to thrombin (151, 168). To exert its powerful anticoagulant action, heparin requires an additional factor which is known to be present in blood, plasma and serum (17, 110, 170). This factor, for which the term heparin complement has been proposed (232), has been shown to be contained in the serum albumin fraction. (170) but to be absent from crystalline serum albumin (116, 231, 232).

A study of the relation between thrombin and heparin complement showed that the heparin complement acted only above a critical concentration (232). When decreasing quantities of albumin were mixed with fixed quantities of thrombin and heparin, and the mixtures added to fibrinogen, the coagulation of the latter was inhibited until a minimum concentration of the albumin was reached, at which the coagulation time fell sharply.* For this reason, the occurrence of clotting in a system containing thrombin, fibrinogen and heparin may depend entirely on the concentration of the heparin complement, which perhaps is the determining factor in individual responses to heparin. The investigation of the distribution of the heparin complement in various albumin fractions from human plasma showed that, while crystalline albumin lacked this factor, it was present in approximately similar concentrations in the fractions insoluble

* A similar critical relationship likewise appears to hold true in the opposite case, *viz.*, with a constant concentration of plasma and a varying concentration of heparin (27).

at an ammonium sulfate saturation of 75 and 100% and even in the small protein portion soluble at 100% saturation. Similar results were obtained with horse serum albumin fractions.

Additional information with respect to the heparin complement and the combination of heparin with the blood proteins was obtained in a study of the influence of heparin on the electrophoretic behavior of human plasma proteins (40). It was found that heparin (and synthetic anticoagulants of the heparin type) reacted with both the plasma albumin and globulin fractions. The addition of heparin to plasma caused the appearance in plasma and isolated plasma albumin preparations of a new electrophoretic boundary (the C component) with a mobility intermediate between that of heparin and albumin. The globulin patterns appeared broken up into a large number of small components. In order to decide whether the heparin complement was identical with, or contained in, the newly formed C component, electrophoretic separation experiments were carried out in which mixtures of albumin and heparin were studied. The contents of the cell after prolonged electrophoresis were divided into three parts: the fast, middle and slow components. When tested in the presence of heparin for heparin complement activity under strictly comparable conditions, the fast and middle fractions were very markedly active while the slow fraction was inactive. The findings indicated that the heparin complement, although not different from albumin in electrophoretic mobility in the absence of heparin, is a specific component of the albumin fraction separable in the presence of the anticoagulant. The natural clotting inhibitor could, therefore, be considered as a complex between the heparin complement and heparin in which the latter perhaps functions as a prosthetic group. It cannot yet be decided whether a certain steric arrangement of the sulfuric acid groups in heparin, enabling it to combine with its complement in a specific manner, is the reason for its so much greater potency as compared with the synthetic anticoagulants. It should certainly be of interest to study the behavior of inactivated heparin samples (see page 50) toward plasma proteins.

If it were proved conclusively that heparin does occur in normal circulating blood, the identity of the antithrombin of normal serum (89, 177) with the heparin–complement complex could be discussed with some degree of assurance. This is, unfortunately, not yet the case, although the presence of both the heparin complement and the normal antithrombin in the serum albumin fraction appears suggestive. But there seem to exist differences between these two agents with respect to their distribution in subfractions of serum albumin (232) and to other properties (63). A recent report (95)

suggests, in fact, that serum antithrombin may owe its activity to a lipide component. If this finding should be confirmed, the lipide anticoagulants, to be discussed in the following paragraphs, would assume additional interest.

4. Lipide Inhibitors

A lipide fraction inhibiting the coagulation of blood and plasma has been found in the brain of sheep and pigs, in the spinal cord of cattle and in the blood cells of sheep (20, 21). A similar lipide inhibitor has also been isolated from the lipides of human spleen in a case of Niemann-Pick disease (24). The lipide inhibitor, which is insoluble in acetone, slightly soluble

TABLE II

ANTICOAGULANT ACTIVITY OF LIPIDE INHIBITOR FROM SHEEP BRAIN (21)

Amount in 0.1 ml. plasma or blood, mg.	Clotting time, min.		
	Activated chicken plasma	Recalcified oxalated human plasma	Human blood
0	9	2	4
0.031	36	...	...
0.062	54	8	...
0.124	90	18	100
0.249	> 250	51	150
0.498	> 250	82	...

in cold pyridine and ether, easily soluble in cold glacial acetic acid and in warm methyl alcohol and ethyl acetate, is found to accompany the crude sphingomyelin fraction from which it can be separated by various methods. The analysis of one fraction is given as an example: carbon, 62.4; hydrogen, 10.8; nitrogen, 2.6; phosphorus, 3.0; sulfur, 0.7. The substances are free of carbohydrates. The activity of one fraction from sheep brain is exemplified in Table II. The solubility characteristics and the general behavior of the lipide inhibitors make the presence in them of heparin extremely unlikely. In view of the difficulties attending the preparation of pure lipides, it cannot be stated whether the presence of sulfur in the lipide inhibitor fractions is connected with their anticoagulant potency. But the preparation of synthetic lipide anticoagulants by the introduction of sulfuric acid groups into cerebrosides (phenosin, kerasin) may be considered suggestive (22).

It cannot yet be asserted that these lipide inhibitors have physiological

significance. But it is possible that they will acquire interest when more is known about the composition of normal serum antithrombin and, in general, about the mechanisms by which the body controls the fluidity of circulating blood.

IV. Some Remarks on the Mechanisms

The student of the phenomenon of blood coagulation will return from a reading of the literature with many ideas about how blood should clot, but with very few about how it does. Too many hypotheses, at once obstinate and feeble, have cluttered up our understanding to make an addition to their number appear inviting. The only hopeful approach would seem to lie in taking stock of what really is known to be true and in adding to the body of facts by critical experiments with well-defined systems. The following remarks, aiming neither at finality nor at completeness, will discuss a few points for which there was no room in the preceding pages.

The Formation of Prothrombin.—This problem is in essence one pertaining to the physiological action of vitamin K and as such does not lie within the range of this article. A review on vitamin K by Dam has appeared in a preceding volume of this series (53). The most obvious explanation for the necessity of vitamin K would, of course, be the assumption that it forms part of the prothrombin molecule. Feeding experiments with prothrombin concentrates have, however, yielded no indication of the presence of vitamin K activity (54). Whether the very crude prothrombin preparations would be expected to show vitamin activity, if vitamin K were linked to the protein in equimolar proportions, could be debated. It has recently been shown that 2-methyl-1,4-naphthoquinone can form conjugates with proteins containing free sulfhydryl groups (77); and it should be of interest to test similar compounds between the quinone and liver protein fractions for prothrombin activity. The final decision will not be reached before pure prothrombin and thrombin are available for chemical and spectroscopic comparison, but the possibility should be borne in mind that vitamin K, if it forms part of the enzyme molecule, could be present in prothrombin in the hydroquinone stage, and that the conversion to thrombin could be accompanied by an oxidation to the quinone form.

The Formation of Thrombin.—It is hard to decide whether the often observed conversion of partially purified prothrombin to thrombin in the absence of added activator (compare Section II, page 34) is really spontaneous. In view of the activating effect of trypsin (page 45), this con-

version is probably due to a contamination with proteolytic enzymes of the plasma (221d). It should be mentioned that the purified thrombin preparations of Seegers (193) exhibited no fibrinolytic activity.

In a discussion of the mechanism of the conversion of prothrombin to thrombin it is advisable to distinguish clearly between the three pathways by which this reaction can be accomplished: (*a*) the thromboplastic protein; (*b*) the thromboplastic lipide; (*c*) proteolytic enzymes. Reactions *a* and *c* have been studied in purified systems. It is not clear whether the activation by lipides ever has been followed in a system composed of purified prothrombin (+ calcium), fibrinogen and purified, analytically defined phosphatide preparations. In the absence of convincing evidence to the contrary, it would appear conceivable that the formation of thrombin by lipides requires the combination of the latter with a plasma protein, so that pathways *a* and *b* mentioned above would be essentially similar.

The explanations of the action of the thromboplastic protein have, in general, centered on one of the following possibilities: (*1*) The thromboplastic protein (or a portion of it) combines with prothrombin and calcium to form thrombin (13); (*2*) the thromboplastic protein combines with a thrombin inhibitor with which thrombin normally is associated, and thereby releases the active thrombin (108); (*3*) the thromboplastic protein catalyzes the conversion of prothrombin to thrombin without itself entering into the reaction. This essentially enzymatic conception has found expression in the term thrombokinase (159).

It must be realized that, at the present stage of our knowledge, it is almost impossible to evaluate these conceptions adequately. A few observations may, however, be offered. The view of a compound formation between prothrombin and the thromboplastic protein, at least as far as the transfer of phosphorus-containing substances is concerned, has been made improbable by experiments with a thromboplastic protein preparation labeled with radioactive phosphorus (38). Conception *2* would appear somewhat more plausible, since it offers the possibility of reconciling the action of the thromboplastic protein with that of trypsin, in the sense that the activation of prothrombin could be accomplished by the destruction of the inhibitor by trypsin or by its removal through compound formation with the thromboplastic protein. This view could be tested by a careful analysis of the products resulting from the reaction between pure prothrombin and thromboplastic protein. In this connection, it should also be of interest to ascertain whether the normal antithrombin of serum is destroyed by trypsin. Conception *3*, finally, which in a way equates trypsin and the tissue activator, suffers from the fact that it has been im-

possible to demonstrate a trypsin-like enzymatic activity even in the most powerful preparations of the thromboplastic protein.

The action of the thromboplastic protein on prothrombin is exemplified in the following experiment taken from a recent publication (32). To 0.1 ml. of a 0.1% saline solution of beef prothrombin (2000 units per mg. of nitrogen, obtained from Dr. W. H. Seegers), 0.1 ml. of the thromboplastic protein from beef lung (Table I, Preparation 3) suspended in saline containing 0.15% calcium nitrate was added. After 20 minutes at 30° C., 0.03 ml. of the mixture was added to 0.2 ml. of a 1.4% human fibrinogen solution in phosphate buffer of *p*H 7. In the following determinations, carried out at 30°, each tube contained the equivalent of 15 γ of prothrombin, 2.8 mg. of fibrinogen and the indicated amounts of the thromboplastic protein.

	γ of thromboplastic protein in experiment (32)							
	3	1	0.33	0.11	0.037	0.012	0.004	0
Clotting time, sec.	14	15	26	33	57	113	148	>7200

It will be seen that the amount of thromboplastic protein necessary for the complete activation of the available prothrombin lay between 0.3 γ and 1 γ.

The conditions governing the thromboplastic effect in plasma have been discussed theoretically by Wöhlisch (222). The kinetics of blood clotting with respect to the relationship between the various factors taking part in this reaction were recently investigated in a careful study by Legler (135).

The Action of Thrombin.—The nature of the chemical reactions that, in the presence of thrombin, lead to the formation of fibrin is largely unknown. Wöhlisch (207) has defined the coagulation of fibrinogen as a denaturation process and thrombin as a "denaturase." But since the denaturation of a protein is an extremely vague term covering more reactions than one would care to enumerate, this semantic transformation will scarcely contribute to the understanding of the reactions involved. Nor will the definition of the formation of fibrin as a proteolytic process help much until it has been possible to identify definite split products formed in the course of the coagulation. For the time being it would appear advisable to limit the definition of the action of thrombin to the statement that it is a *proteotropic enzyme*, *i. e.*, that it brings about irreversible changes in the structure of fibrinogen that lead to the formation of the insoluble protein fibrin, and to defer speculation on the nature of these changes to a time when more experimental data are available.

The experiments on the clotting of fibrinogen by various clotting agents (30), mentioned on page 46, may perhaps give a clue to the direction in which the reactions produced by thrombin proceed. Most of the compounds found to be active are able to oxidize amino acids and peptides

containing free amino groups. The activity of the clotting agents in the coagulation of fibrinogen was, in fact, found to parallel their ability to decarboxylate amino acids. Generally, substances that failed to liberate carbon dioxide from amino acids were inactive in clotting, with the exception of α-naphthoquinonesulfonic acid which, although an active clotting agent, did not produce decarboxylation. There would appear to be several possible mechanisms of the clotting of fibrinogen by the synthetic agents: (*1*) oxidation of aminoacyl groups present in the protein; (*2*) oxidation of other susceptible groupings, such as sulfhydryl; (*3*) combination of the clotting agents with the protein. If the oxidation proceeded by way of an aminoacyl group to a ketoacyl group, the latter must have been stable under the experimental conditions, since no carbon dioxide could be demonstrated following the clotting of large amounts of fibrinogen. Reducing substances, such as sodium bisulfite and glutathione, were found to inhibit the action of the clotting agents. This finding supports the view of an oxidative reaction.

When the conception of the clotting of fibrinogen as an oxidative process is extended to the formation of fibrin by thrombin, it will be understood why the chemical differences between fibrinogen and fibrin are, at least from what little we know about the composition of these proteins, so small. The removal from the large molecule of a few polar groups or the blocking of these groups would not produce a noticeable change in the analytical figures. But a reaction of this type might be sufficient to bring about a conspicuous and irreversible change in the solubility properties of a large molecule.

Attempts to analyze in the mass spectrometer the composition of the atmosphere above a clotting mixture, both before and after the addition of thrombin to large amounts of purified fibrinogen, led to results that were not always reproducible, although in several experiments indications were obtained (30) of the liberation during clotting of very small amounts of carbon dioxide. It was an interesting finding that, in these experiments, rapid coagulation of fibrinogen by thrombin was observed in an atmosphere of water vapor at a nitrogen pressure of 10^{-4} mm. Hg. The nitrogen contained less than 0.1% of oxygen. The action of thrombin, therefore, appeared to require no atmospheric oxygen. The clotting of freshly prepared fibrinogen by thrombin was inhibited by sodium bisulfite. It is at present not possible to decide whether the inhibiting action of reducing agents is exerted on the thrombin or on the fibrinogen, but the first assumption appears more probable.

The groupings in fibrinogen that are attacked by thrombin are completely

a matter of conjecture. By analogy to the action of the synthetic clotting agents, free aminoacyl groups of the protein could be assumed to be involved. Baumberger (7) recently suggested that fibrin may be formed as the result of the formation of S—S bridges produced by the oxidation of the sulfhydryl groups of fibrinogen by thrombin; but the evidence on which these conclusions are based is not made clear. In unpublished experiments of the author with Drs. E. Brand, B. Kassell and M. Ziff, only very small amounts of —SH groups could be found in fibrinogen by reaction with porphyrindin. But in a protein of a particle weight near 500,000 these small amounts may be significant. Their presence would not be excluded by a negative nitroprusside test in fibrinogen (114). Interesting results have been obtained by Jaques (114) in a study of the action of hydrogen peroxide and iodine on fibrinogen and fibrin. Fibrinogen was found to reduce these substances at a fairly rapid rate. Fibrin showed an even greater reducing power towards hydrogen peroxide. It is not clear whether these properties are in any way related to the clotting process, since fibrinogen, following its oxidation with peroxide, still was coagulable by thrombin.

It is quite possible that the clotting of blood represents only one example of coagulation processes of a much more general biological importance. In what manner the living organism controls these coagulation processes is completely unknown. One may assume that the various factors that constitute the clotting phenomenon, although continually formed and destroyed and continually acting on each other, are held in a delicate equilibrium. This is, in fact, what constitutes both the difficulty and the fascination of the problem: the difficulty, because it is a borderline problem, involving some of the most refractory and least explored substances and reactions; the fascination, because in the coagulation of blood there is brought into the open, as it were, one of the innumerable systems through which the organism maintains, by predetermined oscillations, the condition of life.

Bibliography

1. Adams, M. A., and Taylor, F. H. L., *Am. J. Med. Sci.*, **205**, 538 (1943).
2. Apitz, K., *Z. ges. exptl. Med.*, **101**, 552 (1937).
3. Arthus, M., and Pagés, C., *Arch. physiol. norm. path.*, **22**, 739 (1890).
4. Astrup, T., and Darling, S., *Acta Physiol. Scand.*, **2**, 22 (1941).
5. Bailey, K., *Advances in Protein Chemistry.* Vol. I, Academic Press, New York, 1944, p. 289.
6. Bailey, K., Astbury, W. T., and Rudall, K. M., *Nature*, **151**, 716 (1943).

7. Baumberger, J. P., *Am. J. Physiol.*, **133**, P206 (1941).
8. Bergmann, M., and Niemann, C., *J. Biol. Chem.*, **115**, 77 (1936).
9. Bergström, S., *Z. physiol. Chem.*, **238**, 163 (1936).
10. Bering, E. A., Jr., *J. Clin. Investigation*, **23**, 586 (1944).
11. Best, C. H., Cowan, C., and MacLean, D. L., *J. Physiol.*, **92**, 20 (1938).
12. Bleibtreu, M., and Atzler, E., *Arch. ges. Physiol. (Pflügers)*, **181**, 130 (1920).
13. Bordet, J., *Ann. Inst. Pasteur*, **34**, 561 (1920).
14. Bordet, J., and Delange, L., *ibid.*, **27**, 341 (1913).
15. Brand, E., Kassell, B., and Saidel, L. J., *J. Clin. Investigation*, **23**, 437 (1944).
16. Brinkhous, K. M., *Medicine*, **19**, 329 (1940).
17. Brinkhous, K. M., Smith, H. P., Warner, E. D., and Seegers, W. H., *Am. J. Physiol.*, **125**, 683 (1939).
18. Campbell, H. A., and Link, K. P., *J. Biol. Chem.*, **138**, 21 (1941).
19. Campbell, H. A., Roberts, W. L., Smith, W. K., and Link, K. P., *ibid.*, **136**, 47 (1940).
20. Chargaff, E., *Science*, **85**, 548 (1937).
21. Chargaff, E., *J. Biol. Chem.*, **121**, 175 (1937).
22. Chargaff, E., *ibid.*, **121**, 187 (1937).
23. Chargaff, E., *ibid.*, **125**, 671 (1938).
24. Chargaff, E., *ibid.*, **125**, 677 (1938).
25. Chargaff, E., *Advances in Protein Chemistry*. Vol. I, Academic Press, New York, 1944, p. 1.
26. Chargaff, E., *J. Biol. Chem.*, **155**, 387 (1944).
27. Chargaff, E., Bancroft, F. W., and Stanley-Brown, M., *ibid.*, **115**, 149 (1936).
28. Chargaff, E., Bancroft, F. W., and Stanley-Brown, M., *ibid.*, **115**, 155 (1936).
29. Chargaff, E., Bancroft, F. W., and Stanley-Brown, M., *ibid.*, **116**, 237 (1936).
30. Chargaff, E., and Bendich, A., *ibid.*, **149**, 93 (1943).
31. Chargaff, E., and Bendich, A., *Science*, **99**, 147 (1944).
32. Chargaff, E., Bendich, A., and Cohen, S. S., *J. Biol. Chem.*, **156**, 161 (1944).
33. Chargaff, E., and Cohen, S. S., *ibid.*, **129**, 619 (1939).
34. Chargaff, E., Moore, D. H., and Bendich, A., *ibid.*, **145**, 593 (1942).
35. Chargaff, E., and Olson, K. B., *ibid.*, **122**, 153 (1937–38).
36. Chargaff, E., and Ziff, M., *ibid.*, **131**, 25 (1939).
37. Chargaff, E., and Ziff, M., *ibid.*, **138**, 787 (1941).
38. Chargaff, E., Ziff, M., and Cohen, S. S., *ibid.*, **135**, 351 (1940).
39. Chargaff, E., Ziff, M., and Cohen, S. S., *ibid.*, **136**, 257 (1940).
40. Chargaff, E., Ziff, M., and Moore, D. H., *ibid.*, **139**, 383 (1941).
41. Chargaff, E., Ziff, M., and Rittenberg, D., *ibid.*, **144**, 343 (1942).
42. Charles, A. F., Fisher, A. M., and Scott, D. A., *Trans. Roy. Soc. Can. V*, **28**, 49 (1934).
43. Charles, A. F., and Scott, D. A., *J. Biol. Chem.*, **102**, 425 (1933).
44. Charles, A. F., and Scott, D. A., *ibid.*, **102**, 431 (1933).
45. Charles, A. F., and Scott, D. A., *Trans. Roy. Soc. Can. V*, **28**, 55 (1934).
46. Charles, A. F., and Scott, D. A., *Biochem. J.*, **30**, 1927 (1936).
47. Charles, A. F., and Todd, A. R., *ibid.*, **34**, 112 (1940).
48. Cohen, S. S., and Chargaff, E., *J. Biol. Chem.*, **136**, 243 (1940).
49. Cohen, S. S., and Chargaff, E., *ibid.*, **139**, 741 (1941).
50. Cohen, S. S., and Chargaff, E., *ibid.*, **140**, 689 (1941).

51. Cohn, E. J., Oncley, J. L., Strong, L. E., Hughes, W. L., Jr., and Armstrong, S. H., Jr., *J. Clin. Investigation*, **23**, 417 (1944).
52. Conradi, H., *Beitr. chem. Physiol. Path.*, **1**, 136 (1902).
53. Dam, H., in *Advances in Enzymology*. Vol. II, Interscience, New York, 1942, p. 285.
54. Dam, H., Glavind, J., Lewis, L., and Tage-Hansen, E., *Skand. Arch. Physiol.*, **79**, 121 (1938).
55. Demole, V., and Reinert, M., *Arch. exptl. Path. Pharmakol.*, **158**, 211 (1930).
56. Douglas, S. R., and Colebrook, L., *Lancet*, **2**, 180 (1916).
57. Dyckerhoff, H., *Biochem. Z.*, **316**, 397 (1943–44).
58. Dyckerhoff, H., von Behm, W., Goossens, N., and Miehler, H., *ibid.*, **288**, 271 (1936).
59. Dyckerhoff, H., and Gigante, D., *ibid.*, **304**, 334 (1940).
60. Dyckerhoff, H., and Grünewald, O., *ibid.*, **315**, 124 (1943).
61. Dyckerhoff, H., and Kürten, H. F., *ibid.*, **284**, 111 (1936).
62. Dyckerhoff, H., and Marx, R., *ibid.*, **313**, 115 (1942).
63. Dyckerhoff, H., and Marx, R., *ibid.*, **316**, 255 (1943–44).
64. Dyckerhoff, H., and Torres, I., *ibid.*, **316**, 31 (1943–44).
65. Eagle, H., *J. Gen. Physiol.*, **18**, 531 (1935).
66. Eagle, H., *J. Exptl. Med.*, **65**, 613 (1937).
67. Eagle, H., *Medicine*, **16**, 95 (1937).
68. Eagle, H., and Harris, T. N., *J. Gen. Physiol.*, **20**, 543 (1937).
69. Edsall, G., *Am. J. Physiol.*, **134**, 609 (1941).
70. Edsall, J. T., Ferry, R. M., and Armstrong, S. H., Jr., *J. Clin. Investigation*, **23**, 557 (1944).
71. Elsner, H., Broser, W., and Bürgel, E., *Z. physiol. Chem.*, **246**, 244 (1937).
72. Ferguson, J. H., *Physiol. Revs.*, **16**, 640 (1936).
73. Ferguson, J. H., *Am. J. Physiol.*, **117**, 587 (1936).
74. Ferguson, J. H., *Science*, **97**, 319 (1943).
75. Ferguson, J. H., and Erickson, B. N., *Am. J. Physiol.*, **126**, 661 (1939).
76. Ferry, J. D., and Morrison, P. R., *J. Clin. Investigation*, **23**, 566 (1944).
77. Fieser, L. F., and Fieser, M., *Organic Chemistry*. Heath, Boston, 1944, p. 738.
78. Fischer, A., *Biochem. Z.*, **240**, 364 (1931).
79. Fischer, A., *ibid.*, **278**, 133 (1935).
80. Fischer, A., *ibid.*, **278**, 334 (1935).
81. Fischer, A., and Hecht, E., *ibid.*, **269**, 115 (1934).
82. Fischer, A., and Herrmann, H., *Enzymologia*, **3**, 180 (1937).
83. Florkin, M., *J. Biol. Chem.*, **87**, 629 (1930).
84. Folch, J., *ibid.*, **146**, 35 (1942).
85. Folch, J., and Schneider, H. A., *ibid.*, **137**, 51 (1941).
86. Fuchs, H. J., and Hartmann, E., *Z. Immunitäts*, **58**, 1 (1928).
87. Fuld, E., *Beitr. chem. Physiol. Path.*, **2**, 514 (1902).
88. Fuld, E., and Spiro, K., *ibid.*, **5**, 171 (1904).
89. Gasser, H. S., *Am. J. Physiol.*, **42**, 378 (1917).
90. Glazko, A. J., and Greenberg, D. M., *ibid.*, **125**, 108 (1939).
91. Gratia, A., *Compt. rend. soc. biol.*, **83**, 311, 313 (1920).
92. Gratia, A., *Ann. Inst. Pasteur*, **35**, 513 (1921).
93. Gratia, A., and Levene, P. A., *J. Biol. Chem.*, **50**, 455 (1922).

94. Greenberg, D. M., *Advances in Protein Chemistry.* Vol. I, Academic Press, New York, 1944, p. 121.
95. Grüning, W., *Naturwissenschaften*, **31**, 299 (1943).
96. Guidi, G., *Arch. intern. pharmacodynamie*, **37**, 305 (1930).
97. Halliburton, W. D., *Proc. Roy. Soc. London*, **44**, 255 (1888).
98. Ham, T. H., and Curtis, F. C., *Medicine*, **17**, 413 (1938).
99. Hammarsten, O., *Z. physiol. Chem.*, **22**, 333 (1896–97).
100. Heard, W. N., *J. Physiol.*, **51**, 294 (1917).
101. Hobson, F. C. G., and Witts, L. J., *J. Path. Bact.*, **52**, 367 (1941).
102. Holmgren, H., and Wilander, O., *Z. mikroskop.-anat. Forsch.*, **42**, 242 (1937).
103. Horwitt, M. K., *Science*, **92**, 89 (1940).
104. Howell, W. H., *Am. J. Physiol.*, **26**, 453 (1910).
105. Howell, W. H., *ibid.*, **31**, 1 (1912–13).
106. Howell, W. H., *ibid.*, **71**, 553 (1925).
107. Howell, W. H., *Bull. Johns Hopkins Hosp.*, **42**, 199 (1928).
108. Howell, W. H., *Physiol. Revs.*, **15**, 435 (1935).
109. Howell, W. H., and Donahue, D. D., *J. Exptl. Med.*, **65**, 177 (1937).
110. Howell, W. H., and Holt, E., *Am. J. Physiol.*, **47**, 328 (1918–19).
111. Huggett, A. St. G., and Rowe, F. M., *J. Physiol.*, **80**, 82 (1933–34).
112. Jaques, L. B., *J. Biol. Chem.*, **133**, 445 (1940).
113. Jaques, L. B., *Biochem. J.*, **37**, 189 (1943).
114. Jaques, L. B., *ibid.*, **37**, 344 (1943).
115. Jaques, L. B., Charles, A. F., and Best, C. H., *Acta Med. Scand.*, **90**, Suppl., p. 190 (1938).
116. Jaques, L. B., and Mustard, R. A., *Biochem. J.*, **34**, 153 (1940).
117. Jaques, L. B., and Waters, E. T., *J. Physiol.*, **99**, 454 (1941).
118. Jaques, L. B., Waters, E. T., and Charles, A. F., *J. Biol. Chem.*, **144**, 229 (1942).
119. Jorpes, E., *Biochem. J.*, **29**, 1817 (1935).
120. Jorpes, E., *Heparin.* Oxford Univ. Press, New York, 1939.
121. Jorpes, E., *Biochem. J.*, **36**, 203 (1942).
122. Jorpes, E., *Z. physiol. Chem.*, **278**, 7 (1943).
123. Jorpes, E., and Bergström, S., *ibid.*, **244**, 253 (1936).
124. Jorpes, E., and Bergström, S., *J. Biol. Chem.*, **118**, 447 (1937).
125. Jorpes, E., and Bergström, S., *Biochem. J.*, **33**, 47 (1939).
126. Jorpes, E., Holmgren, H., and Wilander, O., *Z. mikroskop.-anat. Forsch.*, **42**, 279 (1937).
127. Jühling, L., *Kolloid-Z.*, **91**, 47 (1940).
128. Jühling, L., and Wöhlisch, E., *Biochem. Z.*, **298**, 312 (1938).
129. Kaplan, M. H., Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Investigation*, **21**, 533 (1942).
130. Karrer, P., Koenig, H., and Usteri, E., *Helv. Chim. Acta*, **26**, 1296 (1943).
131. Kühnau, J., and Morgenstern, V., *Z. physiol. Chem.*, **227**, 145 (1934).
132. Kuizenga, M. H., and Spaulding, L. B., *J. Biol. Chem.*, **148**, 641 (1943).
133. Laki, K., *Z. physiol. Chem.*, **273**, 95 (1942).
134. Leathes, J. B., and Mellanby, J., *J. Physiol.*, **96**, 38P, 39P (1939).
135. Legler, R. G., *Helv. Chim. Acta*, **26**, 1512, 1673 (1943).
136. Lenggenhager, K., *Helv. Med. Acta*, **1**, 527 (1934–35).
137. Loeb, L., *Beitr. chem. Physiol. Path.*, **5**, 191 (1904).

138. Loomis, E. C., and Seegers, W. H., *Arch. Biochem.*, **5**, 265 (1944).
139. Loucks, M. M., and Scott, F. H., *Am. J. Physiol.*, **91**, 27 (1930).
140. Macfarlane, R. G., and Barnett, B., *Lancet*, **2**, 985 (1934).
141. Macfarlane, R. G., Trevan, J. W., and Attwood, M. P., *J. Physiol.*, **99**, 7P (1940–41).
142. Martin, C. J., *ibid.*, **15**, 380 (1894).
143. Martin, C. J., *ibid.*, **32**, 207 (1905).
144. Marx, R., and Dyckerhoff, H., *Biochem. Z.*, **316**, 1 (1943–44).
145. Masamune, H., Suzuki, M., and Kondoh, Y., *J. Biochem. (Japan)*, **31**, 343 (1940).
146. McLean, J., *Am. J. Physiol.*, **41**, 250 (1916).
147. McLean, J., *ibid.*, **43**, 586 (1917).
148. Mellanby, J., *J. Physiol.*, **38**, 28 (1909).
149. Mellanby, J., *Proc. Roy. Soc. London*, **B107**, 271 (1930).
150. Mellanby, J., *ibid.*, **B113**, 93 (1933).
151. Mellanby, J., *ibid.*, **B116**, 1 (1934).
152. Mellanby, J., and Pratt, C. L. G., *ibid.*, **B128**, 201 (1940).
153. Mills, C. A., *J. Biol. Chem.*, **46**, 135 (1921).
154. Milstone, H., *J. Gen. Physiol.*, **25**, 679 (1942).
155. Moore, D. H., and Lynn, J., *J. Biol. Chem.*, **141**, 819 (1941).
156. Morawitz, P., *Beitr. chem. Physiol. Path.*, **5**, 133 (1904).
157. Morawitz, P., *Ergeb. Physiol.*, **4**, 307 (1905). (a) p. 369. (b) p. 367.
158. Morawitz, P., *Beitr. chem. Physiol. Path.*, **8**, 1 (1906).
159. Morawitz, P., in C. Oppenheimer, *Handbuch der Biochemie.* Vol. IV, Fischer, Jena, 1925, p. 44.
160. Mueller, J. H., and Sturgis, S., *Science*, **75**, 140 (1932).
161. Mylon, E., Winternitz, M. C., and de Sütö-Nagy, G. J., *J. Biol. Chem.*, **143**, 21 (1942).
162. Nolf, P., *Arch. intern. physiol.*, **6**, 1 (1908).
163. Nolf, P., *Medicine*, **17**, 381 (1938).
164. Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, **16**, 267, 295, 313 (1932–33).
165. Parfentjev, I. A., *Am. J. Med. Sci.*, **202**, 578 (1941).
166. Patek, A. J., Jr., and Taylor, F. H. L., *J. Clin. Investigation*, **16**, 113 (1937).
167. Pekelharing, C. A., *Deut. med. Wochschr.*, **18**, 1133 (1892).
168. Quick, A. J., *Proc. Soc. Exptl. Biol. Med.*, **35**, 391 (1936).
169. Quick, A. J., *Am. J. Physiol.*, **114**, 282 (1936).
170. Quick, A. J., *ibid.*, **123**, 712 (1938).
171. Quick, A. J., *ibid.*, **131**, 455 (1940).
172. Quick, A. J., *ibid.*, **132**, 239 (1941).
173. Quick, A. J., *The Hemorrhagic Diseases and the Physiology of Hemostasis.* C. C Thomas, Springfield, 1942. (a) p. 66. (b) p. 80.
174. Quick, A. J., *Physiol. Revs.*, **24**, 297 (1944).
175. Quick, A. J., *Ann. Rev. Physiol.*, **6**, 295 (1944).
176. Reiner, L., and Kopp, H., *Kolloid-Z.*, **46**, 99 (1928).
177. Rettger, L. J., *Am. J. Physiol.*, **24**, 406 (1909).
178. Roderick, L. M., *ibid.*, **96**, 413 (1931).
179. Rosenmann, M., *Biochem. Z.*, **112**, 98 (1920).
180. Rosenmann, M., *ibid.*, **128**, 372 (1922).

181. Rosenmann, M., *Biochem. Z.*, **129**, 101 (1922).
182. Rosenmann, M., *ibid.*, **287**, 26 (1936).
183. Rosenmann, M., *ibid.*, **290**, 214 (1937).
184. Rous, P., Gilding, H. P., and Smith, F., *J. Exptl. Med.*, **51**, 807 (1930).
185. Rumpf, F., *Biochem. Z.*, **55**, 101 (1913).
186. Scheuring, H., *ibid.*, **277**, 437 (1935).
187. Schloessmann, H., *Beitr. klin. Chir.*, **79**, 477 (1912). (a) p. 529.
188. Schlossmann, A., *Verh. Gesellsch. f. Kinderheilk*, 1902. Quoted in M. von Pfaundler and A. Schlossmann, *Handbuch der Kinderheilkunde*, Vol. I, 3rd ed., Vogel, Leipzig, 1923, p. 257.
189. Schmidt, A., *Zur Blutlehre.* Vogel, Leipzig, 1892.
190. Schmidt, A., *Weitere Beiträge zur Blutlehre.* Bergmann, Wiesbaden, 1895.
191. Schofield, F. W., *J. Am. Vet. Med. Assoc.*, **64**, 553 (1924).
192. Scott, D. A., and Charles, A. F., *J. Biol. Chem.*, **102**, 437 (1933).
193. Seegers, W. H., *ibid.*, **136**, 103 (1940).
194. Seegers, W. H., *Arch. Biochem.*, **3**, 363 (1944).
195. Seegers, W. H., Brinkhous, K. M., Smith, H. P., and Warner, E. D., *J. Biol. Chem.*, **126**, 91 (1938).
196. Seegers, W. H., Loomis, E. C., and Vandenbelt, J. M., *Proc. Soc. Exptl. Biol. Med.*, **56**, 70 (1944).
197. Seegers, W. H., Loomis, E. C., and Vandenbelt, J. M., Abstracts, Meeting of Am. Chem. Soc., New York, Sept., 1944, p. 23B.
198. Seegers, W. H., and McGinty, D. A., *J. Biol. Chem.*, **146**, 511 (1942).
199. Seegers, W. H., and Smith, H. P., *ibid.*, **140**, 677 (1941).
200. Seegers, W. H., Smith, H. P., Warner, E. D., and Brinkhous, K. M., *ibid.*, **123**, 751 (1938).
201. Smith, H. P., *Ann. Rev. Physiol.*, **4**, 245 (1942).
202. Smith, H. P., Warner, E. D., and Brinkhous, K. M., *Am. J. Physiol.*, **107**, 63 (1934).
203. Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Exptl. Med.*, **66**, 801 (1937).
204. Solé, A., *Klin. Wochschr.*, **14**, 1354 (1935).
205. Stahmann, M. A., Huebner, C. F., and Link, K. P., *J. Biol. Chem.*, **138**, 513 (1941).
206. Steppuhn, O., and Brychonenko, S., *Biochem. Z.*, **140**, 1 (1923).
207. Strughold, H., and Wöhlisch, E., *Z. physiol. Chem.*, **223**, 267 (1934).
208. Tagnon, H. J., *J. Lab. Clin. Med.*, **27**, 1119 (1942).
209. Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Investigation*, **21**, 525 (1942).
210. Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *ibid.*, **22**, 127 (1943).
211. Tocantins, L. M., *Medicine*, **17**, 155 (1938).
212. Trevan, J. W., and Macfarlane, R. G., *Medical Research Council, Report* **1936–37**. H. M. Stationery Office, London, 1938, p. 143.
213. Vincke, E., and Schmidt, E., *Z. physiol. Chem.*, **273**, 39 (1942).
214. Wadsworth, A., Maltaner, F., and Maltaner, E., *Am. J. Physiol.*, **91**, 423 (1929–30).
215. Waldschmidt-Leitz, E., Stadler, P., and Steigerwaldt, F., *Z. physiol. Chem.*, **183**, 39 (1929).

216. Warner, E. D., *J. Exptl. Med.*, **68**, 831 (1938).
217. Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, **114**, 667 (1936).
218. Weitnauer, H., and Wöhlisch, E., *Biochem. Z.*, **288**, 137 (1936).
219. Wöhlisch, E., *Ergeb. Physiol.*, **28**, 443 (1929).
220. Wöhlisch, E., *Naturwissenschaften*, **24**, 513 (1936).
221. Wöhlisch, E., *Ergeb. Physiol.*, **43**, 174 (1940). (a) p. 341. (b) p. 277. (c) pp. 232, 259, 314. (d) p. 216.
222. Wöhlisch, E., *Biochem. Z.*, **304**, 326 (1940).
223. Wöhlisch, E., *ibid.*, **316**, 295 (1943–44).
224. Wöhlisch, E., and Jühling, L., *ibid.*, **297**, 353 (1938).
225. Wöhlisch, E., and Kiesgen, A., *ibid.*, **285**, 200 (1936).
226. Wolfrom, M. L., Weisblat, D. I., Karabinos, J. V., McNeely, W. H., and McLean, J., *J. Am. Chem. Soc.*, **65**, 2077 (1943).
227. Wooldridge, L. C., *Arch. Anat. Physiol., Physiol. Abt.*, **1883**, 389.
228. Wooldridge, L. C., *ibid.*, **1886**, 397.
229. Zak, E., *Arch. exptl. Path. Pharmakol.*, **70**, 27 (1912).
230. Zak, E., *ibid.*, **97**, 499 (1923).
231. Ziff, M., and Chargaff, E., *Proc. Soc. Exptl. Biol. Med.*, **43**, 740 (1940).
232. Ziff, M., and Chargaff, E., *J. Biol. Chem.*, **136**, 689 (1940).
233. Ziff, M., and Chargaff, E., *unpublished data.*
234. Zunz, E., and La Barre, J., *Arch. intern. physiol.*, **18**, 116 (1921).
235. Zunz, E., and La Barre, J., *Compt. rend. soc. biol.*, **85**, 1107 (1921).

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THE AMINO ACID DECARBOXYLASES OF MAMMALIAN TISSUE

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CONTENTS

I. Introduction

The aim of this report is to gather the scattered information available on amine-forming enzymes in animal tissues. Whereas in bacterial metabolism formation of amines is well established, it was not satisfactorily proved to occur in the animal body until recently. Substances like histamine and adrenaline are found in the body, but the site of their formation, the substances from which they are derived and the intermediary steps of their synthesis are not at all clear. In the case of histamine, some observers have even doubted its formation in mammalian tissues; they suggest that it arises in the intestinal lumen as a result of bacterial activity and is then absorbed and stored by the tissues. The work on histidine

decarboxylase in mammalian tissue which will be described below makes unlikely this view of the intestinal lumen as the only or chief source of the body's histamine; it leads to an explanation of why the enzyme histaminase which destroys histamine is present in high concentration in the wall of the intestine. Its function here seems to be to prevent the absorption of histamine formed in the lumen of the intestine, especially under abnormal conditions of an acid *p*H. But the fact remains that amine formation is not as easily demonstrated in animal tissue as in bacteria where, under optimal conditions, considerable amounts of amines are formed from the corresponding amino acids.

The bacterial formation of amines has been a complicating factor in much of the earlier work on mammalian decarboxylases. Tissue extract or mince was incubated over long periods, but in spite of the addition of disinfectants bacterial activity was not always excluded.

Decarboxylation is best studied under anaerobic conditions. In the presence of oxygen amino acids are capable of reacting in many other ways when incubated with tissue extracts, and a stoichiometric relation between amino acid metabolized and amine formed cannot be attained. Moreover, in the presence of oxygen the yield of amines may be low, if the tissues or tissue extracts contain enzymes which metabolize amines in oxidation reactions, *e. g.*, amine oxidase, histaminase or other oxidases.

II. In Vitro Experiments

The formation of amines from amino acids in tissue has been investigated by one of the following methods:

Pharmacological: Pharmacologically active substances are often formed from the inert amino acids on decarboxylation. On incubation the suspensions or extracts will therefore acquire an activity that can be assayed. The method requires small amounts of material, but without proper precautions the degree of specificity is not very high. Tissue extracts often contain substances which modify the response of the test preparations to histamine, a fact which must be borne in mind in assessing the reliability of the quantitative data on histamine formation.

Chemical: (*a*) *Isolation of the amine or a characteristic derivative.*—This method has been employed in two cases. It gives the most satisfactory proof for the formation of a given compound, but requires relatively large amounts of material; and the time course of the reaction cannot easily be followed. Moreover, yields are often far from quantitative, so that no relationship can be established between amino acid metabolized and amine formed. (*b*) *Manometric.*—The formation of carbon dioxide in the reaction is followed. The method is simple and the time course of the reaction is easily followed. It requires more material than the pharmacological methods, but less than is needed for isolation of a compound.

1. *Histidine Decarboxylase*

The formation of a histamine-like substance on incubation of tissue slices with histidine was reported by Werle in 1936 (64). It occurred not only in slices, but also in extracts of rabbit kidney and to a lesser extent of pig kidney (70). The histamine was identified by pharmacological methods: injected into cats or dogs the extracts caused a fall of the arterial blood pressure. On the isolated preparation of the guinea-pig small intestine they caused contraction of the muscle which was not abolished by atropine; but when the muscle had become specifically insensitive to histamine by its administration in large doses the extracts were found to be inactive. This effect is considered typical for histamine (1). The active principle was stable to hydrochloric acid and resembled histamine in its solubility. The activity of the extract disappeared on incubation with histaminase.

The distribution of the enzyme in different tissues was studied by Werle and Krautzun (71). Their results are presented in Table I.

TABLE I

DISTRIBUTION OF *l*(−)-HISTIDINE DECARBOXYLASE (71)

Animal	Kidney	Liver	Pancreas	Spleen	Brain	Lung	Stomach	Heart
Guinea pig	+++	++	+	−		±	−	−
Rabbit	+++	++	+	−	−	−	−	
Hamster	++	+	+					
Rat	±	−						
Mouse	+++	+						
Chick	+	+	−	±				
Sheep	−							
Horse	−							
Cow	−							
Pig	±							
Dog	−							
Cat	−							

The *p*H optimum of histamine formation was found at 8.6–9.0. The enzyme responsible for the formation of histamine was precipitated by half-saturation with ammonium sulfate; it was not inactivated by dialysis and it was destroyed by alcohol and acetone. Histamine was formed from the naturally occurring *l*(−)-histidine, not from *d*(+)-histidine.

The enzyme was reversibly inhibited by cyanide. It was not inhibited by carbon monoxide, but strongly by hydroxylamine—almost 100% inhibition in a 10^{-5} *M* solution—and by semicarbazide (80% inhibition in 10^{-4} *M* solution) (69). The inhibition of histamine formation by hydroxylamine was partly reversed by pyruvic acid (see Table II).

Other ketone reagents such as phenylhydrazine, dinitrophenylhydrazine and those described by Girard and Sandolescu (24) also inhibited the enzyme (65). From these observations Werle (65) concludes that the enzyme contains a carbonyl group. Thiamine and cocarboxylase acted as slight inhibitors; in no concentration did they activate the reaction. Bayer 205 inhibited completely in a concentration of 10^{-5} *M*. The enzyme was inhibited by bile. Recently, Werle (68) reported inhibition of the *l*-histidine decarboxylase by adrenaline and arterenol. The enzyme was also inhibited by *d*-histidine and by both *l*-dopa and *d*-dopa.

A certain degree of purification was obtained by the use of absorbents. Enzyme preparations from different animals behaved differently. Kaolin did not absorb the enzyme from rabbit kidney, but absorbed that from

TABLE II

EFFECT OF PYRUVIC ACID ON THE INHIBITION OF HISTIDINE DECARBOXYLASE BY HYDROXYLAMINE (AFTER WERLE AND HEITZER, 69)

Molar concentration of:		Histamine formed, μg.
Hydroxylamine	Pyruvate	
0	0	7
10^{-4}	0	0.15
10^{-4}	10^{-1}	1.8

guinea-pig kidney (69). The histaminase of rabbit kidney was almost completely absorbed on kaolin which thus could be used to free the histidine decarboxylase of rabbit kidney from the histaminase. This effect proved useful, as it increased the yield of histamine when histidine was incubated with enzyme preparations under aerobic conditions. The enzyme from both rabbit and guinea-pig kidney in neutral solution was absorbed on alumina Cγ and on fuller's earth. The best results in purification were obtained with rabbit kidney; extracts were first shaken three times with kaolin and the enzyme was subsequently absorbed on alumina Cγ; the absorbate was then washed with 0.2 *M* disodium hydrogen phosphate. A 34.5-fold purification was thus obtained.

Another attempt at increasing the yield of histamine is reported in a later paper by Werle (68). It is claimed that even under the approximately anaerobic conditions of his experiments histaminase is still active and responsible for a loss of histamine. The addition of another substrate of histaminase, putrescine, reduced the loss of histamine and thus increased the yield.

Many of Werle's earlier findings were observed independently and at

about the same time by Holtz and Heise (40). They found no histamine formation in extracts from cat lung, spleen, pancreas and duodenum, from rabbit lung, spleen, pancreas, skeletal and cardiac muscle and from dog and calf pancreas. They found a better yield of histamine in nitrogen than in air and ascribed this to the presence of histaminase in the extracts. More histamine was formed in extracts from liver than in those from the kidney (42); this was attributed to a relatively high histaminase activity of the kidney extracts. Slight histamine formation was also obtained with cat liver. The enzyme was present in the wall of the small intestine. but not of the colon and rectum, of the guinea pig. Since the fetal guinea-pig intestine also formed histamine from *l*(−)-histidine, the formation of histamine in the intestinal wall of the adult animal is most likely not due to bacterial activity (38).

These experiments strongly suggest the presence of a *l*(−)-histidine decarboxylase in mammalian tissues. The amounts of histamine formed are small; it has not been isolated. In addition, no experiments have been carried out to show the formation of carbon dioxide in the reaction. But it appears unlikely that the formation of histamine resulted from bacterial contamination, particularly since the *p*H optimum of the bacterial decarboxylase is at 4.0 (19, 20), whereas Werle found an optimum for the enzyme in the mammalian tissue at *p*H 9.0. It is interesting to compare the different rates of amine formation: Gale (19, 21) obtained a Q_{CO_2} of 3 to 60 for various strains. Werle gives an example in which 0.4 g. of rabbit kidney (fresh weight) formed 8.54 μg. of histamine in 2.5 hours. This corresponds to a Q_{CO_2} of 0.00861.

2. *Tryptophan Decarboxylase*

According to Werle and Menniken (72) extracts from the kidney of guinea pigs, when incubated with tryptophan, yielded a substance which when injected into cats and dogs produced a rise of arterial blood pressure characteristic for tryptamine. Extracts from liver, spleen, lungs and pancreas of the same animals and of kidneys of dog, ox, goat, pig and monkey were inactive. The formation of the pressor substance was completely inhibited in 10^{-3} *M* cyanide. The extracts to which toluene had been added were found to be sterile at the end of the incubation. No experiments on stereospecificity have been reported.

3. *Tyrosine Decarboxylase*

Experiments by Emerson (14) and by Heinsen (29) in which autolyzing ox pancreas was incubated with tyrosine over a period of days with a re-

sultant formation of tyramine do not give very satisfactory evidence of an active tyrosine decarboxylase. Neither do the observations of Schuler *et al.* (57, 58) which have been reviewed critically by Heinsen (29) and by Holtz (31). In Heinsen's experiments, no tyramine was formed when liver and kidney brei were similarly allowed to autolyze in the presence of tyrosine; but according to Holtz (31, 32) this negative result was due, not to the absence of a decarboxylase for tyrosine, but to the oxidative destruction of the tyramine formed by the amine oxidase present in these extracts. Holtz (31) studied the distribution of the tyrosine decarboxylase in different tissues; he found that, unlike the histidine decarboxylase, it was present chiefly in the kidney of the rabbit and the guinea pig; tyramine formation was doubtful in the liver of these animals. Holtz did not find the formation of tyramine in the pancreas of the rabbit, cat, dog and ox. The enzyme was absent from the liver of pigs and chicks, from the kidney of rats, pigs and chicks (42) and from the small intestine of guinea pigs (38). When incubated with tyrosine, the extracts containing the enzyme acquired the pressor action characteristic of tyramine; cocaine, which abolishes the effect of tyramine on the arterial blood pressure and sensitizes that of adrenaline, abolished also the action of the incubated extracts (31). In one experiment, the activity on the arterial blood pressure of the cat of 50 g. of rabbit kidney, incubated for 15 hours at 37° C. with $M/20$ *sec*-phosphate buffer and with 0.5 g. of tyrosine, corresponded to 22 mg. of tyramine. In this experiment 15 mg. of the dibenzoyl derivative of tyramine was isolated and characterized by melting point and analysis. In a similar experiment with pig kidney the amount of tyramine was about one-third to one-fourth of that found with rabbit kidney (32).

No attempts at purification of the enzyme have been described. It is not certain if tyramine is formed from $l(-)$-tyrosine only; Holtz, Credner and Reinhold (38) claim to have shown this in their earlier publications, but these publications do not contain experiments on stereospecificity.

The findings of Holtz and his collaborators have been confirmed independently by Werle and Menniken (72), who found, in addition, that in $M/1000$ cyanide no pressor activity developed.

4. Dopa Decarboxylase

The enzyme that catalyzes the decarboxylation of $l(-)$-β-(3,4)-dihydroxyphenylalanine or $l(-)$-dopa to hydroxytyramine differs from all the decarboxylases thus far discussed by the fact that its activity is so great that the formation of carbon dioxide can readily be measured. Since

l(−)-dopa is relatively easily decomposed in the presence of oxygen, it is advisable to perform the experiments in the absence of oxygen.

The enzyme was discovered by Holtz, Heise and Luedtke (41) in the kidney of the guinea pig and the rabbit. It was soon afterwards shown to occur in the liver (4, 43). In the guinea pig, but in no other animal hitherto examined, it was also found in the mucous membrane of the small intestine (38). Holtz, Credner and Walter (39) found the enzyme in the liver and kidney of sheep, goats, pigs and hens, but could not detect it in those of rats, mice and cattle. Later, however, using an improvement of their earlier manometric technique, Holtz and Credner (33) found the enzyme in rat kidney and liver. Table III, taken from Blaschko (6), gives the ac-

TABLE III

DOPA DECARBOXYLASE ACTIVITY OF TISSUE EXTRACTS (6)

Animal	Organ	Number of expts.	Dopa-q_{CO_2}	Hydroxytyramine formed per hr. and g. fresh tissue, mg.
Guinea pig	Liver	9	0.470–0.910	3.20– 6.20
	Kidney	3	1.860–3.390	12.70–49.60
Pig	Kidney	3	0.310–2.000	2.10–13.70
Cat	Liver	2	0.067, 0.069	0.46, 0.47
	Kidney	2	0.176, 0.186	1.20, 1.27
Dog	Liver	3	0.018–0.044	0.12– 0.30
	Kidney	3	0.024–0.043	0.16– 0.29
Rhesus	Liver	1	0.130	0.79
	Kidney	2	1.550, 2.300	10.60, 15.70
Man	Kidney	2	0.200, 0.940	1.37, 6.42
Rana temporaria	Liver*	3	0.031–0.080	0.21– 0.55
	Kidney*	1	0	0

* These experiments were carried out at 18.5–20.5° C.

tivity of dopa decarboxylase in a number of organs, expressed in dopa-$q^{N_2}_{CO_2}$ *i. e.*, as cu. mm. of carbon dioxide formed by 1 mg. (fresh weight) of tissue in 1 hour. These dopa-quotients are determined from the initial rate of formation of carbon dioxide; the amounts of hydroxytyramine formed (in mg.) are obtained by multiplying these values by 6.83.

All mammals hitherto examined show enzymatic activity. It is least in the tissues of the dog, in which animal there is no marked difference in the activity of liver and kidney extracts; in all other animals the kidney has a higher activity. In man, only renal tissue has so far been examined (2, 6, 33). In the frog, the kidney showed no activity but small amounts of the enzyme were present in the liver. The enzyme also occurs in the pancreas (33); the activity per unit of weight is greatest in the guinea pig,

less in the pig and the dog and least in the cat. The enzyme has not been found in *Sepia officinalis* (5).

The high activity of the enzyme makes its investigation relatively easy. Holtz, Heise and Luedtke (41) established the decarboxylation reaction by: (*1*) the pressor action of the hydroxytyramine formed; (*2*) the isolation of the tribenzoyl derivative; and (*3*) the manometric measurement of the carbon dioxide formed.

The enzyme is specific for *l*(−)-dopa; no other substrate has yet been found. Holtz, Heise and Luedtke (41) showed that only half the theoretical amount of substrate was attacked when *dl*-dopa was incubated;

TABLE IV

CARBON DIOXIDE FORMATION* OF TISSUE EXTRACTS WITH AMINO ACIDS (6)

Substrate	Pig kidney	Guinea pig		Rhesus	
		Liver	Kidney	Liver	Kidney
l(−)-Dopa	+	+	+	+	+
d(+)-Dopa	−	−			
dl-N-Methyldopa	−	−			−
l(−)-Tyrosine	−	−		−	−
dl-N-Methyltyrosine (synthetic surinamine)		−			
dl-Phenylalanine			−		
l(−)-Tryptophan		−			
l(−)-Histidine	−	−	−	−	−
d(+)-Histidine	−				
dl-Alanine			−		
dl-Proline			−		
dl-Hydroxyproline			−		
dl-Serine			−		
l(−)-Leucine			−		

* Formation of carbon dioxide is indicated by a plus sign; no formation is indicated by a minus sign.

they concluded that the dextrorotatory stereoisomeride was not decarboxylated. *d*(+)-Dopa, prepared by Harington and Randall (26), was not decarboxylated (4, 6).

Extracts containing dopa decarboxylase, when incubated with other amino acids, did not form carbon dioxide. Table IV gives a number of amino and methylamino acids tested in this way. The fact that neither *l*(−)-tyrosine nor *l*(−)-histidine led to the formation of carbon dioxide in detectable amounts is particularly interesting because it stresses the great difference in the rate of decarboxylation, which is slow with these amino acids as compared with dopa.

The enzyme was not inhibited by octyl alcohol, carbon monoxide or

sodium azide. The latter was tested at *p*H 6.5 and 7.4. It was inhibited by cyanide (41): this inhibition was reversible and not progressive (6). Slight inhibition was found with sodium sulfide—21% in $M/50$ Na_2S. Suramine (Antrypol B.D.H.) and Trypan Blue acted as inhibitors. With Suramine the inhibition was complete at a concentration of 10^{-3} M; at 10^{-4} M it was 57% initially, but became progressively more complete. With 10^{-3} M Trypan Blue inhibition was 33%. Hydroxytyramine, the end product of the reaction, acted as a slight inhibitor (6).

The activity–*p*H curve has not been fully established: at *p*H 7.4 and 8.0 there was no obvious difference in activity; at *p*H 5.0 the amine formation was less or even absent (41).

5. *Cysteic Acid Decarboxylase*

In the liver of some animals (dog, rat, guinea pig and pig) there occurs an enzyme which forms carbon dioxide from *l*(−)-cysteic acid under anaerobic conditions (7). The activity of the enzyme, expressed as cysteic acid quotient (*i. e.*, as μl.CO_2 formed per mg. fresh weight of tissue per hour), was determined by measuring the initial rate of the reaction in liver extracts manometrically. The results are given in Table V.

TABLE V

CYSTEIC ACID DECARBOXYLASE ACTIVITIES OF VARIOUS LIVER EXTRACTS (7)

Animal	No. of expts.	Cysteic acid-$Q_{CO_2}^{N_2}$	Taurine formed per hr. per g. tissue, mg.
Guinea pig	2	0.045; 0.0525	0.25; 0.29
Pig	1	0.089	0.50
Rat	3	0.12 (0.073–0.180)	0.67 (0.41–1.00)
Dog	6	0.22 (0.11–0.33)	1.23 (0.61–1.84)

The enzyme was not found in the liver of the cat, the rabbit and the cod. Neither was it found in the kidneys of those animals hitherto examined (man, dog, rat). This absence from the rat kidney is of interest in connection with a finding by Medes and Floyd (49) of a loss of carboxyl nitrogen without a corresponding loss of amino acid nitrogen in rat kidney brei incubated with cysteic acid under anaerobic conditions. The system responsible for this effect must be different from the hepatic enzyme, as there is no appearance of carbon dioxide.

The enzyme is stereospecific for *l*(−)-cysteic acid from which the theoretical amount of carbon dioxide is formed. From *dl*-cysteic acid only

half the theoretical amount of carbon dioxide is formed, indicating that the *d*(+)-cysteic acid is not attacked.

No carbon dioxide is formed from *dl*-homocysteic acid, but this substance acts as an inhibitor of the enzyme (Blaschko, *unpublished work*). The enzyme is reversibly inhibited by cyanide; octyl alcohol does not act as an inhibitor.

Dialyzed extracts have lost much of their activity and do not regain it when boiled liver extract or dialyzate is added. The enzymatic activity of liver extracts from vitamin-B_1 deficient rats is greatly reduced, but cannot be restored to normal figures by adding either cocarboxylase or thiamine *in vitro*. In paired feeding experiments it appeared likely that the low activity of the deficient animals was not due to the lack of the vitamin, but to the reduced food intake directly: rats on a diet sufficient in vitamin B_1, but insufficient in calories, also gave liver extracts with low decarboxylase activity. It seems likely that the low protein intake of the animals is responsible for the low enzymatic activity.

6. Conclusions

The experiments which have been described in the preceding sections show that all the preparations hitherto available have a relatively slow action. But from the data available there seems to be little doubt that the animal decarboxylases form a group of enzymes with characteristic properties. They are stereospecific; they are reversibly inhibited by cyanide and insensitive to octyl alcohol. Their sensitivity to cyanide might suggest that they are metal-containing catalysts, but the alternative suggestion has been put forward by Werle (65) that they react with cyanide because they contain a carbonyl group. It seems interesting that the histidine enzyme is inhibited by all ketone reagents.

The mammalian enzymes share most of their properties with the bacterial enzymes. A study by Werle (66, 67) shows that the bacterial histidine decarboxylase is also inhibited by cyanide and semicarbazide and by the ketone reagents of Girard and Sandolescu. Werle interprets this as an indication that the prosthetic group of the bacterial enzyme also contains a carbonyl group. The chief difference between bacterial and mammalian enzymes is in their activities, probably due to the difference in the amounts of enzyme present.

Recent work by Gale and Epps (15, 22, 23) has added considerably to our knowledge of the chemistry of the bacterial enzymes. Using acetone-dried preparations they have purified both *l*(+)-lysine and *l*(−)-tyrosine decarboxylase, so that the final products had an activity of about 50 and

100 times that of the original preparations. The preparation acting on tyrosine also decarboxylated *l*(−)-dopa. Both enzymes were split into an inactive apoenzyme, the activity of which could be re-established by a coenzyme which the authors call codecarboxylase. The codecarboxylase preparations were active in restoring both the lysine and tyrosine apoenzymes.

A study of the codecarboxylase (23) shows that it is widely distributed: it was found in all the organs of the rat that were tested, in peas, in cabbage leaves, and in yeast as well as in bacteria. Dried brewers' yeast was used for the purification of codecarboxylase; the final preparations, which were still heterogeneous, were about 15,000 times more active than the starting material. These preparations were characterized by a faint yellow color and an ultraviolet absorption band at 265 mμ. The preparations contained carbon, hydrogen and nitrogen, but no phosphorus or sulfur; they also did not contain any flavine. Gale and Epps give experiments showing that the new coenzyme is different from any of the prosthetic groups or coenzymes hitherto recognized.

That the new coenzyme is so widely distributed in mammalian tissue suggests that the mammalian enzymes may have the same prosthetic group. This is supported by the fact that the two groups of enzymes are inhibited by the same substances.

That the various substrates are metabolized by different mammalian enzymes, each of which is specific for one amino acid (with the possible exception of tyrosine and tryptophan), is chiefly based on the study of the relative activities of the various decarboxylations in different organs. The distribution is most similar in the case of the histidine and the dopa enzyme, and it seems doubtful if quantitative differences in different organs or in different species can be considered as sufficient evidence that the two amino acids are attacked by two different enzymes (39). But according to Holtz and Credner (33), the histidine enzyme occurs in the pancreas of the guinea pig only, whereas the dopa enzyme was also found in the pancreas of the dog, the cat, the pig and the ox. Another difference between these two enzymes is that the *l*-histidine enzyme is strongly inhibited by *d*-dopa, whereas the *l*-dopa decarboxylase is not inhibited by *d*-dopa (65).

III. Amine Formation in the Body

Although there seems no reason why the decarboxylases described in the first section should not be active in the normal animal, direct evidence on this point is scarce. It will be briefly discussed in the following section.

The main pathway of amino acid breakdown is by oxidative deamination, but for some of the amino acids discussed in Section II it is not clear if this is true, as other reactions are known for these compounds. Nevertheless, it seems unlikely that decarboxylation represents the main pathway of catabolism for any of the amino acids.

1. *Formation of Histamine from Histidine*

l(−)-Histidine is one of the amino acids not readily attacked by *l*-amino acid oxidase; the enzyme, histidase, which is present in the liver is probably the catalyst responsible for one of the principal pathways of histidine breakdown. As far as decarboxylation to histamine is concerned, the evidence is still far from complete. Experiments by Bloch and Pinoesch (9) seemed to show that the histamine content of guinea-pig lungs showed a significant increase after the parenteral administration of *l*-histidine, but Mackay (47) has shown that the histamine content of untreated guinea-pig lungs is less uniform than was found by Bloch and Pinoesch.* Mackay's findings were independently confirmed by Holtz and Credner (35), who could not detect a significant increase of the histamine equivalent of guinea-pig lungs after injections of *l*(−)-histidine. In the same paper, however, the authors described observations which they interpret as evidence for the decarboxylation of *l*(−)-histidine in the living animal: After parenteral administration of *l*-histidine in guinea pigs, a substance was excreted in the urine which (*a*) lowered the arterial blood pressure of the cat, and (*b*) stimulated the isolated intestine of the guinea pig. That the substance was in fact histamine is made likely by the observation that the assay against histamine by the two methods, (*a*) and (*b*), gave the same histamine equivalents. It is interesting that they observed only excretion, but not storage, of the histamine surplus.

It is impossible to relate the vast amount of experimental work on release of histamine from animal tissues to the observations on histidine decarboxylase discussed in Section II of this report, but it should be pointed out that most of this work is not directly concerned with the problem of histamine formation. It is well known that many tissues store histamine or a histamine-like substance, and that the tissue histamine can be liberated under the influence of a great number of stimuli; the histamine thus appearing in the tissue fluids or in the circulation will have the pharma-

* Greater histamine equivalents in the guinea-pig lung after injections of both *l*(−)- and *d*(+)-histidine have been reported in a preliminary communication by Edlbacher, Simon and Becker (13), but a promised detailed report on this work is not at present available in this country.

cological effects of histamine in the body. But in most of this work on release of histamine the source of the tissue histamine is not discussed. Where a balance sheet for histamine has been made, *e. g.*, in experiments on the release of histamine from the perfused guinea-pig lung due to snake venom (cobra or Australian copperhead venom), it was shown that the output of histamine in the perfusate plus drainage and leakage fluids could amount practically to the total initial histamine content of the tissues; at the end of the experiment there was a corresponding reduction of the tissue histamine (16).

2. *Formation of Tyramine and Hydroxytyramine*

There is no reliable evidence that tyramine is being formed in the animal body. Claims have repeatedly been put forward that tyramine is responsible for the rise of arterial blood pressure in hypertonia of renal origin, but these claims have not been verified (63). The work on the mammalian tyrosine decarboxylase shows that this enzyme has a very low activity, and one would think that tyramine would be removed by amine oxidase as soon as it is formed.

There are reports of the occurrence of tyramine in invertebrates. It was isolated by Henze (30) from the posterior salivary glands of *Octopus;* it has been held responsible for some of the toxic effects of the saliva (10). Sereni (59) believed tyramine to be a hormone in the octopus. No decarboxylation of *l*-tyrosine was found in the extracts of posterior salivary glands, liver and kidneys of *Sepia officinalis* (5). No similar observations have been made for *Octopus*.

We have seen that the diphenol, *l*-dopa, is much more rapidly decarboxylated than tyrosine; and the question arises whether the dopa enzyme is active in the living organism. That this is so is made likely by observations of Holtz and Credner (34, 36, 37) that, after oral as well as after parenteral administration of *l*-dopa, there appeared in the urine of the cat a substance which raised its arterial blood pressure. The pressor substance shared with hydroxytyramine the property of being destroyed by incubation with kidney extracts. The extracts thus treated acquired a depressor action, due to the action of amine oxidase which attacks hydroxytyramine (8) to form the corresponding aldehyde which, according to Holtz, Heise and Luedtke (41), has a depressor effect.

The pressor substance was found not only in man, but also in rabbits and guinea pigs. In one experiment in which 50 mg. of *l*-dopa was injected intravenously into a human subject, the pressor substance excreted corresponded to 15 mg. of hydroxytyramine, *i. e.*, 39% of the theoretically

possible amount was found. But the total amount of hydroxytyramine formed appears to have been greater than that, as the urine samples had a stronger pressor activity after boiling with acid for a few minutes. Holtz and Credner (34) ascribe this increase to the hydrolysis of some esterified hydroxytyramine present in the urine.

Normal urine usually had no pressor action or, at most, a very feeble one, but after acid hydrolysis a pressor effect was usually obtained. This observation will be discussed below in connection with the significance of dopa decarboxylase in normal metabolism.

Holtz and Credner (36) suggest that the weak hyperglycemic action of *l*-dopa is really an effect of the hydroxytyramine formed in metabolism.

In a later paper (37), Holtz and Credner showed that *dl*-dopa, when injected intramuscularly in rats, led to the appearance of more hydroxytyramine than could be accounted for by the amount of *l*(−)-dopa administered. This is explained as an indication that, in the rat, the dextrorotatory amino acid was being converted to the levorotatory stereoisomer. In the guinea pig, no evidence for the formation of pressor substance from *d*-dopa was obtained. This is explained by the differences in *d*-amino acid oxidase activity in the two species; according to the authors the oxidative deamination of *d*(+)-dopa proceeds more quickly in the rat.

It seems likely that decarboxylation is one of the pathways of metabolism of *l*(−)-dopa, but the importance of this substance in metabolism is not known. *l*-Dopa was first found in *Vicia faba* (25), and it also occurs in other legumes (51) and it is quite possible that it is morely widely distributed in nature than is at present known. But it is likely that dopa arises in the body as an intermediary metabolite in the breakdown of *l*-tyrosine. In the action on tyrosine by the enzyme tyrosinase (monophenol oxidase), dopa is formed. Tyrosinase occurs in plants and in certain invertebrates, but it has never convincingly been shown to be present in mammals. There is, however, some evidence that this reaction does occur in the body: (*1*) The diphenol, adrenaline, is usually believed to be derived from tyrosine (the formation of adrenaline is discussed more fully below). (*2*) *l*-Dopa is also considered to be the precursor of melanin. (*3*) In an isolated case of tyrosinosis, a metabolic disorder of the tyrosine metabolism, *l*-dopa was found in the urine after administration of tyrosine (48). (*4*) The observation of Holtz and Credner on esterified hydroxytyramine in normal human urine may also be quoted.

It therefore seems that the decarboxylation of *l*-dopa to hydroxytyramine may be one step on one of the pathways of tyrosine breakdown. It has

been suggested that this step may be of importance in the synthesis of adrenaline. It was for this reason that the N-methylated derivatives, *dl*-N-methyltyrosine (synthetic surinamine) and *dl*-N-methyldopa were examined. Neither of these compounds was decarboxylated by the dopa enzyme (see Table IV). It would clearly be desirable to extend this study to include more possible precursors of adrenaline, such as 3,4-dihydroxyphenylserine and the corresponding N-methylamino acid, but these two compounds are not available.

Hitherto, the decarboxylation of *l*-dopa to hydroxytyramine has been discussed as one of the intermediary steps (4, 6, 32*a*, 33), but an alternative reaction, the decarboxylation of one of the two serine derivatives by dopa decarboxylase or by a similar enzyme closely related to it, must be considered.

Although it is doubtful that hydroxytyramine is normally circulating in the blood, it seems possible that it might escape into the blood under conditions of oxygen lack. It has already been mentioned that hydroxytyramine is attacked by amine oxidase (8); and this enzyme is dependent on a high partial pressure of oxygen for its activity (45). Bing and Zucker (3) have reported experiments on cats in which *l*(−)-dopa was injected into the partly or completely ischemic kidney. The injection led to a rise of arterial blood pressure which was, like the pressor action of hydroxytyramine, enhanced after cocaine. Schroeder (56) saw an immediate and prolonged blood pressure rise in rats after the injection of 3–10 mg. of dopa, but not after the injection of 10 mg. of tyrosine. According to Oster and Sorkin (53), intravenous injection of *l*(−)-dopa caused a blood pressure rise only in chronically hypertensive cats, and not in normal or acutely hypertensive animals. They also reported a rise of blood pressure in patients with essential hypertension; in normal subjects, the blood pressure rise was absent and the general effects of the injection (nausea) were much less marked.

These observations seem to suggest that, at low oxygen pressures, hydroxytyramine does escape into the general circulation. It seems quite possible therefore that hydroxytyramine might contribute to the condition of hypertension due to oxygen lack of the ischemic kidney, but it must be borne in mind that the assumption that *l*(−)-dopa is a normal metabolite must still be proved.

3. *Formation of Taurine*

In vivo experiments on the biosynthesis of taurocholic acid have been carried out on the dog; and it is known that, in the presence of an ade-

quate amount of cholate, both cystine and cysteic acid increase the amount of taurocholic aid excreted (Bergmann, 1*a*; Virtue and Doster-Virtue, 63*a*). Since dog liver has been found to contain the highest cysteic acid decarboxylase activity, it seems likely that in this animal the decarboxylase is responsible for taurine formation. On the other hand, the enzyme has not been found in some animals which excrete taurocholic acid, *e. g.*, the cat and the cod. Therefore, either there exists an alternative pathway of taurine synthesis or the decarboxylase cannot be so easily extracted in these animals.

Taurine is known to occur in mollusks. Kelly (44) found as much as 5% of taurine in the dry matter of muscle from *Mytilus edulis* and *Pecten opercularis*. It has also been found by Mendel (50) in marine gastropods. Attempts to extract the cysteic acid decarboxylase from *Mytilus* and *Helix* have been unsuccessful (Blaschko, *unpublished*).

4. Conclusions

The experiments on amine formation in the animal body and the experiments on the isolated enzyme systems discussed in Section II show that the function of the mammalian decarboxylases is the elaboration of specialized substances such as histamine, adrenaline or taurine. This function explains the specific character of the enzymes, the relatively weak activity of the crude organ extracts and the fact that only a few of the amino acids are decarboxylated. The discovery of the amino acid decarboxylases in mammals and the recognition that these enzymes form a characteristic group with common properties is of relatively recent date; more enzymes of this class may exist. In the following sections a few examples are given which seem to show that there may be more amino acid decarboxylases than those hitherto described.

Formation of Diamines.—The decarboxylation of the dibasic amino acids, ornithine and lysine, by bacterial enzymes is well known. The formation in autolyzing liver of putrescine from ornithine (11) is probably due to bacteria. Since the discovery by Udránszky and Baumann (61) of the occurrence in the urine of certain cystinuric subjects of the diamines, putrescine and cadaverine, the question has been discussed as to whether the diamines are formed by bacterial action in the intestine or whether they arise in the human body. Despite much work on this point, it has not yet been possible to decide between the two alternatives. It was soon shown by Udránszky and Baumann (62) that the diamines when administered are not excreted, probably because of the action of diamine oxidase (see Zeller, 73). It therefore seems likely that it is the disappearance of the

diamines which is at fault in these cystinuric subjects. And it seems reasonable to ask whether the intermediary formation of the diamines is a normal event and, if so, where they are formed.

Formation of Ethanolamine.—This substance has been shown to be formed in putrefying meat from the corresponding amino acid, serine (52). Recent findings suggest that similar reactions may occur in mammalian metabolism. It was shown that the cephalin fraction of ox brain contains about 40–70% of its nitrogen as an hydroxyamino acid (18). This acid was identified as serine (17) and the phosphatide in which it is contained was called phosphatidylserine. This finding suggested a close relationship between serine and the corresponding amine. That serine is, in fact, the precursor of the ethanolamine in cephalin was shown by Stetten (60), who administered to rats *dl*-serine containing N^{15} and found that it was quickly incorporated into both the serine and the ethanolamine of brain phosphatides. The available evidence makes it impossible to decide whether it is the free amino acid that is decarboxylated or whether decarboxylation occurs only after the serine has been esterified with phosphoric acid.

Formation of Spermine.—This compound was discovered in human sperma by Leeuwenhoek (46), and also occurs in other tissues (12, 27, 28, 54, 55). It seems probable that spermine is derived from an amino acid

$$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$$
Spermine

and that its formation must involve a decarboxylation reaction, but neither its precursors nor its site of origin is known.

Bibliography

1. Barsoum, G. S., and Gaddum, J. H., *J. Physiol.*, **85**, 1 (1935).
1*a*. von Bergmann, E., *Beitr. chem. Physiol. Path.*, **4**, 192 (1904).
2. Bing, R. J., and Zucker, M. B., *Proc. Soc. Exptl. Biol. Med.*, **46**, 343 (1941).
3. Bing, R. J., and Zucker, M. B., *J. Exptl. Med.*, **74**, 235 (1941).
4. Blaschko, H., *J. Physiol.*, **96**, 50P (1939).
5. Blaschko, H., *ibid.*, **99**, 364 (1941).
6. Blaschko, H., *ibid.*, **101**, 337 (1942).
7. Blaschko, H., *Biochem. J.*, **36**, 571 (1942).
8. Blaschko, H., Richter, D., and Schlossmann, H., *ibid.*, **31**, 2187 (1937).
9. Bloch, W., and Pinoesch, H., *Z. physiol. Chem.*, **239**, 236 (1936).
10. Bottazzi, F., and Valentini, V., *Arch. sci. biol.*, **6**, 153 (1924).
11. Dakin, H. D., *J. Biol. Chem.*, **1**, 171 (1906).
12. Dudley, H. W., Rosenheim, M. C., and Rosenheim, O., *Biochem. J.*, **18**, 1263 (1924).

13. Edlbacher, S., Simon, M., and Becker, M., *Naturwissenschaften*, **28**, 411 (1940).
14. Emerson, R. L., *Beitr. chem. Physiol. Path.*, **1**, 501 (1902).
15. Epps, H. M. R., *Biochem. J.*, **38**, 242 (1944).
16. Feldberg, W., and Kellaway, C. H., *J. Physiol.*, **90**, 257 (1932).
17. Folch, J., *J. Biol. Chem.*, **139**, 971 (1941).
18. Folch, J., and Schneider, H. A., *ibid.*, **137**, 51 (1941).
19. Gale, E. F., *Biochem. J.*, **34**, 392 (1940).
20. Gale, E. F., *ibid.*, **34**, 846 (1940).
21. Gale, E. F., *ibid.*, **35**, 66 (1941).
22. Gale, E. F., and Epps, H. M. R., *ibid.*, **38**, 232 (1944).
23. Gale, E. F., and Epps, H. M. R., *ibid.*, **38**, 250 (1944).
24. Girard, A., and Sandolescu, G., *Helv. Chim. Acta*, **19**, 1095 (1936).
25. Guggenheim, M., *Z. physiol. Chem.*, **88**, 276 (1913).
26. Harington, C. R., and Randall, S. S., *Biochem. J.*, **25**, 1028 (1931).
27. Harrison, G. A., *ibid.*, **25**, 1885 (1931).
28. Harrison, G. A., *ibid.*, **27**, 1152 (1933).
29. Heinsen, H. A., *Z. physiol. Chem.*, **245**, 1 (1937).
30. Henze, M., *ibid.*, **87**, 51 (1913).
31. Holtz, P., *Arch. exptl. Path. Pharmakol.*, **186**, 684 (1937).
32. Holtz, P., *Z. physiol. Chem.*, **251**, 226 (1938).
32*a*. Holtz, P., *Naturwissenschaften*, **27**, 724 (1939).
33. Holtz, P., and Credner, K., *Arch. exptl. Path. Pharmakol.*, **199**, 145 (1942).
34. Holtz, P., and Credner, K., *ibid.*, **200**, 356 (1943).
35. Holtz, P., and Credner, K., *Z. physiol. Chem.*, **280**, 1 (1944).
36. Holtz, P., and Credner, K., *ibid.*, **280**, 9 (1944).
37. Holtz, P., and Credner, K., *ibid.*, **280**, 39 (1944).
38. Holtz, P., Credner, K., and Reinhold, A., *Arch. exptl. Path. Pharmakol.*, **193**, 688 (1939).
39. Holtz, P., Credner, K., and Walter, H., *Z. physiol. Chem.*, **262**, 111 (1939/40).
40. Holtz, P., and Heise, R., *Arch. exptl. Path. Pharmakol.*, **186**, 377 (1937).
41. Holtz, P., Heise, R., and Luedtke, K., *ibid.*, **191**, 87 (1938).
42. Holtz, P., Heise, R., and Spreyer, W., *ibid.*, **188**, 580 (1938).
43. Holtz, P., Reinhold, A., and Credner, K., *Z. physiol. Chem.*, **261**, 278 (1939).
44. Kelly, A., *Beitr. chem. Physiol. Path.*, **5**, 377 (1904).
45. Kohn, H. I., *Biochem. J.*, **31**, 1693 (1937).
46. Leeuwenhoek, A., *Trans. Roy. Soc. London*, **12**, 1040 (1678).
47. Mackay, M., *Australian J. Exptl. Biol. Med. Sci.*, **16**, 137 (1938).
48. Medes, G., *Biochem. J.*, **26**, 917 (1932).
49. Medes, G., and Floyd, N., *ibid.*, **36**, 836 (1942).
50. Mendel, L. B., *Beitr. chem. Physiol. Path.*, **5**, 582 (1904).
51. Miller, E. R., *J. Biol. Chem.*, **44**, 481 (1920).
52. Nord, F. F., *Biochem. Z.*, **95**, 281 (1919).
53. Oster, K. A., and Sorkin, S. Z., *Proc. Soc. Exptl. Biol. Med.*, **51**, 67 (1942).
54. Rosenheim, M. C., *J. Physiol.*, **51**, VI (1917).
55. Rosenheim, O., *Biochem. J.*, **18**, 1253 (1924).
56. Schroeder, H. A., *J. Exptl. Med.*, **75**, 513 (1942).
57. Schuler, W., Bernhardt, H., and Reindel, W., *Z. physiol. Chem.*, **243**, 90 (1936).
58. Schuler, W., and Wiedemann, A., *ibid.*, **233**, 235 (1935).

59. Sereni, E., *Biol. Bull.*, **59**, 247 (1930).
60. Stetten, D., Jr., *J. Biol. Chem.*, **144**, 501 (1942).
61. von Udránszky, L., and Baumann, E., *Z. physiol. Chem.*, **13**, 562 (1889).
62. von Udránszky, L., and Baumann, E., *ibid.*, **15**, 76 (1890).
63. Verney, E. B., and Vogt, M., *Quart. J. Exptl. Physiol.*, **28**, 253 (1938).
63*a*. Virtue, R. W., and Doster-Virtue, M. E., *J. Biol. Chem.*, **127**, 431 (1939).
64. Werle, E., *Biochem. Z.*, **288**, 292 (1936).
65. Werle, E., *ibid.*, **304**, 201 (1940).
66. Werle, E., *ibid.*, **306**, 264 (1940).
67. Werle, E., *ibid.*, **309**, 61 (1941).
68. Werle, E., *ibid.*, **311**, 270 (1942).
69. Werle, E., and Heitzer, K., *ibid.*, **299**, 420 (1938).
70. Werle, E., and Herrmann, H., *ibid.*, **291**, 105 (1937).
71. Werle, E., and Krautzun, H., *ibid.*, **296**, 315 (1938).
72. Werle, E., and Menniken, G., *ibid.*, **291**, 324 (1937).
73. Zeller, E. A., in *Advances in Enzymology*, Vol. II. Interscience New York, 1942, p. 93.

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ALCOHOLIC FERMENTATION OF THE OLIGOSACCHARIDES

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CONTENTS

I. Introduction

The fundamental equation for alcoholic fermentation was first exactly formulated by Lavoisier as follows:

$$C_6H_{12}O_6 \longrightarrow 2\ CO_2 + 2\ C_2H_5OH \qquad (1)$$

Strictly, this equation sums up the net result of the reaction sequence of fermentation for hexose substrates only. In nature and in industry, however, alcoholic fermentations are rarely conducted on hexose as such, the

materials *par excellence* of alcoholic fermentation being rather compound sugars: polysaccharides such as starch and glycogen, and oligosaccharides such as maltose, sucrose and lactose. Essentially, the final products of the alcoholic fermentation of these substrates are qualitatively and quantitatively identical. The Lavoisier fermentation equation thus becomes, for disaccharides:

$$C_{12}H_{22}O_{11} + H_2O \longrightarrow 4\ CO_2 + 4\ C_2H_5OH \qquad (2)$$

and generally for all sugars:

$$C_{6n}H_{10n+2}O_{5n+1} + (n-1)H_2O \longrightarrow (2n)CO_2 + (2n)C_2H_5OH \qquad (3)$$

simplifying for polysaccharides (sugars with high n values) to:

$$(C_6H_{10}O_5)_n + (n)H_2O \longrightarrow (2n)CO_2 + (2n)C_2H_5OH \qquad (4)$$

Alcoholic fermentation of a compound sugar thus involves necessarily both (*a*) net entry of water, and, with equal necessity, (*b*) fission of the intersaccharidic linkages.

It is obvious from superficial consideration that the mergence of the fermentation paths of higher sugars with that of the hexose cannot occur previously to step (*b*). The stage in the reaction and the method of the fission of the intersaccharidic linkage is, therefore, the main preoccupation of oligosaccharide fermentation chemistry in distinction to that of the hexoses.

II. Main Historical Trends. Nomenclature

It might have been expected that the progressive resolution of the zymase complex into its constituents and the accompanying identification of the partial reactions and intermediary products of the alcoholic fermentation path would early have provided unequivocal information on the question as to whether there are zymases capable of acting desmolytically on disaccharides as such. It is, however, a striking fact that the growth of our knowledge of fermentation has occurred always from the apex to the base of the sequence. We have been following the reaction train until very recently, not from its starting point to its terminus, but from its terminus backwards; and somehow our arrival at the end of the journey—the substrate as such—is forever being delayed. The early identified intermediaries and partial processes of the sugar fermentation sequence, beginning with the decarboxylation of pyruvate, through the production of pyruvate by the dismutation of trioses, and back to the production of the phosphorylated hexoses from which the C_3 compounds derive, have all belonged to ultimate or penultimate fermentation phases in which the primary di-

vergences which possibly distinguish the transformation of monoses, oligosaccharides and polysaccharides, respectively, are already in all probability erased.

Prior to the discovery of glucose-1-phosphate and glycogen as early products of the fermentation process, reducing phosphate esters of hexose (Robison ester, Harden–Young ester) had to be regarded as the first known products of the action of zymase on all its substrates. It was clear that fission of the intersaccharidic linkage of a compound sugar in fermentation must occur at some point in the reaction sequence prior to the production of phosphorylated intermediaries with a free reducing group.

For the breakdown of intersaccharidic linkages in oligosaccharide fermentation, four alternative sorts of reaction types should in principle have been distinguished: (*1*) hydrolysis carried out on the unchanged compound sugar; (*2*) hydrolysis carried out on a derivative of the original substrate; (*3*) fission of the intersaccharidic linkage by a process other than hydrolysis carried out on the unchanged sugar; (*4*) fission by a process other than hydrolysis carried out on a derivative of the original substrate. In practice, no effective choice between these alternatives was possible—the full alternative choice was never even clearly formulated—as long as the mode of formation of the earliest of the known fermentation intermediaries, the reducing hexose phosphate esters, from the mother sugars was unknown.

The discussion of the mechanism of oligosaccharide fermentation in the earlier phases of fermentation chemistry was arbitrarily narrow, being limited to the question as to whether disaccharides when fermented are first hydrolyzed, or primarily transformed in some way and only then hydrolyzed. The mechanisms of compound sugar fermentation in which the path for attack by a true zymase constituent is prepared by hydrolytic breakdown of the sugar to monose through the intervention of an oligase were termed "indirect fermentation." Mechanisms in which zymase as represented by one or more of its constituent enzymes (phosphatases, enolases, oxidoreductases, carboxylase) is assumed to attack the compound sugar, with consequent removal of a carbon group, intramolecular hydrogen shift, entry of phosphate, or other change, prior to intervention of oligase with consequent hydrolysis, were lumped under the term "direct fermentation."

It is only recently, following the discovery of nonhydrolytic mechanisms for the breakdown of the intersaccharidic linkage, that the full range of primary transformation possibilities open to a compound sugar has gained wider appreciation. As long as hydrolytic fission of the intersaccharidic

linkage was supposed to be the only possible mode of breakdown, the crucial point of mergence of the sequences of fermentation of higher sugars and monoses, respectively, was unanimously identified with the point of the entry of water into the compound sugar molecule. Even this seemingly elementary conclusion must be re-examined, however, when it becomes clear that hydrolysis is only one of several ways in which biological fission of the intersaccharidic linkage can be accomplished.

With the recognition of these possibilities, a new terminology, which promises to displace the older simple terms—"direct" fermentation, "indirect" fermentation—has sprung into being (see pages 113–114).

III. Indirect Fermentation Theory of Emil Fischer

The realization that fermentability is not a general feature of sugars as such but a specific function of certain constitutions and configurations within the carbohydrate group dates from the development of modern sugar chemistry by Emil Fischer in the eighties and nineties of the past century. This investigator first posed clearly the question of the relation between the fermentation of disaccharides and monoses. Always he found in yeasts which fermented a given disaccharide an enzyme which hydrolyzed this disaccharide (*e. g.*, maltase and invertase in maltose and sucrose-fermenting yeast, lactase in lactose-fermenting yeast), whereas in yeast which fermented monose but not disaccharide he noted the absence of a disaccharide-hydrolyzing enzyme. These results clearly established a probability that at least a portion of the fermenting disaccharide is hydrolyzed before it is fermented. But Fischer supposed more than just this: he considered that disaccharide fermentation is *always* preceded by hydrolysis; and only monoses were regarded by him as true substrates of fermentation. He, therefore, postulated by way of generalization that no oligosaccharide whatever can be attacked "directly," *i. e.*, as such, by desmolytic agents.

The fermentation of a compound sugar was accordingly divided by Fischer into two distinct phases, each of which is mediated by a mutually separable group of agents:

$$\text{Compound sugar} \xrightarrow{\text{oligase or polyase}} \text{zymohexose} \xrightarrow{\text{zymase}} CO_2 + C_2H_5OH \quad (5)$$

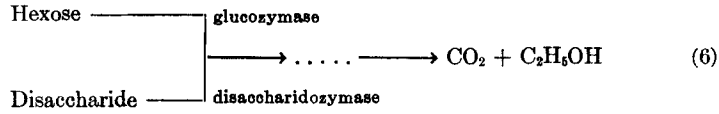
This formulation, entailing always a hydrolase + zymase system, became the classical expression of the theory of the indirect fermentation of compound sugars (20–22).

Fischer's explanation, owing largely perhaps to the high authority of its

author, was widely accepted. Essentially a speculative theory based on an attractive correlation, it assumed in the minds of both chemists and biologists the status of a proved fact. Even though Fischer himself had not even attempted to subject his theory to crucial quantitative test, the theory went almost unchallenged for nearly a generation. Moreover, its scope was broadened and the belief became general that the Fischer mechanism described not only alcoholic fermentation of oligosaccharides by yeast, but also the fermentations of oligosaccharides by bacteria, and equally the glycolysis of compound sugars by tissues of the higher forms of life. Laborde's (64) early criticism of the Fischer view in its application to sucrose metabolism in heterofermentative lactic acid bacteria was simply ignored.

IV. Direct Fermentation Theory of Willstätter

Willstätter was the first to subject the indirect fermentation hypothesis of Fischer to experimental quantitative test. In the early twenties of the century, the Willstätter school carried out quantitative measurements of the oligase content of yeast cells. The oligase contents were found to be far below the minimum necessary on the basis of the indirect fermentation theory to explain the fermentation rates of the oligosaccharides. Willstätter consequently abandoned Fischer's explanation of oligosaccharide fermentation. In its place, the concept was proposed of "direct fermentation" involving intervention of oligosaccharide-specific zymases, *e. g.*, "maltozymase," "lactozymase," "sucrozymase." Schematically, we may represent the viewpoint of Willstätter as follows:



(6)

It is significant here that Willstätter in this phase proposes no positive description for his disaccharidozymase mechanisms; he defines them only negatively in the sense of their nonidentity with the older "hydrolase + zymase" mechanisms of Fischer.

The following observations formed the early basis of Willstätter's work on oligosaccharide fermentation:

(*a*) Lactose-fermenting yeasts may ferment lactose more rapidly than they ferment the monose constituents of lactose, glucose and galactose, or their mixture (123).

(*b*) Moreover, extracts of lactose-fermenting yeast may contain lactase

in an amount which does not suffice to hydrolyze lactose at a rate at which lactose is fermented by the mother yeast in equivalent concentration; in certain cases the lactase content of such extracts is even negligible (123).

(*c*) Similarly, extracts of bakers', of distillers' and of some brewers' yeasts contain maltase in insufficient amount to explain the rate of maltose fermentation by the same yeast (126).

It should be noted that the measurements of oligase content conducted by Willstätter and his collaborators were carried out on extracts rather than on the intact cell itself in which fermentation capacity had been determined. The two measurements could not be carried out on suspensions of intact cells, since hydrolysis by the latter is accompanied by fermentation which inevitably masks the hydrolytic activity. It was assumed, however, that the entire oligase activity of the cells was expressed in the activity of the extracts, and the oligase content of the cells was therefore computed on the basis of the extracted oligase.

The argument upon which the above conclusions were based was found to be vulnerable at several points. In the first place, conclusive proof had not in fact been advanced that the entire oligase activity of the living cell had been preserved in the cell extracts upon which the actual hydrolysis measurements were conducted. Moreover, glucose is an inhibitor of maltase activity. Since glucose accumulates in the action of isolated maltase on maltose but is removed in the action of living cells on maltose, the computation of cell maltase from activity found in the cell extract tends to give a low result (18, 19, 61). However, Willstätter strengthened the probability that the entire maltase content of living yeast had been transferred to the yeast extracts used for measuring oligase content by demonstrating that the maltase content found is essentially the same, independent of variation in the procedure employed for the preparing of the extract. Moreover, the extent of the underestimation of maltase activity due to the inhibiting action on isolated maltase of accumulating glucose could be evaluated, and it was shown that even the corrected maltase activity is still so low that the general situation remains unchanged (119, 120).

Finally, selective treatments known to destroy or inactivate hydrolases were tested for their effect on the fermentation rate of oligosaccharides. It was argued that such treatments should destroy or markedly retard the oligosaccharide fermentation if hydrolase is in fact necessary for producing the actually fermenting substrate. The influence of *p*H was found to be effectively selective in this respect. Using this factor, the following was shown:

(*d*) Maltose is fermented by bakers' and brewers' yeast most vigorously at *p*H 4.0–5.0 and still markedly even at 2.5. The range is one in which maltase activity, at least *in vitro*, is entirely suppressed (119, 120).

(*e*) Distillers' and bakers' yeasts treated with acid or alkali retain the ability to ferment sucrose, but extracts of such yeast contain little invertase. The fermentation rate of sucrose by acid- or alkali-treated yeast is greater than is predictable on the basis of the invertase activity of the cell extracts. It could be shown, moreover, that estimations of the invertase content of yeast cells on the basis of the activity of their extracts have fairly high accuracy (122).

These findings provided further support for the view that maltose and lactose fermentation by yeast may be direct, and suggested, too, that sucrose might be added to the list of disaccharides for which direct fermentation by yeast seemed likely.

It was manifest, nevertheless, that a final decision had not been achieved. Even in the best-established instance of maltose, there was still lacking proof, crucial to the entire argument, that the conditions of maltase in the cell-free milieu of the maltase estimation are pertinently the same as obtained *in vivo* during actual fermentation. A significant element, the *p*H of the intracellular milieu and the nature of its response to extracellular buffers, had not been determined. It was conceivable, furthermore, that the *p*H–activity curves of cell-bound and cell-free maltase, respectively, are different. It could therefore still be claimed that failure of a given cell preparation to hydrolyze disaccharide with the rapidity prescribed by indirect fermentation theory was rather a manifestation of the difference between the conditions prevailing *in vivo* and *in vitro* than proof that the living cell could ferment the disaccharide without the participation of a corresponding hydrolase. In the case of sucrose, furthermore, the puzzling result remained unexplained that, in sucrose–yeast systems, so impoverished in invertase as to suggest that sucrose transformation is due at least in part to a direct fermentation mechanism, transient accumulation of reducing sugar had nevertheless been encountered (7, 122). On the basis of the prevailing knowledge, such accumulation of reducing sugar could only be ascribed to the intervention of a hydrolase which is present in effective excess over zymase, a possibility which the Willstätter interpretation had explicitly denied.

Above all, it could be argued that comparative measurements of separated glycolysis and hydrolysis can *never* guarantee complete identity of the environmental conditions employed in each case, since in the last resort it is always possible to claim that glycolysis and hydrolysis are *in vivo* coupled,

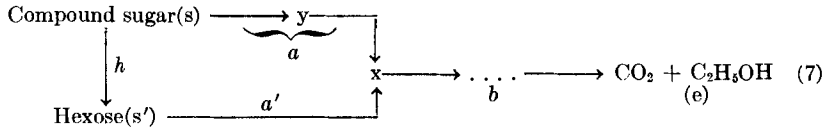
so that in the presence of zymase the hydrolase component is activated. If such a relation exists, it would be readily conceivable that the system hydrolase + zymase *in vivo* can act upon its substrate with greater velocity than the most active of the isolated components of the same system *in vitro* would separately. The very data of Willstätter and Bamann (119, 120) on the regeneration of sucrase and maltase in acid-pretreated cells during sucrose and maltose fermentation support the suspicion that hydrolase is activated by zymase in action.

Repeatedly in subsequent years authoritative investigators reaffirmed their faith in the classical hypothesis of indirect fermentation as expressed by Fischer, despite the criticisms which had been leveled at it by Willstätter (1, 58, 76). In the same decade, also, attempts to strengthen the case for direct fermentation were recorded by a number of original investigators. Sobotka and Holzman (102) contributed a valuable confirmation and extension of Willstätter's findings for maltose. From considerations of a kinetic nature, Wright (128–129) postulated a direct fermentation of lactose by streptococci. On similar grounds, Myrbäck *et al.* (82–84) tentatively suspected the existence of a direct fermentation of trehalose by yeast. Neuberg-Rabinowitsch (88) attempted a new approach to the problem of maltose fermentation, but arrived at no conclusion. Nord and Engel (91) studied phosphorylative and "nonphosphorylative" fermentation by *Fusarium lini* Bolley. They found that both a direct and an indirect fermentation of maltose occur in this organism.

To some extent, the problem of the direct fermentation of oligosaccharides remained unsolved. Significant progress toward a solution necessarily awaited a refinement of the suggested criteria for distinguishing between indirect and direct oligosaccharide fermentation mechanisms. In particular, it awaited, as will be seen, the discovery of new devices whereby cell hydrolases are rendered accessible to measurement *in vivo* and *in situ*, *i. e.*, in the intact and living cell and during the fermentation itself. It awaited also the formulation of precise positive hypotheses on the method of the cleavage of the intersaccharidic linkage in the direct fermentation of the oligosaccharides.

V. Criteria Differentiating the Mechanisms of Oligosaccharide Fermentation

Before proceeding to the consideration of recent experimental findings in the field of oligosaccharide fermentation, a classification is needed of the various criteria which are useful in differentiating different types of oligosaccharide fermentation mechanisms. The following scheme (72), which embodies the alternative of direct fermentation or indirect fermentation, may facilitate this discussion:



where s, s', x, y and e represent substrates, intermediaries and end products and italicized letters (*a*, *a'*, *b*, *h*) represent enzyme systems and the reactions they catalyze. The compound sugar and hexose are s and s', respectively; x is the intermediary first common to the fermentation sequences of both substrate types, *i. e.*, simply hexose (s') in indirect fermentation, and an as yet unidentified intermediary on the theory of direct fermentation; y is an intermediary irrelevant to indirect fermentation but possibly formed prior to x in direct fermentation; e represents the end products of the fermentation; *h* represents oligase and the hydrolysis it mediates; *a* and *b* together form the enzyme system and reaction of direct fermentation; *h* and *a'* and *b* together form the enzyme system and reactions of indirect fermentation.

Four groups of criteria for distinguishing direct and indirect fermentation mechanisms may be distinguished:

(*1*) *Fermentation of compound sugar despite given or induced absence of oligase.* It is immediately evident from perusal of reaction sequence (7) that accomplishment of a fermentation in the absence of *h* refutes the theory of indirect fermentation, and hence by implication establishes direct fermentation as defined. Unfortunately, proof that *h* is in fact absent is not always easy *in vivo*. To overcome this difficulty, special devices described in the succeeding section (page 97) have been proposed.

(*2*) *Isolation of a specific intermediary.* Referring to (7), it is clear that isolation from a disaccharide fermentation reaction mixture of a substance of type y, *i. e.*, of a substance which has no place in the indirect fermentation path ($h + a' + b$) would establish direct fermentation. A positive result in this test would indeed be decisive, but a negative result, *i. e.*, failure to isolate a compound of type y, cannot be considered as proof that there is no such intermediary and hence as proof that there is no direct fermentation ($a + b$).

(*3*) *Fermentation of compound sugar at a velocity greater than the rate of fermentation of its monose constituents.* Considerable and, to some extent mistaken, importance has been attached to this criterion by some workers in the field of oligosaccharide fermentation. It may be seen from reaction sequence (7) that, in indirect fermentation, reaction *h* supplies the substrate of reaction *a'*. It is reasonable to postulate that *a'* cannot,

in the given condition, proceed more rapidly than h as long as the latter reaction supplies the substrate of a', and thus that the entire reaction chain cannot proceed with a velocity greater than link h. It would therefore seem *prima facie* that conversion of s to e at a rate greater than the conversion of s′ to e or than the reaction h itself must mean that a short cut between s and e which dispenses with steps h and a' must exist, *i. e.*, a must exist or, in other words, direct fermentation must take place.

In application, the criterion just outlined has been notoriously treacherous. One of the main sources of error has derived from disregard for the factor of the purity of the substrates employed. Thus, an impressive if inacceptable case of a direct fermentation of cellulose was seemingly established through the finding that cellulose-degrading bacteria may be unable to utilize glucose as a source of carbohydrate although they grow on cellulose. It took nearly a generation to show that the claimed unsuitability of glucose for growth was due, not to an absence of a glucose-fermenting enzyme in the bacteria, but to the presence in glucose solutions of a toxic substance which is formed from glucose during autoclaving (105). Again Gottschalk (26) claimed the existence of a direct fermentation mechanism for glycogen in yeast on the basis of the finding that preparations of *Saccharomyces Ludwigii* ferment glycogen more rapidly than maltose or glucose. Leibowitz (66), however, was able to show that the alleged superior fermentability of glycogen disappears when the polysaccharide is purified and obtained free of dextrins. Numerous investigators (55, 75, 77, 96, 110) have also reported that some yeasts are able to oxidize or utilize maltose even though they are unable under the same conditions to utilize or oxidize glucose. Kluyver and collaborators and others have shown, however, that the alleged superiority of maltose as an oxidation substrate vanishes when traces of foreign activator substances, shown to occur in commercial preparations of "pure maltose," are removed (46, 56, 117).

A second group of errors encountered in the application of criterion (*3*) derives from the circumstance that a substrate being formed in *statu nascendi* may be different in chemical or physical state from the equivalent substance in its definitive form. These differences may express themselves in different fermentation velocities, and thus lead to a mistaken impression that is necessarily, under conditions of the experiment, fermented more rapidly than s′. The case of fructose, which is fructofuranose when liberated from sucrose but is soon converted, at least predominantly, into fructopyranose, is an important illustration of this type of difficulty.

Finally, errors may arise in the application of criterion (*3*) from the fact that a given substrate, though itself fermentable, can nevertheless function as an inhibitor of fermentation, and even as an inhibitor of its own fermentation (72, 101). Velocity ratios of fermentation, established by comparing the fermentation rates of two selected sugars in a given, arbitrarily chosen concentration, cannot therefore be generalized for all concentration relations. The fermentation rate found for s′ in a control experiment

using an arbitrarily selected concentration of s′ need not then necessarily be as high as the rate which is actually achieved by s′ when the latter is being continually formed and removed, *i. e.*, as when it appears in a low "steady-state" concentration as an intermediary of a reaction sequence. Cases of this kind are encountered, for example, in comparisons of the rates of fermentation of maltose and glucose (72). Reverse types of effects, too, can be encountered. Thus, certain substrates become accessible to enzyme attack only when present above a threshold concentration. Thresholds of this kind have been observed in the action of amylase on starch (17). The existence of an analogous threshold for maltase in living yeast is also suggested by the shape of the substrate concentration–activity curve obtained in the fermentation of methyl α-glucoside by brewers' yeast (70). Fortunately, difficulties of this latter type can be eliminated by suitable control tests and therefore need not interfere seriously with the application of criterion (*3*).

(*4*) *Specific accelerators and inhibitors.* Specific, in the sense of this criterion means, with reference to direct fermentation, action on the sequence $a + b$ without similar action on h or on the sequence $a' + b$. With reference to indirect fermentation, it means action on h without similar action on the sequence $a + b$. It is clear that discovery of a substance which meets the requirements of a specific inhibitor or accelerator for step a constitutes proof for the existence of direct fermentation. With an ideal selective inhibitor of indirect fermentation, *i. e.*, a total inhibitor of h or of a' as distinct from a, moreover, the criterion becomes qualitative and can merge with criterion (*1*).

As may be seen, the various types of criteria for differentiating the different mechanisms of oligosaccharide fermentation are subject to a number of serious pitfalls. Great care must therefore be taken in applying the criteria to eliminate all conceivable sources of error by suitable control experiments. It is also wise to base conclusions on the results of several types rather than on one type of test. Finally, it is necessary in interpreting results to give due weight to possible interference by factors such as permeability dependent on the specific organization of the living cell. (For a discussion of the role of cell organization, see pages 119–124).

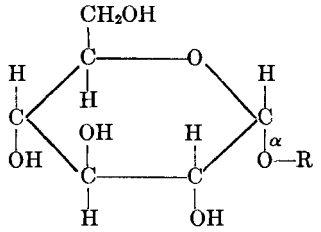
VI. Inadequacy of the Hydrolase–Zymase Mechanism

1. Maltose

In reviewing earlier phases (4) of the development of the chemistry of maltose fermentation, it was made evident that the finding of maltase in-

dicators which could be applied to the living fermenting cell formed a necessary condition of further progress in this field. An attempt to meet this requirement has been made by the present authors (70–72). Two devices have been proposed with the aid of which maltase is made accessible to measurement *in vivo*. One is the fermentation rate of methyl α-glucoside. The other is the effect of glucose on the speed of onset of maltose fermentation.

The use of methyl α-glucoside as a maltase indicator is based on the proof that maltose and methyl α-glucoside are hydrolyzed in conformity with their analogous structure and configuration by one and the same yeast hydrolase—α-glucosidase (121). This view is consistent with all theories of carbohydrase specificity which today hold the field, and is not contradicted by any known fact (39, 40, 69, 74, 114, 115).



(R represents glucose in maltose and the methyl group in methyl α-glucoside)

Methyl α-glucoside is fermented by intact cells of common yeasts, which are known to contain maltase. This proves that methyl α-glucoside can penetrate into the living cell. Owing to the presence of an agluconic component in the molecular structure of methyl α-glucoside, it is extremely improbable that this substrate is fermented except after previous hydrolysis to free glucose. In such conditions, the fermentation rate of methyl α-glucoside cannot exceed the rate of its hydrolysis. Absence of methyl α-glucoside fermentation where zymase is actively present must therefore be attributed to absence or inactivity of α-glucosidase. It follows for any yeast of adequate fermenting power toward glucose that the rate with which it ferments methyl α-glucoside is a relative measure of the maltase activity manifested in the living and actually fermenting cell. If, therefore, maltose is fermented and methyl α-glucoside is not fermented, the fermentation of maltose under the prescribed conditions cannot be assumed to proceed *via* formation of glucose through the action of α-glucosidase.

Brewers' yeast ferments maltose, methyl α-glucoside and glucose at 35° C., and maltose and glucose but not methyl α-glucoside at 4° C.

Brewers' yeast also ferments all three of these substrates at neutral *p*H, and ferments glucose and maltose but not methyl α-glucoside at *p*H 4.5. The fermentation with methyl α-glucoside can be explained as follows: Maltase is contained by the cells, but its activity *in vivo* is limited, as *in vitro*, to *p*H values near neutrality, being completely suppressed below *p*H 5.0; maltase activity *in vivo* is sharply dependent on temperature, being manifest at 35° C. but altogether suppressed at 4° C. Comparison of the *p*H–activity curve of maltase *in vitro* with that of the *p*H–fermentation curve of methyl α-glucoside *in vivo* shows that the two are practically identical. Thus, there is established for the first time a fact first claimed, but never demonstrated, by Willstätter—that the *p*H dependence of maltase *in vivo* and *in vitro* is the same. The fermentation of maltose at acid *p*H or low temperature, in which methyl α-glucoside remains unattacked, must obviously be attributed to a system which is qualitatively insusceptible to factors which determine maltase action. At neutral *p*H and 35° C., furthermore, an important quantitative difference between fermentation of maltase and glucose, on the one hand, and fermentation of methyl α-glucoside, on the other, is demonstrable: The fermentation of methyl α-glucoside under these conditions is found to show a marked dependence on substrate concentration in a range within which the fermentation rates of maltose and glucose are independent of the substrate concentration. Thus, at a low concentration of methyl α-glucoside (2%), its fermentation rate becomes almost negligible, yet maltose in equivalent concentration ferments with undiminished vigor. It seems necessary, therefore, to assume for brewers' yeast a mechanism which ferments maltose without the participation of maltase. This mechanism alone is responsible for maltose fermentation at acid *p*H and at low temperature. On the other hand, at 37° C. and at neutral *p*H, both this and also the indirect maltase–zymase mechanism of fermentation may be assumed to come into play.

In numerous instances, maltose fermentation by brewers' yeast has been found to proceed more rapidly than the parallel fermentation of an equivalent amount of glucose (102, 109, 130). There has been a tendency among supporters of the direct fermentation theory of Willstätter to interpret this finding as striking proof that the fermentation of maltose does not proceed *via* glucose, *i. e.*, it is direct. Some pitfalls of this criterion have been pointed out in a preceding section (see also reference 72).

In fermentation by bakers' yeast, the inadequacy of the maltase–zymase mechanism as an explanation of the experimentally observed facts is particularly evident. The *p*H–activity curve of maltose fermentation by this type of yeast is distinct from that of glucose fermentation, and is actually opposite in trend from that of maltose hydrolysis by either cell-bound or

cell-free maltase (70, 72, 119, 120). Maltose is most vigorous'y fermented by bakers' yeast at acid *p*H values which altogether preclude hydrolysis of maltose by maltase; and maltose is not fermented at all by bakers' yeast in adequately buffered media at neutral *p*H in the range which is optimal for maltase activity (23, 24, 99, 102, 106). The conclusion follows that maltose fermentation by bakers' yeast is in no way related to the presence of active maltase. In accordance with this result is the finding that this yeast fails in intact condition to ferment methyl α-glucoside (72).

The conclusions, reached by the use of a specific maltase inhibitor (acidity) in conjunction with an *in vivo* maltase indicator (fermentability of methyl α-glucoside), are confirmed by the results obtained by the use of a specific activator of maltose fermentation, an activator which serves at the same time as an indicator *in vivo* of the maltase activity. Such a substance, strangely, is glucose itself (70, 99). The zymohexoses but not the nonfermenting galactose all stimulate maltose fermentation by bakers' yeast (72). In a wide range of *p*H and substrate concentration, the presence of minute amounts of glucose brings about a marked activation of maltolysis. Stored bakers' yeast is a particularly poor maltose fermenter (34). In this case, the effect of glucose is particularly pronounced. Factor M for accelerating fermentation and another induction-abolishing factor for maltose fermentation discovered by Blish and Sandstedt (6) are almost certainly glucose and glucose-producing maltase (70). The activation of maltolysis by glucose takes the form mainly of a marked curtailment, but never of the complete abolition, of the time period elapsing between the first contact of the cells with maltose and the time at which the rate of gas evolution becomes maximal. This "induction period" is most prolonged in maltose fermentation and is relatively very brief in hexose fermentation by bakers' yeast. Glucose itself should be an intermediary of maltolysis if this process proceeds through hydrolysis of maltose, *i.e.*, if the mechanism of maltolysis is indirect. In this case, addition of a small amount of glucose from outside to the maltose solution could hardly be expected to bring about any important change in the fermentation course of maltose, since maltase itself in indirect fermentation must be supposed rapidly to liberate a comparable amount of glucose from maltose in any case. Furthermore, it is well known that glucose is an inhibitor, and is certainly not an activator, of yeast maltase. The stimulation of maltose fermentation by added glucose cannot therefore occur through an effect on step *h* of reaction sequence (7). The activation of maltolysis by glucose must mean therefore that normally glucose is not an intermediary of the path of maltolysis by bakers' yeast.

Further support for the above conclusion is provided by the following:

Clearly the induction period preceding onset of gas evolution in pure maltose fermentation can persist only as long as cell maltase in the period of the induction fails to liberate glucose from maltose in a quantity sufficient to cause termination of the induction. The length of the induction period thus furnishes a *maximum* value for possible present maltase activity. Calculations of the maximum value of maltase on this basis have given a figure far below that necessary in the indirect fermentation theory to account for the observed rate of maltose fermentation (70).

Evidence has been furnished that the effect of glucose on maltolysis is not mediated by any induced increase in the permeability of the yeast cell membrane (72). It could be shown, too, that the effect of glucose on maltose fermentation is also obtained when glucose is added to purified maltose. Maltose was purified for this purpose by an elaborate cycle of crystallizations through its derivatives, involving acetylation and deacetylation and nitration and denitration (65).

The effect of glucose on maltolysis thus provides an important corroboration for the view earlier suggested by the results of the use of methyl α-glucoside as an indicator *in vivo* of maltase. But whereas the earlier proof for direct maltolysis rests on the auxiliary assumption that yeast maltase and yeast α-glucosidase are identical, the proofs for direct maltolysis deriving from the effects on maltose fermentation of glucose addition are independent of this auxiliary assumption

A further proof for the inadequacy of the indirect theory of maltose fermentation in application to bakers' yeast derives from the use of methyl α-glucoside, this time not as an *in vivo* indicator of maltase but as a specific inhibitor of the maltolytic mechanism (72, 121). Methyl α-glucoside, in distinction to any of its components (methanol, glucose) or of its isomer methyl β-glucoside, retards the fermentation of commercial "pure," as well as of carefully purified, maltose by stored bakers' yeast, yet is without effect on the fermentation of glucose under the same conditions. The inhibition is induced on previously stored yeast but not on yeast fresh from recent contact with maltose. Since bakers' yeast, in contrast to brewers' yeast, fails to ferment methyl α-glucoside itself at any pH, explanation of the effect of methyl α-glucoside as one of competition with maltase for maltose is out of the question. The effect of methyl α-glucoside can only be explained, therefore, if a specific maltolysis mechanism distinct from maltase–zymase is postulated.

The remarkable effect of oxygen on maltose fermentation by certain yeasts requires consideration here. Kluyver and Custers (57) and Schultz *et al.* (100) have shown that the presence of oxygen, a gas which inhib-

its glucose fermentation, markedly activates maltose fermentation by bakers' and other yeasts. Evidence has elsewhere been advanced that the rate of maltose penetration into the living yeast cell is greater than the fermentation rate of this sugar (72, 101, 103). Hence the action of oxygen cannot be explained as one on permeability (72). Oxygen, moreover, is without demonstrable activating effect on maltose hydrolysis by maltase acting *in vitro*. Its effect *in vivo* cannot plausibly be ascribed without supporting evidence to an activation of maltase. The view that oxygen acts on a direct fermentation mechanism seems indeed to be the most likely alternative.

Some doubt has been cast recently on the view that the products of maltolysis are quantitatively and qualitatively identical with those of glucose fermentation. Guillemet and Leroux (30) found the balance sheet of maltose fermentation by "Fala" yeast to show considerable deviation from

TABLE I

FACTORS IN FERMENTATION OF GLUCOSE AND MALTOSE

Factor	Effect on fermentation of	
	Glucose	Maltose
Methyl α-glucoside	None	Inhibition
Zymohexose	.....	Activation
Oxygen	Inhibition	Activation
*p*H 4.5	Optimum *p*H	Optimum range
*p*H 7.0	Optimum range	Complete inhibition

that of glucose fermentation in the same conditions. Guillemet (29) reports the appearance of a small amount of an unidentified polysaccharide in the fermentation medium when maltose is fermented by this yeast, but finds no such polysaccharide when glucose is fermented. It remains to be shown that this substance was not in fact preformed in the original maltose sample employed, nor due, in formation, to the presence in the maltose sample of specific activators. Provided these objections can be met, the results of Guillemet form definite new support for the view that maltose fermentation proceeds by a path distinct from that of glucose.

A summary (72) of the differential effect of several agents on glucolysis and maltolysis, respectively, by bakers' yeast is given in Table I. None of the agents listed affects maltose hydrolysis in the same direction as they affect maltose desmolysis. In the case of *p*H, the effects on maltolysis and glucolysis are even directly opposed. All these results, therefore, are

clearly opposed to the Fischer view of maltase activity as a necessary condition of maltose fermentation by yeast.

2. Lactose

Lactose (β-1-galactopyranosido-4-glucopyranose) is structurally identical and configurationally isomeric with maltose (α-1-glucopyranosido-4-glucopyranose). This chemical relation is reflected in the generally accepted conception of lactose fermentation by "lactose yeast"—a group which does not attack maltose—as an analogue of maltose fermentation by the common industrial yeasts, which in turn do not act on lactose.

Significant facts regarding lactose fermentation were presented by Willstätter and Oppenheimer (123) in an investigation of *Saccharomyces fragilis* and several other yeasts. The superior fermentation rate frequently found when lactose, rather than monose constituents of this sugar, served as the fermentation carbohydrate, and the low lactase values found in extracts of the same yeast samples, led Willstätter and Oppenheimer to suppose for lactose fermentation a mechanism, "lactozymase," distinct from, but analogous to, the mechanism, "maltozymase," of direct maltose fermentation. Their kinetic findings have since been extended and confirmed for the case of a yeast strain isolated from Palestine "leben" (41), as well as for *Saccharomyces fragilis* and *S. cremoris* (85). It has been shown by Hestrin (41) that the superior fermentability of lactose, as compared with the monose constituents of lactose, is expressed in the relative brevity of the induction period preceding onset of gas evolution in lactose solutions as well as in the maximum fermentation rate finally attained. The results depend, however, on the past history of the yeast material. When cells of the Palestinian strain were grown on lactose medium and then brought into contact with fresh lactose solution, on the one hand, and with solutions of galactose and glucose, on the other, lactose was found to be fermented at a vigorous rate long before fermentation of the hexoses had even begun; and the final rate of fermentation obtained on lactose was distinctly greater that that obtained in solutions of the hexoses. However, previous cultivation of the yeast on glucose modifies the findings decisively, and yeasts so grown are found adapted to glucose, so that they ferment this sugar, as well as galactose, more rapidly than they do lactose.

The superior fermentation rate of lactose, in comparison with glucose and galactose, suggests *prima facie* that the monoses mentioned, the constituents of lactose, are not intermediary to the latter's fermentation. Yet this should not be considered in itself as decisive proof. The possible objection that lactose is only fermented more rapidly because it contains con-

taminants which act as activators and which are absent from preparations of hexose is without experimental support, yet conceivably the following could be argued: Let it be assumed that hexose in high concentration retards its own fermentation by "lactose yeast"; then fermentation of a low "steady-state" concentration of hexose, such as would be established through hydrolysis of lactose and concurrent fermentation of the formed hexoses, might conceivably proceed at a greater speed than fermentation of hexose in an initially high, arbitrarily selected and continuously declining concentration. Myrbäck and Vasseur (85) have shown, however, that galactose ferments more slowly at low than at high concentration. Moreover, they found lactose to ferment steadily at the same superior rate until almost exhausted, whereas glucose and galactose fermented initially more slowly and at a rate which declined as the concentration of hexose progressively diminished. It is therefore difficult to accept the lower fermentation rate of the hexoses compared with lactose as due to an autoinhibition of fermentation by hexose itself. The proof of direct lactose fermentation based on kinetic data of the type mentioned therefore stands.

Further evidence favorable to the theory of direct lactose fermentation may be derived from data of Kluyver and Custers (57), although they have not themselves so interpreted their findings. These investigators observed that the cells of the yeast *Blastodendrium intermedium* utilize, although they fail to respire, lactose, and that both utilize and respire glucose. The absence of respiration in lactose solutions can only mean that glucose, a respiration substrate, is not formed from lactose under the conditions of this experiment. Confirmation that respiration would have been observed had glucose been formed is provided by the fact that the same yeast cells, after cultivation on a lactose medium and accompanying induction of lactase production, acquire the ability to hydrolyze lactose and at the same time begin to respire when suspended in a solution of lactose. Respiratory activity in lactose solutions thus serves with *intermedium* as an indicator of lactase activity *in vivo* and *in situ*, even as the fermentation of methyl α-glucoside serves as a criterion of maltase activity *in vivo*. The use of respiration as an indicator of lactase presents, however, the advantageous feature that it is independent of any auxiliary assumptions concerning the specificity of lactase.

3. *Sucrose*

The experimental material with regard to the manner of fermentation of the important nonreducing natural disaccharide, sucrose, is less clear-cut. The case presented by Willstätter and coworkers (122) for the view

that sucrose fermentation can be "direct" was not considered decisive even by its authors.

It is generally known that the fermentation of sucrose by common industrial yeasts is decidedly slower in rate than is the inversion of sucrose which the same yeasts induce. The fermentation of sucrose in such conditions must be predominantly indirect. Actually it is extremely difficult in view of the great preponderance of sucrase in many yeasts to realize experimental conditions in which the hydrolysis rate can be expected to set the pace of the sucrose fermentation (23). Inhibitors of sucrase such as methyl α-glucoside have frequently failed in our hands to inhibit the rate at which sucrose is fermented. Superficially considered, this finding might seem to point to direct fermentation. Actually, the result is fully explained in the indirect fermentation theory, since even the residual activity left to cell sucrase in the presence of methyl α-glucoside can wholly explain a production of invert sugar sufficient to account for the fermentation rate observed (68). Use of an inhibitor in an amount which does not suppress sucrase activity completely or to a very large degree thus cannot settle the crux of the question: not whether indirect fermentation is predominant, since this is immediately obvious, but rather whether direct fermentation exists side by side with, or as an alternative to, indirect fermentation.

The use of raffinose fermentation as an indicator of sucrase activity in the living cell offers advantages for testing the theory of direct sucrose fermentation analogous to those expounded with reference to the use of methyl α-glucoside as a criterion of maltase activity in tests of the maltolysis mechanism. According to Kuhn (63) and Josephson (51), sucrase and raffinase are identical, sucrose being hydrolyzed by the enzyme about sixteen times as fast as is raffinose. Because of this circumstance, the fermentation of raffinose by yeast, in contrast to that of sucrose, can frequently be found to be strictly governed by the sucrase concentration and activity. In this circumstance, methyl α-glucoside in its function as a sucrase inhibitor was found to inhibit the rate of raffinose fermentation even though it was without effect on the fermentation rate of sucrose (68). These findings are not unexpected and they confirm what was likely *a priori*, that raffinose fermentation is essentially indirect, and that sucrase activity can consequently condition the rate of fermentation of the trisaccharide. They suggest also that the inhibitor susceptibility of sucrase *in vivo* resembles that noted for the enzyme *in vitro*. In agreement with this conclusion is the finding that the *p*H–activity curve of raffinose fermentation by bakers' yeast conforms to that of sucrase, as established directly from inversion either *in vivo* or *in vitro* (65, 87). Hence, in the absence of interfering meli-

biase, fermentation of raffinose constitutes a long-sought-for weapon, a means for measuring sucrase in the living cell and under the conditions of fermentation itself—such a means, moreover, as can be effectively applied even if the sucrase content of the cells is not in excess of the zymase content (41).

The use of raffinose fermentation as a test of sucrase activity has not yet been fully explored, but some results which are not without theoretical interest can be reported.

The *p*H–activity curve of raffinose fermentation by bakers' yeast conforms closely, as has already been mentioned, to the *p*H–activity curve of sucrase *in vitro*, as measured either on sucrose or on raffinose. The *p*H–activity curve of sucrose fermentation, on the other hand, diverges markedly from the raffinose fermentation curve. In a range extending from at least *p*H 7.3 to 2.2, sucrose fermentation is found to be practically independent of *p*H; but at *p*H 8.0 sucrose is still fermented, whereas raffinose fermentation, like sucrase activity *in vitro*, is practically at an end (65). A marked divergence in favor of sucrose fermentation as against either raffinose fermentation or sucrase activity *in vitro* occurs also at *p*H 2.2, at the acid end of the scale (41). The sucrose fermentation observed at *p*H values which already depress raffinose fermentation might perhaps be ascribed to the persistence in the yeast of a small fraction of the original overwhelming sucrase supply. Complete exclusion of sucrase with persistent sucrose fermentation is desirable for definite evidence in support of direct fermentation.

Sought-for conditions can be obtained with acid treatment of bakers' yeast. Following exposure of living bakers' yeast to 0.25 *N* sulfuric acid for a suitable period of time, qualitative separation of sucrose fermentation from raffinose fermentation has been accomplished (41). At *p*H 2.2 such acid-pretreated yeast cells ferment sucrose but not raffinose within a prolonged time interval. Such yeasts appear to lack active raffinase completely, yet they ferment sucrose. Taking the generally held view that sucrase and raffinase are in fact identical, these findings must mean that sucrose is fermentable by a mechanism which is independent of sucrase. A criticism of this conclusion is that it depends on the validity of the accepted view of the nature of sucrase specificity. Certain experimental findings are, however, difficult to fit into the accepted theories of this carbohydrase's specificity (65, 68, 87).

Independent evidence for the possibility that the metabolic paths of sucrose need not necessarily lead to invert sugar derives from work with plant tissue cultures. White (116) has shown that excised tomato roots

thrive better on a medium containing sucrose than on a medium containing any simpler sugar. The superiority of sucrose was not due to the presence in the sucrose samples of contaminating activator substances, nor to the different osmotic pressure of sucrose medium.

Positive evidence that sucrose can be broken down by mechanisms other than hydrolysis to invert sugar derives from work on bacteria. This evidence will be discussed in a later section.

4. Trehalose

The nonreducing, relatively rare, natural disaccharide, trehalose, has been investigated for fermentability by a number of workers (58, 82–84, 92). Trehalose is a constituent of yeast (111) and appears to fulfill in many fungi the role reserved for sucrose in plants. A trehalose monophosphate is known to occur in yeast extracts as a by-product of glucose fermentation (97).

The fact that trehalose is fermented at a greater rate than is glucose by some aged, dry yeast preparations and in particular by cozymase-poor preparations raised the suggestion that trehalose fermentation by a direct mechanism exists (82). However, Myrbäck, in his work on trehalose fermentation, has drawn attention to the inconclusive nature of his proofs for direct trehalose fermentation (83, 84) by yeasts. A convincing observation has been made in fresh cultures of *Fusarium lini* Bolley, which grow better on trehalose than on glucose (92).

In summing up the above discussion, it may be stated that a strong case has been built up for the view that maltose can be fermented by a mechanism which is independent of oligase, that similar proofs apply with reference to lactose fermentation, and that there is evidence, albeit not conclusive, that sucrose and trehalose, too, can be fermented by yeast by nonhydrolytic mechanisms.

VII. Recent Hypotheses on the Nature of Oligosaccharide Fermentation

The term "direct fermentation" as inherited from Willstätter should be assumed to be purely negative in context: a denial of the view that disaccharides ferment only after hydrolysis. Although the term "direct" was selected, it was not used in practice in the sense of a *necessary* assertion that cleavage of the carbon chain is the first step in the sequence of changes of disaccharide fermentation. Kluyver and Custers (57) believe that, in the "direct" fermentation of a disaccharide, intermediate compounds with a number of C atoms, between 7 and 11, and still containing the oxygen

bridge of the disaccharide, must be formed. This definition is arbitrary, however, as it narrows the use of the concept "direct" beyond the limits in which it was actually used. Positive hypotheses concerning the nature of the first step in the desmolysis of disaccharide molecules could not, in fact, be usefully formulated before the first step in hexose fermentation had become in any way known.

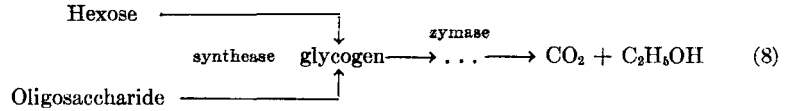
As was pointed out earlier (page 88), all the intermediary products of fermentation recognized until less than a decade ago belonged to fairly late fermentation steps, and seemed to be independent of the nature of the original substrate. Thus, the point of mergence of the fermentation paths of different sugars—monoses, oligosaccharides, polysaccharides, respectively—remained obscure. In the sequel, however, fundamental reactions which define the very earliest steps in sugar fermentation were discovered. They form the basis of the several positive hypotheses of disaccharide cleavage discussed below.

1. Role of Glycogen

The endowment of glycogen with a specific role in the fermentation of simple sugars recurs with strange persistency in the history of fermentation chemistry. Cremer (13*a*) first noticed formation of glycogen in press juice of yeast forty-six years ago. Nine years later, Kohl (59) enunciated clearly, but on purely speculative grounds, the theory of the formation of glycogen as a primary intermediary of hexose fermentation. Pringsheim (95) presented the same hypothesis in a modified form when he reached the conclusion, on the basis of chemical investigations on the nature of glycogen and blood sugar, that there is a close relation between glycogen structure and fermentability. The discovery (80) that an isolated "glycolytic enzyme" of muscle acts on glycogen, starch and some related polysaccharides ("hexosans") but not on the common fermentable sugars (hexoses and oligosaccharides) except in the presence of a factor, hexokinase, present in yeast extracts, was in good conformity with Pringsheim's view. The central position of glycogen in the network of the sugar transformations which characterize the carbohydrate metabolism of the higher organism was evident from the long-known fact that all the metabolized sugars are stored by the liver as glycogen. The assignation to glycogen of the role of an intermediary in alcoholic fermentation was thus but a further reassertion of the widely suspected essential similarity of the carbohydrate metabolisms of yeast and animal cells. Yet the experimental basis for the view that glycogen was an intermediary of hexose fermentation remained scant; in consequence, after enjoying a brief vogue, the theory was once more

largely forgotten. However, it gained new impetus, in 1937, in the hands of Willstätter and Rohdewald (124), who had found that, shortly after the contact of solutions of fermentable sugars with living yeast and previous to any evolution of carbon dioxide, free sugar disappears from yeast suspensions and is recoverable from the cells in theoretical or near-theoretical yield as glycogen. This synthetic step was demonstrated both on glucose and on maltose. It was apparently a starting point of the reaction chain which leads through the known intermediaries to the end products of fermentation. Their findings were summed up by Willstätter and Rohdewald in a startling statement, strangely reminiscent of the old formulation of Kohl: "Unsere Zucker sind nicht direkt gärbar. Die erste Phase der Gärungen ist Glycogensynthese."

In the view proposed by Willstätter and Rohdewald, therefore, glycogen is an essential intermediary of sugar fermentation. It was supposed to be formed both from simple sugars and from oligosaccharides during fermentation by the action of specific "syntheases." In the theory as formulated, the old view that disaccharides must first be reduced to hexoses before desmolysis sets in is clearly unnecessary, the first step in fermentation being conceived not as a degradation but as a polymeric synthesis. This synthetic step would be as feasible from disaccharides as from monoses, and would abolish any difference between them in regard to subsequent fermentation steps. Since formation of glycogen even from nonglucose sugars in normal carbohydrate metabolism in the body is a well-established fact, the mechanism of fermentation proposed by Willstätter and Rohdewald could be extended with suitable refinement to include not only fermentation of glucose, fructose and maltose, but also that of sucrose, trehalose and lactose. It is thus possible to summarize the main elements of the theory of fermentation of Willstätter and Rohdewald simply as follows:



(8)

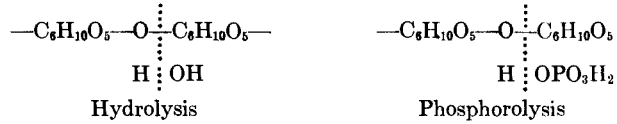
The view advanced by Willstätter and Rohdewald encountered strong opposition. In several laboratories, investigators were unable to demonstrate an accumulation of glycogen in the early phase of desmolysis by yeast (25, 62, 81). Willstätter and Rohdewald (125), on the other hand, were able to substantiate their claim by adding evidence for the early formation of glycogen also in glycolysis by muscle brei. Leibowitz and Kupermintz (73) further demonstrated an early production of polysaccharide in the fermentation of glucose by *Escherichia coli*. It seems, therefore, that glycogen accumulation is a frequent, though not an inevitable, accompaniment of sugar fermentation by *intact cells*.

It is important in the present context to note that the theory of Willstätter and Rohdewald did not in any sense explain the fermentation of higher sugars without hydrolysis. Essentially, it only shifted the difficulty of the problem from the case of oligosaccharides to that of the postulated polysaccharidic intermediary. The answer to the question of the way in which glycogen is transformed into the end products of hexose fermentation without preliminary conversion into hexose by hydrolysis was not even attempted by Willstätter.

2. Phosphorolysis

The discovery of a reaction whereby glucosidic linkages can be broken down by direct interaction with phosphoric acid through the medium of a specific enzyme called phosphorylase introduces a different concept into the fermentation theory of higher sugars. Indeed, it seems probable at the present moment that the history of our knowledge of the fermentation of compound sugars will crystallize into two distinct phases, with the discovery of phosphorylase as their common boundary.

Progress was made by the isolation of glucose-1-phosphate from glycolyzing extracts by Cori and Cori in 1936 (12). In the same year, the theory of the phosphorolytic cleavage of glucosidic linkages was clearly formulated by Parnas (94). In the proposed phosphorolytic fission of the glucosidic linkage, phosphoric acid takes functionally the former role of water, the ions, H^+ and $OPO_3H_2^-$, corresponding in phosphorolysis to the water constituents, H^+ and OH^-, in hydrolysis:



Hydrolysis Phosphorolysis

But, whereas in hydrolysis the result of the reaction is the formation of two free sugar molecules, in phosphorolysis only one of the two sugar components is liberated as free sugar while the other appears as a sugar monophosphate. In the context of the present discussion the significant fact stands out clearly: that in phosphorolysis a long-sought-for biological mechanism for fission of the molecular chain of a compound sugar without entry of water is finally found; the phosphorolytic reaction gives us a definite picture as to how fermentable monose derivatives can be obtained from an oligo- or polysaccharide without the intervention of an hydrolytic agent.

The complex of interactions connecting sugar fermentation and glycogen is sharply illuminated by the new theory. The enzyme, phosphorylase,

which acts phosphorolytically on both glycogen and starch, has been isolated from a wide variety of biological sources and seems to be ubiquitous wherever these polysaccharides are naturally desmolyzed (9). Its action is reversible, leading to an equilibrium:

$$(C_6H_{10}O_5)_n + (n)H_3PO_4 \rightleftharpoons (n)C_6H_{11}O_5{-}OPO_3H_2 \qquad (9)$$

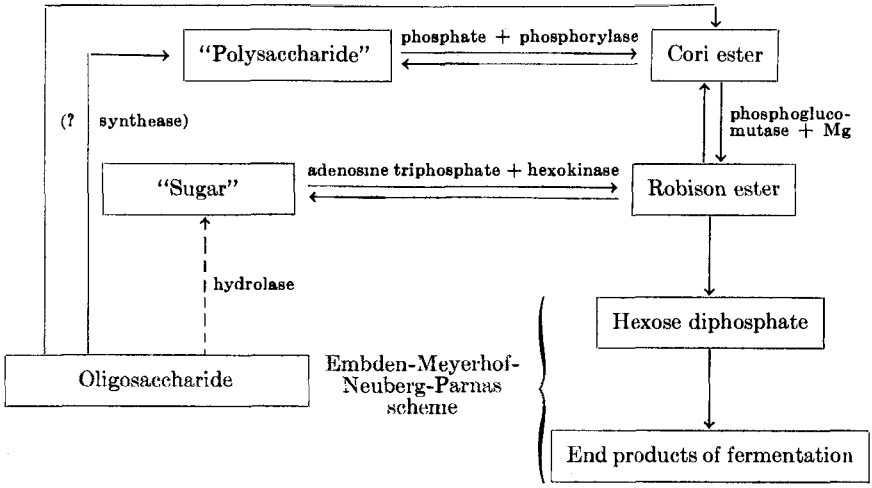
Glycogen or starch — Glucose-1-phosphate (Cori ester)

The Cori ester product of the above equilibrium further links up with Robison ester through the following reversible reaction:

$$\text{Glucose-1-phosphate} \underset{\text{phosphoglucomutase}}{\overset{\text{Mg}}{\rightleftharpoons}} \text{Glucose-6-phosphate} \qquad (10)$$

(Cori ester) (Robison ester)

But the Robison ester is directly derivable from zymohexoses through *phosphorylation* (as distinct from *phosphorolysis*), brought about by the action of adenosine triphosphate in the presence of a specific enzyme, hexokinase. Hence, in summing up (8, 10, 31, 32, 53, 54, 98), we obtain Scheme 1.



SCHEME 1

It is clear that, in the presence of the necessary enzymes and coenzymes and given suitable relative concentrations of catalysts and reactants, the production of glycogen in living cells as a transient by-product of sugar fermentation is bound to occur. Thus, direct polymerization of sugar to glycogen by the intervention of a specific synthease, as supposed by Will-

stätter and Rohdewald, becomes unnecessary for the explanation of glycogen production. The factor, hexokinase, in whose absence muscle enzyme preparations lack a capacity for glycolyzing sugars (80), is now easily identified as an enzyme which transfers phosphate from adenosine triphosphate to hexose. Moreover, the glycogen-producing system of the intact yeast or muscle cell is not a specific individual "synthease," but the sum of hexokinase and phosphoglucomutase and phosphorylase.

In Scheme 1, "sugar" means primarily, of course, zymohexose, and "polysaccharide" means glycogen (or starch). The problem of the direct fermentation of maltose, lactose, sucrose, etc., reduces itself to the question of whether the oligosaccharides can be placed within the frame of this scheme, and if so, whether their place is within the rectangle of "sugar" or in that of "polysaccharide." If the oligosaccharides cannot be placed in either rectangle, they must enter the desmolytic path from outside and link up with the phosphorylating or phosphorolytic mechanisms by some as yet unknown process.

It is necessary now to weigh what direct experimental evidence exists for the hypothesis that disaccharides can be phosphorolyzed as such and thus be fermentable without any preliminary hydrolysis. The mediation by yeast enzymes of a maltose phosphorolysis is an attractive hypothesis, but such a reaction has not been demonstrated. Cori *et al.* (11) found yeast phosphorylase inert toward maltose. That a maltose phosphorolysis may nevertheless exist follows from the claim of Hanes (31) that pea extract slowly phosphorolyzes maltose. The case of lactose has not been investigated; galactose-1-phosphate, the analogue of glucose-1-phosphate, has, however, been shown to be an intermediary of galactose metabolism (60). We also have no positive information concerning a trehalose phosphorolysis. In the case of sucrose, the existence of an exact replica of glycogen phosphorolysis has been demonstrated (15, 16, 35*a*, 52):

$$\text{Sucrose} + H_3PO_4 \rightleftharpoons \text{Cori ester} + \text{fructose}^* \qquad (11)$$

The enzyme mediating this equilibrium has been found in *Pseudomonas saccharophila* Doudoroff and in *Leuconostoc mesenteroides* and has not as yet been detected in yeast. The significant fact is, however, that a way

* Until recently, free fructose was believed to be essentially fructopyranose and hence fundamentally distinct from the fructofuranosidic residue of sucrose. This concept seemed to exclude the possibility of a reversion (lower arrow) such as has been formulated. Isbell and Pigman (48) have, however, brought forward evidence that free fructose is in equilibrium with the pyranose and furanose forms. The discovery of a reversible sucrose phosphorolysis fully substantiates the conclusions of Isbell and Pigman.

has been found in which sucrose can be metabolized without preliminary hydrolysis and yet yield intermediary and end products identical with those found when hexose is fermented. The temptation to attribute analogous mechanisms of breakdown to other disaccharides is strong.

With the discovery of phosphorolysis, an old argument against "direct" fermentation has been answered. As long as only phosphorylation—as distinct from phosphorolysis—was known, it was valid to argue that, in the postulated fermentation of disaccharides without preliminary hydrolysis, a formation of a disaccharide diphosphate (the disaccharidic analogue of the Robison ester) or even of a disaccharide tetraphosphate (the disaccharidic analogue of the Harden–Young ester*) had been implied. The failure to isolate such intermediaries was counted as a proof against direct fermentation (4, 18). But the discovery of the reaction of phosphorolysis whereby a disaccharide can at one step, without intervention of water and with the intervention of only one phosphate molecule, be dissociated into monose monophosphate and free monose removes this objection. It is of interest, nevertheless, that formation of a phosphorylated disaccharide—trehalose monophosphate—has in fact been demonstrated as a normal feature of sugar fermentation by yeast extract (97). Moreover, the formation of trehalose monophosphate is not limited to the case of trehalose fermentation but actually occurs when hexose is the fermentation substrate.

In the newer light of the phosphorolytic fermentation mechanism, the term "direct" as applied to oligosaccharide fermentation stands in need of critical appraisal and, possibly, redefinition. In the Fischer theory, as represented in Scheme 1 by the dotted arrow, the oligosaccharides are undoubtedly less directly connected with the degradation process of desmolysis than hexoses, and the postulated Fischer mechanism of fermentation of oligosaccharides is, therefore, aptly termed "indirect," as compared with the fermentation mechanism of hexoses. In the modern phosphorolytic scheme of fermentation, however, hexose and polysaccharide are, apparently at least, equidistant from the main degradation path, neither category of substrate being an intermediary of the other's fermentation. In an energy sense, moreover, polysaccharide is actually the more directly fermentable, since, while hexose phosphorylation depends on prior accumulation of adenosine triphosphate with the aid of an outside source of energy, polysaccharide phosphorolysis is independent of adenosine triphosphate accumulation and of any external energy-supplying reaction (8, 78). Willstätter and Rohdewald, because they regarded glycogen as a necessary

* The claim (47) that the Harden–Young ester itself is a disaccharide tetraphosphate is completely without support and may be disregarded.

intermediary of sugar fermentation, did not hesitate to reverse the old usage completely, and claimed that only polysaccharide fermentation is direct, hexoses in their paradoxical phrase being "nonfermentable" as such. In this reversal of usage and in the phosphorylative schemes of fermentation, the terms "direct" or "indirect" in reference to disaccharide fermentation lose their old meaning in the sense of a structural description of the status of oligosaccharides in comparison with that of hexose in fermentation. It becomes appropriate indeed to dispense altogether with the term "direct" in the former connotation.

There can remain, however, for the term "direct" the connotation of an energy category. It will perhaps be useful to regard an oligosaccharide fermentation as "direct" when the phosphorolyzing step of this fermentation is found—like that of glycogen in glycolysis—to be independent of any outside energy source; and to reserve the term "indirect" for mechanisms in which phosphorylation depends on an external source of energy, *e. g.*, the classical "oligase + zymase mechanism."

VIII. Specific Catalysts and Inhibitors

A study of the action of specific activators or inhibitors promises to throw new light on the nature of the enzyme systems which mediate fermentations of oligosaccharides. *Specific* in the present context will be regarded as meaning that an agent so described affects disaccharide fermentation but has no bearing of a similar nature on either hexose fermentation or oligase activity. For the present, existing information concerning the mode of action of such agents relates largely to the case of maltose.

In striking contrast to the classical conception of fermentation as *the* anaerobic process inhibitable by oxygen (Pasteur effect), Schultz *et al.* (100) and Kluyver and Custers (57) have found that the onset of fermentation in pure maltose solutions in the presence of bakers' yeast is linked to the availability of oxygen. The suggestion that oxygen is necessary for maltase activity is completely without experimental support. The view that oxygen affects maltose fermentation by increasing the permeability of the cell wall to maltose can be excluded, since the evidence available shows that the rate of penetration of maltose into the cell is not the rate-limiting factor of maltolysis by bakers' yeast (72). It seems necessary to conclude, therefore, that oxygen is a *specific* positive catalyst of the maltolytic process. The precise mechanism of the oxygen effect in maltolysis remains obscure. However, it is noteworthy that many yeasts, and in particular brewers' yeast, ferment maltose also anaerobically. It seems likely, therefore, that the function of oxygen can be performed by some other agent.

The possibility must be borne in mind too that oxygen may be acting, not directly on the main cycle of maltolysis as such, but secondarily on the formation of a catalyst of the maltolytic process.

The accelerating effect of glucose and of other zymohexoses on the onset of maltolysis by bakers' yeast has already been discussed. Galactose, on the other hand, fails to accelerate the fermentation. It is significant that the effect of glucose on maltolysis persists long after glucose itself has disappeared from the solution through the fermentative action of the yeast (72). Therefore, the acceleration cannot be ascribed to the presence of glucose as such but must be attributed to a substance produced from glucose. It has been possible to show that the catalytic action of prior glucose fermentation is not a function of the shift in *p*H which glucose fermentation entails (72). Schultz *et al.* (100) note that in the presence of traces of glucose the effect of oxygen is masked. It is plausible, therefore, that glucose and oxygen are functionally interchangeable. It has been shown that both oxygen and glucose exert a depressant effect on the concentration of phosphate in the yeast cell (49, 79). The possibility that this common stimulative action on maltolysis is mediated through an effect on phosphate therefore deserves investigation.

The inhibitory effect of methyl α-glucoside on the onset of maltolysis in bakers' yeast provides a further weapon for elucidating the catalyst complex of maltose fermentation. The retarding effect of methyl α-glucoside is dependent on prior storage of the yeast, and is not obtained when the methyl α-glucoside is added to yeast which is fresh from a previous maltose fermentation (72). This must mean that methyl α-glucoside acts, like glucose, not on the main maltolytic cycle but on a catalyst-forming side reaction of the main cycle. Yeasts fresh from a maltose fermentation are resistant to methyl α-glucoside inhibition because they already contain a store of the necessary catalyst. In luminescent bacteria, it has been observed that methyl α-glucoside acts as a respiratory inhibitor (50). The possibility that this agent acts on maltolysis by an inhibition of some oxidation process is an hypothesis which needs to be borne in mind.

Prior treatment of bakers' yeast with glucose, in contrast to prior treatment with maltose, fails to abolish the retarding action of methyl α-glucoside on the onset of maltolysis. The action of methyl α-glucoside in inhibiting catalyst production cannot, therefore, be referred to catalysts of maltolysis derived from glucose. Some catalyst derived from maltose must be the agent whose production is affected. That such an agent exists is clearly shown also by the fact that treatment of the yeast with glucose curtails, but does not completely abolish, the induction phase of maltolysis.

The facts of maltose fermentation can be explained only if a complex of specific catalysts is assumed, some of which apparently derive from glucose and some from maltose but not from glucose (72). The failure of maltose fermentation in the purified reconstructed maltase-free zymase system prepared by Winberg and Brandt (127) can be due to the absence from their system of specific enzymes of maltolysis, of specific nonenzymic factors, or even of enzymes needed for the production of the factors necessary to maltolysis.

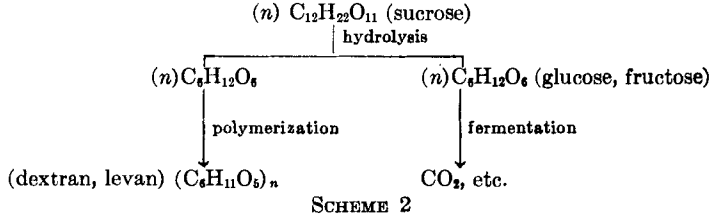
Recent work has shown that traces of primers can determine not only the speed with which a polymeric reaction proceeds, but also in some cases the possibility of the reaction, as well as the nature of the product formed (13, 107). The possibility that oligosaccharide fermentations may involve transient formation of different products, depending on the nature of the catalytic equipment available to the cell, will thus deserve consideration, particularly where polymerization is assumed to occur in the course of the fermentation process. In this connection, also, the amount and nature of activators which accompany substrate preparations as contaminants acquire an added importance.

IX. Polymeric Degradation of Sucrose by Bacteria

Although attention has been rightfully centered in recent years on the role of phosphorolysis in the breakdown of glucosidic linkages, it should not be overlooked that nonhydrolytic cleavages other than phosphorolysis may also play a part in natural fermentations. The form of the fermentation of sucrose by a wide range of microorganisms presents a specific case in that in these fermentations one-half of the disaccharide molecule is not degraded into smaller molecules but, on the contrary, is built up into a larger molecular aggregate, a polysaccharide, while one-half of the substrate molecule ferments to the usual end products. Yet phosphorus, as will be seen, does not enter into the scheme at all.

Two distinct reaction trains proceed in accordance with the principle here outlined. In one reaction, exemplified by the sucrose metabolism of *Leuconostoc mesenteroides*, the end products of fermentation of sucrose are a polysaccharide, dextran, derived from the glucose half of the molecule, and acid and gaseous products which derive from the breakdown of the fructose half of the molecule. In another reaction, exemplified by the sucrose metabolism of *Bacillus subtilis*, the end products of fermentation are a polysaccharide, levan, deriving from the fructose half of the molecule and acid and gaseous products which derive from the glucose half of the molecule (5).

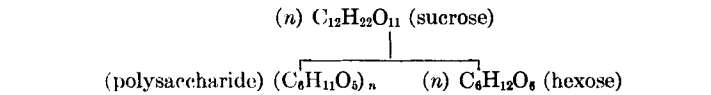
Followers of Fischer must consider the mechanism of these conversions as consisting of a primary hydrolytic inversion, after which the components liberated by hydrolysis pursue different destinies, one—polymerization, and the other—fermentation (Scheme 2). This simple view is, however, in



SCHEME 2

conflict with the fact that cells which produce levan or dextran from sucrose may be completely unable to produce the polysaccharide from either glucose or fructose alone or from their mixture (5, 35, 108).

The polymeric reactions discussed have recently been made accessible to closer investigation by the development of methods whereby the acting system may be separated from the living cell and the reaction chain of polymerization reproduced *in vitro*. A soluble cell-free enzyme which mediates levan production from sucrose has been isolated from *Bacillus subtilis* and *Aerobacter levanicum* by Hestrin *et al.* in this laboratory (2, 3, 44). In accordance with its substrate requirement and end effect, this enzyme is termed levansucrase. On the other hand, cells of *L. mesenteroides* and related organisms have yielded a soluble extract which forms dextran from sucrose (36, 38, 104). In both cases the polymerization of one component of the sucrose molecule is accompanied by accumulation of an equivalent amount of the other component in the form of free hexose, *i. e.*, fructose in dextran production, and glucose in levan production. Disregarding side reactions which may partially mask the main effect, the process may be formulated as in Scheme 3. The substrate specificity of



SCHEME 3

the cell-free enzymes is singularly sharp and fully conforms to that observed earlier in work with the living cells. The polymerizations occur on sucrose but not on glucose, fructose or invert sugar. The findings clearly dispose of the view that polysaccharide production from sucrose depends either on a preceding oligosaccharide hydrolysis with liberation of free

hexose (normal glucose and fructose) or on a parallel desmolytic breakdown of sugar.

It might have been argued in opposition to this conclusion that hydrolysis was involved but that only hexoses in *statu nascendi*, *i. e.*, at the moment of their liberation from sucrose linkage, are susceptible to polymerization. In the case of dextran production, little can be said in favor of this *ad hoc* hypothesis. In the case of levan production, however, this explanation seemed to have fair probability as long as free fructose was generally supposed to be exclusively fructopyranose, different and distinct from the fructofuranosidic residue present in the sucrose molecule. This explanation was further strengthened, moreover, by the fact that raffinose, a substrate which contains fructose in the same furanosidic form as sucrose, proved a suitable substrate for levan production (5, 35, 42). In the meantime, however, Isbell and Pigman (48) have adduced evidence that free fructose in solution is an equilibrium mixture of the pyranose and furanose forms. In the light of this finding, no difference in principle should exist between the behavior of fructofuranose as liberated from sucrose and of fructose in stable solution.

As a further test of the hydrolysis hypothesis, which postulates a substrate specificity for levansucrase depending on the presence of the fructofuranose configuration as a terminal part of the molecule, Hestrin and Avineri-Shapiro (42) tested the following substrates with a terminal fructofuranosidic group for suitability for levan production by cell-free enzyme: fructose-6-phosphate (Neuberg ester), fructose-1,6-diphosphate (Harden–Young ester), methyl γ-fructoside and inulin. None was transformed into levan. Moreover, addition of powerful yeast invertase to the levan-producing system failed to elicit levan production from methyl γ-fructoside although the substrate was then rapidly hydrolyzed (43), and failed to augment the rate of production of levan from either sucrose or raffinose (41*a*). Addition of yeast inulase to levansucrase similarly failed to induce levan production from inulin. Thus, levan production clearly does not proceed from fructose, even if the latter is present or is liberated as fructofuranose. The levan polymerization proceeds, therefore, only on the sucrose grouping which is also present as such in raffinose, while dextran production is strictly limited to the intact sucrose molecule.

The processes of polymerizations from sucrose proceed in accordance with equations (12) and (13):

$$(n)\ \text{glucosido} < > \underset{(\text{sucrose})}{\text{fructoside}} \longrightarrow \underset{(\text{dextran})}{(\text{glucosan})_n} + (n)\ \text{fructose} \qquad (12)$$

$$(n)\ \text{fructosido} < > \underset{(\text{sucrose})}{\text{glucoside}} \longrightarrow \underset{(\text{levan})}{(\text{fructosan})_n} + (n)\ \text{glucose} \qquad (13)$$

The reaction is thus formally analogous to the synthesis of glycogen by phosphorylase:

$$(n)\ \underset{\text{(Cori ester)}}{\text{glucosido} < > \text{phosphate}} \rightleftharpoons \underset{\text{(glycogen)}}{(\text{glucosan})_n} + (n)\ H_3PO_4 \qquad (14)$$

But neither levansucrase (43) nor dextransucrase (37) acts on the Cori ester, and the ordinary phosphorylase does not act on sucrose. Whereas the action of phosphorylase is reversible, no sign of reversion has been detected in the sucrose polymerization (42). It has been possible further to show that phosphate does not participate in levan production (43). While the exact mechanism of the polymeric degradation of sucrose remains obscure, one negative fact emerges clearly: that the process does not involve a primary hydrolysis and therefore may be termed direct.

X. Role of Cell Organization

Since the living cell is of primary interest to students of fermentation, some emphasis has been placed throughout the preceding sections on experiments carried out with living cells. We must now discuss whether, and in what circumstances, fermentation measurements on cell preparations or extracts can be translated into terms of the living cell. Conversely, it is also necessary to ask whether, and in what degree, results obtained with living cells need apply to the action of enzyme systems isolated from the cells.

1. Oligases Active in Cell Extracts but Inactive in the Intact Cell

When desmolysis of oligosaccharides by living cells is compared with that of extracts or preparations, the puzzling fact emerges that an oligase, which has been shown to be inactive within the living cell, can make an active appearance in preparations of the same cell. In fact, numerous instances of striking discrepancy between the behavior of living cells and their preparations can be cited. Not all of them can be explained as due to the specific intervention *in vivo* of permeability factors or to the absence in death of enzyme components which are characteristic of the intact cell.

The case of yeast maltase (= α-glucosidase) is particularly instructive in this respect.

Thus it has been shown (70) that cell α-glucosidase becomes inactive *in situ* at a temperature of 4° C.; as a result, living brewers' yeast at this temperature ferments glucose and maltose but not methyl α-glucoside, whose fermentability, unlike that of maltose, depends on hydrolysis. On

the other hand, extracts of the same yeast both hydrolyze and ferment methyl α-glucoside at 4° C. Outside the cell, therefore, α-glucosidase seems to remain quite active at 4° C., whereas within the cell at this temperature it is inactive.

An even clearer illustration of α-glucosidase activity manifested *in vitro* but absent *in vivo* is provided by a bakers' yeast (72). Intact fresh cells of this yeast fail to ferment either maltose or methyl α-glucoside at *p*H 7.0, a *p*H optimal for maltase activity. Hence, they must be regarded as free of active maltase. Yet the cells after drying ferment both maltose and methyl α-glucoside, and autolyzates of the same yeast contain typical maltase (= α-glucosidase). The finding (57) of maltase in extracts from a yeast which failed *in vivo* to glycolyze maltose anaerobically agrees with these results.

It has been possible similarly to extract cellobiase from lactose yeast which failed *in vivo* to ferment cellobiose (89), sucrase from *Schizosaccharomyces octosporus* strains which failed *in vivo* to attack sucrose (45), trehalase from yeast which *in vivo* was inert to added trehalose (82) and hydrolases of melibionic acid and maltose carbonic acid from yeast which had failed to act on these substrates (88). Also, there have been obtained: lactase from *Escherichia coli mutabile* variants which are inert to lactose (14), and glycogenase from autolyzates of *Corynebacterium diphtheriae, mitis* type, which is inert to glycogen *in vivo* (67). The last case is further noteworthy because the different behavior to glycogen of the two strains of diphtheria bacteria, *mitis* and *gravis*, both fermenting glucose, seems to be connected with a difference between the phosphate-transporting systems in the two types of microorganism: only in autolyzates of the *gravis* strain could active phosphatase be detected (67). Although living *mitis* cells do not ferment glycogen, they are a source of glycogenase, which can produce fermentable sugar from glycogen *in vitro*. This fact suggests that glycogenase is not actually employed as long as the cell organization is preserved.

The nonfermentability by living yeast of the normal intermediary of sugar fermentation, hexose diphosphate, although the same yeast obviously contains enzymes which act on this ester, is well known and has been much discussed (93). Some authors assume a wholly unexplained inability on the part of the Harden–Young ester to penetrate the cell membrane. A similar difference in behavior *in vivo* and *in vitro* is manifested with reference to the Robison ester (glucose-6-phosphate) which is oxidized by extracts of erythrocytes but not by the intact blood cell (113). Willstätter and Rohdewald (125) have further drawn attention to differences as regards polysaccharide production between glycolyzing muscle tissue and extracts of

the same tissue. Marked differences between fermentation by living yeast and by lifeless preparations of the same yeast have been recognized by Nord (90). The susceptibility of cell-bound zymase to inactivation by narcotics and the relative resistance of dissolved zymase to the same agents are commonly encountered examples of these differences.

To assume, in the cases cited above, that enzymes in question owe their existence, not to preformation in the living cell, but to *post mortem* formation in processes incidental to the destruction of the cell substance during autolysis is surely to court improbability. It seems easier, by far, to assume that the enzymes in question are actually present in the living cell, as well as in its extracts, but that they are prevented there from acting by specific structural or functional interrelationships. Therefore, too, it is risky to draw conclusions about chemical mechanisms *in vivo* solely from studies of mechanisms found operative in isolated enzyme complexes. In the specific context of this discussion, the corollary of the above is that findings on disaccharide fermentation obtained by a study of lifeless preparations cannot in themselves be decisive for the case of the living cell. This circumstance has been kept in view throughout the present discussion; and it was with this consideration in mind that proofs which have been advanced for the existence in yeast cells of mechanisms of nonhydrolytic breakdown of oligosaccharides were based, not on work with extracts, but on experiments carried out *in vivo* and *in situ*, *i. e.*, in the living cell, in the presence of fermentable substrate and during the progress of an actual fermentation.

2. *Nonhydrolytic Mechanisms of Oligosaccharide Fermentation in Yeast Extracts*

While glycogen fermentation by yeast extracts has been widely investigated, comparatively little work on alcoholic oligosaccharide fermentation by yeast preparations has so far been reported. Some evidence suggests that nonhydrolytic mechanisms of oligosaccharide fermentation may be present in certain types of yeast preparations, but the nature of these mechanisms can only be conjectured.

The failure of the yeast phosphorylase which acts on glycogen to attack maltose (11) has already been mentioned. Nevertheless, Sobotka and Holzman (102) observed vigorous fermentation of maltose by a brewers' yeast maceration juice shown to be practically free from maltase. Guillemet (28), furthermore, observed that aged yeast extracts can sometimes retain feeble ability to produce alcohol from maltose after ability to ferment glucose has already been lost. The possible presence of zymase activators

in maltose preparations tends to detract from the significance of this finding. No general method for the preparation of maltolyzing yeast systems free from maltase and cell-free has so far been made known; the preparation of cell-free soluble yeast systems capable of glycolyzing, without initial hydrolysis, oligosaccharides other than maltose does not appear so far even to have been attempted.

The degree of cell organization persisting in dried yeast is still controversial (33), so that the bearing of evidence obtained with dried yeast samples on questions of oligosaccharide fermentation by soluble cell-free systems is necessarily doubtful. There is evidence, suggestive but not decisive, that nonhydrolytic mechanisms of oligosaccharide fermentation can persist in dried yeast cells. One such proof relating to the case of maltose fermentation depends on the action of hexose diphosphate (70). As is well known, the length of the induction period preceding gas evolution in glucose fermentation by dry yeast expresses the speed with which initial formation of hexose diphosphate proceeds from glucose. Additions of traces of the phosphate ester to suspensions of dry yeast in glucose solution markedly curtails the length of the induction period found. In the fermentation of maltose and methyl α-glucoside by a preparation of dry brewers' yeast, we observed inductions similar in length to those found in a parallel glucose fermentation. Addition of traces of hexose diphosphate to the test mixtures practically abolished the induction periods preceding the fermentation of maltose and glucose, but failed significantly to affect the induction period of the fermentation of methyl α-glucoside. In the latter case, the factor governing the beginning of fermentation must obviously be the rate of the hydrolysis of the glucoside. The mutual similarity, in response to hexose diphosphate addition, of the fermentation curves of maltose and glucose, and their common dissimilarity from that of methyl α-glucoside, suggest that maltase limited the rate of fermentation only in the case of the last-named substrate. A second type of proof depends on comparison of the rate of fermentation of disaccharide and hexose, respectively, under different conditions (92). Myrbäck and Örtenblad (82,83) have applied this criterion to the study of trehalose fermentation by dried yeast. In conformity with theories of nonhydrolytic oligosaccharide fermentation, they believed they observed greater fermentation rates with trehalose than with glucose when aged or cozymase-impoverished yeast preparations were employed. The simplest explanation of these findings is to suppose that trehalose is fermented without previous hydrolysis and hence is fermentable by yeast preparations which have been rendered relatively inactive toward glucose by loss of adenosine triphosphate, a factor essential for

hexose fermentation but unnecessary for initiation of the direct fermentation of higher sugars.

In summarizing, it seems clear that the question of the presence in cell-free yeast preparations of nonhydrolytic mechanisms of oligosaccharide breakdown deserves further examination. The mentioned appearance in yeast extracts of oligases inactive in the original living cell partly explains the slow rate of progress which has so far been made in this field of study. Nevertheless, the fact remains disturbing that all the well-defined mechanisms of nonhydrolytic oligosaccharide breakdown—levansucrase, dextransucrase, sucrose-phosphorylase—are derived from sources other than yeast.

3. *Metabolic Regulation in the Living Cell*

The attempt to reproduce nonhydrolytic oligosaccharide fermentations in isolated enzyme systems is but one aspect of the general problem of the role of cell organization in directing and determining the enzymatic processes of the cell. There is still a spirited controversy as to whether phenomena observed in isolated enzyme complexes can truly depict the nature of processes *in vivo*. In this controvery, two opposed extremes have been championed, by Willstätter and by Warburg, respectively. Yet both these leaders have factually contradicted their own first articles of faith in the course of their experimental work.

Willstätter originally (118) postulated the identity of chemical processes in isolated enzyme systems with those prevailing in living cells:

"Die chemischen Reaktionen, die bei der Einwirkung von Gewebsenzymen auf aus denselben Geweben isolierte Stoffe festgestellt werden, sind als wirkliche Teilreaktionen des chemischen Geschehens im Gewebe anzusehen."

Nevertheless, the last experimental undertaking made by Willstätter was devoted to proof (125) that there are intrinsic differences between glycolysis by living and by lifeless systems.

Warburg, again, sweepingly denied all possibility of investigating mechanisms of catalysis in the living substance by methods of preparative chemistry (112):

"Da die Erfahrung lehrt, dass man die Katalysatoren der lebendigen Substanz—die Fermente—von ihren inaktiven Begleitstoffen nicht trennen kann, so liegt es nahe, auf die Methoden der praeparativen Chemie zu verzichten, und die Fermente unter ihren natuerlichsten Wirkungsbedingungen, in der lebenden Zelle selbst, zu untersuchen."

Yet it was Warburg who identified and isolated enzymes and coenzymes of cell respiration as definite chemical entities, and by this achievement sup-

plied preparative biochemistry with its best justification. This tendency to reversal rather than unification of viewpoint on the part of two leading investigators clearly demonstrates the complexity and uncertain status of the entire problem.

Notwithstanding differences of opinion exemplified above, a fundamental theorem was long common to all preparative biochemists: that a cell preparation can possibly fall short of, but can never exceed the capacity of, the cell from which it derives. Thus it was most generally admitted that a negative result *in vitro* is no proof that the living cell lacks a sought-for enzyme; but a positive result *in vitro* was held to give proof that the demonstrated enzyme activity is present in life. Warburg, even in his most radical mood, stated (112):

"Ich setze . . . voraus, dass Stoffe, die im Reagensglas reagieren, unter sonst gleichen Bedingungen auch in der lebenden Zelle reagieren."

Even this assumption is, however, untenable. In the light of facts, some of which have been enumerated above, it has become clear that the risk involved in translating results from lifeless to living systems is a two-way one: not only may mechanisms which operate *in vivo* be absent *in vitro;* mechanisms may be present *in vitro* and yet not necessarily function *in vivo*. In fermentation physiology, as in biology generally, *selective* and *restrictive activity* by the living organism must always be taken into account. It seems highly probable that, in life, regulative and coordinating factors restrict and limit the functioning of the metabolic potentialities of the cell (27, 86, 90). The chemical paths actually taken by the cell are therefore but the final result of selective competition between a larger number of initial possibilities. Some of these indeed are realized in the living cell and not outside it; but others are realized in the uncoordinated and unrestricted play of catalytic activities which characterize the life-free extracts, and are never realized in life. The enzymatic processes of cell extracts, therefore, disclose potential mechanisms of, but not necessarily mechanisms operative in, the living organism. Thus, the difference between the behavior of an enzymatic apparatus *in vivo* and *in vitro* must be regarded in principle not as one of substance but rather as of a functional difference in regulation.

Bibliography

1. Armstrong, E. F., and Armstrong, K. F., *The Carbohydrates.* 5th ed., Longmans, Green, New York, 1934.

2. Aschner, M., Avineri-Shapiro, S., and Hestrin, S., *Harefua (Palestine)*, **22**, 1 (1942).

3. Aschner, M., Avineri-Shapiro, S., and Hestrin, S., *Nature*, **149**, 527 (1942).
4. Baba, T., *Biochem. Z.*, **273**, 207 (1934).
5. Beijerinck, M., *Folia Microbiologica (Delft)*, **1**, 377 (1912).
6. Blish, M., and Sandstedt, R., *J. Biol. Chem.*, **118**, 765 (1937).
7. Cohn, R., *Z. physiol. Chem.*, **168**, 92 (1927).
8. Colowick, S. P., and Sutherland, E. W., *J. Biol. Chem.*, **144**, 423 (1942).
9. Cori, C. F., *Cold Spring Harbor Symposia Quant. Biol.*, **7**, 260 (1939).
10. Cori, C. F., *ibid.*, **5**, 131 (1941).
11. Cori, G. T., Colowick, S. P., and Cori, C. F., *J. Biol. Chem.*, **123**, 375 (1938).
12. Cori, G. T., and Cori, C. F., *Proc. Soc. Exptl. Biol. Med.*, **34**, 702 (1936); **36**, 119 (1937).
13. Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, **151**, 57 (1934).
13*a*. Cremer, M. *Ber.*, **32**, 2062 (1899).
14. Deere, C., Dulaney, A., and Michelson, I., *J. Bact.*, **37**, 355 (1939).
15. Doudoroff, M., *J. Biol. Chem.*, **151**, 351 (1943).
16. Doudoroff, M., Kaplan, N., and Hassid, W., *ibid.*, **148**, 67 (1943).
17. Eadie, G., *Biochem. J.*, **20**, 1016 (1926).
18. Euler, H. von, and Brunius, E., *Z. physiol. Chem.*, **160**, 242 (1926).
19. Euler, H. von, and Josephson, K., *ibid.*, **120**, 42 (1922).
20. Fischer, E., *Ber.*, **27**, 3479 (1894).
21. Fischer, E., *Z. physiol. Chem.*, **26**, 72 (1898).
22. Fischer, E., and Lindner, P., *Ber.*, **28**, 3034 (1895).
23. Genevois, L., *Ann. fermentaticas*, **3**, 600 (1937).
24. Genevois, L., and Pavloff, M., *Bull. soc. chim. biol.*, **17**, 991 (1935).
25. Goda, T., *Biochem. Z.*, **298**, 431 (1938).
26. Gottschalk, A., *Z. physiol. Chem.*, **152**, 132 (1926); **153**, 215 (1926).
27. Green, D. E., in Needham, J., and Green, D. E., *Perspectives in Biochemistry*. Macmillan, New York, and Cambridge Univ. Press, London, 1937, p. 175.
28. Guillemet, R., *Bull. soc. chim. biol.*, **18**, 932, 941, 1132 (1936).
29. Guillemet, R., *Compt. rend. soc. biol.*, **130**, 1402 (1939).
30. Guillemet, R., and Leroux, H., *ibid.*, **128**, 74 (1938).
31. Hanes, C., *Proc. Roy. Soc. London*, **B128**, 421 (1940).
32. Hanes, C., *ibid.*, **B129**, 174 (1940).
33. Harden, A., *Alcoholic Fermentation*. Longmans, Green, New York, 1932.
34. Harding, V., and Nicholson, T., *Biochem. J.*, **27**, 1082 (1933).
35. Harrison, F. C., Tarr, H., and Hibbert, H., *Can. J. Research*, **3**, 449 (1930).
35*a*. Hassid, W., Doudoroff, M., and Barker, H., *J. Am. Chem. Soc.*, **66**, 1416 (1944).
36. Hehre, E., *Science*, **93**, 237 (1941).
37. Hehre, E., *Proc. Soc. Exptl. Biol. Med.*, **54**, 240 (1943).
38. Hehre, E., and Sugg, G., *J. Exptl. Med.*, **75**, 339 (1942).
39. Hestrin, S., *Enzymologia*, **8**, 193 (1940).
40. Hestrin, S., *Biochem. J.*, **34**, 213 (1940).
41. Hestrin, S., *Dissertation*, Hebrew Univ., Jerusalem, 1941.
41*a*. Hestrin, S., *Nature*, **154**, 581 (1944).
42. Hestrin, S., and Avineri-Shapiro, S., *Nature*, **152**, 49 (1943).
43. Hestrin, S., and Avineri-Shapiro, S., *Biochem. J.*, **38**, 2 (1944).
44. Hestrin, S., Avineri-Shapiro, S., and Aschner, M., *ibid.*, **37**, 450 (1943).
45. Hofmann, E., *Biochem. Z.*, **272**, 417 (1934).

46. Hoogerheide, J., *Dissertation, Delft*, 1935.
47. Hvistendahl, B., *Z. angew. Chem.*, **46**, 335 (1933).
48. Isbell, H., and Pigman, W., *J. Research Natl. Bur. Standards*, **20**, 773 (1938).
49. Johnson, F. H., *Science*, **94**, 200 (1941).
50. Johnson, F. H., and Anderson, R., *J. Cellular Comp. Physiol.*, **12**, 273, 281 (1938).
51. Josephson, K., *Z. physiol. Chem.*, **136**, 62 (1924).
52. Kagan, B., Lyatker, S., and Tszafman, E., *Biokhimiya*, **7**, 92 (1942).
53. Kalckar, H., *Chem. Revs.*, **28**, 71 (1941).
54. Kiessling, W., *Biochem. Z.*, **302**, 50 (1939).
55. Kita, G., *Chem. Zentr.*, **1912, II**, 1938.
56. Kluyver, A., *Biochem. Z.*, **52**, 486 (1913).
57. Kluyver, A., and Custers, M., *Antonie van Leeuwenhoek J. Microbiol. Serol.*, **6**, 121 (1940).
58. Kluyver, A., and Roosmalen, F., *Biochem. Z.*, **245**, 13 (1932).
59. Kohl, F., *Die Hefepilze.* Quelle & Meyer, Leipzig, 1908, p. 46.
60. Kosterlitz, H., *J. Physiol.*, **93**, 34 (1938).
61. Krieble, V., Skau, E., and Lowering, E., *J. Am. Chem. Soc.*, **49**, 1728 (1927).
62. Kruyk, K., and Klingmüller, V., *Biochem. Z.*, **300**, 343 (1939).
63. Kuhn, R., *Z. physiol. Chem.*, **125**, 28 (1923).
64. Laborde, I., *Compt. rend.*, **138**, 228 (1904).
65. Leibowitz, J., *unpublished work.*
66. Leibowitz, J., *Z. physiol. Chem.*, **173**, 84 (1928).
67. Leibowitz, J., and Avineri-Shapiro, S., *Nature*, **147**, 745 (1941).
68. Leibowitz, J., and Frankenthal, L., *unpublished work.*
69. Leibowitz, J., and Hestrin, S., *Nature*, **141**, 552 (1938).
70. Leibowitz, J., and Hestrin, S., *Enzymologia*, **6**, 15 (1939).
71. Leibowitz, J., and Hestrin, S., *Nature*, **145**, 671 (1940).
72. Leibowitz, J., and Hestrin, S., *Biochem. J.*, **36**, 772 (1942).
73. Leibowitz, J., and Kupermintz, N., *Nature*, **150**, 233 (1940).
74. Leibowitz, J., and Mechlinski, P., *Z. physiol. Chem.*, **154**, 64 (1926).
75. Lindner, P., *Chem.-Ztg.*, **34**, 1144 (1910).
76. Lindner, P., *Mikroskopische und biologische Betriebskontrolle in den Gärungsgewerben.* Parey, Berlin, 1930, p. 245.
77. Lindner, P., and Saito, K., *Wochschr. Brau.*, **27**, 509 (1910).
78. Lipmann, F., in *Advances in Enzymology*, Vol. I. Interscience, New York, 1941, p. 99.
79. Macfarlane, M., *Biochem. J.*, **30**, 1369 (1936).
80. Meyerhof, O., *Biochem. Z.*, **178**, 462 (1926).
81. Mirski, A., and Wertheimer, E., *Enzymologia*, **7**, 58 (1939).
82. Myrbäck, K., and Örtenblad, B., *Biochem. Z.*, **288**, 329 (1936).
83. Myrbäck, K., and Örtenblad, B., *ibid.*, **291**, 61 (1937); **292**, 230 (1937).
84. Myrbäck, K., Örtenblad, B., and Ahlborg, K., *Enzymologia*, **3**, 210 (1937).
85. Myrbäck, K., and Vasseur, E., *Z. physiol. Chem.*, **277**, 171 (1943).
86. Needham, J., *Order and Life.* Yale Univ. Press, New Haven, and Cambridge Univ. Press, London, 1936, p. 120.
87. Nelson, J., *Chem. Revs.*, **12**, 1 (1933).
88. Neuberg-Rabinowitsch, I., *Enzymologia*, **3**, 41 (1937).
89. Neuberg, C., and Hofmann, E., *Biochem. Z.*, **256**, 450 (1932).

90. Nord, F. F., *Chem. Revs.*, **26**, 442 (1940).
91. Nord, F. F., and Engel, W., *Biochem. Z.*, **296**, 153 (1938).
92. O'Connor, R. C., *Biochem. J.*, **34**, 1008 (1940).
93. Paine, S., *Proc. Roy. Soc. London*, **84**, 289 (1911).
94. Parnas, J. K., *Enzymologia*, **5**, 166 (1938).
95. Pringsheim, H., *Biochem. Z.*, **156**, 109 (1925).
96. Pringsheim, H., and Zemplén, G., *Z. physiol. Chem.*, **62**, 367 (1909).
97. Robison, R., and Morgan, W., *Biochem. J.*, **22**, 1277 (1928).
98. Schäffner, A., *Naturwissenschaften*, **27**, 195 (1939).
99. Schultz, A., and Atkin, L., *J. Am. Chem. Soc.*, **61**, 291 (1939).
100. Schultz, A., Atkin, L., and Frey, C., *ibid.*, **62**, 2271 (1940).
101. Slator, A., *J. Chem. Soc.*, **T93**, 217 (1908).
102. Sobotka, H., and Holzman, M., *Biochem. J.*, **28**, 734 (1934).
103. Sobotka, H., Holzman, M., and Reiner, M., *ibid.*, **30**, 933 (1936).
104. Stacey, M., *Nature*, **149**, 639 (1942).
105. Stanier, R. Y., *Soil Sci.*, **53**, 479 (1942).
106. Stark, I., and Somogyi, M., *J. Biol. Chem.*, **142**, 579 (1942).
107. Sumner, J. B., Somers, G., and Sisker, E., *ibid.*, **152**, 479 (1944).
108. Tarr, H., and Hibbert, H., *Can. J. Research*, **5**, 414 (1931).
109. Trautwein, K., and Wassermann, J., *Biochem. Z.*, **215**, 293 (1929).
110. Trautwein, K., and Weigand, K., *ibid.*, **240**, 423 (1931).
111. Veibel, S., *ibid.*, **252**, 305 (1932).
112. Warburg, O., *Über die katalytischen Wirkungen der lebendigen Substanz.* Springer, Berlin, 1928, p. 1.
113. Warburg, O., and Christian, W., *Biochem. Z.*, **242**, 206 (1931).
114. Weidenhagen, R., *Ergeb. Enzymforsch.*, **1**, 169 (1932).
115. Weidenhagen, R., in Nord-Weidenhagen, *Handbuch der Enzymologie.* Akadem. Verlagsgesellschaft, Leipzig, 1940, p. 512.
116. White, P., *Plant Physiol.*, **15**, 355 (1940).
117. Williams, R., Mosher, W., and Rohrman, E., *Biochem. J.*, **30**, 2036 (1936).
118. Willstätter, R., *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **22**, 553 (1937).
119. Willstätter, R., and Bamann, E., *Z. physiol. Chem.*, **151**, 242 (1926).
120. Willstätter, R., and Bamann, E., *ibid.*, **152**, 202 (1926).
121. Willstätter, R., Kuhn, R., and Sobotka, H., *ibid.*, **134**, 224 (1924).
122. Willstätter, R., and Lowry, C., *ibid.*, **150**, 287 (1925).
123. Willstätter, R., and Oppenheimer, G., *ibid.*, **118**, 168 (1922).
124. Willstätter, R., and Rohdewald, M., *ibid.*, **247**, 269 (1937).
125. Willstätter, R., and Rohdewald, M., *Enzymologia*, **8**, 1 (1940).
126. Willstätter, R., and Steibelt, W., *Z. physiol. Chem.*, **115**, 211 (1921).
127. Winberg, W., and Brandt, K., *Svensk Chem. Tid.*, **48**, 213 (1936).
128. Wright, H., *J. Path. Bact.*, **42**, 31 (1936); **43**, 487 (1936).
129. Wright, H., *ibid.*, **45**, 117 (1937); **46**, 261 (1938).
130. Yamasaki, I., *Biochem. Z.*, **228**, 127 (1930).

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PYRUVATE METABOLISM

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CONTENTS

I. Introduction

Of the many substances involved in intermediary metabolism, pyruvic acid, an α-keto acid, is most reactive and diverse in the reactions in which it can participate. Barrón (8) has pictured pyruvate as "the hub toward which converge carbohydrate, fats and proteins in their catabolic and anabolic reactions." Insofar as carbohydrate is concerned, pyruvate is also a natural dividing point between the "glycolytic" phases of metabolism and further oxidative processes. This keto acid is an obligatory product of hexose phosphate breakdown, but it seems clear that it is the last outpost on the main pathway of carbohydrate breakdown, after which several

pathways divide the flow of sugar catabolism. In reviewing the subject it becomes clear that metabolic steps prior to pyruvate are relatively well understood and the enzymes responsible carefully studied and isolated, but beyond this stage the field is characterized by divergent opinions, difficultly correlated results from the use of varieties of biological material, and only a limited knowledge of the enzymes concerned in the postulated reactions.

A sharper focus on pyruvate, both in isolated tissue studies and in the intact organism, has recently and quite properly displaced the emphasis formerly placed on lactate. The latter arises secondarily by reduction of pyruvate, and there is no evidence that lactate can have any other immediate fate than to be reconverted to pyruvate. The level of lactate in intact muscle or in the blood stream appears largely dependent on the oxygen supply of the tissue.

The great bulk of the details of carbohydrate metabolism have necessarily been realized from experiments on extracts and homogenates of tissues. It is generally recognized that such experiments can only hope to demonstrate what reactions are at least possible in tissues *in situ*, that is, to demonstrate what machinery the cell has available for metabolism of the substances in question. The various schemata of carbohydrate oxidation are divorced from the probable directive influences of cell structure and remain theories until the individual steps are demonstrable in the intact tissue or the animal. The development of accurate and specific analytical methods for individual components has in general lagged behind the rapidly changing picture of carbohydrate metabolism. An outstanding exception to this generalization has been that of pyruvate, for which analytical methods are available. The application of these has given much credence to existing theories of pyruvate metabolism and has posed new problems in the whole of metabolism.

It is the primary objective of the reviewer to consider the more recene advances in pyruvate metabolism. To accomplish this and build the framework for support of the new contributions, it is inevitable that thert will be some overlapping with older reviews. Of these, the reviews of Barrón (8) and Krebs (107) are particularly to be recommended. Special attention will be devoted, wherever possible, to phases which can be correlated with recent physiological studies of carbohydrate metabolism.

II. Pyruvate Anabolism

1. Glycolysis

The glycolytic process, or anaerobic glycolysis, usually refers to the series of enzymatic reactions by which glucose is transformed to lactate.

Credit for the elucidation of the complex series of reactions belongs to many, but special honor is accorded to Cremer, Meyerhof, Parnas, Embden, Lohmann, Cori and Warburg. An indication of the research energies devoted to this subject becomes evident with the realization that seven of the enzymes involved in glycolysis have been isolated as crystalline proteins. In the series of glycolytic reactions phosphate which is initially taken up by glucose, occurs in each intermediate compound and is finally released during the formation of pyruvate. In the meantime it has been shuttled from compound to compound by means of adenylic acid, the latter being often termed the coenzyme of fermentation or glycolysis. In the shuttling process, phosphate establishes special (energy-rich) chemical bonds (see 123) which store the energy derived from the stepwise degradation of the glucose molecule. It is a growing opinion that phosphate bonds may be the only form of energy which the tissues recognize for their synthetic or mechanical work.

Hexokinase.—The enzyme hexokinase is the heat-labile factor widely distributed in yeast, bacteria and animal tissues which catalyzes the formation of glucose-6-phosphate from glucose. Donation of the necessary phosphate is from adenosine triphosphate, denoted by ATP (63, 146).

$$\text{Glucose} + \text{ATP} \longrightarrow \text{glucose-6-phosphate} + \text{ADP}$$

Adenosine diphosphate (ADP), itself unavailable for phosphate transfer to glucose (39), can be made available in muscle by the enzyme myokinase (39), which converts two molecules of ADP to adenylic acid and ATP (92). Myokinase is unusually stable to high temperatures and to precipitation with trichloroacetic acid; it is inactivated by hydrogen peroxide and reactivated by sulfhydryl compounds.

Phosphoglucomutase.—This enzyme catalyzes the transformation of glucose-6-phosphate to glucose-1-phosphate (42, 190). As such it is of primary importance in the process of glycogen storage, and in the reverse direction in making glycogen available for metabolic breakdown:

$$\text{Glucose} \rightarrow \underset{\substack{\swarrow \quad \searrow \\ \text{oxidation} \quad \text{glycolysis}}}{\text{glucose-6-phosphate}} \rightleftharpoons \underset{\downarrow\uparrow}{\text{glucose-1-phosphate}}$$
$$\text{glycogen}$$

The enzyme has been freed of other proteins affecting the 1- and 6-phosphates and establishes an equilibrium at room temperatures of approximately 95% glucose-6-phosphate to 5% glucose-1-phosphate (38). Phosphoglucomutase requires either magnesium or manganese ions for its activity.

Phosphorylase.—The formation of glucose-1-phosphate from glycogen

and inorganic phosphate and the reverse reaction (phosphorolysis) are catalyzed by the enzyme phosphorylase. These reactions have been studied in great detail by Cori and coworkers (41, 44). The active form of the enzyme has been isolated from rabbit muscle and obtained in a crystalline state (77). By enzymatic means the prosthetic group, adenylic acid, can be dissociated from the protein to give an inactive protein, and active phosphorylase can be reconstituted by addition of adenylic acid. The occurrence of an enzyme in muscle and spleen which releases adenylic acid from phosphorylase and the preponderance of the inactive phosphorylase in stimulated muscle may have an important physiological significance.

Phosphohexose Isomerase.—This enzyme has been studied by Lohmann (128) and shown to catalyze the reaction glucose-6-phosphate $\rightleftarrows$ fructose-6-phosphate. At equilibrium there is present 70% of the glucose derivative (Robison ester) and 30% of the fructose phosphate (Neuberg ester).

Phosphohexokinase.—The formation of hexose (fructose)-diphosphate from fructose-6-phosphate is catalyzed by phosphohexokinase and requires the donation of phosphate from ATP (164). The enzyme is water-soluble but has not been purified. Oxidizing agents are reported to inhibit greatly the action of phosphohexokinase (60).

Aldolase and Isomerase.—Fructose diphosphate is the form of carbohydrate prepared for scission by aldolase to give the aldo and keto triose phosphates (144, 145). Herbert and coworkers (82) have prepared this enzyme (then called zymohexase) from rabbit skeletal muscle in a form that was homogeneous by cataphoresis and ultracentrifuge tests. The enzyme from rat muscle has been crystallized by Warburg and Christian (205). The equilibrium between phosphodihydroxyacetone and 3-phosphoglyceraldehyde is established by triose phosphate isomerase (147), and is greatly in favor of the keto component.

The isolation of 1,3-diphosphoglycerate as the product of triose phosphate oxidation has led Warburg and coworkers (157, 202) to postulate the formation of 1,3-diphosphoglyceraldehyde from the mono ester. The diphospho ester of glyceraldehyde has been prepared only as a synthetic dimer (3), and was biologically inactive. From the failure of phosphate, diphosphopyridine nucleotide or Warburg's diphosphoglyceraldehyde dehydrogenase to influence the equilibrium value of either aldolase or isomerase, Meyerhof and Junowicz-Kocholaty (150) have concluded that the addition of a second phosphate to 3-phosphoglyceraldehyde can only be of a loose physical nature.

Phosphoglyceraldehyde Dehydrogenase.—This enzyme, previously termed triose phosphate dehydrogenase, has been prepared in the pure state and studied by Warburg and coworkers (157, 202). The product of its action on phosphoglyceraldehyde has been established as 1,3-diphosphoglycerate, this being the first reaction of the glycolytic series in which diphosphopyridine nucleotide is involved. The coenzyme is reduced to the dihydro form by the simultaneous oxidation of aldehyde to acid:

$$\text{3-Phosphoglyceraldehyde} + \text{phosphate} + \text{DPN} \longrightarrow \text{1,3-diphosphoglycerate} + \text{DPN-H}_2$$

The carboxyl phosphate (energy-rich) of 1,3-diphosphoglycerate is particularly labile and transfers its phosphate nonenzymatically to ADP to regenerate ATP:

$$\text{1,3-Diphosphoglycerate} + \text{ADP} \longrightarrow \text{3-phosphoglycerate} + \text{ATP}$$

Phosphoglyceromutase.—3-Phosphoglycerate is converted enzymatically to 2-phosphoglycerate by phosphoglyceromutase (143). It appears to be inhibited by magnesium ions (150), and establishes an equilibrium in favor of the 3-phospho component (148, 150, 204).

Enolase.—This enzyme (129, 203) catalyzes the conversion of 2-phosphoglycerate to 2-phosphopyruvate by removal of the elements of water. The latter compound, containing an enol phosphate, is "energy-rich." Enolase has been prepared as a mercury salt (inactive) from yeast by Warburg and Christian (204). This salt becomes active when freed of mercury and combined with magnesium, manganese or zinc. Dissociation constants of the magnesium complex have been measured. The equilibrium established between phosphopyruvate and 2-phosphoglycerate is in favor of the former.

Dephosphorylation of Phosphopyruvate.—The formation of pyruvate from phospho-(enol) pyruvate (111) involves an enzymatic transfer of phosphate to adenylic acid or ADP to regenerate ATP (165). Phosphate transfer to ADP is the more rapid; magnesium (or manganese) and potassium ions are reported as essential in the reaction (14). The dephosphorylation of phosphopyruvate is believed by many to be irreversible; and there are several studies (to be discussed later) to indicate that the C_4 acids are involved in the synthesis of phosphopyruvate and glycogen from pyruvate.

Adenosinetriphosphatase.—Regeneration of ATP by the dephosphorylation of pyruvate makes this compound available for continued phosphorylation of glucose, fructose-6-phosphate or creatine. Adenosinetriphosphatase is involved in these transfers and the energy released in

ATP splitting is generally regarded as the direct source for muscular contraction. It has still been found impossible to separate the adenosinetriphosphatase activity from the most highly purified preparation of muscle myosin (58, 154, 192), and the terms myosin and adenosinetriphosphatase are now often used synonomously. Myosin is strongly activated by calcium ions (192), less so by manganese (4). The stability of myosin to heat is greatly increased by ATP; and this is believed to be the result of an enzyme–substrate type of compound. Since myosin is believed to be the protein of muscle responsible for its elastic and contractile properties, the close association (or identity) of adenosinetriphosphatase with myosin is of great physiological interest.

Lactic Dehydrogenase.—Without a compensating reaction for reoxidation of the diphosphopyridine nucleotide which is reduced during the oxidation of phosphoglyceraldehyde, the progress of carbohydrate metabolism would be limited by the quantity of coenzyme present. In the usual acetone extract of muscle or in more complete experimental systems in the absence of oxygen, reoxidation of the coenzyme occurs linked with reduction of pyruvate to lactate:

$$\text{Pyruvate} + \text{DPN-H}_2 \longrightarrow \text{lactate} + \text{DPN}$$

The activating protein responsible for this reaction is lactic dehydrogenase. Straub (188) isolated this enzyme from heart muscle in crystalline form, and Kubowitz and Ott (110) prepared the crystalline mercury complex of lactic dehydrogenase from rat sarcoma, which becomes active upon separation of the mercury. DPN is the prosthetic group of the enzyme or, according to preference of terminology, lactic dehydrogenase requires the coenzyme DPN for its activity.

In more complex tissue suspensions and under aerobic conditions, other agents compete with pyruvate for the oxidation of diphosphopyridine nucleotide. Oxidation by oxaloacetate or other systems dependent on DPN, and by flavoproteins (hence to cytochromes and oxygen) are the principal possibilities.

2. Inhibitors of Glycolysis

Specific inhibitors of glycolysis were not only instrumental in identifying intermediate stages of the process in muscle extracts, but also promise to seek their target in the complete organism, tissue slice or crude homogenate and indicate the relative contribution to respiration of the reaction which they inhibit. The inhibitor technique, although offering a valuable tool in this direction, has met considerable criticism. Inhibitors can only be

employed with a full appreciation of their limitations and specificity characteristics.

Fluoride.—The immediate precursors of phosphopyruvate have been revealed in large part by the use of the inhibitor, sodium fluoride. The inhibition of lactic acid formation by fluoride (54) from either glycogen or hexose diphosphate was found to be accompanied by accumulation of a phosphate ester (127). This ester was identified by Embden and Deuticke (55) as phosphoglycerate. Thus it was early concluded that fluoride acted on enolase, and probably by combination with a metal component of the enzyme (117, 171). Warburg and Christian later showed that fluoride formed a fluorophosphate with the magnesium of enolase (203, 204). Active enolase containing magnesium is more strongly inhibited by fluoride than the manganese-containing complex due to a greater stability of the magnesium–fluoride complex (150).

Iodoacetate.—Iodoacetate, even at low concentrations, is an inhibitor of glycolysis. This inhibitor affects primarily those enzymes which contain sulfhydryl groups essential for their activity. The activating protein for phosphoglyceraldehyde is such an enzyme (168), and appears to be the principal point of attack in the glycolytic series for low concentrations of iodoacetate.

Phlorhizin.—Lundsgaard (134) showed that phlorhizin inhibited lactic acid formation in minced muscle by blocking phosphorylation of the glycogen. That this action involved phosphorylase was indicated by Ostern and coworkers (164) and by Cori, Colowick, and Cori (43), and proved by studies with the pure enzyme (41).

However, Beck (9) has found that phlorhizin inhibits oxidative phosphorylation of glucose in kidney preparations, the system first described by Kalckar (90), and in concentrations likely to exist during phlorhizin poisoning. It seems probable therefore that phlorhizin also acts on some step of metabolism involving a phosphate transfer to glucose. An inhibitory action of phlorhizin on adenosinetriphosphatase has been reported (59). Kaplan and Greenberg (96) found that phlorhizin poisoning in rats prevented the normal increase of P^{32} in the liver ATP after feeding glucose and radioactive phosphate. Since fluoride and malonate acted in a similar manner to phlorhizin, these experiments support the theory that the primary action of phlorhizin might be in the formation of ATP during reactions liberating energy-rich phosphate. Such a mechanism is in harmony with the fact that the absorption of glucose by the kidney tubule is an aerobic process and would require ATP in the formation of the absorbed hexose phosphate (hexokinase action).

3. *Alternative Mechanisms of Carbohydrate Oxidation*

Nonphosphorylating.—In extending the reviewed material to more complex biological systems, there seems little reason to doubt the general role of phosphate in the anaerobic metabolism of carbohydrates. Although occasional claims have been made for a nonphosphorylating glycolysis in some tissues, for example in brain (2) and embryonic tissue (155, 156), these were later made untenable by subsequent demonstration of phosphorylated intermediates in the same tissues (149). It seems probable that destruction of pyridine nucleotides by phosphatases liberated in the maceration of tissues (140) might be at least one reason for the apparent absence of a phosphorylating glycolysis in certain tissues. Indeed, the frequent inability to demonstrate a reaction in macerated tissue which is readily demonstrable in the intact tissue will possibly be traced in many instances to destruction of vital coenzymes by the action of liberated enzymes.

The apparently slow rate of radioactive phosphorus (P^{32}) turnover in the organic phosphorus fractions of muscle after injection of radioactive inorganic phosphate, which was recorded by Sacks (172), was interpreted by this investigator as evidence that phosphorylating glycolysis can play only a minor role in muscle metabolism. The experimental basis for these conclusions has been criticized by Kalckar (91) and Lipmann (123). They have pointed out that the rate of P^{32} incorporation into the organic phosphate fractions of the muscle was erroneously determined on the basis of the radioactivity of the total inorganic phosphate of the muscle. Since a large share of the total inorganic phosphate is extracellular, and the rate of penetration of phosphate into the cell is slow (84), the turnover calculations must be based on the intracellular phosphate concentration. Sacks and Altschuler (173) later attempted to correct the experimental deficiencies by applying corrections for the extracellular phosphate, and found that the P^{32} concentration of the pyrophosphate fraction (ATP) reached a level equal to or greater than that of the intracellular phosphate. Rather than reversing an earlier conclusion, it was stated that, although phosphorylating glycolysis may play a part in the resting metabolism of muscle, it cannot be extended to "activity" metabolism. Both Bollman and Flock (13) and Furchgott and Shorr (74) have shown, however, that the penetration and uptake of radioactive phosphorus is the same in resting and exercising muscle.

Kalckar and coworkers (93) have reinvestigated the experimental approach to this problem. They have found it necessary to remove the highly radioactive extracellular phosphate by perfusion of the organ before meas-

uring the radioactivity of intracellular inorganic and organic phosphate. A second point of experimental interest is the use of only a short (20-minute) incubation period after injection of P^{32} before perfusion and fixing of the tissue, the longer incubation periods used by other workers being found unnecessarily long for equilibrium of the inorganic and organic intracellular phosphate. In the case of liver, for example, it was found that the labile phosphate of adenosine triphosphate had reached essentially the level of the intracellular inorganic phosphate in as little as 6–7 minutes. Rates of rejuvenation of labile phosphate groups of 20–30 micrograms of phosphorus per minute per gram of muscle, and in liver 15 micrograms per minute per gram, were calculated from the recent data. These figures are much higher than previously recorded and are in full support of phosphorylating mechanisms in carbohydrate metabolism.

Direct Oxidation of Hexose Phosphate.—There is increasing evidence that, although glycolysis is the principal mechanism in the anaerobic breakdown of carbohydrate, a different series of reactions, possibly starting with direct oxidation of glucose-6-phosphate, may account for the initial phases of carbohydrate metabolism under aerobic conditions.

In animal tissues there is evidence that glycolysis can account for only a portion of carbohydrate breakdown, and that oxidative reactions must intervene early in the course of glucose metabolism. Lundsgaard (133) noted that the respiration (oxygen consumption) of muscle remained essentially intact in the presence of iodoacetate, an inhibitor of glycolysis. Barker, Shorr and Malam (5) found that the respiration of heart muscle slices or of brain slices and homogenates was rarely inhibited more than 30% by iodoacetate concentrations which completely blocked anaerobic glycolysis. They have eliminated the possibility that the respiration remaining in the presence of iodoacetate could be due to preformed substrates of the tissue, such as lactate. These observations have been confirmed with brain homogenates in the writer's laboratory (unpublished), and the possibility of iodoacetate destruction occurring aerobically was eliminated by observing a complete inhibition of anaerobic glycolysis after a preliminary aerobic incubation with iodoacetate. Furthermore, fluoride gave results similar to iodoacetate.

Since it is probable that phosphoglyceraldehyde dehydrogenase is the principal enzyme involved in anaerobic glycolysis which is affected by the low concentration of iodoacetate, these experiments imply that this enzyme is not involved in iodoacetate-resistant respiration. Thus if the glycolysis intermediates are at all involved, they must be "shunted" before the triose phosphate stage.

Fazekas and Himwich (67) have performed interesting experiments which have a bearing on this problem. They found that one-day-old rats, which survive for approximately 50 minutes in nitrogen, live only 1–5 minutes when injected with iodoacetate or fluoride. Litter mates kept in air and injected with the same quantity of iodoacetate or fluoride lived approximately an hour. The most obvious conclusion drawn was that in nitrogen the energy for survival is obtained from anaerobic glycolysis and is blocked by the glycolysis inhibitors, while in oxygen another mechanism of carbohydrate breakdown becomes available.

The direct oxidation of glucose-6-phosphate has been demonstrated in animal tissue by Warburg and coworkers (200, 201). The enzyme hexose monophosphate dehydrogenase (*Zwischenferment*) has been isolated in the pure state and, in conjunction with triphosphopyridine nucleotide, oxidizes glucose-6-phosphate to phosphogluconic acid. Proteins prepared from yeast, with the same coenzyme, were found to react with phosphogluconic acid, resulting in oxygen uptake and carbon dioxide liberation. Among the products of the reaction was a compound giving the color reactions of a pentose, and one having the composition of phosphoglyceric acid. The latter, however, was without optical rotation or activity with yeast extracts. There have been no further reports from Warburg's laboratory on this subject since 1937.

Lipmann (118) demonstrated an oxidative fermentation (oxygen uptake and carbon dioxide evolution) of phosphogluconic acid in yeast macerate, during which reaction the organic phosphate remained intact. Carbon dioxide production was nearly absent in nitrogen. It was believed that phospho-α-ketogluconic acid was the first product of the reaction, which then readily lost carbon dioxide. Engelhardt and Barchash (57) also noted the aerobic (but not anaerobic) fermentation of phosphogluconic acid by yeast. Dickens (46) isolated two compounds from phosphogluconic acid oxidation by yeast maceration juice which he identified as a phosphoketogluconic acid and a phosphopentonic acid. Employing a less pure sample of triphosphopyridine nucleotide, he isolated a compound which was thought to be phosphoerythronic acid. Kidney slices were found to oxidize 2-keto-*d*-gluconic acid, and yeast maceration juice fermented *d*-ribose-5-phosphate. The results taken together indicate that the direct oxidation of hexose phosphate involves successive oxidations and decarboxylations.

Emerson, Stauffer and Umbreit (56), in presenting supporting evidence for their interesting theory of chlorophyll function in the creation of energy-rich phosphate bonds, have also found the glycolysis mechanism inadequate to account for their experimental findings in *Chlorella*. In cer-

tain aerobic bacteria which can oxidize glucose but not ferment it, glycolysis is apparently entirely supplanted by a direct oxidation mode of carbohydrate metabolism.

Engelhardt and Barchash (57) were early to introduce the "hexose monophosphate shunt" model into the perennial discussions of the mechanism of the Pasteur effect. (For an attractive display of the 1939 models, see Burk, 29.) It is believed that the blocking of the anaerobic glycolysis pathway by redox dyes (57) and by oxygen (60) is due to the sensitivity of phosphohexokinase (fructose-6-phosphate → fructose diphosphate) to oxidizing agents. Hexose phosphate is thereby shunted through phosphogluconic acid.

4. Glucose–Pyruvate–Lactate Relations

Blood Pyruvate.—Until adequate methods for the determination of blood pyruvate were available, exact relations of lactate and pyruvate *in vivo* could not be studied. There has been no lack of methods suggested for the determination of pyruvate. Among these may be mentioned the zinc–copper couple reduction method of Wendel (210), the carboxylase method of Westerkamp (214), the α-methylindole color reaction of Dische and Robbins (47), and the ceric sulfate method of Fromageot and Desnuelle (73). Most of these methods have been found to be deficient either in specificity or sensitivity when applied to blood or animal tissues. The Clift and Cook (33) bisulfite titration method has been generally useful, and most of the facts gained by the application of this method remain substantiated by use of more specific methods. The present most commonly used methods of pyruvate determination involve the formation of the 2,4-dinitrophenylhydrazone derivative. In these methods, the basic pattern of which was described by Case (31), the hydrazone derivatives of aldehydes and ketones present in a trichloroacetic filtrate of tissues are successively extracted by ethyl acetate and sodium carbonate. Final estimation is by formation of a red color by addition of alkali to the hydrazone. Bueding and Wortis (22) and Friedemann and Haugen (71, 72) have studied the phenylhydrazone method extensively, from both the standpoint of the precautions required in the collection of blood samples and the analytical method itself. The former investigators have made a follow-up study of their observation of rapid pyruvate loss in fresh blood, and indicate that pyruvate is reduced to lactate by oxidoreduction with glyceraldehyde phosphate as in muscle extracts (23).

Lactate–Pyruvate Ratio.—It has been known for some time that increases in blood lactate occur after exercise (86) and anoxia (130, 138).

Elevations of blood pyruvate have also been noted even after mild exercise (22, 132, 184), anoxia (184) and various pathological conditions (27, 221). Friedemann and Barborka (70) have noted a definite relation or ratio of lactate and pyruvate levels in blood after exercise. Their work indicates that a short period of time is required immediately after exercise to establish the "normal" relation of these two substances in the blood. Stotz and Bessey (184) have further studied the lactate–pyruvate relation both in man and in the rat, and have arrived at formulas to describe the normal relation of these substances in the blood. They advocate the use of the blood lactate–pyruvate ratio to distinguish true abnormalities of pyruvate metabolism from the simple but often difficultly controlled blood pyruvate fluctuations due to factors such as exercise, anoxia and food consumption. Further illustrated is the use of the ratio in experimental thiamine deficiency in pigeons, where it was possible to note a disturbance of pyruvate metabolism early in acute deficiency and to distinguish smaller degrees of chronic thiamine deficiency despite different degrees of activity.

The observed relation between blood lactate and pyruvate constitutes direct evidence for at least this phase of anaerobic glycolysis in the intact animal. The results are interpreted to mean that, by imposing relatively anaerobic conditions, either by a lag in oxygen supply to the muscles immediately after exercise or directly by anoxia, an increase in the products of glycolysis occurs in the tissues which is reflected in the blood stream.

Evidence is available for the *in vivo* functioning of an oxidoreduction type of reaction which is coupled through diphosphopyridine nucleotide. The coupled reaction of alcohol and pyruvate is such a coenzyme-linked reaction, since both the oxidation of alcohol (62, 167) and the reduction of pyruvate (78) are dependent upon diphosphopyridine nucleotide.

$$\underset{\text{Ethyl alcohol}}{CH_3CH_2OH} + \underset{\text{Pyruvic acid}}{CH_3COCOOH} \rightleftharpoons \underset{\text{Acetaldehyde}}{CH_3CHO} + \underset{\text{Lactic acid}}{CH_3CHOHCOOH}$$

Leloir and Muñoz (114) reported an increased oxidation of alcohol in liver slices when pyruvate was added, and attributed the effect to oxidoreduction. More recently Westerfeld, Stotz and Berg (212, 213) have demonstrated this reaction in the dog by blood studies following the administration of the individual components of the reaction. These investigators first noted (212) that administration of pyruvate caused marked increases in the rate of alcohol oxidation as measured by blood alcohol changes. At the same time, pyruvate was utilized more rapidly if alcohol had been previously administered. These experiments demonstrated an interaction of alcohol and pyruvate as required by the oxidation-reduction reaction.

After administration of alcohol there is an increased concentration of blood acetaldehyde both in dogs (213) and in man (185). In dogs having previously received alcohol, the administration of pyruvate causes a sharp rise in blood acetaldehyde. This can only mean that acetaldehyde is a product of the interaction of alcohol and pyruvate as required by the oxidation-reduction reaction. Finally the increase in blood lactate following alcohol administration observed by Clark and coworkers (32) is further evidence of the coenzyme-linked reaction.

5. Pyruvate from Glucose

The availability of analytical methods to determine intermediates in the complicated series of chemical reactions comprising glucose metabolism has made possible several studies of abnormal carbohydrate metabolism. The use of blood pyruvate determinations has at least severed the metabolic chain into two pieces and permitted studies of the isolated portions. It is entirely true that the level of a given intermediate such as pyruvate must represent a balance between its formation and removal; but when there is reason to believe that a given imposed condition has no effect on, for example, the removal, then fluctuations of the intermediate can justifiably be attributed to changes in its rate of formation.

Thus it is believed that the elevated blood pyruvate after glucose administration can be attributed to an increased rate of pyruvate formation from the extra glucose. This was the conclusion of Bueding, Stein and Wortis (24), who noted in normal human subjects an average rise in blood pyruvate of 0.46 milligram per cent (from approximately 1 mg. per cent) at the end of 1 hour after the ingestion of 1.75 grams of glucose per kilogram of body weight. On the other hand, the higher fasting value and greater rise noted by the same investigators in subjects with clinical evidence of thiamine deficiency is attributed to a decreased rate of pyruvate removal. This apparent reversal in the interpretation of the pyruvate level is warranted, at least for the present, since thiamine is known to be concerned with the enzymes causing pyruvate removal, and not involved in the glycolysis reactions.

Insulin Action and Diabetes.—Bueding and coworkers (25) later made the significant observation that the injection of glucose (2 g./kg.) into depancreatized dogs failed to produce a rise in blood pyruvate, while nearly doubling the fasting level in normal dogs. When insulin was administered with the glucose to depancreatized dogs, a marked rise in blood pyruvate occurred. These observations were extended to diabetic patients (26, 97), where it was again shown that there was little or no rise in blood pyruvate

following glucose ingestion, but a significant rise when insulin was also administered. The effect of insulin plus glucose in causing an increased oxidation of alcohol in man has also been reported (32, 75). In conjunction with the demonstration that pyruvate also increases the rate of alcohol oxidation (114, 212, 213), these results gain explanation and at the same time increase the probability that the pyruvate response from insulin is due to enhanced pyruvate formation rather than decreased removal.

A pyruvate response from insulin plus glucose is apparently difficult to demonstrate unless the insulin supply is already low, as in depancreatized animals or diabetic subjects, or unless the glucose is administered in great excess. Bueding and Goldfarb (28) were able to note a pyruvate response from insulin in normal subjects only during continuous infusion of glucose. The pyruvate level of diabetic patients is also essentially normal. The effect of insulin may thus be of an auxiliary rather than of a critical nature. The indispensability of insulin for carbohydrate oxidation has been questioned by the glucose balance studies of Soskin and coworkers (180) and studies with depancreatized-hypophysectomized dogs (88, 179), these animals showing an essentially normal glucose metabolism.

The pyruvate response from glucose in normal and diabetic subjects and the effect of insulin, nevertheless, have an important bearing on theories of insulin action and diabetes. The work cited would, at first consideration, imply that insulin played an enzyme or activator role in one of the series of reactions comprising glycolysis. The experiments of Levine and coworkers (116), which demonstrated an increased phosphorylation of glucose *in vitro*, might also be interpreted as support of an insulin action on glycolytic phases. Yet, with the intensive study that has been accorded to the isolated phases of glycolysis, there have been no confirmed or generally accepted reports of an action of insulin on the enzyme systems that are involved.

We are now well aware that the phosphorylation of glucose is dependent on adenine nucleotides (ATP) and that phosphate transfers can result from phases of metabolism beyond pyruvate (9, 90). Pertinent in this respect are the experiments of Kaplan and Greenberg (96), which demonstrated an increase of P^{32} in the ATP of liver after the administration of insulin and radioactive phosphate to rats, as well as those of Sacks (174), which revealed a marked increase of P^{32} turnover in the ATP and phosphocreatine of muscle after insulin plus glucose and radioactive phosphate administration to cats. Rice and Evans (169) have shown that the ability of insulin to sustain the respiration of pigeon breast muscle is due to a maintenance of pyruvate oxidation. Reactions in which fumarate or oxaloace-

tate could act as catalysts seemed to be especially involved. Further study of pyruvate catabolism and phosphate transfers in seeking the specific action of insulin would seem at present to offer the best opportunities for solution of the problem.

The older "under-utilization" theory of diabetes held that glucose was inadequately oxidized as a result of insufficient insulin action, a view upheld by the classical experiments on the glucose–nitrogen ratio and the respiratory quotient in diabetes. This evidence has been assailed by proponents, particularly Soskin (181), of the glucose "over-production" theory of diabetes. Comments on the inadequacy of R.Q. measurements to determine the relative intensity of carbohydrate oxidation are entirely justified in that other metabolic transformations could also contribute to or account for the lowered R.Q. noted in diabetes. However, Stotz, Root and Carpenter (187) have combined R.Q. and pyruvate measurements in diabetic subjects after the administration of glucose with or without insulin. A negative response in R.Q. was nearly invariably accompanied by a lack of pyruvate response, and when a significant rise in R.Q. was found, particularly as a result of previous insulin administration, blood pyruvate and lactate levels also increased. The under-utilization theory of diabetes gains considerable support from the nature of the pyruvate response to glucose and insulin in diabetes, and these facts will now have to receive account in any generally accepted theory of diabetes.

III. Pyruvate Catabolism

1. General

The principal avenues of pyruvate metabolism, dismutation, oxidation, decarboxylation and condensation, all appear to require diphosphothiamine (8). The partial loss of this coenzyme during thiamine deficiency results in a great accumulation of pyruvate in the blood and other tissues; and this has been traced to decreased pyruvate catabolism in these tissues. The characteristic opisthotonos of acute thiamin deficiency in pigeons, often occurring before any definite neurologic lesions can be observed, has been described as a biochemical or functional disturbance. The rapid reversibility of this critical condition by administration of thiamin suggests that pyruvate breakdown is intimately concerned with a vital factor in nerve function. The most interesting suggestion in this connection comes from the work of Mann and Quastel (139), who showed that pyruvate catabolism was necessary for the synthesis of acetylcholine in brain tissue. Here again it seems probable that reactions involved in pyruvate

oxidation are utilized to form the ATP involved directly in the synthesis of acetylcholine (152, 153).

In addition to thiamin it is indicated that pantothenic acid and biotin are involved in the metabolism of pyruvate (49, 166, 189), but at which stage or by what mechanism is not yet clear.

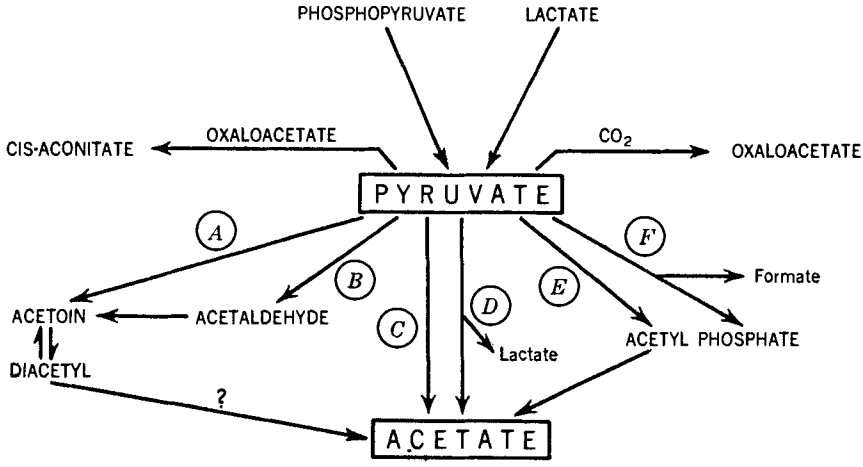


Fig. 1.—Channels of pyruvate catabolism. *A* and *B*, decarboxylation; *C*, oxidative decarboxylation; *D*, dismutation; *E*, oxidative (dehydrogenation); *F*, phosphoroclastic.

All animal tissues tested and a variety of bacteria can metabolize pyruvate (52, 53, 102, 103, 106), and probably by a diversity of reactions. Barrón and Lyman (6) have shown that the catabolism of pyruvate in various animal tissues is considerably faster aerobically than under anaerobic conditions.

2. Dismutation

The formation of acetic acid from pyruvate has long been recognized in "acetic" fermentations. In animal tissues, acetate has also been identified as a product of pyruvate catabolism, and under anaerobic conditions principally by a dismutation reaction (103, 119, 206):

$$\underset{\text{(pyruvic)}}{2\,CH_3COCOOH} + H_2O \longrightarrow \underset{\text{(lactic)}}{CH_3CHOHCOOH} + \underset{\text{(acetic)}}{CH_3COOH} + CO_2$$

This reaction is commonly measured by the extra carbon dioxide liberated upon addition of pyruvate.

Another type of anaerobic splitting of pyruvate to yield acetate and formate has been described in certain bacteria (6, 105) but not in animal tissues:

$$\underset{\text{(pyruvic)}}{CH_3COCOOH} + H_2O \longrightarrow \underset{\text{(acetic)}}{CH_3COOH} + \underset{\text{(formic)}}{HCOOH}$$

Studies from Werkman's laboratory (94) first showed that phosphate was necessary for this "hydroclastic" splitting in cell-free preparations of *Escherichia coli*, and later (197) that ATP was formed during the reaction if adenylic acid was used as an acceptor. In the absence of adenylic acid, an acid-labile phosphate accumulated which was presumed to be acetyl phosphate. Utter, Werkman and Lipmann (198) have now identified acetyl phosphate as a product of the renamed "phosphoroclastic" splitting of pyruvate by *E. coli*, and have shown that acetate arises secondarily from the splitting of acetyl phosphate:

$$\text{Pyruvate} + \text{water} + \text{phosphate} \rightleftharpoons \text{formic} + \text{acetyl phosphate} \downarrow \text{acetic}$$

Lipmann and Tuttle (126) have also demonstrated the reverse of this reaction, the formation of pyruvate from acetyl phosphate and formate, again with an *E. coli* preparation.

Phosphate was also found necessary in the splitting of pyruvate to acetate, carbon dioxide and hydrogen by *Clostridium butylicum* (100).

3. Decarboxylation

The decarboxylation of pyruvate in various biological materials apparently yields different products under aerobic and anaerobic conditions. In the former case, at least in certain bacteria, acetate and carbon dioxide are formed with the intermediate formation of acetyl phosphate. Anaerobically, the formation of acetaldehyde—in yeast (159), and acetoin—in bacteria and animal tissues (79, 175), occurs.

Yeast carboxylase has been highly purified and contains diphosphothiamine as a prosthetic group. It is activated considerably by cysteine (30). A simple decarboxylation to acetaldehyde and carbon dioxide is indicated.

Green and coworkers (79) have described a carboxylase preparation in animal tissues which is a diphosphothiamin enzyme and converts pyruvate to acetylmethylcarbinol (acetoin) and α-ketoglutarate to succinic semialdehyde:

$$\underset{\text{(pyruvic)}}{2\,CH_3COCOOH} \longrightarrow \underset{\text{(acetoin)}}{CH_3COCHOHCH_3} + 2\,CO_2$$

$$\underset{(\alpha\text{-ketoglutaric})}{HOOC{-}CH_2{-}CH_2{-}CO{-}COOH} \longrightarrow \underset{\text{(succinic semialdehyde)}}{HOOC{-}CH_2{-}CH_2{-}CHO} + CO_2$$

Although free acetaldehyde could not be detected as an intermediate in the pyruvate reaction, it was presumed that the mechanism involved preliminary decarboxylation of the pyruvate, since (*a*) the formation of acetoin was accelerated fourfold when acetaldehyde was present in addition to the pyruvate, (*b*) the yield of acetoin from a given amount of pyruvate in the presence of acetaldehyde was twice that obtained with pyruvate alone and (*c*) when acetaldehyde was replaced by propionaldehyde the corresponding acetylethylcarbinol was formed. The detailed mechanism is therefore proposed as follows:

$$CH_3COCOOH \longrightarrow CH_3CHO + CO_2$$

$$CH_3CHO + CH_3COCOOH \longrightarrow CH_3COCHOHCH_3 + CO_2$$

Acetaldehyde has been "trapped" by aldehyde fixatives in autolyzing tissues (87, 158, 196, 217), and acetoin formation from pyruvate has been demonstrated in minced heart muscle (76), skeletal muscle, liver and kidney (193). The nearly quantitative formation of acetoin in brain homogenates from acetaldehyde in the presence of pyruvate has been demonstrated (186), while smaller amounts of acetoin were formed from pyruvate or acetaldehyde alone. The relative stability of the acetoin formed in brain homogenates appears to pose a serious question as to the further metabolism of this substance, a consideration in which the optical activity of the product may be involved. Tankó and coworkers (193), although demonstrating principally the formation of *l*-acetoin in animal tissue preparations, suspect the presence of admixture with the unnatural isomer. Martius (142) refers to the formation of racemic acetoin in yeast maceration juice, and advances the hypothesis that biacetyl is actually the first product formed, followed by reduction to racemic acetoin.

The *in vitro* work on acetoin formation from acetaldehyde was supplemented by the *in vivo* demonstration of this reaction (186). Injection of acetaldehyde into rats with measurements of blood acetaldehyde indicated its rapid metabolism, and the simultaneous formation of acetoin was demonstrated by its detection in the blood stream. A further consideration of the optical activity of the acetoin arises in these experiments, since, although the disappearance of injected *dl*-acetoin in dogs (81) and rats (186) appears to be a slow process, the acetoin formed *in vivo* from acetaldehyde

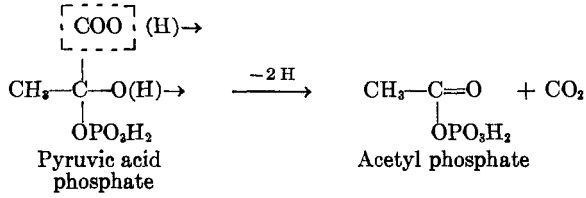
appears to be rapidly metabolized because it could only be detected immediately after acetaldehyde injection.

4. *Oxidation*

Studies on the oxidation of pyruvate to acetate and carbon dioxide have been restricted largely to bacteria, although it seems probable that the reaction occurs in animal and other tissues (104, 119, 131, 206). The pyruvate oxidation system in *Lactobacillus delbrückii* particularly has been studied by Lipmann (122) because of its relative stability. With oxygen as a hydrogen acceptor, several factors are necessary to complete the reaction: (*a*) a protein; (*b*) diphosphothiamin; (*c*) a flavin (120); (*d*) manganese, magnesium or cobalt; and (*e*) inorganic phosphate. Acetate and carbon dioxide are products of the reaction, and acetyl phosphate is an intermediate (124). In the presence of the bacterial enzyme preparation, energy-rich phosphate from acetyl phosphate can be transferred to adenylic acid. This strongly suggests that acetyl phosphate may also be an intermediate in all reactions of coupled oxidation of pyruvate and phosphorylation. Such a coupling has been illustrated by Ochoa in cell-free heart muscle extracts (162) and in brain (161), but there has been no conclusive evidence thus far of acetyl phosphate in animal tissue; in fact, Ochoa, Peters and Stocken (160) doubt its significance in animal tissue because acetyl phosphate was not found to donate phosphate to adenylic acid in brain extracts. The excellent study of the properties, synthesis and determination of acetyl phosphate by Lipmann and Tuttle (125) promises to facilitate physiological studies with this compound.

A sharp distinction was made by Lipmann (122) between oxidation and decarboxylation in *L. delbrückii*. These bacteria oxidize pyruvate readily aerobically, but have little action on pyruvate anaerobically. The oxidation is conceived as a dehydrogenation of a postulated pyruvic phosphate followed by carbon dioxide elimination with formation of acetyl phosphate (reaction *A*), rather than a decarboxylation followed by oxidation (oxidative decarboxylation) (reaction *B*).

A. By pyruvic dehydrogenase:



B. By carboxylase and oxidation:

$$CH_3COCOOH \xrightarrow[-CO_2]{(O)} CH_3COOH + CO_2$$

It follows from the concept of pyruvic dehydrogenation that the enzyme involved (pyruvic dehydrogenase) would have thiamin as the prosthetic group, and further that thiamin should undergo a reversible oxidation-reduction. The latter possibility has been studied, but without positive results.

The extension of this picture to the oxidation of pyruvate in animal tissue is at present a little tenuous. "Pyruvic dehydrogenase" has not been sufficiently isolated to be assured that it is a single protein, and acetyl phosphate formation has not yet been demonstrated in animal tissues. On the other hand, a carboxylase, although its significance is not clear, has been demonstrated in animal tissue (79).

Martius (142) has proposed an interesting hypothesis to account for anaerobic and aerobic decarboxylation. In the presence of carboxylase, pyruvate yields the hypothetical radical (CH_2—C=O), carbon dioxide and available hydrogen:

$$CH_3COCOOH \longrightarrow \underset{|\quad|}{CH_2—C=O} + (2\,H) + CO_2$$

(*a*) In simple decarboxylation, the radical is simply stabilized by the H to form free acetaldehyde:

$$\underset{|\quad|}{CH_2—C=O} + 2\,H \longrightarrow CH_3CHO$$

(*b*) With oxygen as acceptor for the hydrogen (flavin necessary?) and addition of water, acetic acid is formed:

$$\underset{|\quad|}{CH_2—C=O} + H_2O \longrightarrow CH_3COOH$$

Anaerobically, pyruvate might act as the hydrogen acceptor to form lactate (dismutation).

(*c*) In the presence of acetaldehyde, biacetyl would be formed;

$$\underset{|\quad|}{CH_2—C=O} + CH_3CHO \longrightarrow \underset{\text{Biacetyl}}{CH_3COCOCH_3}$$

and reduced to acetoin:

$$\underset{\text{Biacetyl}}{CH_3COCOCH_3} + 2\,H \longrightarrow \underset{\text{Acetoin}}{CH_3CHOHCOCH_3}$$

It is further suggested that this radical might actually be in combination, perhaps as an imide linkage, with thiamine. It must be pointed out that Martius' hypothesis has little experimental support, but should stimulate further work on the mechanism of oxidative decarboxylation. Weil-Malherbe's hypothesis (208), closely related to this, provides for oxidation and reduction of thiamin as a thiamin–pyruvate Schiff base, and accounts both for a simple decarboxylation and an oxidative one. An acetyl derivative of diphosphothiamin is suggested as one of the intermediates in the oxidation of pyruvate.

Acetylation.—Pyruvate seems to be a primary source of acetyl groups for the acetylation of choline (139) and sulfanilamide (141). There is a bulk of evidence that acetate is directly involved as the source of acetyl groups (10, 11, 12, 98). The failure to find an increased acetylation by acetate while demonstrating a positive effect with pyruvate (141) or acetoin (48) leaves open the possibility, however, that a substance other than acetate itself may be the direct agent. In support of this is the work of Fishman and Cohn (68), who concluded that the direct acetylating agent should exchange deuterium readily with that of the body water, since deuterium was found in the acetylated sulfanilamide and *p*-aminobenzoic acid excreted after administration of heavy water (D_2O) with these substances. Pyruvate or acetate themselves did not appear to exchange readily.

The feeding of phenylaminobutyric acid with deuterio derivatives of acetate, ethyl alcohol, butyric acid, alanine, valeric acid and myristic acid all resulted in the appearance of deuterium in the acetyl group of the excreted acetylphenylaminobutyric acid (12).

5. Acetate Metabolism

The disappearance of acetate added to most animal tissues appears quite slow, only kidney showing a rate of acetate removal commensurate with a principal pathway of pyruvate metabolism. Acetate fed to rats is rapidly metabolized, however (20). It seems anomalous that the tissues which can form acetate from pyruvate cannot readily metabolize it. It seems not improbable that the acetate arising naturally during metabolism is in a reactive form which decomposes during the usual methods of identification of acetate. Acetyl phosphate (121), acetyldiphosphothiamine, biacetyl and Martius' $CH_2{-}C{=}O$ radical are among the possibilities of an "active" acetate.

Acetate to Acetoacetate.—Most of the available evidence indicates

that acetate is converted principally to acetoacetate. Edson (50) and Jowett and Quastel (89) have demonstrated the formation of acetoacetate and β-hydroxybutyrate from acetate in liver tissue. The tracer studies of Swendseid and coworkers (191) leave little doubt that this conversion takes place. The enzyme described by Lehninger (113), which was demonstrated to convert acetoacetate to acetate, may also be of significance in acetoacetate formation. Kleinzeller (99), from studies of acetate disappearance in kidney slices and homogenates, concluded that acetate undergoes condensation, and that dicarboxylic acids appear to be involved since malonate inhibited acetate oxidation. Wieland and Rosenthal attribute the rapid disappearance of acetate from kidney tissue (215) to condensation to acetoacetate, followed by further condensation with oxalacetate to form citric acid (216). (The latter mechanism is discussed under "Relations with Fat Metabolism" on pages 156–157.) Lynen (135) finds that acetic acid is metabolized in yeast by way of the tricarboxylic acids, involving condensation of acetate with oxalacetate. Krebs and Johnston (104) also concluded that acetoacetate was formed from acetate, but by condensation with pyruvate to form acetopyruvic acid. Lehninger (112) has found that acetopyruvic acid is ketogenic.

Acetate to Succinate.—Thunberg (195) suggested in 1920 that succinate might be formed by the dehydrogenation of two molecules of acetate. There was little adequate proof for this reaction until the recent work of Slade and Werkman (176). These investigators, employing C^{13} as a tracer, have proved that cell suspensions of *Aerobacter indologenes* can perform this reaction. Some 13% of the acetate added was transformed to succinate by this reaction.

6. Carbon Dioxide Fixation

As early as 1938, Wood and Werkman (218) noted carbon dioxide utilization during glycerol fermentation in propionic acid bacteria, and equimolar formation of succinate. It was clearly formulated that a C_3 intermediate might unite with carbon dioxide to form oxalacetate, which could be reduced to succinate. Krebs and Eggleston (106), in noting the formation of α-ketoglutarate, fumarate, malate, succinate and citrate (and in the same proportions) from pyruvate and oxalacetate, also postulated an interchange of the latter two substances. The enhancing effect of carbon dioxide in the production of α-ketoglutarate led them to the belief that pyruvate might be first converted to oxalacetate:

$$\underset{\text{(pyruvic)}}{CH_3COCOOH} + CO_2 \longrightarrow \underset{\text{(oxalacetic)}}{HOOCCOCH_2COOH}$$

Carbon dioxide fixation has now been thoroughly established, particularly by the isotope experiments of Wood and coworkers (219, 220) and of Evans and Slotin (65). Both working with liver tissue, the former investigators located the isotopic carbon of the assimilated carbon dioxide in malate and fumarate, and the latter workers, concentrating more on the role of oxalacetate in the citric cycle, established the presence of radioactive carbon in α-ketoglutarate.

It has been difficult to produce the final proof of oxalacetate as the first product of carbon dioxide assimilation by pyruvate. Oxalacetate seems to be more actively metabolized than pyruvate, and is only present in trace quantities. Krampitz and Werkman (101) have demonstrated the enzymatic reversal of this reaction, the β-decarboxylation of oxalacetate, by *Micrococcus lysodeikticus*. Evans and coworkers (66) have also studied the enzymatic decarboxylation of oxalacetate in pig liver. Most recently, Kalnitsky and Werkman (95) have been able to detect the anaerobic formation of small quantities of oxalacetate from pyruvate and carbon dioxide by an enzyme preparation from *E. coli*. Two different methods (Straub's hydrazone–nitrous acid color reaction and the aniline citrate method) were employed to identify the oxalacetate, but the quantities formed were too small either for more positive identification or for isotope analysis. The reader is referred to the review of Werkman and Wood (211) for greater details of the carbon dioxide fixation reaction.

Since the ultimate goal in carbohydrate oxidation is in the energy gained consequent to successive losses of carbon dioxide, the existence of a carbon dioxide fixation reaction must have a special metabolic significance. It now seems clear that this reaction provides for the synthesis of intermediates which play a catalytic role in catalytic "cycles" and which are involved in the resynthesis of carbohydrates. It was early indicated (52) that the C_4 dicarboxylic acids were involved in the synthesis of glycogen from pyruvate, and later that phosphopyruvate originated from these substances by oxidative pathways. Kalckar (90) has demonstrated the formation of phosphopyruvate in the oxidation of malate by kidney extracts, presumably by way of a phospho (enol) oxalacetate, and Epshein has reported the synthesis of phosphopyruvic acid in muscle during the oxidation of citric acid (61). That pyruvate must become involved in condensation reactions depending on diphosphothiamin before synthesis to glycogen has been shown by Barrón and Lyman (6) in experiments with kidney slices of thiamin-deficient rats. Even more direct proof is supplied by

Buchanan, Hastings and Nesbett (21), who showed with radioactive carbon that the synthesis of glycogen from pyruvate by rabbit liver slices involved fixation of carbon dioxide.

Of all the mechanisms outlined for the metabolism of pyruvate, the carbon dioxide fixation reaction has the strongest support of physiological experiments. Employing lactate as a source of pyruvate, Hastings and coworkers (40) have shown, in rats, that injected $C^{11}O_2$ (bicarbonate) is assimilated and occurs in the liver glycogen. Employing carboxyl-labeled

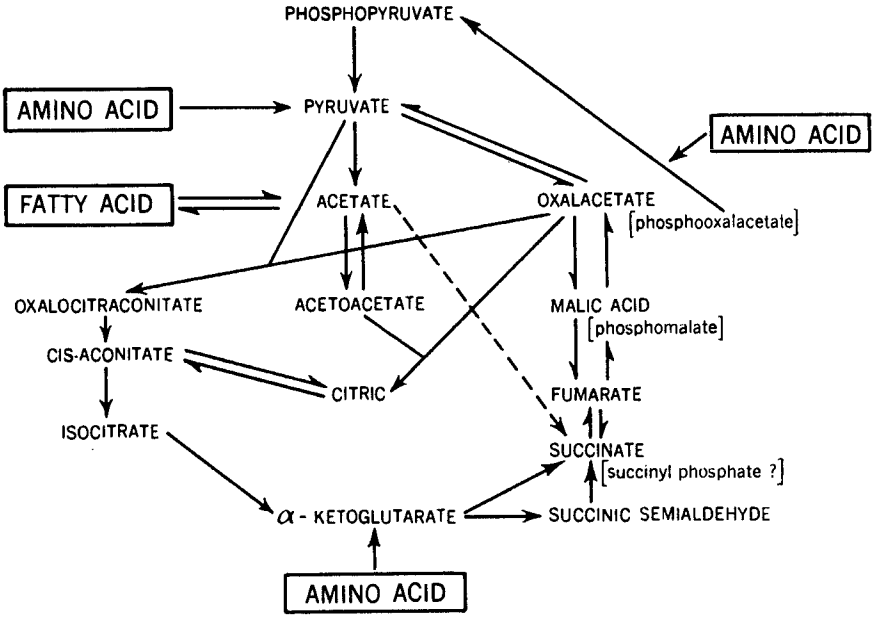


Fig. 2.—The metabolic pool and relations with protein and fat.

lactic acid, it was found that the liver glycogen averaged over 30% of the lactate fed, but contained only 1.6% of the radioactivity (178). A theory was formulated that fixation of carbon dioxide occurred to form oxalacetate and other C_4 dicarboxylic acids, from which a phosphorylated intermediate (phosphooxalacetate?) was formed. The latter yields phosphopyruvate for the synthesis of glycogen. Administration of lactate with α- or β-labeled carbon resulted in glycogen containing approximately twice the radioactivity as when carboxyl-labeled lactic acid was fed (199). Due to the symmetry of the succinate and fumarate molecule, the C^{11} of carboxyl labeled lactic acid would be half lost in decarboxylation to phosphopyru-

vate, while that in α or β carbon positions would be completely retained. Largely from these studies has developed the concept of a "metabolic pool" into which may drain derivatives of carbohydrate, fat and protein metabolism, and from which may be drawn substances for resynthesis, while the major outlet is complete oxidation for energy needs.

Of the recognized pathways of pyruvate catabolism, we are still very ignorant of the relative importance of these in any given tissue, or for that matter in any given species. One of the few reports from which such deductions might be made is that of Buchanan, Hastings and Nesbett (20). Although the radioactive carbon of lactate and pyruvate (21, 178) can be traced to glycogen, that in acetate was not recovered in this form. If acetate were a major product of pyruvate catabolism, it should also yield liver glycogen.

7. *Citric Acid Cycle*

A detailed discussion of the "citric acid cycle" has been presented by Krebs (107) and will not be attempted here. Citrate, *cis*-aconitate, isocitrate, α-ketoglutarate, succinate, fumarate, malate and oxalacetate, members of either the "citric acid cycle" or the "C_4 dicarboxylic acid cycle," have all been reported to increase the respiration of minced muscle, although the accelerating effect of citrate itself has been challenged (18, 183, 194). The formation of α-ketoglutarate from pyruvate has been adequately proved by Evans and Slotin (65); and these experiments have been extended by Wood and coworkers (220) to demonstrate the transfer of radioactive carbon dioxide to several intermediates of the cycle.

Since the α-ketoglutarate synthesized from pyruvate in the presence of radioactive carbon dioxide contains the isotope only in the carboxyl group adjacent to the keto group, citrate (a symmetrical molecule) cannot be the direct product of pyruvate and oxalacetate condensation (65). Wood and coworkers (220) have offered a mechanism most consonant with the facts by postulating oxalocitraconitate as the primary condensation product, followed by conversion to *cis*-aconitate. Breusch (19) and Wieland and Rosenthal (216) attach a new significance to citric acid as a product of acetoacetate condensation with oxalacetate (see pages 156–157). The enzyme, aconitase, widely distributed in animal tissues, causes a reversible hydration of *cis*-aconitate to citrate or isocitrate. Isocitric dehydrogenase (1) is responsible for conversion of isocitrate to α-ketoglutarate.

Ochoa (163) has studied the properties of α-ketoglutarate dehydrogenase present in a cell-free suspension of washed heart muscle. An earlier study of α-ketoglutarate oxidation by Barrón and coworkers (7) indicates that

the enzyme contains diphosphothiamine. It is now reported that the activity of the enzyme is coupled with phosphorylation since inorganic phosphate, adenylic acid and magnesium are required. The enzyme or enzyme complex described by Ochoa appears to be distinct from the carboxylase of Green and coworkers (79) because both anaerobic decarboxylation of α-ketoglutarate and oxidation of succinic semialdehyde were slower with this preparation than α-ketoglutarate oxidation. Oxidation was limited to the succinate stage by the use of malonate. It was indicated that succinyl phosphate might be the primary reaction product since it was rapidly dephosphorylated by the enzyme system.

Formation of energy-rich phosphate with the oxidation of α-ketoglutarate to succinate (163), succinate to fumarate (37) and malate to oxalacetate (151) has been demonstrated.

IV. Relationships of Carbohydrate, Protein and Fat Metabolism

1. Conversion of Amino Acids

The amino acids, alanine, aspartic acid and glutamic acid, yield pyruvate, oxalacetate and α-ketoglutarate, respectively, as direct products of oxidative deamination; others yield these keto acids indirectly. Green and coworkers (80) have prepared an *l*-amino acid oxidase from rat liver and kidney which catalyzes the oxidative deamination of several natural amino acids to the corresponding keto acids. The enzyme preparation was free of *d*-amino acid oxidase.

It has been pointed out that some 40–50% of the casein molecule is composed of amino acids (glucogenic) which may yield the keto acids common to carbohydrate metabolism. In reverse, carbohydrate yields the α-keto acids required for the synthesis of the nonessential amino acids.

Transamination.—Transamination is the reaction by which an amino group of an α-amino acid may be transferred to a keto acid to form the corresponding amino and keto acids:

$$R_1\text{—}\underset{\displaystyle NH_2}{\underset{|}{CH}}\text{—}COOH + R_2\text{—}\underset{\displaystyle O}{\overset{\displaystyle O}{\overset{\|}{C}}}\text{—}COOH \rightleftharpoons R_1\text{—}\underset{\displaystyle O}{\overset{\displaystyle O}{\overset{\|}{C}}}\text{—}COOH + R_2\text{—}\underset{\displaystyle NH_2}{\underset{|}{CH}}\text{—}COOH$$

Of a long series of amino and keto acids tested which might engage in this reaction, Cohen (34) found that *l*-aspartic acid, *l*-alanine, oxalacetic acid and pyruvic acid were most active in pigeon breast muscle. The enzyme "transaminase" from both pig heart and pigeon breast muscle has been studied by Cohen (35). Although not extensively purified, this enzyme appears not to be dependent on diphosphopyridine nucleotide, on di-

phosphothiamin or in fact on any heat-stable factors (64). Transaminase was most active with the systems glutamate–oxalacetate and α-ketoglutarate–aspartate. According to Kritzman (109), the transaminating power of pigeon breast muscle and pig heart is due to at least two enzymes, one concerned with glutamic acid, termed glutamic aminopherase, and the other with aspartic acid, aspartic aminopherase. A coenzyme present in muscle extracts is claimed for both enzymes by this investigator; and that for aspartic aminopherase has been further purified (17) but not yet identified.

Although a rapid transamination would appear to be restricted to relatively few combinations of amino and keto acids, Braunstein and coworkers (15, 16) have demonstrated a catalytic influence of glutamate or α-ketoglutarate which might extend the power of transferring amino groups to several other amino acids. For example, in pigeon breast muscle there was no reaction between lysine and pyruvate, but with a trace of added glutamate the reaction proceeded rapidly. In this reaction, the glutamate is thought to be converted to α-ketoglutarate by pyruvate and regenerated by lysine.

The exact role of transamination in tissue metabolism is yet uncertain, but it may be significant that the keto acids principally involved in the reaction are those already established as intermediates in carbohydrate metabolism. Since glutamic dehydrogenase depends on both di- and triphosphopyridine nucleotides, glutamate might couple with other systems dependent on these coenzymes.

For further details on the transamination reaction, the reader is referred to the reviews of Cohen (36) and Herbst (83).

2. *Relations with Fat Metabolism*

Catabolism of Fat.—There has been little proof for the transformation of fat to carbohydrate in the intact animal, but ample evidence that metabolic products of fat are common with those also recognized as products of carbohydrate metabolism.

Davies (45) has described an enzyme from *Clostridium acetobutylicum* which specifically catalyzes the decarboxylation of acetoacetate to acetone; but even if this enzyme should be demonstrated in animal tissue it seems unlikely that such a step would have any significance other than in a detoxification sense. The fate of the ketone bodies, acetoacetate and β-hydroxybutyrate, is rather obscure, although it is recognized that these substances can be rapidly metabolized in a variety of tissues (69, 207). The enzyme of muscle described by Lehninger (113), which catalyzes the reaction, acetoacetate → acetate, is of particular interest in this connection.

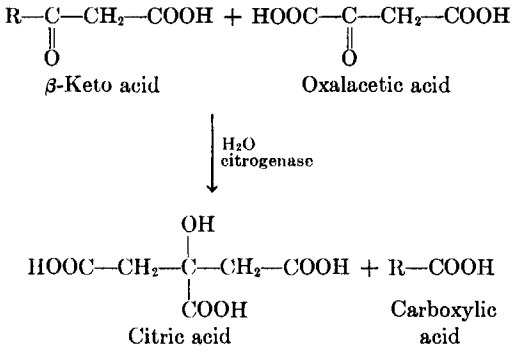
As previously discussed (see pages 149–150), however, the probable pathway of acetate metabolism is conversion to acetoacetate. This enzyme may therefore be most significant in its reverse reaction, the formation of acetoacetate from acetate.

The extensive formation of free acetic acid residues in fat catabolism has been definitely contraindicated by the reports of Weinhouse, Medes and Floyd (209). These investigators incubated *n*-octanoic acid containing carboxyl C^{13} with rat liver slices. The resulting acetoacetate was decomposed to acetone and carbon dioxide, and their C^{13} content measured to determine the distribution of C^{13} between carbonyl and carboxyl carbons. Starting with C^{13} only in the carboxyl group of octanoic acid, the resulting acetoacetate should contain no C^{13} according to the classical β-oxidation theory. The multiple alternate oxidation theory, which calls for the direct splitting into four-carbon units, should yield acetoacetate with C^{13} only in the carboxyl group. Finally, if two-carbon units were removed by β-oxidation and these condensed at random to the ketone bodies, the isotope should be evenly distributed between carbonyl and carboxyl carbons of the acetoacetate, but none should occur in the methyl carbons. The demonstrated presence of C^{13} in both the acetone (found restricted to the carbonyl carbon) and carbon dioxide residues of acetoacetate decarboxylation clearly indicate the latter mechanism. The β-oxidation condensation theory also gains earlier support from studies on the five-carbon fatty acid, valeric acid. This acid apparently donates two carbons for acetoacetate formation and the remaining three for carbohydrate synthesis (136).

It is not clear whether the intermediate two-carbon unit released by β-oxidation previous to condensation is free acetic acid. The latter is known to give rise to ketone bodies both *in vitro* (50, 89, 115) and *in vivo* (137); and the occurrence of isotopic carbon in acetoacetate after administration of carboxyl-labeled acetate (191) leaves little doubt that a direct synthesis can occur. The possibility that a more active two-carbon derivative is the actual condensing residue is certainly not excluded.

The clearest concept in understanding the further metabolism of acetoacetate has been presented by Wieland and Rosenthal (216) and by Breusch (19). The former investigators demonstrated an aerobic formation of citric acid from oxalacetate and acetoacetate in kidney tissue. In the presence of barium or magnesium ions, as much as 80% of the acetoacetate added was accounted for as citrate. The same reaction was found in heart, but not in liver. Indeed, Breusch believes citric acid to be more important as a product of acetoacetate metabolism than of pyruvate condensation with oxalacetate. He has designated an enzyme, "citrogenase," which cata-

lyzes the reaction between a β-keto acid and oxalacetate to form citric acid and a carboxylic acid containing two carbon atoms less than the original β-keto acid:



These conclusions were reached from a demonstration of an increased rate of acetoacetate removal by oxalacetate, and the greater yield of citrate obtained from a combination of these substances than from either alone. The action of citrogenase was extended to other β-keto acids containing up to ten carbon atoms. The distribution of the enzyme (large amounts in muscle, kidney and brain, but small amount in liver) harmonizes with the *in vivo* experiments of Snapper and Grünbaum (177), who noted a rapid utilization of acetoacetate on perfusing muscle, kidney and brain, but a slow utilization by liver. Citrogenase is sensitive to selenic and arsenic acids, but not to iodoacetate.

The acetoacetate-oxalacetate condensation has already been challenged in a note from Krebs' laboratory (108). Although Breusch's results were confirmed, it is stated that the extra acetoacetate which disappears upon addition of oxalacetate can be recovered as β-hydroxybutyric acid. The latter is believed to be formed by oxidoreduction of the acetoacetate with malic acid or by reaction with α-ketoglutarate to form succinic acid and carbon dioxide.

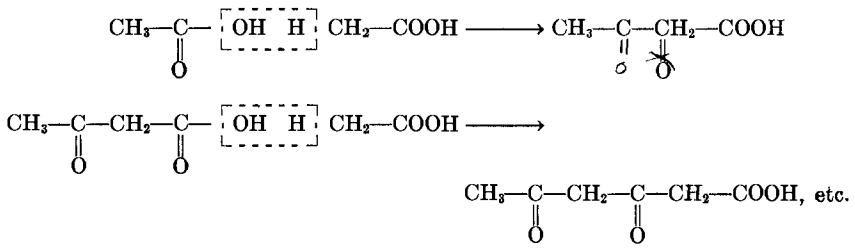
According to Breusch, oxalacetate thus becomes the meeting place of fat and carbohydrate oxidation. It accepts —CH_2COOH from β-keto fatty acids (to form citrate) or H from sugar derivatives (to form malate). The higher concentration of oxalacetate stated to be necessary for condensation with β-keto acids than for reduction by "sugar hydrogen" regulates the preferential oxidation of carbohydrate. A relative absence of carbohydrate oxidation with decreased pyruvate and consequently less oxalacetate (pyruvate + carbon dioxide) would thus lead to accumulation of keto acids.

This picture is in close harmony with the present concept of "competitive" utilization of carbohydrate and fat and of antiketogenesis (51, 182).

Anabolism of Fat.—The synthesis of fats from carbohydrates, thoroughly established *in vivo*, is completely lacking in details from work *in vitro*. The exclusive occurrence in nature of fatty acids with an even number of carbon atoms has long supported the belief that two-carbon carbohydrate residues represent the starting materials for the synthesis of fatty acids. Condensation of acetaldehyde units of the aldol type has been suggested as a mechanism as well as condensation of acetaldehyde with pyruvate and subsequent loss of carbon dioxide. A most significant advance in this problem is the work of Rittenberg and Bloch (170), which clearly demonstrated the synthesis of fat from acetic acid in the rat. Sodium acetate, containing both 19 atom per cent excess of C^{13} in the carboxyl group and a 77 atom per cent excess of deuterium in the methyl group, was fed to rats for a period of eight days. The liver fatty acids were separated and the saturated fatty acids isolated. In addition to analysis of the fatty acid for C^{13} and D, it was decarboxylated to determine the C^{13} in the carboxyl group. Isotopic carbon was found in the carboxyl of the fatty acid and in approximately twice the concentration as in the whole molecule. These results indicate that the fatty acid was synthesized by successive condensation of C_2 units. Utilization of the CH_3 as well as the COOH group must have occurred, since the fatty acid had 1.5 to 4.5 times the deuterium concentration of the body fluids. This study therefore indicates the general reaction:

$$CD_3—C^{13}OOH \longrightarrow CD_3—C^{13}H_2—CD_2—C^{13}H_2C\cdots D_2—C^{13}OOH$$

Again, these results cannot prove whether the acetate molecule or a more active derivative is immediately involved in the condensation. In view of these findings and in conjunction with the now well-established synthesis of acetoacetate from acetate (see pages 149–150), it may be pertinent to consider the possibility of reversal of the β-oxidation mechanism in the synthesis of fats:



Subsequent or simultaneous reduction of the keto groups and removal of water would result in unsaturated fatty acids. Hilditch (85) has emphasized that theories of fat synthesis should account not only for the occurrence of an even number of carbon atoms and the unsaturation in naturally occurring fatty acids, but also for the quantitatively important oleic acid with its centrally located double bond.

There are thus several intermediates common to carbohydrate, protein and fat metabolism. Although pyruvate has had particular attention by virtue of being an experimental stopping point in carbohydrate breakdown, it is evident that other substances such as acetate, oxalacetate and α-ketoglutarate are equally important members of the pool draining the metabolic flow of the principal food stuffs. Details of the interconversion within this metabolic pool await identification of the enzymes involved; and, to aid in distinguishing possibilities from actualities, a close correlation of such studies must be made with the fate of the intermediates as revealed by *in vivo* studies.

Bibliography

1. Adler, E., von Euler, H., Günther, G., and Plass, M., *Biochem. J.*, **33**, 1028 (1939).
2. Ashford, C. A., *ibid.*, **28**, 2229 (1934).
3. Baer, E., and Fischer, H. O. L., *J. Biol. Chem.*, **150**, 213 (1943).
4. Bailey, K., *Biochem. J.*, **36**, 121 (1942).
5. Barker, S. B., Shorr, E., and Malam, M., *J. Biol. Chem.*, **129**, 33 (1939).
6. Barrón, E. S. G., and Lyman, C. M., *ibid.*, **127**, 143 (1938).
7. Barrón, E. S. G., Goldinger, J. M., Lipton, M. A., and Lyman, C. M., *ibid.*, **141**, 975 (1941).
8. Barrón, E. S. G., in *Advances in Enzymology*, Vol. III. Interscience, New York, 1943, page 149.
9. Beck, L. V., *Proc. Soc. Exptl. Biol. Med.*, **49**, 435 (1942).
10. Bernhard, K., *Z. physiol. Chem.*, **267**, 99 (1940).
11. Bernhard, K., and Steinhauser, H., *ibid.*, **273**, 31 (1942).
12. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **155**, 243 (1944).
13. Bollman, J. L., and Flock, E. V., *ibid.*, **147**, 155 (1943).
14. Boyer, P. D., Lardy, H. A., and Phillips, P. H., *ibid.*, **146**, 673 (1942); **149**, 529 (1943).
15. Braunstein, A. E., *Enzymologia*, **7**, 25 (1939).
*16. Braunstein, A. E., and Kritzman, M. G., *Biokhimiya*, **4**, 168 (1939).
*17. Braunstein, A. E., and Kritzman, M. G., *ibid.*, **8**, 1 (1943).
18. Breusch, F. L., *Biochem. J.*, **33**, 1757 (1939).
19. Breusch, F. L., *Science*, **97**, 490 (1943).
20. Buchanan, J. M., Hastings, A. B., and Nesbett, F. B., *J. Biol. Chem.*, **150**, 413 (1943).
21. Buchanan, J. M., Hastings, A. B., and Nesbett, F. B., *J. Biol. Chem.*, **145**, 715 (1944).

* Original reference not available.

22. Bueding, E., and Wortis, H., *ibid.*, **133,** 585 (1940).
23. Bueding, E., and Goodhart, R., *ibid.*, **141,** 931 (1941).
24. Bueding, E., Stein, M. H., and Wortis, H., *ibid.*, **140,** 697 (1941).
25. Bueding, E., Fazekas, J. F., Herrlich, H., and Himwich, H. E., *Science,* **95,** 282 (1942); *J. Biol. Chem.,* **148,** 97 (1943).
26. Bueding, E., Wortis, H., and Fein, H. D., *Am. J. Med. Sci.,* **204,** 838 (1942).
27. Bueding, E., Wortis, H., Stern, M., and Esteronne, D., *J. Clin. Investigation,* **21,** 85 (1942).
28. Bueding, E., and Goldfarb, W., *J. Biol. Chem.,* **147,** 33 (1943).
29. Burk, D., *Cold Spring Harbor Symposia Quant. Biol.,* **7,** 420 (1939).
30. Cajori, F. A., *J. Biol. Chem.,* **143,** 357 (1942).
31. Case, E. M., *Biochem. J.,* **26,** 753 (1932).
32. Clark, B. B., Morrissey, R. W., Fazekas, J. F., and Welch, C. S., *Quart. J. Studies Alc.,* **1,** 663 (1941).
33. Clift, F. P., and Cook, R. P., *Biochem. J.,* **26,** 1788 (1932).
34. Cohen, P. P., *ibid.,* **33,** 1478 (1939).
35. Cohen, P. P., *J. Biol. Chem.,* **136,** 565, 585 (1940).
36. Cohen, P. P., in *A Symposium on Respiratory Enzymes.* Univ. Wisconsin Press, Madison, 1942, p. 210.
37. Colowick, S. P., Kalckar, H. M., and Cori, C. F., *J. Biol. Chem.,* **137,** 343 (1941).
38. Colowick, S. P., and Sutherland, E. W., *ibid.,* **144,** 423 (1942).
39. Colowick, S. P., and Kalckar, H. M., *ibid.,* **148,** 117 (1943).
40. Conant, J. B., Cramer, R. D., Hastings, A. B., Klemperer, F. W., Solomon, A. K., and Vennesland, B., *ibid.,* **137,** 557 (1941).
41. Cori, C. F., Cori, G. T., and Green, A. A., *ibid.,* **151,** 39 (1943).
42. Cori, G. T., Colowick, S. P., and Cori, C. F., *ibid.,* **123,** 375 (1938).
43. Cori, G. T., Colowick, S. P., and Cori, C. F., *ibid.,* **127,** 771 (1939).
44. Cori, G. T., and Cori, C. F., *ibid.,* **151,** 57 (1943).
45. Davies, R., *Biochem. J.,* **37,** 230 (1943).
46. Dickens, F., *ibid.,* **32,** 1626, 1645 (1938).
47. Dische, Z., and Robbins, S. S., *Biochem. Z.,* **271,** 304 (1934).
48. Doisy, E. A., Jr., and Westerfeld, W. W., *J. Biol. Chem.,* **149,** 229 (1943).
49. Dorfman, A., Berkman, S., and Koser, S. A., *ibid.,* **144,** 393 (1942).
50. Edson, N. L., *Biochem. J.,* **29,** 2082 (1935).
51. Edson, N. L., *ibid.,* **30,** 1862 (1936).
52. Elliott, K. A. C., Benoy, M. B., and Baker, Z., *ibid.,* **29,** 1936 (1935).
53. Elliott, K. A. C., Greig, M. E., and Benoy, M. B., *ibid.,* **31,** 1003 (1937).
54. Embden, G., and Haymann, C., *Z. physiol. Chem.,* **137,** 154 (1924).
55. Embden, G., and Deuticke, H. J., *ibid.,* **230,** 50 (1934).
56. Emerson, R. L., Stauffer, J. F., and Umbreit, W. W., *Am. J. Botany,* **31,** 107 (1944).
*57. Engelhardt, W. A., and Barchash, A. P., *Biokhimiya,* **3,** 500 (1938).
58. Engelhardt, W. A., and Lyubimova, M. N., *Nature,* **144,** 668 (1939).
*59. Engelhardt, W. A., and Lyubimova, M. N., *Biokhimiya,* **7,** 205 (1942).
*60. Engelhardt, W. A., and Sakov, N. E., *ibid.,* **8,** 9 (1943).
*61. Epshein, S. F., *Biochem. J. (Ukraine),* **17,** 139 (1941).
62. von Euler, H., and Adler, E., *Z. physiol. Chem.,* **226,** 195 (1934).
63. von Euler, H., and Adler, E., *Z. ibid.,* **235,** 122 (1935).

64. von Euler, H., Adler, E., Günther, G., and Das, N. B., *Z. physiol. Chem.*, **254**, 61 (1938).
65. Evans, E. A., Jr., and Slotin, L., *J. Biol. Chem.*, **136**, 301 (1940).
66. Evans, A. E., Jr., Vennesland, B., and Slotin, L., *ibid.*, **147**, 771 (1943).
67. Fazekas, J. F., and Himwich, H. E., *ibid.*, **139**, 971 (1941).
68. Fishman, W. H., and Cohn, M., *ibid.*, **148**, 619 (1943).
69. Friedemann, T. E., *ibid.*, **116**, 133 (1936).
70. Friedemann, T. E., and Barborka, C. J., *ibid.*, **141**, 993 (1941).
71. Friedemann, T. E., and Haugen, G. E., *ibid.*, **144**, 67 (1942).
72. Friedemann, T. E., and Haugen, G. E., *ibid.*, **147**, 415 (1943).
73. Fromageot, C., and Desnuelle, P., *Biochem. Z.*, **279**, 174 (1935).
74. Furchgott, R. F., and Shorr, E., *J. Biol. Chem.*, **151**, 65 (1943).
75. Goldfarb, W., Bowman, K. M., Parker, S., and Krautman, B., *J. Clin. Investigation*, **18**, 581 (1939).
76. Gorr, G., *Biochem. Z.*, **254**, 12 (1932).
77. Green, A. A., and Cori, G. T., *J. Biol. Chem.*, **151**, 21 (1943).
78. Green, D. E., and Brosteaux, J., *Biochem. J.*, **30**, 1489 (1936).
79. Green, D. E., Westerfeld, W. W., Vennesland, B., and Knox, W. E., *J. Biol. Chem.*, **140**, 683 (1941).
80. Green, D. E., Nocito, V., and Ratner, S., *ibid.*, **148**, 461 (1943).
81. Greenberg, L. A., *Quart. J. Studies Alc.*, **3**, 347 (1942).
82. Herbert, D., Gordon, H., Subrahmanyan, V., and Green, D. E., *Biochem. J.*, **34**, 1108 (1940).
83. Herbst, R. M., in *Advances in Enzymology*, Vol. IV. Interscience, New York, 1944, p. 75.
84. Hevesy, G., and Rebbe, O., *Acta Physiol. Scand.*, **1**, 171 (1940).
85. Hilditch, T. P., *The Chemical Constitution of Natural Fats*. Wiley, New York, 1941, p. 281.
86. Hill, A.V., and Long, C. N. H., *Ergeb. Physiol.*, **24**, 43 (1925).
87. Hirsch, J., *Biochem. Z.*, **134**, 415 (1922).
88. Houssay, B. A., and Biasotti, A., *Endocrinology*, **15**, 511 (1931).
89. Jowett, M., and Quastel, J. H., *Biochem. J.*, **29**, 2159 (1935).
90. Kalckar, H. M., *ibid.*, **33**, 631 (1939).
91. Kalckar, H. M., *Chem. Revs.*, **28**, 71 (1941).
92. Kalckar, H. M., *J. Biol. Chem.*, **148**, 127 (1943).
93. Kalckar, H. M., Dehlinger, J., and Mehler, A., *ibid.*, **154**, 275 (1944).
94. Kalnitsky, G., and Werkman, C. H., *Arch. Biochem.*, **2**, 113 (1943).
95. Kalnitsky, G., and Werkman, C. H., *ibid.*, **4**, 25 (1944).
96. Kaplan, N., and Greenberg, D. M., *J. Biol. Chem.*, **150**, 479 (1943).
97. Klein, D., *ibid.*, **145**, 35 (1942).
98. Klein, J. R., and Harris, J. S., *ibid.*, **124**, 613 (1938).
99. Kleinzeller, A., *Biochem. J.*, **37**, 674 (1943).
100. Koepsell, H. J., and Johnson, M. J., *J. Biol. Chem.*, **145**, 379 (1942).
101. Krampitz, L. O., and Werkman, C. H., *Biochem. J.*, **35**, 595 (1941).
102. Krebs, H. A., and Eggleston, L. V., *ibid.*, **31**, 299 (1937).
103. Krebs, H. A., and Johnson, W. A., *ibid.*, **31**, 645 (1937).
104. Krebs, H. A., and Johnson, W. A., *ibid.*, **31**, 772 (1937).
105. Krebs, H. A., *ibid.*, **31**, 2095 (1937).

106. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, **34**, 1380 (1940).
107. Krebs, H. A., in *Advances in Enzymology*, Vol. III. Interscience, New York, 1943, p. 191.
108. Krebs, H. A., and Eggleston, L. V., *Nature*, **154**, 209 (1944).
109. Kritzman, M. G., *ibid.*, **143**, 603 (1939).
110. Kubowitz, F., and Ott, P., *Biochem. Z.*, **314**, 94 (1943).
111. Lehmann, H., *ibid.*, **281**, 271 (1933).
112. Lehninger, A. L., *J. Biol. Chem.*, **143**, 147 (1942).
113. Lehninger, A. L., *ibid.*, **148**, 393 (1943).
114. Leloir, L. F., and Muñoz, J. M., *Biochem. J.*, **32**, 299 (1938).
115. Leloir, L. F., and Muñoz, J. M., *ibid.*, **33**, 734 (1939).
116. Levine, R., Feinstein, R. N., and Soskin, S., *Federation Proc.*, **1**, 50 (1942).
117. Lipmann, F., *Biochem. Z.*, **206**, 171 (1929).
118. Lipmann, F., *Nature*, **138**, 588 (1936).
119. Lipmann, F., *Skand. Arch. Physiol.*, **76**, 255 (1937).
120. Lipmann, F., *Nature*, **143**, 436 (1939).
121. Lipmann, F., *ibid.*, **144**, 381 (1939).
122. Lipmann, F., *Cold Spring Harbor Symposia Quant. Biol.*, **7**, 248 (1939).
123. Lipmann, F., in *Advances in Enzymology*, Vol. I. Interscience, New York, 1941, p. 99.
124. Lipmann, F., *Federation Proc.*, **1**, 122 (1942).
125. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **153**, 571 (1944).
126. Lipmann, F., and Tuttle, L. C., *ibid.*, **154**, 725 (1944).
127. Lohmann, K., *Biochem. Z.*, **222**, 324 (1930).
128. Lohmann, K., *ibid.*, **262**, 137 (1933).
129. Lohmann, K., and Meyerhof, O., *ibid.*, **273**, 60 (1934).
130. Long, C. N. H., *J. Physiol.*, **58**, 455 (1924).
131. Long, C., and Peters, R. A., *Biochem. J.*, **33**, 759 (1939).
132. Lu, G. D., *ibid.*, **33**, 774 (1939).
133. Lundsgaard, E., *Biochem. Z.*, **161**, 219 (1925).
134. Lundsgaard, E., *ibid.*, **264**, 209 (1933).
135. Lynen, F., *Ann.*, **552**, 270 (1942).
136. Mackay, E. M., Wick, A. N., and Barnum, C. P., *J. Biol. Chem.*, **136**, 503 (1940).
137. Mackay, E. M., *J. Clin. Endocrinol.*, **3**, 101 (1943).
138. Macleod, J. J. R., *Am. J. Physiol.*, **55**, 184 (1921).
139. Mann, P. J. G., and Quastel, J. H., *Nature*, **145**, 856 (1940).
140. Mann, P. J. G., and Quastel, J. H., *Biochem. J.*, **35**, 502 (1941).
141. Martin, G. J., and Remebaum, E. H., *J. Biol. Chem.*, **151**, 417 (1943).
142. Martius, C., *Z. physiol. Chem.*, **279**, 96 (1943).
143. Meyerhof, O., and Kiessling, W., *Naturwissenschaften*, **22**, 838 (1934).
144. Meyerhof, O., and Lohmann, K., *Biochem. Z.*, **271**, 89 (1934).
145. Meyerhof, O., and Lohmann, K., *ibid.*, **273**, 73 (1934).
146. Meyerhof, O., *Naturwissenschaften*, **23**, 850 (1935).
147. Meyerhof, O., Lohmann, K., and Schuster, P., *Biochem. Z.*, **286**, 319 (1936).
148. Meyerhof, O., and Schulz, W., *ibid.*, **297**, 60 (1938).
149. Meyerhof, O., and Perdigon, E., *Enzymologia*, **8**, 353 (1940).
150. Meyerhof, O., and Junowicz-Kocholaty, R., *J. Biol. Chem.*, **149**, 71 (1943).
151. Muñoz, J. M., and Leloir, L. F., *ibid.*, **147**, 355 (1943).

152. Nachmansohn, D., Cox, R. T., Coates, C. W., and Machado, A. L., *J. Neurophysiol.*, **6**, 383 (1943).
153. Nachmansohn, D., and Machado, A. L., *ibid.*, **6**, 397 (1943).
154. Needham, D. M., *Biochem. J.*, **36**, 113 (1942).
155. Needham, J., and Lehmann, H., *ibid.*, **31**, 1210, 1913 (1937).
156. Needham, J., and Nowinski, W. W., *ibid.*, **31**, 1165 (1937).
157. Negelein, E., and Brömel, H., *Biochem. Z.*, **303**, 40 (1939).
158. Neuberg, C., and Gottschalk, A., *ibid.*, **146**, 185 (1924).
159. Neuberg, C., and Simon, E., *Ergeb. Enzymforsch.*, **2**, 116 (1933).
160. Ochoa, S., Peters, R. A., and Stocken, L. A., *Nature*, **144**, 750 (1939).
161. Ochoa, S., *J. Biol. Chem.*, **138**, 751 (1941).
162. Ochoa, S., *ibid.*, **151**, 493 (1943).
163. Ochoa, S., *ibid.*, **155**, 87 (1944).
164. Ostern, P., Guthke, J. A., and Terszakowéc, J., *Z. physiol. Chem.*, **243**, 9 (1936).
165. Parnas, J. K., and Ostern, P., *Biochem. Z.*, **279**, 94 (1935).
166. Pilgrim, F. J., Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, **145**, 237 (1942).
167. Quibell, T. H., *Z. physiol. Chem.*, **251**, 102 (1938).
168. Rapkine, L., *Biochem. J.*, **32**, 1729 (1938).
169. Rice, L., and Evans, E. A., Jr., *Science*, **97**, 470 (1943).
170. Rittenberg, D., and Bloch, D., *J. Biol. Chem.*, **154**, 311 (1944).
171. Rothschild, P., *Biochem. Z.*, **206**, 186 (1929).
172. Sacks, J., *Am. J. Physiol.*, **129**, 227 (1940).
173. Sacks, J., and Altschuler, C. H., *ibid.*, **137**, 750 (1942).
174. Sacks, J., *Science*, **98**, 388 (1943).
175. Silverman, M., and Werkman, C. H., *J. Biol. Chem.*, **138**, 35 (1941).
176. Slade, H. D., and Werkman, C. H., *Arch. Biochem.*, **2**, 97 (1943).
177. Snapper, I., and Grünbaum, A., *Biochem. Z.*, **181**, 410, 418 (1927); **185**, 223 (1928); **201**, 464 (1928).
178. Solomon, A. K., Vennesland, B., Klemperer, F. W., Buchanan, J. M., and Hastings, A. B., *J. Biol. Chem.*, **140**, 171 (1941).
179. Soskin, S., Mirsky, I. A., Zimmerman, L. M., and Heller, R. C., *Am. J. Physiol.*, **114**, 648 (1936).
180. Soskin, S., Levine, R., and Taubenhaus, M., *Proc. Soc. Exptl. Biol. Med.*, **44**, 257 (1940).
181. Soskin, S., *Physiol. Revs.*, **21**, 140 (1941).
182. Stadie, W. C., *Ann. Internal Med.*, **15**, 783 (1944).
183. Stare, F. J., Lipton, M. A., and Goldinger, J. M., *J. Biol. Chem.*, **141**, 981 (1941).
184. Stotz, E., and Bessey, O. A., *ibid.*, **143**, 625 (1942).
185. Stotz, E., *ibid.*, **148**, 585 (1943).
186. Stotz, E., Westerfeld, W. W., and Berg, R. L., *ibid.*, **152**, 41 (1944).
187. Stotz, E., Root, H. F., and Carpenter, T. M., to be published.
188. Straub, F. B., *Biochem. J.*, **34**, 483 (1940).
189. Summerson, W. H., Lee, J. M., and Partridge, C. W. H., *Science*, **100**, 250 (1944).
190. Sutherland, E. W., Colowick, S. P., and Cori, C. F., *J. Biol. Chem.*, **140**, 309 (1941).
191. Swendseid, M. E., Barnes, R. H., Hemingway, A., and Nier, A. O., *ibid.*, **142**, 47 (1942).

192. Szent-Györgyi, A. v., and Banga, I., *Science*, **93**, 158 (1941).
193. Tankó, B., Munk, L., and Abonyi, I., *Z. physiol. Chem.*, **264**, 91 (1940).
194. Thomas, Q., *Enzymologia*, **7**, 231 (1939).
195. Thunberg, T., *Skand. Arch. Physiol.*, **40**, 1 (1920).
196. Utevski, A., *Biochem. Z.*, **204**, 81 (1929).
197. Utter, M. F., and Werkman, C. H., *Arch. Biochem.*, **2**, 491 (1943).
198. Utter, M. F., Werkman, C. H., and Lipmann, F., *J. Biol. Chem.*, **154**, 723 (1944).
199. Vennesland, B., Solomon, A. K., Buchanan, J. M., Cramer, R. D., and Hastings, A. B., *ibid.*, **142**, 371 (1942).
200. Warburg, O., Christian, W., and Griese, A., *Biochem. Z.*, **282**, 157 (1935).
201. Warburg, O., and Christian, W., *ibid.*, **292**, 287 (1937).
202. Warburg, O., and Christian, W., *ibid.*, **301**, 221 (1939).
203. Warburg, O., and Christian, W., *Naturwissenschaften*, **29**, 589 (1941).
204. Warburg, O., and Christian, W., *Biochem. Z.*, **310**, 384 (1942).
205. Warburg, O., and Christian, W., *ibid.*, **314**, 149 (1943).
206. Weil-Malherbe, H., *Biochem. J.*, **31**, 2202 (1937).
207. Weil-Malherbe, H., *ibid.*, **32**, 1033 (1938).
208. Weil-Malherbe, H., *Nature*, **145**, 106 (1940).
209. Weinhouse, S., Medes, G., and Floyd, N. F., *J. Biol. Chem.*, **153**, 689; **155**, 143 (1944).
210. Wendel, W. B., *ibid.*, **94**, 717 (1932).
211. Werkman, C. H., and Wood, H. G., in *Advances in Enzymology*, Vol. II. Interscience, New York, 1942, p. 135.
212. Westerfeld, W. W., Stotz, E., and Berg, R. L., *J. Biol. Chem.*, **144**, 657 (1942).
213. Westerfeld, W. W., Stotz, E., and Berg, R. L., *ibid.*, **149**, 237 (1943).
214. Westerkamp, H., *Biochem. Z.*, **263**, 239 (1933).
215. Wieland, H., and Rosenthal, C., *Ann.*, **548**, 255 (1941).
216. Wieland, H., and Rosenthal, C., *ibid.*, **554**, 241 (1943).
217. Wohlgemuth, J., and Nakamura, Y., *Biochem. Z.*, **175**, 232 (1926).
218. Wood, H. G., and Werkman, C. H., *Biochem. J.*, **32**, 1262 (1938).
219. Wood, H. G., Werkman, C. H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **139**, 377, 483 (1941).
220. Wood, H. G., Werkman, C. H., Hemingway, A., and Nier, A. O., *ibid.*, **142**, 31 (1942).
221. Yanov, Z. A., *Arch. Internal Med.*, **72**, 239 (1943).

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RECENT PROGRESS IN THE BIOCHEMISTRY OF FUSARIA*

By

F. F. NORD and ROBERT P. MULL

New York, N. Y. and Summit, N. J.

CONTENTS

Wenn man dem Prinzip der Vollständigkeit in der Ausarbeitung huldigt, kommt man langsam voran.

—ADOLF BAEYER†

I. Introduction

It has been passed over in silence repeatedly and pointed out occasionally, but timidly, that much of the newer findings in the forging ahead of the physiology of cells were obtained in studies conducted with isolated or structureless enzymes. However, it is a well-known fact that living systems very often react in a quite different and more complicated manner

* Contribution No. 41.

† Quoted from a letter to Adolf Spiegel, dated January 11, 1907.

than enzymes and organisms separated from them do *in vitro* (5, 46, 51, 57). Otherwise the terms "life" and "living" would be meaningless. Consequently, it would appear that what we do not know yet is how the harmonious cooperation comes about of the factors present in the cell which characterize life itself.

The magnitude of difficulties to be overcome in understanding the available findings may be sensed from a recent review (78), which includes the following statement: "The results are not inconsistent with the operation *in vivo* of a mechanism similar to that in cell-free yeast preparations, including the function of diphosphopyridine nucleotide." This passage is supported by citing a paper (23) which contains the following sentence: "As a result there is but scanty evidence to provide a basis for the assumption that the fermentation of sugar by living cells takes place as in cell-free extracts through the formation and decomposition of phosphoric esters, phosphorus transference, and oxidation-reduction reactions."

This seemingly paradoxical interpretation of reaction mechanisms (31) said to occur in living systems is not at all astonishing if we take into consideration that, ever since the discovery by Cremer (10) of glycogen formation by means of yeast (30*a*) juice, the media used in the study of vital reactions were extracts of muscles or maceration juices of yeasts, which are enzymatically incomplete (68).

It remains, however, so far unexplained, that without regard to one or two recent investigations, the behavior of frozen extracts, first obtained by Dixon and Atkins (15), which, above all, have the advantage of containing also the enzymes otherwise left behind in the cell fragments, is except from a physicochemical standpoint (55) hardly studied.

In view of these undeniable inconsistencies in regard both to observations made and tools used in investigations, scattered studies were carried out on a few living systems to coordinate results obtained from their action on different substrates which might be helpful in attempts to build up a "spectrum" of the cell as the foundation of permissible generalizations. Besides the observations gathered with *Thermobacterium mobile* Lindner and *Zymosarcina ventriculi*, the findings accumulated with certain *Aspergillae* (75) are especially noteworthy and deserve more attention. In addition, studies were carried out in Canada, Germany and the United States with the genus *Fusarium;* this report will attempt to discuss the results of some of these investigations as they appear in the literature since 1938.*

In the last decades *Fusaria* have received much attention from workers

* For earlier reports see: J. H. Birkinshaw, *Biol. Rev. Cambridge Phil. Soc.*, **12**, 357 (1937). W. Frankenburger, *Katalytische Umsetzungen in homogenen und enzymatischen Systemen*, Akad. Verlagsgesellschaft, Leipzig, 1937. H. Raistrick, *Ergeb. Enzymforsch.*, **1**, 345 (1932). F. F. Nord, *Chem. Revs.*, **26**, 423 (1940). F. F. Nord, in Nord-Weidenhagen, *Handbuch der Enzymologie*, Akad. Verlagsgesellschaft, Leipzig, 1940, p. 963. H. W. Wollenweber, *Zentr. Bakt. Parasitenk., II*, **106**, 171 (1943).

in the plant sciences. As a consequence, they are known by plant pathologists (21*a*, 26, 29, 73, 82*a*) as one of the most difficult genera in which to distinguish species. Perhaps the major cause of difficulties in classification is the capacity of the species to vary in morphological characteristics and in intensities of pigmentation which sometimes are affected by slight modifications in culture technique. It is, however, improbable but not impossible that in culturally distinct types demonstrating frequently the dual phenomenon, *i. e.*, one producing conidia in abundance and scant mycelium, the other developing fewer conidia and more abundant mycelium (25), the extent of enzymatic distribution would vary much under normal conditions.

F. lini Bolley is characterized biochemically by a high thiamin content (70) and enzymatically by the presence of, for example, a negligible amount of organic phosphorus donors, or a powerful dehydrogenating system, which is capable of acting on methyl alcohol, ethylene glycol, fatty acids, etc. Moreover, it ferments hexoses as well as pentoses, slowly but completely, giving rise also to the accumulation of significant amounts of intermediary products. The prevailing state of enzymatic "unsaturation" in the rugged *Fusarium* cells, in contradistinction, for example, to yeasts, in turn prevents a too close integration of the activity of the enzymes present, thus making their artificial separation to a certain extent dispensable. Besides, *Fusaria* are able to utilize potassium cyanide as a carbon and nitrogen source; they contain a lipase and are capable of synthesizing a variety of pigments. Accordingly they constitute an almost perfect, chlorophyll-free, natural system for the study of enzymatic and fungistatic (90) reaction mechanisms in the intact, heterogeneous and structurally organized cell.

II. Alcoholic Fermentation of Pentoses, Hexoses and Trehalose

Since establishing the first facts (54) concerning their over-all similarity to the action on hexoses of enzymes present in the yeast cell, the study of the intermediary phases of carbohydrate metabolism by means of different *Fusaria* influenced basically our information in this field (56). An active *Fusarium* juice (84) was also successfully prepared. In spite of these studies, which were concerned mainly with the problem of a possible introductory rearrangement of carbohydrate molecules (see page 179), there remained some gaps and undetermined phases in the observations on the course of the breakdown which might be regarded as decisive and common in the mechanism of alcoholic fermentation of *hexoses* as well as of *pentoses*.

1. *Possible Role of Nitrogen Source*

In previous investigations of this series, the nitrogen required by the organism was supplied in the form of asparagine and hydrogen cyanide (11, 13). The shift in the *p*H of the nutrient medium as observed when nitrates were used led to the application of inorganic nitrogen sources to the study of the alcoholic fermentation of hexoses and pentoses (85). Accordingly, ammonium nitrate, potassium nitrate and ammonium sulfate were introduced with the purpose of investigating whether a possible reducibility of a part of the nutrient medium or a difference in the permeability of cells for anions of varying length would be helpful in throwing light upon the subsequent course of the breakdown of these carbohydrates.

Fermentation experiments utilizing glucose, fructose, mannose and galactose were carried out with potassium nitrate as a nitrogen source and tests were made for various conceivable intermediates. The tests indicated the absence of acetaldehyde, methylglyoxal, dihydroxyacetone and glyceraldehyde. However, considerable quantities of pyruvic acid were detected and the compound was isolated and identified as its 2,4-dinitrophenylhydrazone. Simultaneously, tests were made in the course of the fermentations for the possible occurrence of reduction products of nitrate, with the result that the appearance of nitrite was established. It seemed to be obvious at once that the striking accumulation of pyruvic acid in the presence of nitrate, in contrast to its appearance only in smaller quantities when ammonium sulfate was employed, was related to the reduction of nitrate to nitrite. Experiments with glucose, fructose and mannose were carried out with potassium nitrate and ammonium sulfate as nitrogen sources, for the latter, naturally, cannot give rise to nitrite. With all three carbohydrates there was a considerable accumulation of pyruvic acid in the nitrate media but only smaller quantities in the medium containing ammonium sulfate.

Determinations of the cocarboxylase content revealed that there is actually more cocarboxylase in the dried mycelia of *Fusaria* when grown on a medium in which the nitrogen was supplied in the form of potassium nitrate than when ammonium sulfate was used. This indicates that the accumulation of pyruvic acid in the nitrate medium, as contrasted to its appearance in the ammonium sulfate medium, cannot be due to a deficiency in the carboxylase system of the organism.

2. *Influence of Thiamin and Nicotinic Acid*

Because pyruvic acid was found to accumulate under conditions that may be designated as normal for the organism, since a vigorous growth was ob-

tained on the synthetic media employed, studies were made of the effect produced by the addition of substances known to be components of the several enzyme systems involved in alcoholic fermentation. Accordingly, the nutrient medium was supplemented by varying amounts of vitamin B_1 hydrochloride.

From the analytical results obtained, it was clear that the addition of this factor to the nutrient medium profoundly influenced the reaction effected by the decarboxylating system of the organism, for when the amount of pyruvic acid accumulated had reached a maximum the ratio between the amounts in the vitamin-supplemented and nonsupplemented media was approximately one to seven. Furthermore, when analyses were run, on only the sixth day was the amount of pyruvic acid appreciable in the

TABLE I

EFFECT OF VARYING CONCENTRATION OF VITAMIN B_1* ON ACCUMULATION OF PYRUVIC ACID (85)

Day	0	50	100	250	0	50	100	250	0	50	100	250
	Glucose fermented, g.				Pyruvic acid, mg.				Mycelium weight, mg.			
2	0.15	0.25	0.32	0.24	5	Trace	Trace	Trace				
4	1.73	1.35	1.31	1.23	159	8	6	6	132 ± 5	91 ± 4	99 ± 7	93 ± 1
6	3.47	3.39	3.41	3.36	171	26	24	20				
8	4.09	4.07	4.07	4.08	155	Trace	Trace	Trace	259 ± 3	135 ± 3	144 ± 4	130 ± 3

* Concentration in γ per 100 ml.

vitamin-containing media. The maximum effect was observed by an addition as low as 50 γ per 100 ml. It was found, however, that there was a significant increase in the accumulation of pyruvic acid when the concentration was reduced to the 10 γ level; the quantity accumulated when the amount in the control was a maximum was 135, 44 and 16 mg., respectively, for the control and the media supplemented with 10 and 50 γ per 100 ml. Accordingly, an inhibitory or chemical effect of the nitrite ions (36) produced in the course of the reduction of nitrate appears to be compensated for by the addition of thiamin.

In contrast to these observations, made on *Fusarium lini* Bolley, Gould *et al.* (79) could not demonstrate any stimulating effect of thiamin on alcohol production or increased cocarboxylase content of a strain which they regarded as closely allied to *F. tricothecoides*. Their conclusions are to be taken into account accordingly.

The addition of nicotinic acid has no influence on the rate of disappear-

ance of glucose, whereas the amounts of pyruvic acid accumulated in the latter stages of the experiments are increased in some cases by 15, 30 or 50% above those amounts which were obtained without the addition of nicotinic acid. There thus appears to be a basis for the assumption that the presence of nicotinic acid in the nutrient medium makes it possible for the organism to synthesize dehydrogenating enzymes more readily. The possibility of an augmented dehydrogenase effect may result in an additional reduction of the acceptor–nitrate to nitrite ions.

3. Influence of Nitrate Concentration

If one could assume that a hypothetical phase sequence in the degradation, by means of isolated yeast systems, from hexoses to alcohol is also followed in the case of living *Fusaria*, then for an accumulation of pyruvic acid to occur some hydrogen acceptor or acceptors other than acetaldehyde must be operative so that a corresponding oxidation can proceed at a faster

TABLE II

EFFECT OF VARYING CONCENTRATIONS OF KNO_3* ON ACCUMULATION OF PYRUVIC ACID (85)

Day	2.00	5.00	8.00	2.00	5.00	8.00	2.00	5.00	8.00
	Glucose fermented, g.			Pyruvic acid, mg.			Mycelium weight, mg.		
3	0.44	0.35	0.36	76 = 17.7%	83 = 24.3%	91 = 25.9%			
5	2.24	2.48	2.87	123 = 0.56%	202 = 0.83%	249 = 0.88%			
7	3.76	3.78	3.92	93 = 0.25%	202 = 0.57%	271 = 0.71%	206 ± 3	239 ± 7	265 ± 2

* Concentration in g. per 1000 ml.

rate than the subsequent steps. The assumption of some other hydrogen acceptors was verified by the finding of both nitrite and hydroxylamine as reduction products of nitrate, and also by the observation of a relationship between the amount of pyruvic acid accumulating, both in absolute quantities and percentage yields (if we are to assume that all of the glucose was converted to pyruvic acid), and the nitrate concentration.

4. Mechanism of Pentose Breakdown

It will be recalled that *Fusaria* are able to dissimilate pentoses (56), and that in the case of xylose, at least, considerable quantities of alcohol

are formed. Contrary to the breakdown of pentoses by distillation in alkaline medium (18), and to the observation of minute quantities of carbon dioxide and alcohol during the utilization of xylose by certain Fleischmann's yeast cakes (1), nothing was known concerning the later intermediate phases of their alcoholic breakdown. In conjunction with previous (56) phosphorylation studies, it was of great interest, therefore, to determine whether pyruvic acid could be found during the fermentation of this class of carbohydrates as well.

Employing a nutrient medium with a *d*-xylose concentration of 3%, it was found that pyruvic acid did accumulate, but to a somewhat less marked extent. This fact is easily understandable if we assume that from each pentose molecule only one pyruvic acid molecule can be formed on the basis of a C_3—C_2 split. Such a split is not unlikely, as we have succeeded in actually isolating a C_3 compound, although efforts to trap or isolate glycolaldehyde, which may be regarded as the C_2 moiety, have been thus far unsuccessful. An inability to trap glycolaldehyde in the course of pentose fermentation, in contrast to its trapping during dehydrogenation of ethyleneglycol (page 185), could be related to the consideration that the rate of the enzymatic conversion of the glycolaldehyde formed from the pentoses within the cell is higher than are the possible rates of diffusion out of the cell or of permeation of added glycolaldehyde into the cell. This, in fact, served as a weak carbon source* in contradistinction to its convenient reducibility by fermenting yeast (50).

5. *Mechanism of Carboxylase Blockade*

It had been previously noted that nitrite ions were being formed during the course of the fermentation of glucose. As a possible explanation for the accumulation of pyruvic acid, we may assume an inhibitory or chemical action of nitrite on the NH_2 group of the carboxylase system present in *Fusaria*. Therefore, if as with hexoses a nitrogen source such as ammonium sulfate is employed, there should be less accumulation of pyruvic acid. Also, since it has been shown (37) that, when ammonium nitrate is present as a sole nitrogen source in culture media for *Fusaria*, the nitrate ions are preferentially used. Therefore, to test this consideration, parallel experiments were run with various inorganic nitrogen sources. Ammonium sulfate, potassium nitrate and ammonium nitrate were present in the nutrient

* Judging from their ability to dehydrogenate ethyleneglycol, their inability to utilize glycolaldehyde and the mechanism of degradation of hexoses and pentoses by *Fusaria*, it would appear that this mechanism differs basically from that assumed by Stanier and Adams (74) for degradation of pentoses in the case of *Aeromonas* fermentation.

media in such concentrations that the quantities of nitrogen in the three were identical. In the case of ammonium nitrate, the amount of this substance was calculated on the basis of nitrogen in the nitrate radical for the reason previously mentioned. A nutrient medium of the earlier introduced composition was used, but with a xylose concentration of 3%, to compensate for the *p*H changes mentioned above.

TABLE III

PENTOSE FERMENTATION RELATED TO NITROGEN SOURCE (85)

Day	5.00 g. KNO_3			3.26 g. $(NH_4)_2SO_4$			3.96 g. NH_4NO_3		
	Xylose fermented, g.	Ethyl alcohol, g.	Pyruvic acid, mg.	Xylose fermented, g.	Ethyl alcohol, g.	Pyruvic acid, mg.	Xylose fermented, g.	Ethyl alcohol, g.	Pyruvic acid, mg.
7	1.81	0.35	29	1.46	0.29	Trace	1.64	0.38	38
9	2.63	0.56	34	1.78	0.37	Trace	2.08	0.53	36
12	2.89	0.59	16	2.74	0.41	Trace	2.74	0.59	10
	MYCELIUM WEIGHT ON TWELFTH DAY								
	138 ± 3 mg.			285 ± 9 mg.			166 ± 6 mg.		

From the data recorded, it can be seen that with pentoses as well as with hexoses the accumulation of pyruvic acid depends on the formation of nitrite ions because with ammonium sulfate only traces of this intermediate were detected. On the other hand, the amount of pyruvic acid accumulated was of the same magnitude when both potassium and ammonium nitrate were used as nitrogen sources. However, the ammonium nitrate gave rise to slightly higher percentage yields of ethyl alcohol from the xylose. But ammonium sulfate is a less satisfactory source of nitrogen with respect to both rate of disappearance of xylose and the final formation of ethyl alcohol. It was during the progress of these studies that the course of the nitrate → nitrite reaction was further investigated, and it was established that the reduction of nitrate proceeds beyond the state of nitrite insofar as positive hydroxylamine tests were obtained.

According to Meyer and Janny (38), pyruvic acid forms with hydroxylamine isonitrosopropionic acid:

$$CH_3—CO—COOH + H_2N—OH \longrightarrow CH_3—\underset{\underset{N—OH}{\|}}{C}—COOH \qquad (1)$$

which may be further reduced to alanine. In this case, in biological systems, the cycle of utilization of nitrate by the cell is closed. It may be

noted here that, in experiments with *Fusaria* which were conducted with amino acids as the sole source of carbon and nitrogen, the formation of pyruvic acid from alanine was established (86).

The fact that the nitrates present in the media were easily reduced as far as hydroxylamine indicates that they may serve as an indirect acceptor for the hydrogen freed by the dehydrogenases and supplied by one or more hydrogen donators. The nitrite, as well as ammonium sulfate, in turn serves as a blockader of the carboxylase system, furnishing a means for disclosing a member of the phase sequence of carbohydrate breakdown. The limited accumulation of pyruvic acid and the partial functioning of the carboxylase system involve a continuous oscillation between the nitrite- or sulfate-inhibited and the free enzyme, indicating that the nitrate → nitrite reaction proceeds at a faster rate than the reduction of the latter to hydroxylamine.

From simultaneous investigations carried out with yeasts or *B. turcosum*, it was deduced (9, 40, 67) that there is a great difference between the permeability of cells for longer, polyvalent anions and that for shorter substrate anions. Consequently, it would also seem to be conceivable that, when carbohydrates and nitrates or sulfates are supposed to reach the actual location of enzymatic activity in moulds, the rates of penetration will be favorable to the latter. Because of this competition, it is probable that some of the carboxylase present in the *Fusaria* will be blocked when the step of pyruvic acid in a hypothetical phase sequence of degradations is reached. This "blockade" may be limited, and consequently will give rise to an accumulation of *varying* amounts of pyruvic acid.

All quantities of pyruvic acid obtained and isolated in carbohydrate fermentations by *Fusaria* in the presence of nitrites or ammonium sulfate represent a total amount which is derived from the pyruvic acid obtained through the inhibitory action on carboxylase of the salts mentioned. But from the standpoint of analytical interpretation, it remains insignificant whether the inhibitions occur through chemical action on the prosthetic group characterized by the NH_2 group present in its molecule, or by affecting the carrier protein, or by causing a separation of both. In turn, the nitrate → nitrite reduction may ensue from *two* reactions: (*a*) By the action of hydrogen freed by the dehydrogenases and carried over to the acceptor nitrate from hydrogen donors formed in the course of the initial degradation of the hexoses and pentoses present; and (*b*) by the action of a hypothetical reductase, hitherto regarded as HCN-sensitive, effecting the direct reduction of nitrate to nitrite. Under appropriate experimental conditions it should be possible, therefore, to separate the two modes of

action; and consequently less pyruvic acid should be found in media which are prepared with potassium nitrate to which potassium cyanide has been added.

Compared with the pyruvic acid values of the blank, the amounts isolated from experiments which were carried out in the presence of excessive and increasing quantities of potassium cyanide exhibit a steady drop (71).

TABLE IV

ACCUMULATION OF PYRUVIC ACID IN PRESENCE OF POTASSIUM CYANIDE USING POTASSIUM NITRATE AS A NITROGEN SOURCE (71)

Day	KCN added, mg.			Pyruvic acid accumulated, mg.			
	1	2	3	Blank	1	2	3
4	20	30	50	119	110	118	96
5	70	80	100	156*	90*	102*	128*
7	70	80	100	168	100	50	95
8	..	..	...	172	79	19	6
9	..	..	...	186	80	10	40
ANALYSIS ON NINTH DAY							
Glucose fermented, g.....				2.65	2.57	3.10	3.45
Alcohol, mg.....				763	705	753	534
Mycelium, mg.....				1067	1113	987	1255

Nutrient medium: 40.00 g. glucose, 5.00 g. KNO_3, 5.00 g. KH_2PO_4, 0.75 g. $MgSO_4 \cdot 7\ H_2O$, and water to 1 liter.

* Analyzed on the sixth day.

Despite this observation, most of the values representing the amount of glucose fermented increased and the alcohol values closely approached that obtained in the blank. We know from earlier investigations, however, that this slight diminution of the alcohol is not due to inhibited fermentation but to subsequent dehydrogenation of the alcohol previously formed (see pages 181, 189). Consequently, it would appear that there is a reductase operative in *Fusaria* which, judging from the quantities of potassium cyanide tolerated in these experiments, must be highly KCN-insensitive. A similar enzyme is claimed by Yamagata (88) to be present in *Bacterium pyocyaneum.*

6. *Mechanism of Carbohydrate Breakdown*

In the experiments carried out with a medium in which potassium nitrate was replaced by ammonium sulfate, the absolute amounts of isolated

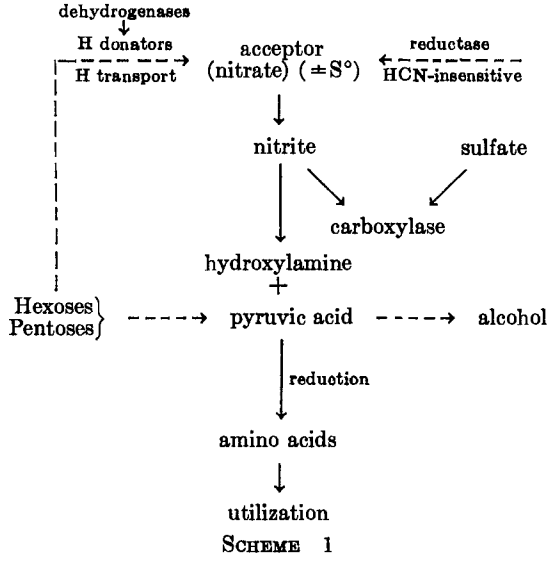
TABLE V

ACCUMULATION OF PYRUVIC ACID WITH KCN USING AMMONIUM SULFATE AS A NITROGEN SOURCE (71)

Day	KCN added, mg.		Pyruvic acid, accumulated, mg.		
	1	2	Blank	1	2
4	20	30	21	18	21
5	75	10	..	..	..
6	...	...	24	20	23
7	100	200	33	27	30
8	...	...	33	33	33
9	...	...	30	40	43
ANALYSIS ON NINTH DAY					
Glucose fermented, g.....			0.81	0.88	1.08
Alcohol, mg.....			287	211	199
Mycelium, mg.....			357	305	457

Nutrient medium: 40.00 g. glucose, 3.2 g. $(NH_4)_2SO_4$, 5.00 g. KH_2PO_4, 0.75 g. $MgSO_4 \cdot 7\ H_2O$, and water to 1 liter.

pyruvic acid are much smaller and remain unchanged on addition of increasing quantities of potassium cyanide. In spite of this, the alcohol values are comparable with those of the blank. Since the reductase referred



SCHEME 1

to before does not act on the ammonium sulfate, only the formation of that amount of pyruvic acid which is originated by the slight inhibitory effect of the sulfate on the carboxylase present can be measured. This would signify that the above-mentioned total value of pyruvic acid consists of only that part which is derived from the direct action of the sulfate ions on the carboxylase in the presence of ammonium sulfate. As neither the reductase nor the hydrogen freed from intermediary hydrogen donors by the dehydrogenases present is capable of reducing ammonium sulfate, there is no possibility that an inhibition of the aforementioned reductase would influence the pyruvic acid values which, in contrast to the "NO_2"–pyruvic acid values, remain undiminished when potassium cyanide is added to the media containing ammonium sulfate. The mechanism involved in these reactions may be represented as in Scheme 1.

7. *Elementary Sulfur as a Hydrogen Acceptor*

Applying elementary sulfur (70) in the course of the alcoholic fermentation of carbohydrates, it was possible to make decisive observations as to its role in this phase sequence. It could be observed that the presence of sulfur brings about an increase in the disappearance of glucose as compared with the blank. The intermediary key product in fermentations, pyruvic acid, accumulates to a lesser extent in the experiments with sulfur and decreases proportionally with the quantity of sulfur added. Accordingly, and as is to be expected, the mat weights and the amounts of alcohol produced are increased. This would indicate that some of the hydrogen freed by the dehydrogenases, and acceptable either by oxygen, nitrates or some intermediates present, is diverted to the elementary sulfur available, which is taking a competitive part in the chain of reactions. Hence, a smaller amount of nitrate is reduced to nitrite, and the inhibition of carboxylase becomes less predominant. In turn, the isolable part of the total amount of pyruvic acid becomes diminished. Evolution of hydrogen sulfide was established in both glucose and xylose fermentations. In connection with earlier findings (56), this observation seems to signify also that, in contradistinction to yeast, the "cyanide-stable" or metal-free respiration in *Fusaria* accounts for a much larger amount of the oxygen consumption than does the cyanide-sensitive part of the enzyme system.

From the data presented it is evident that *elementary* sulfur so introduced participates stoichiometrically in the prevailing reactions and, consequently, shifts the originally established equilibrium between hydrogen donors and acceptors. It seems to be doubtful, however, whether there

TABLE VI

EFFECT OF SULFUR ON GLUCOSE FERMENTATION (70)

Day	Blank			500 γ			1000 γ			10,000 γ		
	Glucose, g.	PA, mg.	Alcohol, mg.	Glucose, g.	PA, mg.	Alcohol, mg.	Glucose, g.	PA, mg.	Alcohol, mg.	Glucose, g.	PA, mg.	Alcohol, mg.
0	4.03	...	..	4.01	...	..	4.01	...	..	4.00	...	..
7	1.43	128	442	1.53	127	451	1.63	127	490	1.59	104	504
10	3.26	159	1052	3.23	151	1049	3.53	141	1136	3.42	108	1150
MAT WEIGHTS												
	1529 mg.			1570 mg.			1935 mg.			1803 mg.		
THIAMIN VALUES												
	21.7 γ/g.			21.5 γ/g.						20 γ/g.		

Glucose expressed in g. per 100 ml. Pyruvic acid (PA) and alcohol expressed in mg. per 100 ml. Sulfur expressed in gamma per 700 ml.

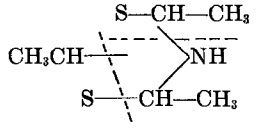
exists a loose connection, if any, between the function of elementary sulfur in dehydrogenations by *Fusaria:*

$$S^0 + 2\,H \longrightarrow H_2S \tag{2}$$

$$2\,H_2S + O_2 \longrightarrow 2\,S + 2\,H_2O \tag{3}$$

$$2\,H_2O + H_2S + 4\,O \longrightarrow SO_4^{--} + 2\,H_3O^+ \tag{4}$$

and the mechanism of the phytochemical reduction of sulfur-*containing* compounds such as thioaldehydes, by fermenting yeasts (47, 48, 53):



$$= 2\,CH_3CHS + CH_3CH{=}NH \tag{5}$$

$$CH_3CHS \longrightarrow C_2H_5SH \tag{6}$$

The amount of thiamin synthesized in the presence of elementary sulfur remained unchanged, in accordance with observations made with certain *Torulae* (17), when its single components were administered during the course of fermentation.

It therefore appears justifiable to emphasize that, in the case of *Fusaria,* according to the nitrogen source present, there exists an organic interaction between the dehydrogenating enzyme system and the zymatic system. The fact that fermentable sugars and sugars designated not long ago as nonfermentable possess the same key intermediate, in the course of their alcoholic

fermentation, seems to be all the more significant, because in the latter case it leads also to the realization of the second component of the C_5 chain, which may be glycolaldehyde.

Since preceding work of this series (56) had shown that *Fusaria* can enzymatically degrade both *d*- and *l*-arabinose, a search for pyruvic acid as an intermediate in the dissimilation of these compounds as well as of *d*-ribose was undertaken. In the case of both *d*-isomers, the presence of a keto acid was indicated when the Lu test was employed. However, the extent of accumulation of this acid was such that no derivative could be isolated for purposes of identification. With *l*-arabinose, on the other hand, large amounts of pyruvic acid were isolated.

TABLE VII

FERMENTATION OF *l*-ARABINOSE (85)

Day	*l*-Arabinose fermented, g.	Pyruvic acid accumulated, mg.
4	0.17	Trace
6	0.55	Trace
8	1.06	Trace
10	1.47	43
12	2.00	115

8. *Fermentation of Trehalose*

Unlike living yeasts, living *F. lini* Bolley ferments trehalose (64) more rapidly than glucose. Growth of the organism is more rapid and more abundant on trehalose than on glucose. Since the rate of trehalose dissimilation is more rapid than that of glucose, the occurrence of the latter as an intermediary is excluded, for in such a case the dissimilation rate of glucose would be the controlling step. The indications thus far observed point toward a *direct* fermentation of this disaccharide. Glucose has never been observed in a fermenting trehalose mixture and, consequently, we cannot say that under these experimental conditions there was a trehalase active or present in *F. lini* Bolley. These observations are at variance with the contradictory data available concerning the fermentability and the mechanism of degradation of trehalose by yeasts.

9. *No Glycerol in Pentose Fermentation*

In fermentation studies conducted with *hexoses* by means of yeast juices, much importance is ascribed to the formation of the acid group of glyceric acid phosphate (34) from the aldehyde group of a triose phosphate, sym-

bolizing an oxidation within the essential oxidation-reduction system postulated by Lavoisier in 1864 (33).

Fermentation experiments (41) carried out with all three *pentoses*, by means of *F. lini* Bolley and *F. oxysporum*, and in the presence of freshly prepared calcium sulfite (49), revealed, after the elapse of 4 to 5 days, the presence of acetaldehyde in the media. Accordingly, in view of the slower rate of dehydrogenation of glycerol than that of the fermentation of pentoses, a temporary accumulation of at least traces of glycerol should have permitted its detection. But attempts to discover glycerol on the seventh, eleventh and fourteenth days were always negative, indicating that glycerophosphate, which might be split, if present, and could have given rise to glycerol, was not formed. Certainly, it should be reasonable to establish the presence of glycerol (42) more readily in a trapping experiment than to accumulate large amounts of pyruvic acid in hexose or pentose fermentation in the absence of any interceptor.

III. Phosphorylation and the Phase Sequence of Carbohydrate Breakdown in *Fusaria*

In an earlier study (56, 61), data on the extraordinarily low content of adenosinetriphosphoric acid and muscle adenosinetriphosphoric acid in *Fusarium* cells were established. Differences were recorded in these respects between *F. lini* Bolley and *F. graminearum* Schwabe and were also related to yeast and *Escherichia coli*. Excess amounts of these phosphorus donors added to the media are deposited in the *Fusarium* mycelia, and had no influence either on the course or the quantitative relations of the enzymatic reactions concerned. It is not surprising, therefore, that no phosphoglyceric acid could be found in the course of the carbohydrate metabolism of these *Fusaria*. This should be kept in mind when attempting to assess the effect of *added* amounts of factors such as pyridine nucleotides or organic phosphorus donors to enzymatically unsaturated systems. This would indicate that phosphoglyceric acid need not be considered as the indispensable mother substance of the abundantly isolated pyruvic acid and of the unchanged amounts of alcohol found. This interpretation also seems to be borne out by the analyses recorded in the course of phosphorylation studies with *Chaetomium funicola Cke* (72) if they are discussed (58) in an unbiased manner. Furthermore, it is also corroborated by the observation that, in the presence of potassium cyanide, higher amounts of alcohol are isolated in *Fusarium* fermentations than in the case of its absence from the nutrient medium.

Consolidating, therefore, results of earlier investigations with the fact that, in the course of fermentations by *Fusaria*, phosphoglyceric acid was neither (61) isolated in the presence of fluoride nor utilized as a carbon source to any measurable extent (83), this phase sequence, interrelated with the action of nitrate, may be represented on the basis of experimental evidence now available as follows:

$$C_5H_{10}O_5 \longrightarrow C_3H_6O_3 + CH_2OH{-}CHO\ (?) \tag{7}$$

$$C_3H_6O_3 + \underset{\text{(cozymase)}}{Co} \longrightarrow CoH_2 + CH_2{=}\underset{\displaystyle OH}{\underset{|}{C}}{-}COOH \tag{8}$$

$$\rightleftharpoons CH_3{-}CO{-}COOH \longrightarrow CH_3{-}CHO + CO_2 \tag{9}$$

$$CoH_2 + \left\{\begin{array}{ll} NO_3^- & \longrightarrow NO_2^- \\ NO_2^- & \longrightarrow H_2N{-}OH \\ CH_3{-}CHO & \longrightarrow CH_3{-}CH_2OH \end{array}\right\} + Co \tag{10}$$

It should be noted here that there appears to be a tendency to generalize the pathways of carbohydrate degradation and to convey the impression that phosphorylation must be regarded as an indispensable phase in all microorganisms or enzyme systems. Readers are, for example, often referred to investigations of Meyerhof and Perdigon (39), but it is not mentioned or not understood that in these experiments enormous quantities of coenzyme were added, thus creating abnormal conditions and, accordingly, carrying little or no convictions to prove the authors' contentions.* This was pointed out also by Needham, Lehmann and Nowinski (45). In addition to the above-mentioned negligible amounts of organic phosphorus donors present in living *Fusaria*, both *Fusaria* and *Chaetomium funicola* seem to be deficient in phosphoglyceromutase, required for the transformation of 3-phosphoglyceric acid to 2-phosphoglyceric acid. This deficiency could then be responsible for the failure of enolase (if present) to act on 2-phosphoglyceric acid to form phosphopyruvic acid.

Furthermore, Heitzmann (28) reported no accumulation of phosphorylated compounds during β-hydroxybutyric acid fermentation when *Bacillus megatherium* and *B. subtilis* were poisoned with sodium fluoride and toluene. Huszák (30), in studying carbohydrate breakdown in the central nervous system, stated: "Eine Phosphorylierung von Glucose kommt in der *weissen* Substanz nicht zustande, weil hier keine Hexokinase vorhanden

* Compare the sentiment of 'Omar Khayyām:

> *"Myself when young did eagerly frequent*
> *Doctor and Saint, and heard great Argument*
> *About it and about: but evermore*
> *Came out by the same Door as in I went."*

ist." This is in marked contrast to his findings with the *gray* material. Recently, *Aspergillus oryzae* and *Aspergillus niger* were also considered (4, 37*a*) as such moulds in which nonphosphorylating glycolysis occurs.

In the case of the enzyme systems of *Fusaria*, there appears to be, because of their predominant hydrogen cyanide resistance, no place for the action of a HCN-sensitive nitrate reductase and no necessity for the assumption of an exclusive and/or continuous turnover according to a phosphate bond energy potential (3). On the other hand, pyruvic acid takes a central position in the entire metabolism of this unique organism: as an intermediate in the degradation of pentoses, hexoses and alanine (86) and as a probable intermediate in the synthesis of amino acids used in the anabolism of the cell proteins.

Moreover, the earlier discovery of the presence of an aeroglucosedehydrogenase and aeropentosedehydrogenase (27, 60) in *Fusaria* indicates that carbohydrate dissimilation can occur under the influence of these moulds in three ways: (*a*) by oxidation (without participation of phosphorus); (*b*) by splitting of carbon chains; and (*c*) by the lingering detour of phosphorylation.

IV. Dehydrogenations

As far as it is possible to ascertain, the use of an integral member of a nutrient medium for a triple purpose—to act simultaneously as (*1*) an indispensable nitrogen source, (*2*) an expedient for the elucidation of the metabolism of an organism and (*3*) a substrate itself—has not been applied before. The experimental technique so introduced served, as could be seen in the preceding section, as a means of clarifying the mechanism of the degradation of hexoses as well as of pentoses by living *Fusaria*, in contrast to the yeasts (65), thereby enabling us to survey facts valid for *both* groups of carbohydrates.

By this means the paramount importance of transient and arbitrarily introduced hydrogen donors and of a powerful dehydrogenase system operative in *Fusaria* was also evidenced.

Investigation has shown that the enzymatic degradation of carbohydrates by *Fusaria* does not stop with the production of ethyl alcohol and carbon dioxide, but proceeds with dehydrogenation (66) of the alcohol produced. A continued study of dehydrogenations in the sense of the classical postulation (8) has been made with propylene glycol and some of the homologues of ethyl alcohol as substrates. Among the latter were both primary and secondary alcohols, which rendered possible an evaluation of

the relative rates of dehydrogenation of these different alcoholic groups, along with the establishment of the course of their degradation (20).

1. *Course of Dehydrogenation of Alcohols*

Qualitative experiments in the case of the normal alcohols indicated that acid production via the aldehydes occurred to a very small extent, since only traces were observed. However, in those experiments in which isopropyl alcohol and propylene glycol were used as carbon sources, indications of the further transformation of the products of dehydrogenations were discernible.

TABLE VIII

QUALITATIVE ANALYSIS OF SUBSTRATES (20)

Substrate	Dissimilation product	Method	Identification
n-Propyl alcohol	Propionaldehyde	Trapping	Dimedon deriv., m. p. 154–156° (aq. alcohol)
Isopropyl alcohol	Acetone	Isolation	2,4-Dinitrophenylhydrazone, m. p. 125–126° (alcohol); semicarbazone, m. p. 188–189°
	Formaldehyde	Trapping	Dimedon deriv., m. p. 186–187° (aq. alcohol)
Acetone	Formaldehyde	Trapping	Dimedon deriv., m. p. 186–187° (aq. alcohol)
n-Butyl alcohol	Butyraldehyde	Isolation	2,4-Dinitrophenylhydrazone, m. p. 122–124° (alcohol)
		Trapping	Dimedon deriv., m. p. 140–141° (aq. alcohol)
sec-Butyl alcohol	Ethyl methyl ketone	Isolation	2,4-Dinitrophenylhydrazone, m. p. 111–113° (alcohol); semicarbazone, m. p. 136–138° (water)
Propylene glycol	Acetol	Isolation	2,4-Dinitrophenylhydrazone m. p. 127–131° (alcohol); semicarbazone, m. p. 198–199° (water)
	Formaldehyde	Trapping	Dimedon deriv., m. p. 186–187° (aq. alcohol)

When isopropyl alcohol was dehydrogenated in the presence of dimedon, the dimedon derivative of formaldehyde was obtained after a lapse of two weeks, giving rise to the opinion that this product appeared only after the initial dehydrogenation of isopropyl alcohol had occurred. To check this possibility, a 0.4% solution of acetone was used as the carbon source in the presence of dimedon. Within a week the derivative of formaldehyde was obtained.

One explanation of the formation of formaldehyde from acetone is that

the latter is broken down by a hydrolytic split to acetaldehyde and methyl alcohol, which is then dehydrogenated to yield formaldehyde. The finding of only the pure dimedon derivative of formaldehyde and not a mixture of derivatives was to be expected under the experimental conditions. In extending the work of Vorländer (80), Yoe and Reid (89) have shown that the sensitivity of the reaction between dimedon and acetaldehyde is much less than that with formaldehyde and this reagent, and further that the experimental conditions greatly affect the acetaldehyde derivative formation in contrast to that with formaldehyde. When this reagent was used for trapping purposes, the procedure given earlier was the only one found suitable, because of the growth-inhibiting action it exerted toward the organism.

TABLE IX
MAXIMUM YIELDS OF DEHYDROGENATION PRODUCTS OBSERVED (20)*

Substrate	Dehydrogenation product	Yield, %
n-Propyl alcohol	Propionaldehyde	Trace
Isopropyl alcohol	Acetone	20–25
n-Butyl alcohol	Butyraldehyde	Trace
sec-Butyl alcohol	Ethyl methylketone	17–20
Propylene glycol	Acetol	10–13

* The values were calculated on the basis of the maximum amount of dehydrogenation product produced with reference to an initial concentration of 0.4% of substrate.

When propylene glycol is used as the carbon source, the later path of the dehydrogenation is still not definitely established. When *F. lini* Bolley acts upon this substrate in the presence of dimedon, nine days after the addition of the reagents, or roughly two weeks after inoculation, the precipitate of the formaldehyde-dimedon derivative appeared and accumulated over a period of one to two weeks.

This finding indicates an unusual mode of degradation of acetol, which is the dehydrogenation product isolated from propylene glycol experiments. The actual conversion of this compound is evidently not that hypothetically attributed to it by the acetic acid bacteria, *i. e.*, formation of methylglyoxal, pyruvic acid, acetaldehyde, etc. Methylglyoxal neither accumulates in the media nor can be trapped with *m*-nitrobenzhydrazide, a reagent specific for this compound in the presence of acetol. Furthermore, neither pyruvic acid nor acetaldehyde could ever be detected or trapped. The experiments carried out with these trapping reagents substantiate the view that the initial dehydrogenation of propylene glycol takes place via acetol and not by the alternative path of lactic aldehyde.

Quantitative experiments carried out on the various substrates indicated that accumulation of the aldehydes did not occur to any appreciable extent, whereas the contrary was true of the ketones. This accumulation of the ketones can be understood in any of three ways. In the first place, it is quite possible that the aldehydes produced, owing to their highly divergent reactivity, can be immediately transformed. Second, the rate of dissimilation of the different alcohols may vary considerably. Finally, the energy requirements of the organism may be partially met to a greater degree by the secondary dissimilation products of the primary alcohols.

It was thought worth while to investigate these considerations further. As a result, the determination of the decrease of substrate was carried out. For this purpose, the normal alcohol was inoculated in various ways: with a spore suspension grown on glucose agar, with bits of mycelium grown on flax seeds and with bits of mycelium grown in the stock alcohol solution. These variations resulted in wide differences in the respective rates of utilization and clearly indicate an adaptative enzymatic effect.

TABLE X

DISAPPEARANCE OF ALCOHOL (20)*

Alcohol	Type of inoculation	2nd day	4th day	6th day	8th day	10th day	12th day
n-Propyl	Glucose–spore	No growth					
	Flax–mycelium				2.4	5.6	10.2
	Adapted mycelium	7.6	18.8	21.8	25.4	37.2	62.5
Isopropyl	Glycose–spore	13.5	26.7	40.5	52.1	75.6	88.3

* Measured in mg. per 50 ml. 0.4% solution.

The rate of utilization of the iso alcohol, when inoculated from a glucose spore suspension, was appreciably greater than the best case with the normal alcohol, *i. e.*, when the latter was inoculated with mycelium from the stock alcohol cultures. This more rapid utilization of the secondary alcohol may make possible the observed accumulation of the dehydrogenation product.

2. Course of Dehydrogenation of Methyl Alcohol, Ethylene Glycol, Glycerol, etc.

The qualitative course of the degradation of various homologues of methyl alcohol and other hydroxylated compounds is presented in Table XI. An examination of the table indicates that, in general, the manner of utilization of these compounds parallels those investigated previously.

As a result of the study of the dissimilation of *n*-propyl, *iso*-propyl, *n*-butyl and *sec*-butyl alcohols and propylene glycol, it had been observed

TABLE XI

QUALITATIVE ANALYSIS OF SUBSTRATES (21)

Substrate	Dissimilation product	Method	Identification
Methyl alcohol	Formaldehyde	Trapping	Dimedon deriv., m. p. 186–187° (aq. alcohol)
Ethyl alcohol	Acetaldehyde	Trapping	Dimedon deriv., m. p. 140–141° (aq. alcohol)
Ethylene glycol	Glycolaldehyde	Trapping	Dimedon deriv., m. p. 224–226° (aq. alcohol)
2,3-Butylene glycol	Acetylmethylcarbinol	Isolation	*p*-Nitrophenylosazone, m. p. 318° (pyridineacetic acid)
Glycerol	Triose	Isolation	2,4-Dinitrophenylosazone, m. p. 264–265° (pyridineacetic acid)
	Pyruvic acid	Isolation	2,4-Dinitrophenylhydrazone, m. p. 214–215° (acetic acid)
	Ethyl alcohol	Isolation	Iodoform and dichromate–nitric acid test
Erythritol	Erythrulose	Isolation	Phenylosazone, m. p. 163° (aq. alcohol)
Glucose	Acetaldehyde	Trapping	Dimedon deriv., m. p. 140–142° (aq. alcohol)
Sorbitol	Hexose mixture	Isolation	Phenylosazone, m. p. 163–164° (aq. acetone)
Mannitol	Pyruvic acid	Isolation	Lu test
Dulcitol	Pyruvic acid	Isolation	Lu test
Inositol	No dissimilation products detected		
tert-Butyl alcohol	No growth observable		

TABLE XII

YIELDS OF DIMEDON DERIVATIVES OBTAINED BY TRAPPING (21)

Substrate	Percentage composition	Dissimilation product	Precipitate weight,* g.
Methyl alcohol	0.2–0.4	Formaldehyde	0.03
Ethyl alcohol	0.4	Acetaldehyde	1.00
Ethylene glycol**	2.0	Glycolaldehyde	0.15
Glucose	0.5	Acetaldehyde	0.10
Erythritol-glucose	1.0	Acetaldehyde	0.12

* Quantity obtained from 10 flasks each containing 100 ml. of nutrient media.

** Because of inhibition by dimedon, this experiment had to be carried out over a period of 12–15 weeks.

that their dehydrogenation followed definite paths, depending upon the alcoholic group present in the substrate molecule. When the alcohols investigated contained but secondary alcoholic groupings, there was marked

accumulation of the intermediate products, while the contrary was true of the primary alcohols. In addition, a compound containing a mixture of the two groups, when acted upon by *F. lini* Bolley, resembled a secondary alcohol in this respect.

No accumulation of intermediates was observed with either ethyl alcohol or ethylene glycol. Rather, the substances shown to be present had to be trapped in order to be determined. When 2,3-butylene glycol, glycerol and erythritol were used as carbon sources, not only isolation of the primary dehydrogenation products was possible without trapping but also compounds other than these were detectable in the glycerol experiments.

TABLE XIII

MAXIMUM YIELDS OF DEHYDROGENATION PRODUCTS OBSERVED (21)

Substrate	Percentage composition	Dissimilation product	Percentage yield
2,3-Butylene glycol	0.4	Acetylmethylcarbinol	25–30
Glycerol	2.0	Triose	0.3–0.5
Erythritol	1.0	Erythrulose	3–4
Sorbitol	4.0	Hexose mixture	3–4

One path of the glycerol dissimilation appears to be via dihydroxyacetone, pyruvic acid and ethyl alcohol, since small amounts of the same could be isolated along with approximately 75 mg. of alcohol from 1 l. of a solution of glycerol nutrient medium. This series of reactions is one which may take place also in the latter phases of the degradation of a hexose in alcoholic fermentation. The glycerol serves as a hydrogen donator for the reduction of the acetaldehyde obtained after the decarboxylation of pyruvic acid:

$$\begin{array}{l} CH_2(OH)—CH(OH)—CH_2—OH \\ \qquad\qquad \downarrow -2\,H \qquad\qquad (11) \\ CH_2(OH)—CO—CH_2—OH \rightleftharpoons CHO—CH(OH)—CH_2—OH \\ \qquad\qquad \downarrow \\ CH_3—CO—COOH \\ \qquad\qquad \downarrow -CO_2 \qquad\qquad (12) \\ CH_3—CHO \\ \qquad\qquad \downarrow +2\,H \qquad\qquad (13) \\ CH_3—CH_2—OH \end{array}$$

Gould (22) is of the opinion that the enzymes of *Fusaria* causing alcoholic fermentation are "constitutive," regardless of the substrate upon which they are grown, and that the amount present varies with the compound employed.

The utilization of hexitols by *F. lini* Bolley gave rise to variable results. When dulcitol and mannitol were used as sole carbon sources, only pyruvic acid could be established as a degradation product. Neither reducing compounds nor ethyl alcohol could ever be detected. Inositol proved totally negative in respect to the analyses for all the compounds just mentioned. Sorbitol yielded a hexose mixture composed of sorbose along with other undeterminable compounds.

3. *Training of Fusaria*

The dimedon fixation method was employed and the simultaneous use of glucose was applied in experiments with methyl alcohol; 0.01 ml. of methyl alcohol was added one week after inoculation to a 0.5% glucose fermentation. This addition was continued weekly for a period of three to four weeks and resulted in isolation of small quantities (approximately 20–30 mg.) of the dimedon derivative of formaldehyde from 10 culture flasks, each containing 50 ml. of media (21).

As controls in these experiments, a series of sterile, uninoculated, glucose–dimedon media was treated in a similar manner and no precipitate was observed. In addition, 0.5% glucose–dimedon medium was inoculated and the derivative of acetaldehyde was obtained after the fermentation had proceeded one week. At the time the precipitation was first noted, the glucose initially present had practically disappeared and only traces of pyruvic acid were detectable. Hence, the formation of acetaldehyde was due to a dehydrogenation of the alcohol formed in the fermentation of glucose. As is known, this compound is an intermediate in the yeast fermentation of hexoses also; but under the experimental conditions used here no trapping of the intermediate of this process was evidenced since the dimedon derivative isolated appeared only after the glucose initially present had been removed. This derivative disappeared over a period of three to four weeks.

In erythritol experiments, 1% solutions of erythritol along with 0.5% glucose were prepared with the usual inorganic nutrient medium. The solutions were then measured into flasks, sterilized, inoculated and periodically analyzed for a reducing compound present and for mycelium weight.

A number of explanations can be offered for the effective role of glucose in bringing about the changes observed when both methyl alcohol and erythritol were used as carbon sources. The most probable is that the use of glucose results in the production of a large quantity of this organism. Consequently, the number of mutants probably present is increased and they then measurably utilize the compound previously considered unattackable. This consideration appears to be substantiated by the marked increase in the mycelium weights over that of the glucose controls observed in the ex-

periments with erythritol–glucose mixtures. With respect to this it should be noted that Braun and Cahn-Bronner (7) utilized bulk inoculations to bring about the same effect in the case of *Eberthella typhosa* requirement for tryptophane. The effects observed can be ascribed to a training brought about by means of a special technique.

An adequate approach to the solution of the mechanism of dehydrogenation catalyzed by *F. lini* Bolley has as its essential part an understanding of the enzyme system concerned. As a contribution to the solution of this problem, a study was undertaken of chemical conversions effected by the various enzymes present. Evaluation of the course of the dehydrogenation with primary, secondary and tertiary alcoholic groups in the molecule indicates that the first two groups of compounds can be dehydrogenated, whereas the third cannot. The failure of the tertiary alcohol (Table XI) to serve as a carbon source may be attributed to the concurrent necessity for a rupture of the molecule along with the dehydrogenation process. Certain differences in the dissimilation of the primary and secondary alcohols were discernible which were enough to make valid distinctions between the two.

When additional compounds containing more than one hydroxyl group and with longer carbon chains were investigated, the deviations observed between the primary and secondary alcohols held with compounds having as many as four carbon atoms in the molecule. When hexitols were used as sole carbon sources, this difference disappeared.

4. Significance of Identification of Glycolaldehyde

A particularly interesting finding, helpful to the interpretation of studies of alcoholic fermentation, was the protracted blocking with dimedon of glycolaldehyde as a dehydrogenation product of ethylene glycol. Glycolaldehyde has been occasionally considered (13, 14) as an intermediate in pentose fermentations; but the identification of even small quantities has not been experimentally shown to date. This observation is at a noteworthy variance with speculations of Dickens (12), who considered that glycolaldehyde, if formed, is supposed to undergo dismutation when fermented by a dialyzed Lebedev (yeast) preparation.

It has been assumed that the alcohol values of *Fusaria* fermentations were low because of the fungal utilization of alcohol and that acetaldehyde was determinable as an intermediate in the fermentation of glucose to alcohol. The results presented indicate that formation of acetaldehyde was observable only after the glucose had virtually disappeared. Hence, the formation of acetaldehyde, as isolated by means of dimedon, was due to a

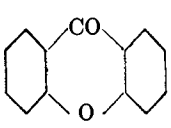
dehydrogenation of the alcohol produced from the glucose and not as an intermediate in the conversion of the hexose to alcohol.

The omnivorous nature of *Fusarium* rendered the evaluation of the stepwise degradation of the intermediates formed from the various substrates difficult if not impossible. Of the compounds investigated, only a very small number were found to be unsuitable as carbon sources, and of these the application of a specialized technique resulted in the "training" of this organism to dehydrogenate both methyl alcohol and erythritol.

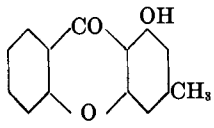
V. Role of Xanthones and Elementary Sulfur in Dehydrogenations by *Fusaria*

In the course of these studies, an attempt was made also to approach the problem of the function of certain "waste materials" such as pigments deposited in plant cells or in microorganisms, since it was observed that the addition of nicotinic acid makes it possible more readily to synthesize dehydrogenating enzymes abundantly present in *Fusaria*. It seemed justifiable to investigate whether a functional relation exists between the two structurally different groups of substances (63).

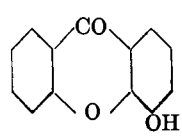
Starting with an isopropyl alcoholic solution of the pigment extracted from *Fusarium graminearum* Schwabe, the effect of solutions upon the rate of dehydrogenation by *F. lini* Bolley of the following substituted and basic xanthones was investigated:



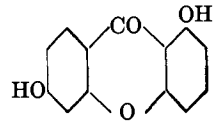
Xanthone



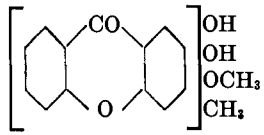
1-Hydroxy-3-methylxanthone



4-Hydroxy-xanthone



1,6-Dihydroxy-xanthone



Rubrofusarin

Table XIV shows the extent of effect observed on the rate of dehydrogenation of isopropyl alcohol and accumulation of acetone in the presence of given amounts of these compounds as compared with the magnitude of the rate of the same reaction effected by adding nicotinic acid. Mat weight determinations at the end of each series of experiments complement the observations.

TABLE XIV

DEHYDROGENATION OF ISOPROPYL ALCOHOL (63)*

Day	Blank		Xanthone (1000)		1-Hydroxy-3-methyl-xanthone (1000)		4-Hydroxy-xanthone (1000)		1,6-Dihydroxy-xanthone (1000)		Rubrofusarin (1000)		Blank		Nicotinic acid (500)		Nicotinic acid (1000)		Nicotinic acid (5000)		Nicotinic acid (10,000)	
	P**	A	P	A	P	A	P	A	P	A	P	A	P	A	P	A	P	A	P	A	P	A
0	180.0	..	181.6	..	182.7	..	186.0	..	183.0	..	180.0	..	183.5	..	187.5	..	187.0	..	189.0	..	185.0	..
7	63.0	14.7	72.1	15.2	72.2	15.2	68.0	17.0	65.4	12.0	60.3	12.2	69.5	14.5	72.3	11.7	72.8	13.7	72.5	12.6	74.0	14.0
8	73.2	..	82.6	..	81.5	..	81.0	..	77.1	..	71.0	..	80.5	..	79.0	..	80.5	..	80.5	..	81.4	..
9	86.3	17.3	91.6	19.3	90.6	21.0	94.1	18.7	88.9	17.2	80.3	14.2	88.8	16.8	89.0	15.8	85.0	16.0	93.2	17.3	90.7	15.3
10	106.0	..	110.3	..	112.2	..	110.0	..	104.3	..	96.3	..	101.7	..	103.3	..	104.1	..	98.5	..	104.3	..
11	108.8	22.7	111.1	24.5	119.2	26.7	124.0	32.2	119.3	28.5	100.3	19.8	108.0	20.2	112.5	20.0	114.7	20.5	120.1	22.5	120.4	25.0
12	120.8	..	122.9	..	123.2	..	130.5	..	129.7	..	116.7	..	121.2	..	123.0	..	127.5	..	129.0	..	132.0	..
13	131.8	33.8	139.8	32.6	138.2	29.0	134.3	35.5	144.0	28.1	126.8	32.9	130.5	25.8	133.5	24.3	135.8	28.0	137.2	28.5	139.8	26.3
MYCELIUM WEIGHT ON THIRTEENTH DAY																						
	26 mg.		29.4 mg.		35.0 mg.		39.9 mg.		41.7 mg.		35.5 mg.		31.7 mg.		29.3 mg.		35.4 mg.		32.4 mg.		42.5 mg.	

* Concentrations (in parentheses) of various xanthones and nicotinic acid are expressed in gamma per 1000 ml.

** P represents isopropyl alcohol; A represents acetone.

It can be seen that, in accordance with the structural formulas of the xanthones, the following relations exist: xanthone (*e. g.*, eighth day, 12.84%) and nicotinic acid (in accordance with the concentrations, 1.99%, 2.69%, 4.13%, 5.69%) measurably increase the rate of dehydrogenation as compared with the blank. The three intermediates tend, in general, to show an increase (*e. g.*, eighth day, 11.34%, 10.66%, 5.33%) in this rate

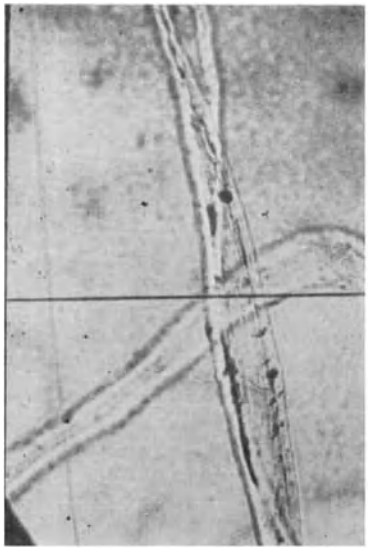


Fig. 1.—*F. lini* Bolley grown on an isopropyl alcohol solution of xanthone (ninth day, × 400) (63).

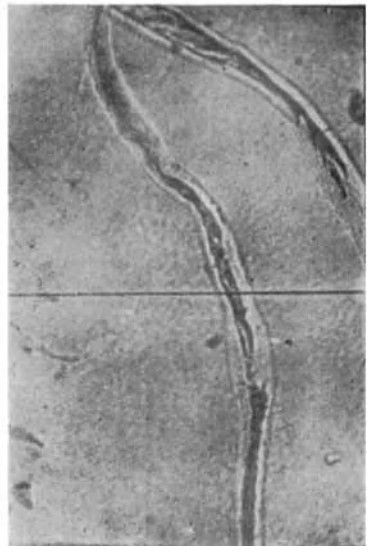


Fig. 2.—*F. lini* Bolley grown on an isopropyl alcohol solution of rubrofusarin (ninth day, × 400) (63).

to a distinctly lesser extent, and the natural pigment definitely retards the progress of dehydrogenation (*e. g.*, eighth day, −3.01%) in accordance with the increasing number of hydroxyl groups and complexity of the xanthone molecule.

In evaluating the analytical data it must be borne in mind that two dehydrogenations proceed parallel to one another. The dehydrogenation of isopropyl alcohol is accompanied by that of the acetone formed. The latter reaction leads to formaldehyde via methanol as intermediary:

$$CH_3COCH_3 + H_2O \longrightarrow CH_3CHO + CH_3OH \qquad (14)$$

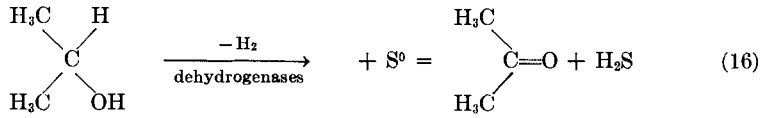
$$CH_3OH \xrightarrow{-H_2} HCOH \qquad (15)$$

The extremely high rate of this reaction is recognizable toward the end, and consequently, the terminal isopropyl alcohol values appear to be higher in the case of extended experiments.

Although the increase in mat weight in the case of basic xanthone is slight, its influence was greatest on the rate of dehydrogenation. In the case of the pigment, however, an approximately 40% increase of the final mat weight was still accompanied by a definite retardation of the rate of dehydrogenation. Contrary to Figure 1, in Figure 2 the fungus shows a visible deposition of the pigment excreted by *Fusarium graminearum* Schwabe, which is not utilized by *F. lini* Bolley. This deposition is accompanied by enhanced growth.

On the other hand, quantitative experiments on the dehydrogenation of isopropyl alcohol carried out in the presence of elementary sulfur (70) tend to show an increase in the rate of disappearance of the substrate when the growth of the organism, evaluated on the basis of mat weights, is taken into consideration. For example, after administration of 1000 γ sulfur, the mat weight determined on the fourteenth day shows a decrease of 56% as compared with a decrease in dehydrogenation of only about 9%. In another case, using 25,000 γ sulfur, the decrease in mat weight on the seventeenth day amounted to 90% as compared with the blank and with a decrease in dehydrogenation of about 32%.

Equation (16) may picture in a simplified way the course of this reaction:



(16)

In utilizing glycerol, which was an excellent carbon source for dehydrogenations, its superiority as compared with the strongly inhibitory isopropyl alcohol served to overcome the detrimental effects of sulfur, hydrogen sulfide, or both, on the growth of the organism.

VI. Rubrofusarin from *Fusarium graminearum* Schwabe

Observations concerning the nature and possible action of the cause of the photosensitizing effect of a fluorescing dye, *i. e.*, hypericin (76), on microorganisms have been made. And in a recent paper (19) it was also indicated that phoenicin, the red pigment present in *Penicillium phoenicium*, is capable of exerting an influence on the respiration of *Bacterium pyocyaneum*. Efforts were consequently made to isolate and identify such compounds present in *F. oxysporum*, *F. lycopersicii* and *F. graminearum* Schwabe.

Growth experiments upon Raulin-Thom and Czapek-Dox nutrient media, covering a *p*H range from 2 to 8, indicated that the maximum color formation of *F. oxysporum*, which was comparatively slight, occurred when using the first of these media at a *p*H of 8. After three weeks' growth in a sterilincubator, and extraction of the mycelium with numerous organic solvents, ethanol was found to be the only solvent capable of removing pigment from the mats. Because of the extremely small amount present, however, attempts to isolate the coloring matter from the solution proved futile.

The results of a series of growth experiments designed to obtain pigments from *Fusaria* are shown in Table XV.

TABLE XV

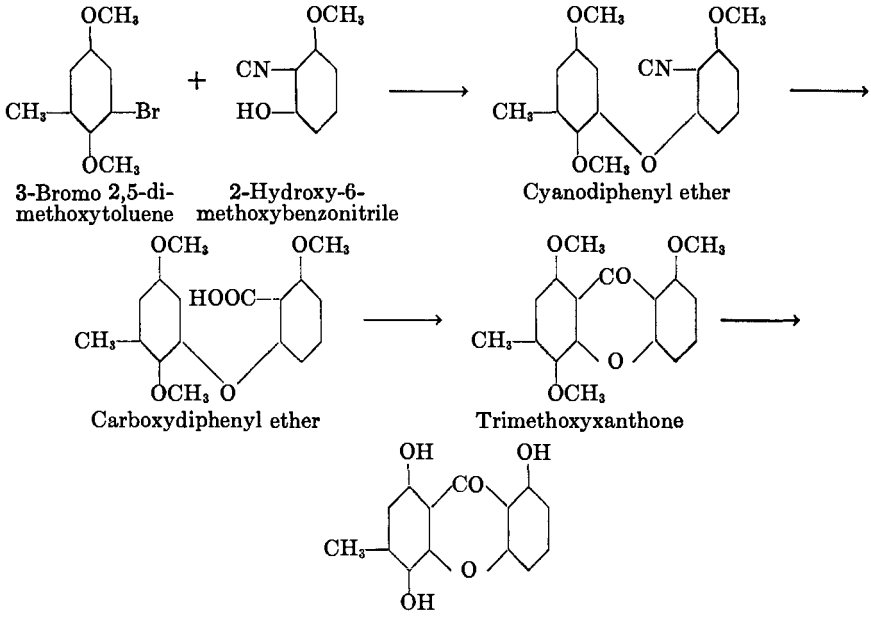
PRODUCTION OF PIGMENTS AND FATS FROM *Fusaria* (43)

Species of *Fusarium*	Initial *p*H	Final *p*H	Incubation period, days	Weight of mycelia, g.	Weight of crude pigment, g.	Weight of crude fat, g.
oxysporum	8.0	7.3	21	380.4	0.002	..
	8.0	6.5	24	405.6	0.003	..
lycopersicii	6.3	6.6	21	305.2	0.004	..
	6.5	6.8	21	300.4	0.003	..
graminearum	4.0	2.8	21	310.0	26.900	32.0
	8.0	7.2	21	405.5	0.231	38.2
	8.0	6.3	23	401.2	0.220	39.0
	8.0	4.3	25	344.6	0.188	31.1
	8.0	5.4	26	350.5	0.186	32.4
	8.0	4.4	32	287.9	0.171	29.6

1. Synthesis of Ravenelin

Although rubrofusarin is believed to be a monomethyl ether of a methyl trihydroxyxanthone, the position of the groups in the complex molecule is not certain, partially because of the small quantity of pigment obtainable and the difficulty encountered in attempting to degrade the compound by ring cleavage. While the presence of certain groups may be indicated by chemical tests, the positions of such constituents are not necessarily shown by this means. However, by comparing the absorption spectra of synthesized compounds of known similar structure with the spectrum of the unknown compound, one can often eliminate certain structural possibilities. It was found necessary, therefore, to prepare a number of xanthones containing hydroxy, methoxy and methyl groups in various positions of the nucleus and to determine their absorption spectra. The synthesis of ravenelin, which is isomeric, but not identical, with rubrofusarin, was also undertaken, and may be represented by the steps (43) given in Scheme 2.

Since nor-rubrofusarin is isomeric, although not identical, with ravenelin (Fig. 3), their absorption spectra were compared as the first step in an



SCHEME 2
SYNTHESIS OF RAVENELIN

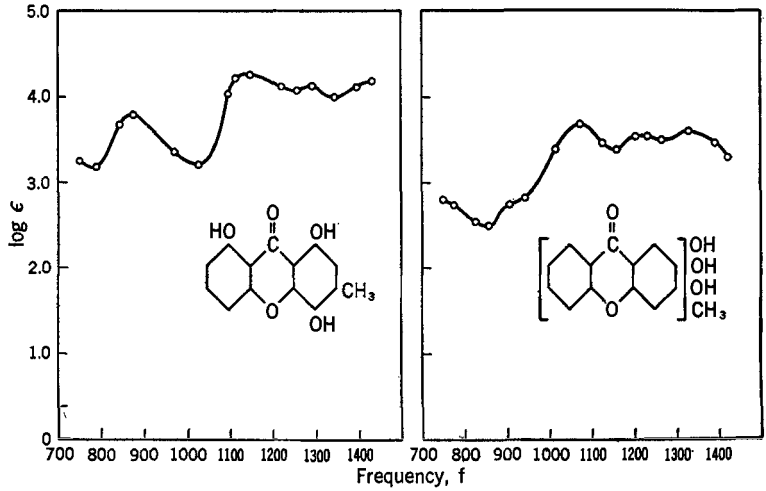


Fig. 3.—Absorption spectrum of ravenelin, 0.0988–0.0079 gram per liter in ethanol (43).

Fig. 4.—Absorption spectrum of norrubrofusarin, 0.2103–0.0263 gram per liter in ethanol (43).

attempt to determine the structure of methylated nor-rubrofusarin (rubrofusarin) from purely spectroscopic findings. The similarity between the spectra of nor-rubrofusarin and rubrofusarin (Figs. 4 and 5) is most noticeable in the range of 1200–1400 f, indicating the presence of an hydroxy group in position 1 of the former.

Positions 3 and 6 as well as 4 and 5 are equivalent in the xanthone nucleus. It seems likely, therefore, that the methyl and third hydroxy groups are in positions 2 and 7. A possible structure for nor-rubrofusarin would thus be 1,2,8-trihydroxy-7-methylxanthone.

From a comparison of the hydroxy- and methoxyxanthones, it is evident that the elimination of the sharpness of a maximum appears to be a characteristic alteration caused by methylating the former compounds. In the spectrum of rubrofusarin this phenomenon is apparent in the region of 1150–1250 f, the range indicating the presence of an hydroxy group in position 1. Rubrofusarin, therefore, may possess either a 2,8-dihydroxy-1-methoxy-7-methyl- or 2,3-dihydroxy-8-methoxy-7-methylxanthone structure.

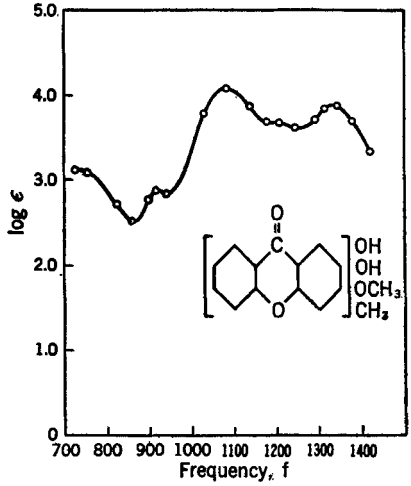


Fig. 5.—Absorption spectrum of rubrofusarin, 0.2241–0.0140 gram per liter in ethanol (43).

2. *Possible Mode of Action of Xanthones*

The living cell is acknowledged to be a complex system capable of forming a wide variety of organizers and substances, yet many of its metabolic products deposited within the cell have been designated as waste materials, and a study of their function in the cell has been largely neglected. Tappeiner (76, 77), in reference to the photodynamic action of such fluorescing compounds as hypericin, has stated that: ". . .Zusatz von fluoreszierender Substanz in geringer Menge hingegen hebt das spezifische Wirkungsvermögen (of enzymes) rasch und dauernd auf." On the other hand, Friedheim (19) claims to have observed a 200–300% increase in the respiration of *Bacterium pyocyaneum* upon the addition of phoenicin. Thus there are recorded two specific instances of "waste" material's exhibiting, not only

diversified, but also completely contrary functions, the one manifesting an irreversible effect upon the enzyme system concerned and the other an intensification of the respiratory action.

According to Schöpf (69), the formation of natural substances in the living cell may be of three types. The cell may have an enzymatic system designed (*a*) for the highly specific synthesis of a certain substance or (*b*) for processes of general application performed by enzymes, *e. g.*, hydrogenations, dehydrogenations, decarboxylations, etc. Finally (*c*) there are those syntheses which take place without the action of enzymes, and which are characterized by the formation of reactive organic substances during the growth of the cell. These compounds upon contact in the cell yield isolable products, which are, in fact, chance products or intermediates if their further conversion within the cell proceeds slower than the aforementioned syntheses.

The pigments present in microorganisms may be considered as resulting from method of formation *a* or *c;* but their relation, if any, to type *b* is not always clear. In the case of one of the pigments obtainable from *Fusarium graminearum* Schwabe, however, the compound had a marked effect upon the activity of a dehydrogenating enzyme system present.

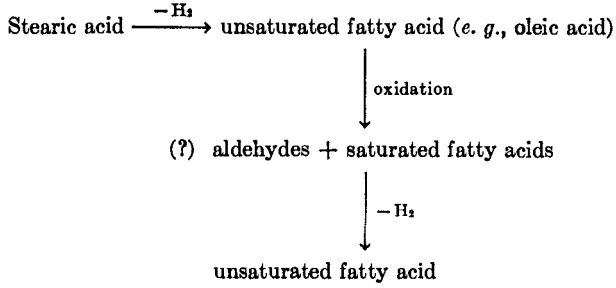
This mode of action of rubrofusarin (see page 190) and the related xanthones, which, contrary to the former, in accordance with their structure, increase the rate of dehydrogenation from 5.33 to 12.84%, indicates that the xanthones have a dual but interdependent effect upon the dehydrogenating enzyme systems present in *Fusaria.* Thus, in a manner analogous to that of nicotinic acid, they may enhance but may also inhibit the synthesis and/or the action of dehydrogenating enzymes by the microorganism, depending upon the structure of the xanthone employed, while simultaneously promoting or retarding the growth of the mould.

VII. Fat Formation in *Fusaria*

In the course of earlier studies (59) it was noticed that, though the mycelia of some *Fusaria* contain exceedingly small quantities of various pigments, the fat content (see Table XV, last column) amounted to as much as 10% of the dry mat weight.

Since a relationship was demonstrated between pigment structure and the rate of dehydrogenation of isopropyl alcohol to acetone, it seemed desirable to determine the chemical composition of this fat in order to reveal any relation between a possible mechanism of its formation through the action of enzymes so far not accounted for in *Fusaria.*

The problem of the synthesis of lipides from hexoses and especially from pentoses is rather complex and intriguing. The large quantity of fat (59) produced in the course of growth of *Fusaria* which appears in all culture media studied (52) seems to have a definite function. No doubt these fats are synthesized by means of a lipase system; from the information thus far available (44), the fat consists mainly of an unsaturated fatty acid similar to oleic acid. The study of an enzymatic action of *Fusarium lini* Bolley upon a likely precursor, such as stearic acid, indicates that the saturated acid may be dehydrogenated by an enzyme system similar to that by which it was formed in *F. graminearum*, perhaps giving rise to aldehydes (generally expressed), in accordance with the findings of Feulgen and Bersin (16), and to a saturated acid. The saturated fatty acid thus obtained can be further acted upon by the enzyme system present (Scheme 3):



SCHEME 3

Thus, presumably, the precursor of the end product, formed, perhaps, in agreement with the postulation of Witzemann (87), is not a static entity but in reality undergoes a series of changes, thereby playing an integral role in the total metabolism of the organism.

From a comparison of the analytical constants it would appear that the composition of yeast fat (81) and of *Fusaria* fat are similar (see Table XVI). Moreover, *Fusaria* possess also a fatty acid dehydrogenase, and are able to

TABLE XVI

COMPARISON OF *Fusaria* AND YEAST FATS

Analysis	*Fusaria* fat	Yeast fat (81)
Iodine number	84.68	71.1
Saponification number	189.28	162
Unsaponifiable matter	2.1%	15.6%
Saponifiable matter	96.3%	ca. 84.4%

split olive oil, just as was observed with yeasts. On the other hand, the earlier established ability of *Fusaria* to utilize elementary sulfur as a hydrogen acceptor in distinction to certain chemautotrophic bacteria, and their failure to give rise to the formation of phosphoglyceric acid while fermenting pentoses via pyruvic acid, seem to demonstrate clearly the great variety of general pathways which nature is able to resort to in microbiological syntheses and degradations.

VIII. Technical Applications

1. *Fermentation of Sulfite Waste Liquors,* Wheat Hydrolyzates and Wood Hydrolyzates*

It was Braconnot (6) who first called attention to the fact that, by treating cellulose with 90% sulfuric acid, a quantitative transformation into a mixture of carbohydrates (hexoses and pentoses) takes place. Later on it was demonstrated by Arnould (2) that, when wood was subjected to the same procedure, considerable amounts of sugars were also obtained. According to Klason (32), 10% of the wood content of wood can be extracted by hot water (amounting to 20% of the cellulose) as wood gum. This gives rise to about 25% xylose (equal to 5% of the cellulose); and from the rest are formed the hexoses present in wood waste liquors.

The production of paper pulp is carried out basically by cooking wood chips with calcium bisulfite under direct (America) or indirect (Europe) application of steam. The aforementioned waste liquors are a by-product of these processes and contain considerable amounts of carbohydrates fermentable by yeasts and *Fusaria*, which gave rise, especially in Sweden, Finland, Germany and Canada, to a large industry for additional production of vast amounts of ethanol.

If in the course of the cooking process the progress of the formation of carbohydrates (hexoses and pentoses) is pursued analytically (24), it can be observed that in the first 6–8 hours only a small amount of sugars is found in the solution, and of this portion again only an insignificant part is fermentable by yeasts. The bulk of the sugars so formed consists of pentoses, which are preferentially taken into solution and which can be subjected to alcoholic fermentation only by *Fusaria*. In the course of continued cooking the amount of yeast-fermentable hexoses increases rapidly. Two reasons are given as an explanation for this rapid increment: (*a*)

* The work described in this section was done under two contracts, recommended by the Wheat-Alcohol Research Committee, between the War Production Board and Fordham University.

the increase of temperature, and (*b*) the composition of the cooking liquor. Besides these, it is claimed that the water content of the wood plays an important role. It is probable that the rate of diffusion of the fresh cooking liquor into the wood chips is controlled by their moisture content.

Additional amounts of hexoses and pentoses should become available when their production will be carried out through acid hydrolysis of sawdust. It must be borne in mind that, *e. g.*, the mash obtained from conifer hydrolyzates contains about 0.6–0.7% pentoses and pentosans corresponding to the pentoses amounting to approximately 10% of the dry weight of the wood. About the same content of pentoses is available in the slops, which at present remain unused after the yeast fermentation of the hexoses has been terminated. This waste represents, in a plant for wood saccharification having a capacity of about 400,000 cubic meters of slops per year, 2500 metric tons of pentoses lost. Conditions in the paper pulp industry seem to be even more unfavorable. The pentose content of a beechwood waste liquor amounts to about 4.5%, that of a spruce waste liquor to about 2%. At a medium-sized plant producing about 350,000 metric tons of slops, this may aggregate to 15,000 or 7000 tons, respectively, of pentoses lost per year.

In the course of confirming earlier investigations from this laboratory (56), an attempt was made recently (13) to attack the problem of the utilization of these wastes. The result was described as fully negative; no alcoholic fermentation of pentoses present in waste liquors and no growth of *Fusaria*, when these liquors served as carbohydrate source, were noticeable. Accordingly, this problem was reinvestigated by the present authors in collaboration with B. Kramer. In order to eliminate the inhibitory material present in the waste liquor, several methods were employed. Both sulfur dioxide and lignin compounds were removed by precipitation with calcium hydroxide (82) at *p*H 10.5 and 12.2, respectively. The calcium ion was displaced as the insoluble sulfate and a final adjustment to *p*H 4.5 was made with sulfuric acid before inoculation. Both the spruce and hemlock waste sulfite liquors were clearer and lighter in color after this procedure, although retaining their characteristic odor. When the liquor was treated in this manner, augmented with inorganic salts, and diluted 1:5 or 2:5 with distilled water, growth of *Fusarium lini* Bolley upon the liquor occurred.

In the course of this operation, the loss of sugar, as shown by the following analytical figures, was negligible:

Before treatment with calcium hydroxide	13.6 mg./ml.
After treatment with calcium hydroxide	10.96 mg./ml.

This general procedure was also used after first concentrating waste sulfite liquor to a thick syrup and then treating with calcium hydroxide. Growth under these conditions was good at a 1:5 dilution.

An alternative method was found to be successful when employed with waste hemlock sulfite liquor. Most resinous materials are capable of taking up varying amounts of water. The process of absorption may depend on different colloidochemical phenomena and probably controls the accumulation of substances dissolved or suspended in a liquor. Accordingly, a resin prepared by autoclaving the following constituents at 15–17 pounds pressure for 45 minutes was applied in this procedure:

53 parts ammonium chloride or 66 parts ammonium sulfate
60 parts formaldehyde
44 parts acetaldehyde

In addition to these introductory operations, supplementing the media with potassium cyanide retarded the disappearance of alcohol and was in agreement with earlier observations (56). Although these methods assisted in the removal of most of the inhibitory substances present, a small amount remained which diminished an optimum utilization by *Fusarium lini* Bolley of the carbohydrates present. When concentrating waste sulfite liquor, some volatile material was detected in the distillate. Based upon qualitative evidence and melting points, it appears that the inhibitory material is one or more of the following substances: dimethyl ketone, formaldehyde, propionaldehyde or N-propylaldehyde.

Judging from the figures recorded in Table XVII, it can be seen that best results were obtained by a calcium hydroxide treatment of the waste sulfite liquor obtained from spruce after concentration to half the original volume. The successful application of a resinous composition to remove inhibitory material is also shown in the same table.

TABLE XVII

ALCOHOL VALUES* FOR WASTE LIQUOR** INOCULATED WITH F. LINI BOLLEY

Waste sulfite liquor	Treatment	Seventh day	Eleventh day	Fourteenth day	Mat† weight
Spruce	$Ca(OH)_2$	167.95	None	None	332
	$Ca(OH)_2$ + KCN	164.4	109.58	99.58	351
	Concentration and $Ca(OH)_2$	211.2	24.18	24.18	...
Hemlock	$Ca(OH)_2$	24.18	None	None	...
	$Ca(OH)_2$ + KCN	24.18	None	None	...
	Concentration and $Ca(OH)_2$	5.45	45.75	46.5	345
	Resin	56.97	None	None	271
	$Ca(OH)_2$ and resin	39.18	3.33	None	285

* Values expressed in mg. per 100 ml. of treated liquor. All the waste liquor used in this investigation was obtained through the courtesy of Dr. Richter Salvesen, Marathon Chemical Co., Rothschild, Wis.

** All dilutions of waste liquor were 1:5.

† Represents the combined weights of 4 flasks containing 50 ml. of nutrient media per flask.

Attempts to improve the yield of ethyl alcohol obtained by calcium hydroxide treatment of the spruce sulfite liquor and inoculation with *F. lini* Bolley were successful. When potassium cyanide was added to the inoculated liquor on the fifth day to give a final concentration of $M/200$ KCN, the disappearance of ethyl alcohol on the seventh day was appreciably decreased. These results signify a reduction of the time required to obtain a maximum yield of ethyl alcohol (56).

It should be mentioned here that, by the application of a fusarial pentose fermentation to sulfuric acid hydrolyzed wheat mashes, an increased yield of alcohol amounting to 6–12% was obtained over that derived from yeast fermentation of the hexoses (62).

Fermentation of Douglas-fir hydrolyzates prepared with dilute sulfuric acid by *Fusarium lini* Bolley indicated (36*a*) that the "pentose" alcohol obtainable from these wood hydrolyzates amounts to about 1% of the alcohol derived from yeast fermentation of hexoses present.

2. *Decomposition of Chemical Lignin*

Fungi are generally regarded as being among the most active of the microorganisms that break down the native lignin in plant materials. In investigations of Ledingham and Adams (35), the effect of 13 different species of *Fusarium* on calcium lignosulfonate medium was studied. Their results are given in Table XVIII. The *Fusarium* cultures all brought about rapid

TABLE XVIII

ANALYTICAL RESULTS FROM *Fusarium* CULTURES GROWN ON CALCIUM LIGNOSULFONATE MEDIUM (35)

Fusarium species	Incubation time, days					
	Twenty			Forty		
	pH	Sugar fermented, %	Lignin decomposed, %	pH	Sugar fermented, %	Lignin decomposed, %
F. culmorum	2.92	83.9	13.4	4.25	90.0	12.5
F. coeruleum	3.40	90.2	8.9	3.60	93.1	11.8
F. orthocera	4.61	93.1	5.2	3.89	92.9	10.9
F. concolor	4.15	94.2	4.8	3.49	94.1	10.7
F. sp.	3.80	93.6	4.9	4.49	94.8	9.5
F. culmorum	4.40	93.5	4.1	4.02	93.5	8.4
F. equiseti	3.63	93.2	8.3	5.25	91.5	7.3
F. avenaceum	3.65	92.5	6.9	4.67	93.6	7.2
F. solani	3.70	92.3	8.2	5.10	95.1	6.1
F. oxysporum	3.80	92.5	6.1	4.92	91.8	5.9
F. oxysporum	3.52	92.3	9.0	5.41	92.4	5.6
F. sp.	3.40	93.6	8.2	4.55	92.9	4.0
F. sp.	4.88	94.8	4.4	5.15	94.7	1.4

fermentation of the sugar present in the medium, but they did not carry the lignin decomposition farther than about 12.5–13.4%.

3. *Nutritive Value of Fusaria*

Reference was made on page 177 to the comparatively high thiamin content of *F. lini* Bolley. These values (70), as contrasted with the toxicity of scabby barley infected with *F. graminearum* Schwabe (29) to certain animals, gave rise to the idea of investigating the possible role of *Fusaria* in nutrition. It is well-known that brewers' yeast at a 10% level in a purified diet supports good growth, reproduction and lactation in mice. The present authors, in cooperation with Cerecedo and Vinson (79*a*), and in order to study conditions of the utilization of *Fusaria* in animal feeding (40*a*), replaced the yeast of the purified diet by two strains of the genus *Fusarium*, *i. e.*, *F. lini* Bolley and *F. graminearum* Schwabe. The *Fusaria* were grown on an artificial stock culture medium containing glucose, potassium nitrate, potassium dihydrogen phosphate and crystalline magnesium sulfate. After an incubation period of about three weeks, the mycelia were dried and ground up into a powder. This powder was incorporated into the diet. To confine the studies to the possible effect of the vitamin-B complex present in *Fusaria*, the fat-soluble vitamins in the diet were supplied liberally by lard, Crisco and cod-liver oil. Mice used in these experiments were albino and Rockland black strains.

The diet containing *F. graminearum* was totally inadequate for growth for both strains of mice. The animals ate very little of the diet and succumbed within two or three weeks. The diet containing *F. lini* proved, over a period of 30–35 days, to be excellent for growth of the albino strain of mice. The growth during this period was superior to that obtained with diets containing 10% yeast. The food intake averaged 3–3.5 grams daily. Immediately after this period of superior growth, a loss in weight set in with unusual suddenness. The daily food intake fell to about 1–1.5 grams. Thereupon a supplement of thiamin (10 γ daily) was either injected or fed. An immediate resumption of growth occurred and the food intake was tripled overnight. This effect of vitamin B_1 is highly significant, since it has been shown that *F. lini* Bolley contained about 17–21 γ of the vitamin per gram of dried material or 1.7–2.0 γ per gram of diet. Such an amount should be adequate for growth. Similar results were observed with the black mice. It should also be noted that the females of both strains were the first to show the collapse in growth on the *F. lini* diet. The addition of any other member of the B complex had no beneficial effect similar to that of vitamin B_1. Three female mice on *F. lini* diet which were given 100 γ

of vitamin B_1 were successfully mated and gave birth to three large litters.

The conclusion may be drawn, that a diet prepared with 10% *F. lini* Bolley as a source of vitamins and containing about 37% fusarial proteins, when incorporated with crystalline vitamin B_1 is an excellent food for mice throughout growth, reproduction and lactation.

Bibliography

1. Abbott, O. D., *Agr. Expt. Sta., Columbia, Mo., Res. Bull.* No. 85 (1926).
2. Arnould, G., *Compt. rend.*, **39**, 807 (1854).
3. Barrón, E. S. G., in *Advances in Enzymology*, Vol. III. Interscience, New York, 1943, p. 149.
4. Barrón, E. S. G., *Science*, **97**, 18 (1943).
5. Beiler, M., *Dissertation*, Fordham University, New York, 1944.
6. Braconnot, H., *Ann. Chim. Phys.*, **12**, 172 (1819); **25**, 81 (1827). See also Ost, H., *Chem. Ztg.*, **34**, 461 (1910).
7. Braun, H., and Cahn-Bronner, C., *Biochem. Z.*, **131**, 272 (1922).
8. Bredig, G., and Sommer, F., *Z. physik. Chem.*, **70**, 35 (1910).
9. Collander, R., *Naturwissenschaften*, **30**, 484 (1942).
10. Cremer, M., *Ber.*, **32**, 2062 (1899).
11. Dammann, E., Rotini, O. T., and Nord, F. F., *Biochem. Z.*, **297**, 184 (1938).
12. Dickens, F., *Biochem. J.*, **32**, 1652 (1938).
13. Dietrich, K. R., and Klammerth, O. O. L., *Chem.-Ztg.*, **63**, 763 (1939).
14. Dische, Z., *Naturwissenschaften*, **26**, 252 (1938).
15. Dixon, H. H., and Atkins, W. R. G., *Sci. Proc. Roy. Dublin Soc.*, **14**, No. 1 (1913).
16. Feulgen, R., and Bersin, Th., *Z. physiol. Chem.*, **260**, 217 (1939).
17. Fink, H., and Just, F., *Biochem. Z.*, **311**, 287 (1942).
18. Fischler, F., and Böttner, R., *Z. physiol. Chem.*, **177**, 264 (1928).
19. Friedheim, E. A. H., *Helv. Chim. Acta*, **21**, 1464 (1938).
20. Goepfert, G. J., *J. Biol. Chem.*, **140**, 525 (1941).
21. Goepfert, G. J., and Nord, F. F., *Arch. Biochem.*, **1**, 289 (1942).
21*a*. Gordon, W. L., *Can. J. Research*, **C22**, 282 (1944).
22. Gould, B. S., and Tytell, A. A., *J. Gen. Physiol.*, **24**, 655 (1941).
23. Gould, B. S., Tytell, A. A., and Jaffe, H., *J. Biol. Chem.*, **146**, 219 (1942).
24. Hägglund, E., *Die Sulfitablauge und ihre Verarbeitung auf Alkohol.* Vieweg, Braunschweig, 1915, pp. 12, 13.
25. Hansen, H. N., *Mycologia*, **30**, 442 (1938).
26. Harter, L. L., *J. Agr. Research*, **62**, 97 (1941).
27. Hayasida, A., *Biochem. Z.*, **298**, 169 (1938).
28. Heitzmann, P., *Bull. soc. chim. biol.*, **23**, 453 (1941).
29. Hoyman, W. G., *Phytopathology*, **31**, 871 (1941).
30. Huszák, I., *Biochem. Z.*, **312**, 326 (1942).
30*a*. Jeanloz, R., *Helv. Chim. Acta*, **27**, 1501 (1944).
31. Johnson, M. H., *Chem. Eng. News*, **21**, 1581 (1943).
32. Klason, P., *Arkiv Kemi Mineral. Geol.*, **3**, No. 6 (1908).
33. Lavoisier, A. L., *Traité élémentaire de Chimie.* Paris, 1864, p. 101.
34. Lebedev, A. v., and Griaznov, N., *Ber.*, **45**, 3256 (1912).

35. Ledingham, G. A., and Adams, G. A., *Can. J. Research*, **C20**, 13 (1942).
36. Loew, O., *Biochem. Z.*, **31**, 166 (1911).
36a. Loughran, G. A., Soodak, M:, and Nord, F. F., *Arch. Biochem.*, **6**, 163 (1945).
37. Luz, G., *Dissertation*, Zürich, 1934.
37a. Mann, T., *Biochem. J.*, **38**, 342 (1944).
38. Meyer, V., and Janny, A., *Ber.*, **15**, 1527 (1882).
39. Meyerhof, O., and Perdigon, E., *Enzymologia*, **8**, 353 (1940).
40. Michaelis, L., *Naturwissenschaften*, **14**, 33 (1926).
40a. Miessner, H., and Schoop, G., *Deut. tierärztl. Wochschr.*, **37**, 167 (1929).
41. Mull, R. P., *unpublished data.*
42. Mull, R. P., *Arch. Biochem.*, **2**, 425 (1943).
43. Mull, R. P., and Nord, F. F., *Arch. Biochem.*, **4**, 424 (1944).
44. Mull, R. P., and Nord, F. F., *ibid.*, **5**, 283 (1944).
45. Needham, J., Lehmann, H., and Nowinski, W. W., *Compt. rend. soc. biol.*, **133**, 6 (1940).
46. Neuberg, C., *Am. Brewer*, **75**, 28 (May, 1942).
47. Neuberg, C., and Nord, F. F., *Ber.*, **47**, 2264 (1914).
48. Neuberg, C., and Nord, F. F., *Biochem. Z.*, **67**, 46 (1914).
49. Neuberg, C., and Nord, F. F., *ibid.*, **96**, 133 (1919).
50. Neuberg, C., and Schwenk, E., *ibid.*, **71**, 114 (1915).
51. Nilsson, R., *Naturwissenschaften*, **34**, 25 (1943).
52. Niethammer, A., *Arch. Mikrobiol.*, **13**, 140 (1942).
53. Nord, F. F., *Ber.*, **52**, 1207 (1919).
54. Nord, F. F., *Protoplasma*, **2**, 303 (1927).
55. Nord, F. F., *Ergeb. Enzymforsch.*, **2**, 23 (1933).
56. Nord, F. F., *ibid.*, **8**, 149 (1939).
57. Nord, F. F., in *A Symposium on Respiratory Enzymes.* Univ. of Wisconsin Press, Madison, 1942, p. 264.
58. Nord, F. F., *Iowa State Coll. J. Sci.*, **19**, 225 (1945).
59. Nord, F. F., Dammann, E., and Hofstteter, H., *Biochem. Z.*, **285**, 254 (1936).
60. Nord, F. F., and Engel, W., *ibid.*, **296**, 153 (1938).
61. Nord, F. F., Hofstetter, H., and Dammann, E., *ibid.*, **293**, 236 (1937).
62. Nord, F. F., Sciarini, L. J., and Wirth, J. C., *Cereal Chem.*, **22**, 11 (1945).
63. Nord, F. F. *et al.*, *Proc. Natl. Acad. Sci. U. S.*, **29**, 121 (1943).
64. O'Connor, R. C., *Biochem. J.*, **34**, 1008 (1940).
65. Pirschle, K., *Biochem. Z.*, **218**, 430 (1930).
66. Rotini, O. T., Dammann, E., and Nord, F. F., *ibid.*, **288**, 414 (1936).
67. Runnström, J. *et al.*, *Arkiv Kemi Mineral. Geol.*, **B13**, No. 10 (1939).
68. Compare, for example, Schlenk, F., in *Advances in Enzymology*, this volume, p. 227.
69. Schöpf, C., *Congr. intern. quim. pura aplicada, 9th Congr., Madrid*, Vol. V, 189 1934).
70. Sciarini, L. J., and Nord, F. F., *Arch. Biochem.*, **3**, 261 (1943).
71. Sciarini, L. J., and Nord, F. F., *ibid.*, **5**, 435 (1944).
72. Semeniuk, G., *Iowa State Coll. J. Sci.*, **18**, 347 (1944).
73. Snyder, W. C., and Hansen, H. N., *Am. J. Botany*, **27**, 64 (1940); *Proc. Pacific Sci. Congr., 6th Congr.*, Vol. IV, 749 (1940).
74. Stanier, R. Y., and Adams, G. A., *Biochem. J.*, **38**, 168 (1944).

75. Tamiya, H., *Acta Phytochim. Japan*, **6**, 1 (1932).
76. von Tappeiner, H., *Ergeb. Physiol.*, **8**, 698 (1909).
77. von Tappeiner, H., and Jodlbauer, A., *Die sensibilisierende Wirkung fluoreszierender Substanzen*. Vogel, Leipzig, 1907.
78. Tatum, E. L., *Ann. Rev. Biochem.*, **13**, 694 (1944).
79. Tytell, A. A., and Gould, B. S., *J. Bact.*, **42**, 513 (1941).
79*a*. Vinson, L. J., Cerecedo, L. R., Mull, R. P., and Nord, F. F., *Science*, **101**, 388 (1945).
80. Vorländer, D., *Z. anal. Chem.*, **77**, 241 (1929).
81. Weiss, G., *Biochem. Z.*, **243**, 269 (1931).
82. Wiley, A. J., Johnson, M. J., McCoy, E., and Peterson, W. H., *Ind. Eng. Chem.*, **33**, 606 (1941).
82*a*. Wilson, I. M., *Nature*, **154**, 709 (1944).
83. Wirth, J. C., *Dissertation*, Fordham University, New York, N. Y., 1943, p. 53.
84. Wirth, J. C., and Nord, F. F., *Science*, **92**, 15 (1940).
85. Wirth, J. C., and Nord, F. F., *Arch. Biochem.*, **1**, 143 (1942).
86. Wirth, J. C., and Nord, F. F., *ibid.*, **2**, 463 (1943).
87. Witzemann, E. J., *J. Phys. Chem.*, **25**, 55 (1921).
88. Yamagata, S., *Acta Phytochim. Japan*, **11**, 145 (1939).
89. Yoe, J., and Reid, L., *Ind. Eng. Chem., Anal. Ed.*, **13**, 238 (1941).
90. Zentmyer, G. A., *Science*, **100**, 294 (1944).

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ENZYMATIC REACTIONS INVOLVING NICOTINAMIDE AND ITS RELATED COMPOUNDS

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CONTENTS

I. Introduction

The study of nicotinamide and its related compounds, especially the codehydrogenases I and II, has attracted many investigators during the past decade. We are dealing with a field which branches out so widely that the significance of any finding revealed by laboratory experiments is not confined to a narrow subject; instead it will affect a wide variety of related fields, some of which are stressed in the chapters of this review.

During the past few years numerous reviews have discussed the nicotinamide compounds from many angles (27, 95, 122, 126, 142, 149, 150). Here emphasis will be laid on their relation to enzymatic reactions. The reactions which will be stressed particularly are dehydrogenations in which nicotinamide participates as part of the coenzymes, and those in which the synthesis and destruction of these derivatives result. Because of the broad scope of this field, the discussion will be confined mainly to problems

which have not been treated extensively by other reviewers. Some basic facts will be underlined in order to provide connecting links between the different topics and to make the review understandable for those less familiar with the subject.

II. Nicotinamide Nucleotide Dehydrogenases

1. Nomenclature

Biochemists who have not done much work in this field find the terminology employed by the different laboratories confusing. Therefore the more commonly used terms will be defined here.

The nicotinamide nucleotides (prosthetic groups or coenzymes) are designated by the following names, which are interchangeable:

(*a*) Cozymase (Harden's coferment of alcoholic fermentation); codehydrogenase I; coenzyme I (sometimes abbreviated Co I); diphosphopyridine nucleotide (or DPN);

(*b*) Coferment of Warburg (see formula below); codehydrogenase II; coenzyme II (abbreviated Co II); triphosphopyridine nucleotide (TPN).

The specific protein moieties (apoenzymes) are designated by the following names: apodehydrogenases (or simply but somewhat inaccurately, dehydrogenases), dehydrases, reductases and protein. The substrate specificity is indicated by using combinations such as lactic acid apodehydrogenase, malic acid apodehydrogenase or alcohol apodehydrogenase. The latter is concerned with the formation of alcohol from acetaldehyde in the process of alcoholic fermentation. Therefore, Warburg and his coworkers prefer to call it "acetaldehyde reductase." Later Warburg proposed a terminology (142) by which this enzyme should be termed

Dihydropyridine proteid
(Acetaldehyde).

Names for all other enzymes listed in Table I could be formed correspondingly. This nomenclature, however, has the disadvantage of being difficult to use colloquially.

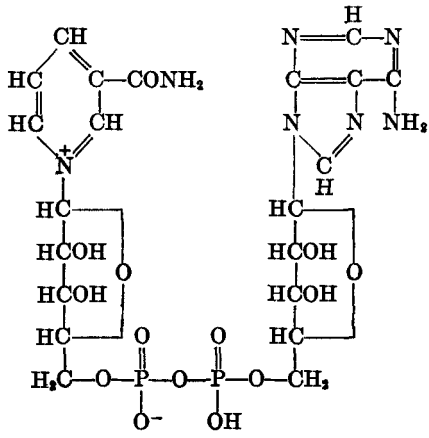
2. The Codehydrogenases

The structure of codehydrogenase I is shown below (cozymase). The chemical evidence for this formulation (125) and for the properties of this coenzyme has been reviewed elsewhere (123). As yet no one has performed experiments that would indicate which of the two phosphoric acid molecules

TABLE I

NICOTINAMIDE NUCLEOTIDE DEHYDROGENASES

No.	Reaction	Coenzyme	Protein	Ref.
1	1,3-Diphosphoglyceraldehyde $\rightleftharpoons$ 1,3-diphosphoglyceric acid	Co I	Prepared in crystallized form from yeast by Warburg and Christian	146
2	Lactic acid $\rightleftharpoons$ pyruvic acid	Co I	Crystalline preparation obtained from heart muscle by Straub; from rat muscle and Jensen sarcoma by Kubowitz and Ott	133 83
3	Alcohol $\rightleftharpoons$ acetaldehyde	Co I	Obtained from yeast in crystalline form by Negelein and Wulff	107
4	Malic acid $\rightleftharpoons$ oxaloacetic acid	Co I	Prepared by Straub from heart muscle in presumably pure form; the protein from bacteria and higher plants can use either Co I or Co II as the prosthetic group	134 93
5	Triose phosphate $\rightleftharpoons$ phosphoglyceric acid	Co I	Crude preparations from animal tissues	6 98
6	α-Glycerophosphate $\rightleftharpoons$ phosphoglyceraldehyde	Co I	Crude preparations from yeast and from tissues	5
7	Methyl / Propyl / Amyl } alcohol $\uparrow\downarrow$ Form- / Propion- / Valer- } aldehyde	Co I	Crude preparations from animal tissues	90
8	$2\,RCHO + H_2O \rightarrow RCOOH + RCH_2OH$	Co I	"Aldehyde mutase"; crude preparations from liver	24
9	β-Hydroxybutyric acid $\rightleftharpoons$ acetoacetic acid	Co I	Crude preparations from animal tissues	43 85
10	Formic acid $\rightleftharpoons CO_2$	Co I	Crude preparations from seeds and bacteria	7
11	Glucose $\rightleftharpoons$ gluconic acid	Co I	Purified preparations from animal tissues	93
12	Betaine aldehyde $\rightleftharpoons$ betaine	Co I	Crude preparations from animal tissues	79
13	Glucose-6-monophosphate (Robison ester) $\rightleftharpoons$ phosphohexonic acid	Co II	(*Zwischenferment*); obtained from yeast in presumably pure form by Negelein and Gerischer	106
14	Phosphohexonic acid $\rightarrow$ decarboxylation and dehydrogenation	Co II	Purified preparation obtained from yeast	144
15	Isocitric acid $\rightleftharpoons$ α-keto-β-carboxyglutaric acid	Co II	Crude preparations from seeds, animal tissues, and yeast	4
16	Glutamic acid $\rightleftharpoons$ iminoglutaric acid	Co I or Co II	Crude preparations only; coenzyme specificity depends on source of protein; for protein from yeast and *E. coli*—Co II; for protein from animal tissues both coenzymes can act as the prosthetic group	28 29 48
17	Luciferin (bioluminescence)	Co I or Co II	"Luciferase" from luminescent organisms. Crude preparations available	71
18	Reduction of nitrate to nitrite	Co I or Co II	Various strains of *Fusarium*	151

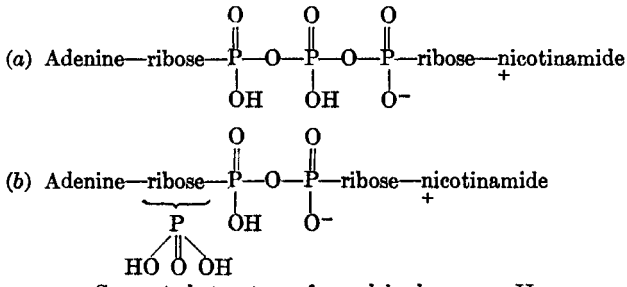


Cozymase

serves as the partner of the betaine linkage or which of the two ortho positions of the pyridine ring is involved in the biological reduction. Highly purified preparations of the codehydrogenases contain considerable amounts of nicotinic acid instead of nicotinamide; and an investigation is needed to show whether this is due to a chemical decomposition of the acid amide part (splitting *a* in Scheme 7, page 231) or to the existence of such coenzyme derivatives in nature. One might expect a slight deviation in the coenzymatic properties of these compounds.

Alternatives for reduction of the nicotinamide nucleus

Codehydrogenase II seems to occur in nature as frequently as codehydrogenase I, but usually in much smaller concentrations. This scarcity may explain why much less work has been done on its chemical structure. Fortunately, most of its configuration is apparent from the fact that the codehydrogenases are interconvertible by reversible enzymatic phosphorylation (3). The question about the position of the third phosphoric acid molecule in codehydrogenase II is not yet solved. For some time a structure represented by (*a*) has been assumed (122), but later experi-



Suggested structures for codehydrogenase II

mental data make a structure such as is tentatively indicated by (*b*) more likely. Definite proof will depend on the preparation of sufficient amounts of the coenzyme to permit the isolation and identification of split products (128).

It seems possible that a nicotinamide mononucleotide, as well as the two nicotinamide-adenine dinucleotides, may have biological significance. If it exists, this substance would be analogous to riboflavin phosphate. Its preparation has been attempted by splitting cozymase with various enzyme preparations (57). Treatment with Kunitz's ribonuclease does not cleave the pyrophosphate linkage (124). A systematic search for the mononucleotide in source material known to be rich in nicotinamide compounds has not yet been carried out.

From a chemist's viewpoint, the synthesis of the codehydrogenases would be most desirable to confirm the structure. Some synthetic steps leading to the isolation of important structural units, notably adenosine and adenylic acid (16, 47, 68, 89), have been performed successfully. Fischer (38) and, later, Karrer (72–74) showed that carbohydrate chains can be attached to the pyridine nucleus. Combining the two nucleosides or nucleotides in the correct fashion offers almost insurmountable difficulties for the methods of organic chemistry known at present. Some of the linkages in the coenzymes are so labile that chemical processing in the course of one synthetic step is likely to destroy other parts of the molecule.

3. The Apodehydrogenases

The dehydrogenases which operate with nicotinamide nucleotides as prosthetic groups are listed in Table I. Each of the coenzymes combines with several proteins; and therefore it may be stated that the codehydrogenases have only a limited specificity. It is the protein part which makes the dehydrogenases strictly specific. Reference is made in this table to recent work on these proteins. While results of experimental studies with

pure coenzymes and apoenzymes can be considered as reliable, well-established information, much uncertainty is encountered in investigations in which impure proteins have been used. Thus, earlier work on glucose dehydrogenase seemed to indicate that either one of the coenzymes could function as the prosthetic group. Lynen and Franke, however, have shown that purified preparations of the protein are activated by codehydrogenase I alone (93). A phosphatase present in impure dehydrogenase preparations can rapidly transform codehydrogenase II into codehydrogenase I; in such a case activation by the former is simulated. It is not impossible that further purification of malic acid dehydrogenase from plant material and of glutamic acid dehydrogenase will lead to similar revisions. In other instances, studies of dehydrogenase systems using impure components make it difficult to state whether the dehydrogenation proceeds at a rate sufficiently large to be considered biologically significant.

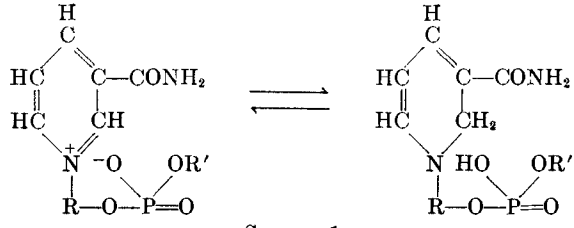
Negelein and Gerischer have pointed out that the protein of a dehydrogenase may vary in its chemical character according to the type of source material from which it has been isolated (106). Robison ester apodehydrogenase from yeast maceration juice was found to have the isoelectric point at *p*H 4.82, while Theorell (137) reported *p*H 5.85 for the corresponding protein from erythrocytes. Such individuality of enzymes isolated from different sources has been known for some time. For example, catalase (135) and pepsin (108) show variations for different animals. The data on malic and glutamic acid dehydrogenases as compiled in Table I are in agreement with this concept. Here, the variation of the protein is so large that even the coenzyme specificity may be altered. In one instance, however, the protein parts of a dehydrogenase isolated from two different sources were found to be identical. Kubowitz and Ott prepared lactic acid dehydrogenase both from Jensen rat sarcoma and from rat muscle (83), and a careful comparison revealed that the two proteins are exactly the same (see also page 218).

Warburg and coworkers have reported data for the elementary composition and other properties of several apodehydrogenases (146), the molecular weight of which presumably is around 75,000; accurate determinations are still lacking.

While it is understood that the nicotinamide nucleotides exert their function by reversible reduction of the pyridine nucleus, there is no information available on the particular structure or combination of groups in the proteins which would explain their specificity for substrate and prosthetic group and for the mode of their activation. Scanty and indirect conclusions may be drawn from the configuration of the coenzymes, which certainly

have several points of attachment in their structure. The predilection of a protein for one of the two coenzymes can depend only upon the different number of phosphoric acid radicals in the prosthetic group. Nicotinamide riboside, which is capable of undergoing reversible reduction, does not combine with apodehydrogenases (121). This characteristic emphasizes the important role of the phosphoric acid radicals; and it is analogous to riboflavin, which cannot function as the prosthetic group of the "yellow enzymes." The pyridinium ring nitrogen, as well as the phosphoric acid group, must have something to do with linking the coenzymes to the protein. Its reduction to the dihydrocoenzyme (49, 143, 148), changing the ring nitrogen from pentavalent to trivalent and providing an additional acid group (see Scheme 1), also alters the affinity for the protein. The amino group of the adenine is essential, and its removal by nitrous acid (splitting *f* in the formula on page 231) results in a decrease in the activity of the codehydrogenases (127, 148).

The following facts pertaining to the reactions listed in Table I may be informative: Reaction 1 represents the oxidative step in alcoholic fermentation of yeast and in muscle glycolysis. The structure of the triose ester, its phosphorylation, oxidation and dephosphorylation have been subject to numerous investigations (12, 17, 97, 146). The reduced coenzyme formed



SCHEME 1

REVERSIBLE REDUCTION OF THE NICOTINAMIDE NUCLEOTIDES

in this step combines with protein No. 2 in the process of glycolysis and reduces pyruvic acid to lactic acid; in alcoholic fermentation, protein No. 3 catalyzes the reduction of acetaldehyde to alcohol. Reversibility, equilibria, dissociation constants, etc., have been studied by Warburg and coworkers (83, 146) in elaborate and elegant investigations.

The work of Straub on malic acid dehydrogenase (134) is of particular interest. *In vitro* the equilibrium of reaction 4 is much in favor of malic acid, but this can be changed by adding glutamic acid and transaminase. Oxaloacetic acid is then consumed and yields aspartic acid. Under certain conditions the reduced cozymase resulting from reaction 4 is reoxidized by

diaphorase (Straub's yellow enzyme). Malic acid apodehydrogenase and diaphorase are in a fixed position in the cellular structure (desmo-enzymes, according to the Willstätter nomenclature). Their successful separation and the availability of both in the pure state have made it possible to decide whether they are located close together in the cell, and in a particular orientation so as to favor the hydrogen shift. Straub compared the reaction rate in the intact cell material (muscle suspension) with the rate in solutions containing the components in biological concentration, and found them to be equal in both instances. Cozymase can alternate rapidly between the two proteins without necessitating a particular steric arrangement of the latter.

TABLE II

CONCENTRATION OF SOME APODEHYDROGENASES IN SOURCE MATERIAL

Dehydrogenase	Source material	Concentration of pure protein, %	Ref.
1,3-Diphosphoglyceraldehyde dehydrogenase	Dried brewers' yeast	0.24	146
Alcohol dehydrogenase (acetaldehyde reductase)	Dried brewers' yeast	0.25	107
Hexose-6-monophosphate (Robison ester) dehydrogenase	Dried brewers' yeast	About 0.1	106
Malic acid dehydrogenase	Heart muscle	0.4	134
Lactic acid dehydrogenase	Heart muscle	0.36	133
Lactic acid dehydrogenase	Jensen rat sarcoma	About 0.1	83
Lactic acid dehydrogenase	Rat muscle	About 0.1	83

The role of reaction 5 in nature is questionable. It may be that data on it are superseded by reaction 1, which was discovered later. Negelein and Brömel (105) studied reaction 6, and came to the conclusion that it is presumably insignificant in nature. Reduction of phosphoglyceraldehyde by dihydrocozymase is catalyzed by protein No. 3; and the existence of a specific protein for reaction 6 is open to question. The reduction is about 20,000 times slower than that of acetaldehyde.

Although the components have not been subjected to close scrutiny, the importance of reactions 15 and 16 is generally acknowledged; but the opposite holds for reactions 11, 13 and 14. They are steps in an alternate pathway in the breakdown of hexoses, which so far has not had the extensive consideration it seems to merit. Much remains to be done concerning reactions 7, 8, 9, 10, 12, 17 and 18, and the place of some of them in the group of nicotinamide nucleotide dehydrogenases has not been established too well. The discovery of further enzymes of this type appears possible and probable, however.

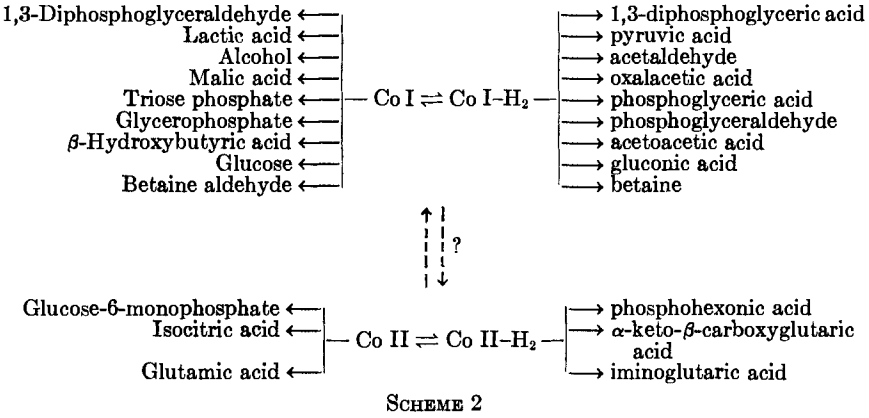
In Table II the concentration of a few apodehydrogenases in source material is given. In view of the astounding multiplicity of enzymatic reactions in living cells, these values seem surprisingly high.

4. The Mode of Action of Codehydrogenases I and II as Prosthetic Groups of Dehydrogenases

The dehydrogenases under discussion here differ from most other enzymes with a known prosthetic group in their high degree of dissociation. They are analogous in this respect to the adenosine phosphates concerned with phosphate transfer. Parnas has coined the descriptive phrase "mobile coenzymes" in contrast to "fixed coenzymes" (110).

Meyerhof has called attention to the fact that under biological conditions the nicotinamide nucleotides are found in great excess, while in other enzymes with a prosthetic group (fixed coenzymes) the ratio of the latter to the protein is 1:1 (95). This particular feature has been emphasized especially in the discussions of Dixon and Zerfas (25), of Parnas (110) and of Waters (149).

In Scheme 2 the central position of the nicotinamide coenzymes is illustrated. They may react in the presence of the specific proteins with the substrates listed. The reduced (or oxidized) coenzymes resulting from this



SCHEME 2

THE CENTRAL POSITION OF COENZYMES I AND II IN PROCESSES OF DEHYDROGENATION

can then combine with another protein and react with any of the components listed on the opposite side. The concentration of the various components and, perhaps, special regulatory mechanisms determine the course and direction of the reaction.

In addition to this, the following ways are important for the biological reoxidation of the dihydro coenzymes: Straub's flavoprotein, diaphorase, oxidizes dihydrocoenzyme I; and Haas's cytochrome reductase, which is also a flavoprotein, oxidizes dihydrocoenzyme II (21, 44, 51, 133). There are several other riboflavin enzymes capable of oxidizing the reduced nicotinamide coenzymes, but their biological role is doubtful (62, 145). The oxidation by *o*-quinones, which was observed by Kubowitz (82), deserves further attention.

5. *Methods for Quantitative Determination*

A large variety of procedures has been used for the determination of nicotinamide dehydrogenases. In the beginning, Thunberg's vacuum-tube method with dyes such as methylene blue as the hydrogen acceptor played an important role, and to it we owe the discovery of numerous dehydrogenases (138). For the estimation of cozymase, Euler and Myrbäck have evolved a fermentation test which is still widely used in the course of its purification and for studies concerning its distribution in nature (100). The principle is the removal of cozymase from a yeast preparation, rendering it incapable of fermentation. The fermenting ability is then restored by adding either known amounts of the coenzyme or solutions to be tested, and the rate of fermentation is compared with standard experiments. The nonfermenting yeast, which is devoid of cozymase only, has been called "apozymase." Since cozymase cannot be extracted from fresh yeast by washing alone, dried brewers' yeast must be used. The process of drying changes the permeability of the cells so that cozymase can be removed by washing at or below room temperature. Several improvements of this procedure have been reported recently (9, 42, 75, 129). Using Warburg manometers, amounts of 2 to 40 gamma can be accurately determined. A test developed by Jandorf, Klemperer and Hastings involves the activation of glycolysis by codehydrogenase I *in vitro* (70). This technique has been adapted for the Cartesian diver method of Linderstrøm-Lang, which permits the determination of 0.001 to 0.006 gamma of coenzyme, according to Anfinsen (8). For quantitative assays of codehydrogenase II in impure extracts, the activation of hexose-6-monophosphate dehydrogenase can be employed (50, 148).

Kohn and others (55, 80, 81, 112) have made wide use of the growth-promoting properties of codehydrogenases I and II for cultures of *Hemophilus influenzae* and *H. parainfluenzae* discovered by Lwoff and Lwoff (91, 92). The density of the cultures is proportional to the amount of pyridinium compounds present. As a measure of growth, Hoagland takes the amount

of nitrite formed by *H. influenzae* (59, 60). Bioassay with *Hemophilus* has the advantage of requiring extremely small quantities of coenzyme, but is not very specific because it does not distinguish between coenzyme I, coenzyme II and nicotinamide riboside. Although a variety of factors complicate this method, as Gingrich (40) recently pointed out, the Duke University group has used it successfully in a series of outstanding investigations.

There have been a great many reports on the distribution of codehydrogenase I in tissues, but the findings are not very conclusive. Wide discrepancies in values for one and the same tissue are found in different papers. In some instances the rapid inactivation of cozymase in tissue brei (see page 230) may have been overlooked, but unsuitable reference standards are the main source of error. The purity of reference preparations is often judged with great optimism. The use of such standards will not only compensate for poor extraction methods, but also simulate coenzyme values for tissues which are far above the actual content.

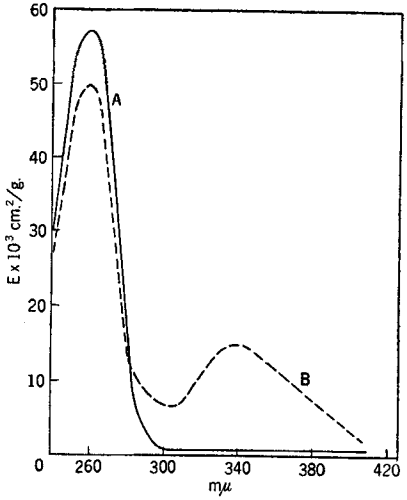


Fig. 1.—Absorption of codehydrogenase I. The ultraviolet absorption of equimolar solutions of codehydrogenase II was found to be identical (142). Curve *A*, oxidized form; curve *B*, reduced form.

For the study of pure or highly purified dehydrogenases, the above procedures have been replaced by the spectrophotometric technique developed by Warburg and his associates (142, 143, 148). Reduction of the nicotinamide coenzymes to their dihydro derivatives gives rise to an absorption band with a maximum at about 340 mμ (see Fig. 1), the intensity of which is proportional to the amount of dihydrocoenzyme present. The photoelectric registration of the appearance or disappearance of this band makes it possible to study reaction rates, specificity and equilibria of the compounds of dehydrogenase systems. Moreover, this technique has also made possible the quantitative measurements of some related reactions by coupling them with nicotinamide dehydrogenases (147).

An interesting modification of the spectrophotometric technique has been developed by Haas (49*a*). It is based on the reduction of 2,6-di-

chlorophenolindophenol by the dihydrocoenzymes and permits the investigation of coenzymes, dehydrogenases and substrates in the visible region of the spectrum (49*a*).

6. *Nicotinamide Enzyme Systems and Malignant Growth*

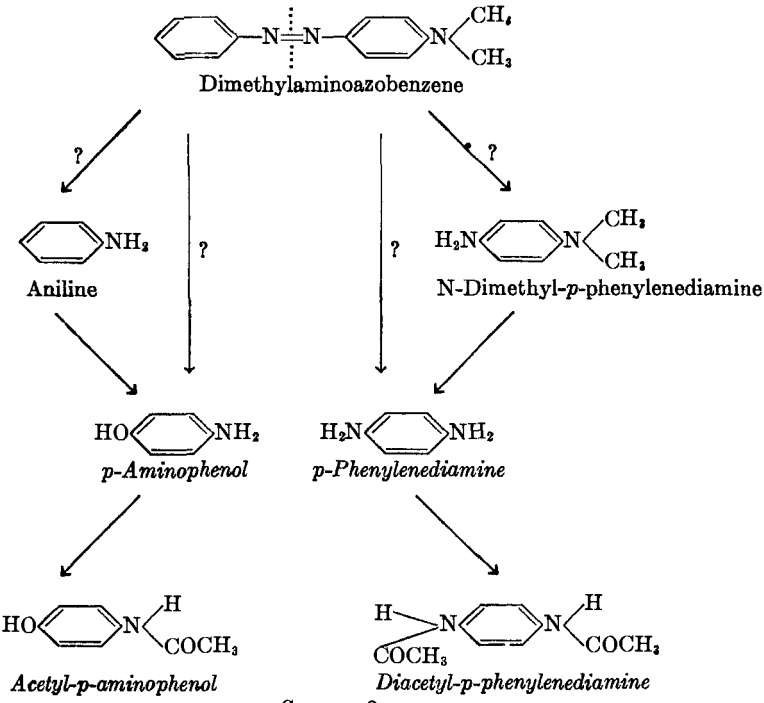
Although the presence of cozymase in malignant tissue was observed as early as 1925 (34), reports on the determination of the codehydrogenase content of these tissues are rather limited. They have the same shortcomings as determinations in other tissues, notably a lack of well-established reference standards, uncertainties in the methods and rapid deterioration of the coenzymes while the tissue is being processed; but in general it appears that cancerous tissues contain smaller amounts of the coenzymes than do normal tissues (14, 37, 45).

With the improved technique for quantitative determination, it became possible to ascertain the ratio of oxidized coenzyme to reduced coenzyme. For this, a portion of the tissue to be examined is extracted with dilute acid, which destroys all dihydro coenzyme immediately and leaves the oxidized form intact. Another sample is extracted with weak alkali, which yields the reduced form while the oxidized part is inactivated (2). Euler and coworkers found that the oxidized form is in excess in most tissues; however, Jensen rat sarcoma showed a considerable excess of the dihydro coenzyme (33). Great significance was attributed to this discovery and much speculation arose, stimulated by the results of Warburg (141) and many subsequent investigators who had established variations in the carbohydrate metabolisms of cancerous and normal tissue. A thorough scrutiny of the ratio is still needed. The author did not find an excess of dihydro coenzyme in a variety of human and animal cancer tissues (124).

Kubowitz and Ott (83) have studied lactic acid dehydrogenase from Jensen rat sarcoma recently, using 3000 grams of sarcoma (from 300 rats), free from necrotic material. The yield was about 50 milligrams of crystalline protein, which was compared with the lactic acid dehydrogenase prepared from muscle tissue of rats. Crystal form and chemical composition were the same, nor was any difference found in the rate of enzymatic activity, the coenzyme specificity, the absorption spectrum or the *p*H–activity curve. Immunochemical studies further corroborated the identity of the two proteins. Hence it is evident that the peculiarities in the carbohydrate metabolism of cancerous tissue are not caused by a difference in the protein which catalyzes the reduction of pyruvic acid.

There have been numerous attempts in cancer research to correlate the action of carcinogenic compounds with various enzyme systems. One of

the most interesting investigations along such lines has been reported by Kensler and his associates (75, 76). They found the codehydrogenase I content of liver from rats fed dimethylaminoazobenzene (butter yellow) to be less than one-half that of liver from control animals fed the same diet without the carcinogen. Therefore, it was tempting to study the effect of



SCHEME 3

METABOLISM OF DIMETHYLAMINOAZOBENZENE BY THE RAT (75)
ITALICIZED COMPOUNDS WERE ISOLATED FROM THE URINE BY STEVENSON, DOBRINER AND RHOADS (132)

butter yellow and its decomposition products on nicotinamide dehydrogenases *in vitro*. Earlier experiments had shown that feeding butter yellow to rats resulted in the excretion of degradation products in the urine, particularly *p*-aminophenol and *p*-phenylenediamine. Cleavage at the azo linkage seems to occur in this and in related compounds (see Scheme 3). The yeast fermentation test was chosen by Kensler for the *in vitro* studies. He found that butter yellow itself does not have any influence on the rate of fermentation, but its degradation products, dimethyl-*p*-phenylenediamine

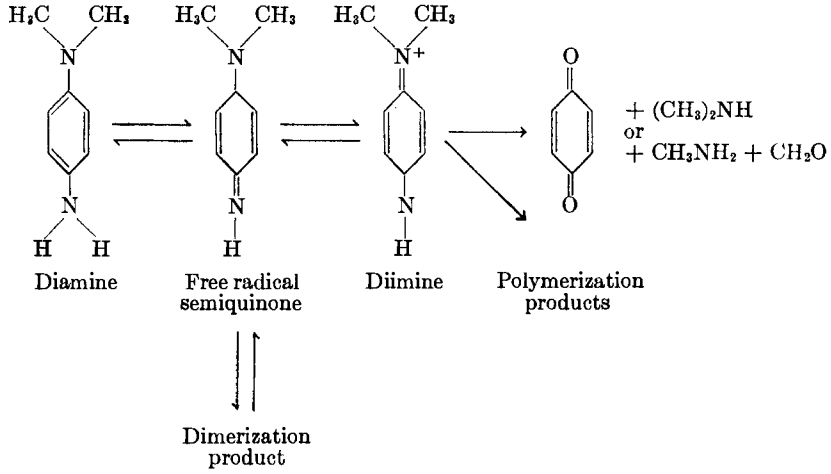
and *p*-phenylenediamine, have a strongly inhibiting effect (see Table III). These studies were extended to closely related compounds which have the ability to undergo partial oxidation to semiquinoid radicals that resemble

TABLE III

EFFECT OF VARIOUS COMPOUNDS ON FERMENTATION OF DIPHOSPHOPYRIDINE NUCLEOTIDE SYSTEM (75)

Compound	Molar concentration $\times 10^{-4}$	Per cent inhibition
N,N-Dimethylaminoazobenzene	Saturated solution	10
Aniline	8.0	15
p-Aminophenol	2.3	6
N,N-Dimethyl-*p*-aminophenol	1.6	5
N,N-Dimethyl-*p*-phenylenediamine	1.8	100
p-Phenylenediamine	2.3	75
Phenol	8.0	25
p-Cresol	7.0	0
Hydroquinone	2.2	0
Catechol	2.2	0
Naphthoresorcinol	2.5	0
β-Naphthylamine	2.1	20
1-Amino-2-naphthol	2.8	28
2-Amino-1-naphthol	2.8	0
Methylene blue	5.0	0

Wurster's salts (see Scheme 4) (99). The inhibition of the yeast fermentation seems to parallel the stability of the free radicals (see Table IV). The



SCHEME 4

STEPS IN THE OXIDATION OF N-DIMETHYL-*p*-PHENYLENEDIAMINE

concentration of the inhibitors is usually about ten to fifty times higher than that of cozymase. The inhibiting effect could be eliminated by increasing the concentration of cozymase to approximate that of the inhibitors (see Table V). This indicates that the degradation products of butter yellow act as competitive inhibitors and that the protein part of the dehydrogenase is subject to the attack. Attempts to establish the speci-

TABLE IV

INHIBITION OF FERMENTING SYSTEM BY COMPOUNDS OF THE TYPE OF WURSTER'S SALTS (75)

Compound	Molar concentration $\times 10^{-4}$	Per cent inhibition	Free radical stability
N,N,N′,N′-Tetramethyl-*p*-phenylenediamine	1.5	100	Weeks
N,N,N′-Trimethyl-1,4-phenylenediamine	1.7	70	7 days
N,N-Dimethyl-*p*-phenylenediamine	1.8	57	7 days
Diaminodurene	1.5	62	2–3 days
2-Methyl-N^4,N^4-dimethyl-1,4-phenylenediamine	1.7	50	2 days
N-Methyl-*p*-phenylenediamine	2.1	42	1 day
N,N′-Dimethyl-*p*-phenylenediamine	1.8	37	1 day
2-Methyl-1,4-phenylenediamine	2.1	35	4–8 hrs.
p-Phenylenediamine	2.3	23	4–8 hrs.
2,6-Dimethyl-N^1,N^1-dimethyl-1,4-phenylenediamine	1.2	0	2 hrs.
2-Methyl-N^1,N^1-dimethyl-1,4-phenylenediamine	2.3	0	5 min.
2,6-Dimethyl-N^1,N^4-dimethyl-1,4-phenylenediamine	1.2	0	1 min.
2,3-Dimethyl-N^1,N^1-dimethyl-1,4-phenylenediamine	1.3	0	0
2,3,5-Trimethyl-N,N,N^1,N^1-tetramethyl-1,4-phenylenediamine	1.2	0	0
2,3,5,6-Tetramethyl-N,N,N′,N′-tetramethyl-1,4-phenylenediamine	1.1	0	0
2,6-Dimethyl-N,N,N′,N′-tetramethyl-1,4-phenylenediamine	1.3	0	0
N,N,N′,N′-Tetramethyl-1,2-phenylenediamine	1.5	0	0

TABLE V

EFFECT OF INCREASING AMOUNTS OF DIPHOSPHOPYRIDINE NUCLEOTIDE ON INHIBITION OF FERMENTATION (75)

Added to system	Fermentation, cu. mm. CO_2	Per cent inhibition
15 μg. DPN*	790	..
300 μg. DPN	2750	..
525 μg. DPN	3130	..
15 μg. DPN + N,N-dimethyl-*p*-phenylenediamine (2.4×10^{-4} molar)	20	97
300 μg. DPN + N,N-dimethyl-*p*-phenylenediamine (2.4×10^{-4} molar)	2500	9

* DPN = diphosphopyridine nucleotide.

ficity of the inhibition revealed that several enzymes were not inhibited; however, yeast carboxylase (77), transaminase (20), urease (114) and succino dehydrogenase (115) were found to be inhibited. An interesting interpretation of Kensler's results has been published recently by Kuhn (84).

The outstanding feature of Kensler's experiments is that they clearly establish interference of carcinogenic products with enzymes *in vitro*. Considerable extension of the experimental work will be needed before the significance of the dehydrogenase inhibition in the metabolism and growth of butter yellow cancer can be interpreted. The limited specificity suggests the examination of other enzymes, while the use of purified dehydrogenases may change the concept of the concentration necessary for the effect of the inhibitor.

A promising field seems to be open here.

III. Enzymatic Synthesis and Decomposition of the Nicotinamide Nucleotides

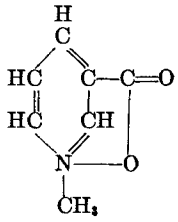
1. Origin and Fate of Nicotinic Acid

The enzymatic synthesis of nicotinamide and the codehydrogenases is still an almost unknown quantity, and the few facts on hand are rather recent discoveries. In contrast to this, the processes of biological degradation of the coenzymes and the fate of surplus nicotinic acid have been studied extensively. We know much more about the breakdown of these compounds than we do about their build-up. This situation is true in most branches of enzyme chemistry. At the same time the processes of disintegration deserve the attention they receive since it may be assumed that they essentially represent the reverse of synthetic steps.

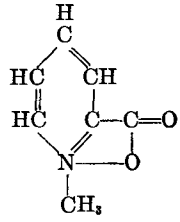
Some lower organisms such as *Chilomonas paramecium* can synthesize nicotinamide and the codehydrogenases from such simple sources of carbon and nitrogen as acetate and ammonia (67). Others, including many species of bacteria (39) and insects (116, 139) as well as most higher animals (27), cannot build the pyridine nucleus. For them it has the character of a vitamin and must be supplied from outside sources, notably the plant kingdom.

Amino acids are believed to be the building stones for the synthesis of nicotinic acid by plants. Bovarnick (15) accomplished a laboratory synthesis of nicotinamide from amino acids by using a rather alchemistic procedure. For this, she placed asparagine and glutamic acid in a water solution containing traces of manganese sulfate within a boiling water bath

with aeration for nine days. The yield was very small, but the reaction may resemble one of nature's routes. The utilization of amino acids by plants for the purpose of producing a pyridine derivative has been reported by Klein and Linser (78). Injection of proline, ornithine and glutamic acid into the stem of rice plants (*Oryza sativa*) increased the yield of trigonelline



Trigonelline

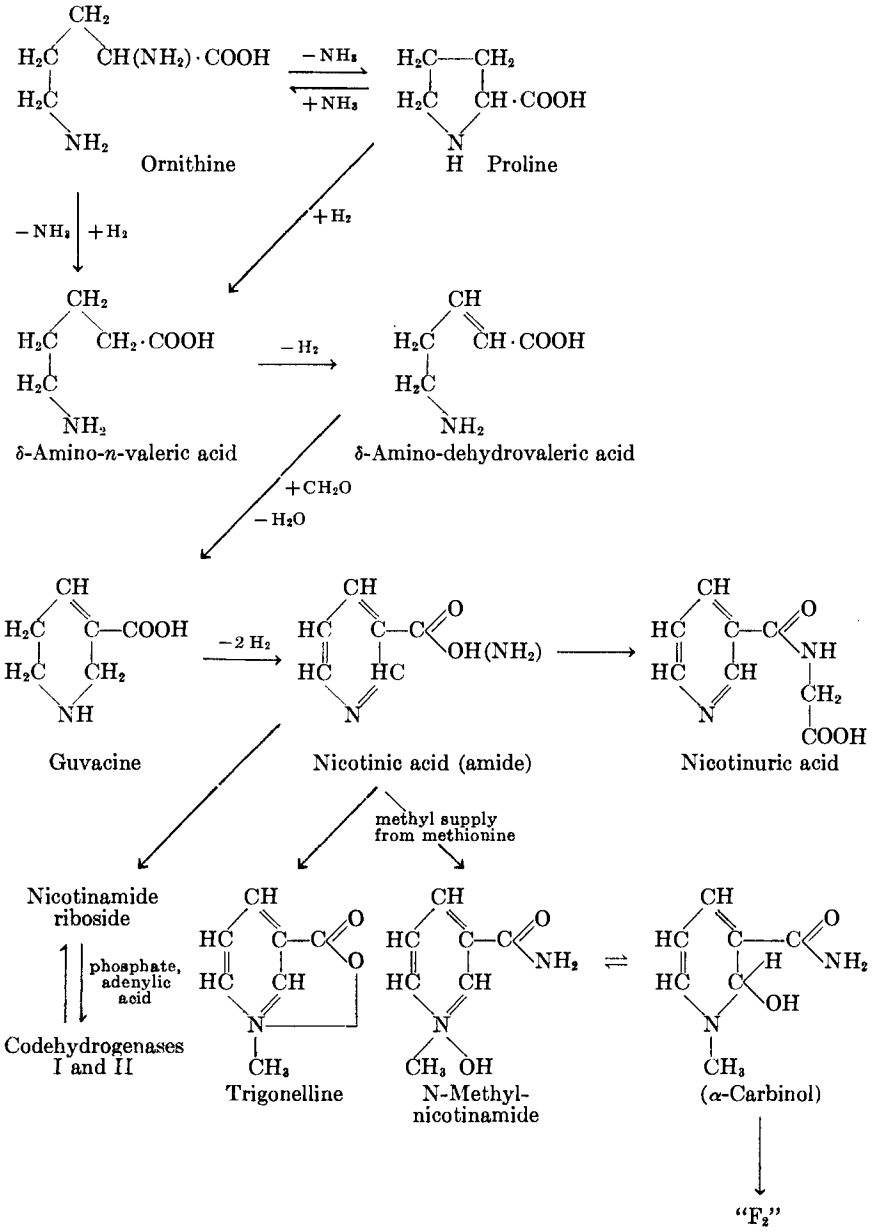


Homarine

from the juice of this plant. Trigonelline and nicotinuric acid had been found in urine long before the biochemical importance of certain pyridine compounds was realized (1). Their occurrence was ascribed to ingestion of vegetable food. Coffee beans, for example, were found to contain a considerable amount of these substances (131). Today we know that most higher organisms excrete the major part of the excess nicotinic acid in the methylated form as trigonelline and N-hydroxymethyl nicotinamide.

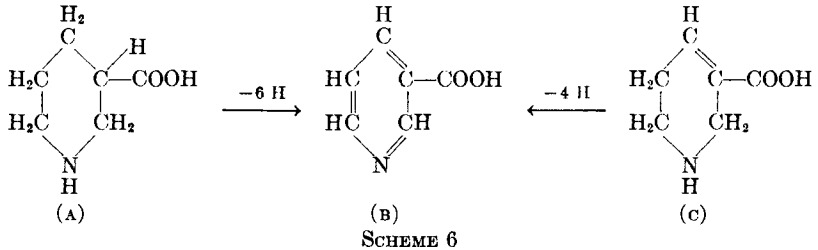
Mention should be made here of homarine, an isomer of trigonelline, which has been isolated from lobster muscle (*Homar homerus*) and from mussels (*Arca noae*) (63). Its origin and biochemical function are still obscure. Pyridine-α-carboxylic acid (picolinic acid), which might be the mother substance of homarine, cannot replace pyridine-β-carboxylic acid (nicotinic acid) as a vitamin.

The present knowledge of the genesis and fate of nicotinic acid is outlined in Scheme 5. A pathway for its synthesis from ornithine has been suggested by Guggenheim (46) and verified by Huff and Perlzweig (64). Among the higher animals, rats are one of the very few forms which are independent of an outside supply of nicotinamide. Parenteral as well as oral administration of glycine and *dl*-δ-amino-*n*-valeric acid to these animals resulted in a prompt increase of nicotinic acid derivatives in their bodies and their excretions. Euler, Högberg, Karrer and their associates (32) have corroborated the existence of biochemical routes for the production of nicotinic acid from guvacine and even from hexahydronicotinic acid. They found that growth of *Staphylococcus aureus* and of *Proteus vulgaris* is promoted by guvacine as well as by hexahydronicotinic acid. Dehydrogenation as in-



SCHEME 5
ORIGIN AND FATE OF NICOTINIC ACID

dicated in Scheme 6 may be assumed, although attempts to bring about this reaction *in vitro* by liver and kidney have failed so far.



SCHEME 6

(A), HEXAHYDRONICOTINIC ACID (B), NICOTINIC ACID (C), GUVACINE

Nothing is known about possible intermediates of a synthesis of nicotinamide starting from asparagine and glutamic acid.

Krehl, Strong, and Elvehjem recently reported the existence of a new nicotinic acid derivative in extracts of wheat bran (81*a*). This substance is characterized by extreme lability toward alkali which liberates nicotinic acid from it. A comparison with the earlier known nicotinic acid derivatives showed that it is not identical with any of them (81*b*).

For some time it was believed that nicotinic acid and nicotinamide could be transformed into each other so universally and so readily that no difference in utilization should be expected. Some limitations to this are apparent now from experimental data. A difference between the two compounds has been observed in their effect on coenzyme synthesis by erythrocytes *in vivo* and *in vitro* (55, 58, 61). The ingestion of adequate amounts of nicotinic acid results in a considerable increase of the coenzyme content, but nicotinamide does not have this effect. There seem to be different routes for the two processes, since nicotinamide results from the enzymatic decomposition of the coenzymes (54) whereas nicotinic acid is used for their synthesis.

The biochemical role of nicotinamide as part of the codehydrogenases had been established before its vitamin nature was discovered. It is not surprising, therefore, that its utilization in the synthesis of the coenzymes seemed beyond doubt, especially since considerable data have been reported in substantiation of this point. While no voices have been raised denying this, some investigators believe there may be a function of nicotinamide in metabolism apart from simply being a structural unit of the codehydrogenases. Data which justify such a claim are meager and are based mainly on balance experiments concerning the fate of ingested nicotinic acid and its excretion as well as its effect on the metabolism of intact

cells (61, 94, 120). Clarification of this alternative by further experimental work is most desirable.

Very little is known about any intermediates between nicotinic acid and the codehydrogenases. With one exception all organisms examined so far have the ability to accomplish the synthetic steps involved. The growth factor requirement of *Hemophilus influenzae* and *H. parainfluenzae* cannot be satisfied by nicotinamide. Lwoff and Lwoff (91, 92) have found that the codehydrogenases promote growth and are identical with what had been termed "V factor" earlier. Even more recently, Gingrich demonstrated that nicotinamide riboside is sufficient (40). Combination of the pentose with the pyridine nucleus is the only synthetic step which cannot be brought about by these organisms.

According to the concept of the dynamic state of body constituents which we owe to Schoenheimer (130), a permanent rejuvenation of the coenzymes in the organism is to be expected; and the continuous need for the building stone, nicotinamide, is proof of this. The rate of replacement has not yet been investigated; however, the exchange of adenylic acid was found to be relatively slow (13, 113).

Our knowledge concerning the excretion of surplus nicotinic acid and nicotinamide was greatly advanced recently by investigations of the Duke University group. In higher animals, such as the rat, dog and man, only a limited amount is excreted as such; most of it undergoes methylation on the ring nitrogen (26, 65, 66, 117–119), yielding trigonelline and N-methyl-nicotinamide (nicotinamide hydroxy methylate) (see Scheme 5). This reaction does not occur in rabbits and guinea pigs (52). The methylation seems to be irreversible. Handler and Dann found that methionine serves as methyl donator in this process (53). The administration of a large excess of the base (1% of the diet) to rats results in a depletion of the methyl supply with consequent nutritional disturbances. Using rat liver, Perlzweig, Bernheim and Bernheim were able to bring about the methylation of nicotinamide in the presence of methionine *in vitro* (111). Kidney and muscle were found to be inactive. The process is aerobic and is dependent on the intact structure of the liver cell.

There is a possibility that trigonelline undergoes further changes in the organism, being transformed into unidentified compounds which are not determined by the analytical methods available so far. Huff and Perlzweig observed that only 20 to 40% of the trigonelline given to rats by mouth is recovered. The fate of the rest is obscure (64). It may be excreted as products other than those discussed above. In an extensive study, Najjar and coworkers have reported the occurrence of a fluorescent compound

(termed F_2) which is present in normal urine in small amounts. The ingestion of large quantities of nicotinamide increases the output of this substance (101, 102, 104). Huff and Perlzweig have presented experimental evidence to prove that F_2 is identical with N-methylnicotinamide (66), a claim contested by Najjar (103) who attributes the formation of fluorescent substances to chemical changes during the process of isolating F_2 from urine. In water solution, part of the N-hydroxymethylnicotinamide undergoes rearrangement to the corresponding α-carbinol. The carbinol reacts with the butanol used in the isolation procedure to form a carbinol ether which is highly fluorescent (23). Ellinger and Coulson have made an extensive and careful investigation of the urinary elimination of N′-methylnicotinamide (26). For this an analytical procedure was elaborated which permits the assay of a large number of urine samples at the same time (26*a*).

2. *Enzymatic Decomposition of the Codehydrogenases*

The codehydrogenases are very labile when the cell structure of the source material is disorganized. Enzymatic breakdown in a variety of ways is responsible for this. Harden and Young in their first papers (56) called attention to the lability of codehydrogenase I in yeast juice. Soon afterwards, Buchner and Klatte (18) stated that impure preparations of castor bean lipase contained enzymes which inactivated the coenzyme. When Meyerhof first demonstrated the presence of cozymase in a variety of tissues and in plant material, he also observed gradual inactivation in minced tissue, and subsequently used boiling water for extraction in order to render the destructive enzymes inactive (96). In the early phase, the enzymatic decomposition of cozymase attracted the interest of investigators in a purely negative sense, as it necessitated precautions against loss in isolation and determination procedures.

When Euler and Myrbäck started their extensive research on the nature and function of cozymase (35), they studied the action of a number of enzymes to obtain information about the character of this compound. Their findings remained inconclusive in many instances, however (100). At this time very crude preparations of cozymase were all that were available, and the purity of the enzymes was also limited.

As soon as pure cozymase could be obtained, the study of its enzymatic degradation was renewed (22, 30). This work could then be extended to the newly discovered codehydrogenase II; and though research was limited by the meager supply, an analogy was established with codehydrogenase I in respect to the actions of disintegrating enzymes (31). First the lability of the coenzymes when added to minced tissues was confirmed. Then pure

cozymase and dihydrocozymase were considered as substrates for various enzymes. It became apparent that dihydrocozymase is more readily attacked by nuclease and phosphatase preparations than is the oxidized form (22). Since the reduced form of the coenzyme is a dibasic pyrophosphoric acid ester and the oxidized form is a monobasic acid, the latter is believed to be less susceptible to the action of the hydrolyzing enzymes.

The action of purified enzyme preparations on cozymase is used in the isolation of important structural units, notably adenosine and nicotinamide riboside (121). Attempts to prepare the nucleotide, consisting of nicotinamide, ribose and phosphoric acid, in this way appear to be promising. The enzymatic interconversion of the two codehydrogenases should be mentioned in this connection (3).

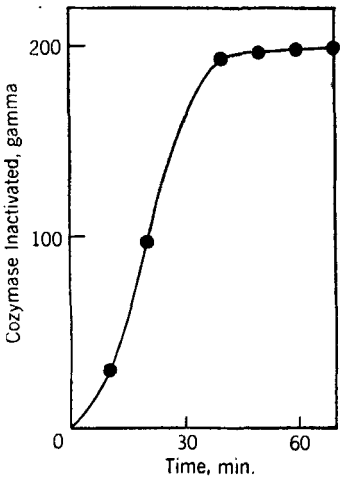


Fig. 2.—Inactivation of cozymase by incubation with apozymase suspension; 200 gamma of cozymase were used in each experiment (88).

In an interesting survey, Chain (19) showed that cozymase is rapidly destroyed by the enzymes present in the venom of the black tiger snake (*Notechis scutatus*). He discussed his finding as a partial explanation of the pharmacological action of the venom.

The revival of interest in the fate of the codehydrogenases in surviving tissue, minced tissue, blood and yeast preparations is a most important development. Since the routes of decomposition vary in each of these materials, they will be discussed separately.

Effect of Yeast Preparations.—The nicotinamide nucleotides are destroyed by yeast enzymes present in press juice, maceration juice and apozymase.

Ohlmeyer (109) found that codehydrogenase I is split to adenylic acid by enzymes present in maceration juice. The inactivation of cozymase by apozymase (studied by Lennerstrand, 86, 88) is quite different. Cozymase, if added to apozymase suspensions in a phosphate buffer containing glucose and hexose diphosphate, activates fermentation at a constant rate for several hours, indicating that the coenzyme remains intact. On the other hand, if cozymase is added to a water suspension of apozymase alone, rapid inactivation occurs (see Fig. 2). It is evident that the presence of

phosphate and substrate protect the coenzyme from attack by the enzymes of the disorganized yeast cells. In the living yeast cell the cozymase is not affected by the lack of substrate. So far it has not been possible to find out what products of inactivation are formed. If phosphate and substrate are added to a mixture of apozymase and cozymase after complete inactivation of the latter, a gradual reactivation of the coenzyme occurs after a few hours (see Fig. 3). As yet, the synthesis of coenzyme I by adding to apozymase preparations the split products available at present has not been accomplished.

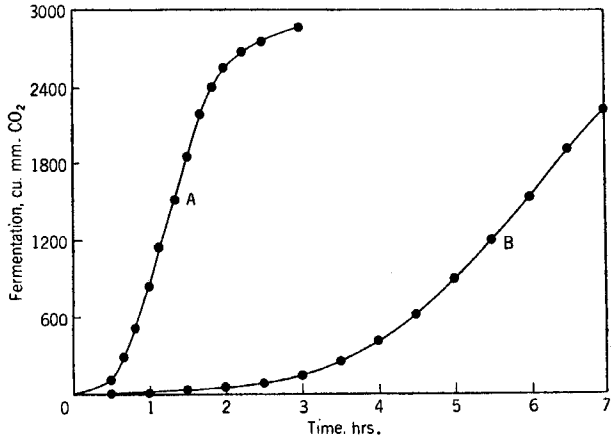


Fig. 3.—Reactivation of cozymase after incubation with apozymase (88). *A*, fermentation without previous incubation; *B*, fermentation after incubation of cozymase with apozymase in water solution for 60 minutes.

The attempts to demonstrate the synthesis of the coenzyme from its structural units by living yeast have been more successful. An interesting fact is that the addition of substrate (glucose) increases the yield of cozymase, while addition of fluoride, which inhibits fermentation, causes a considerable decrease in the cozymase content of the yeast. Lennèrstrand concludes from his results that the processes of cozymase synthesis in living yeast are linked with the processes of carbohydrate metabolism in a fashion similar to that observed by him in the apozymase system. The following picture of the living cell has been developed (88):

"A dynamic equilibrium exists between energy-requiring synthetic processes and spontaneous processes of degradation. The concentration of cozymase accordingly depends on an antagonistic effect between the carbohydrate metabolism and a nuclease

system. In the presence of adenine, adenosine and nicotinamide, the dynamic equilibrium is changed in favor of cozymase formation, provided that simultaneous and unimpeded carbohydrate metabolism can proceed. When the fermentation is inhibited by fluoride, the nuclease effect becomes apparent. Cozymase is broken down, and the addition of adenine, adenosine and nicotinamide cannot influence the balance."

Stability in Blood.—Since no codehydrogenase is found in plasma, it appears to be concentrated in the erythrocytes, and much work has been done on their coenzyme content (10, 11, 58, 80, 81, 140). Coenzyme synthesis by erythrocytes is discussed on page 225. The stability of codehydrogenase I has been studied extensively by Lennerstrand (87); some of his data are compiled in Table VI. Codehydrogenase II behaves in the same fashion. The codehydrogenases are fairly stable in whole blood, but washing with saline, which leaves the cells intact, nevertheless decreases the stability considerably. Some protective principle seems to be present in the serum. Laking of the cells also results in the rapid destruction of the coenzymes.

TABLE VI

INACTIVATION OF COZYMASE IN ERYTHROCYTES BY INCUBATION AT 37° C. (87)

Source of extract	Fermentation test, cu. mm. CO_2 evolved in 2 hrs.	
	Before incubation	After incubation for 3 hrs.
Whole blood	2685	2535
Erythrocytes, washed twice with saline	2775	550
Erythrocytes, washed twice and hemolyzed	2385	197

Destruction by Tissues.—Cozymase is stable in tissues for a limited period of time after the death of the animal (41, 69, 136). As soon as the cellular structure is destroyed, rapid inactivation of both codehydrogenases is observed (31, 36, 100).

A comparison of the mode of nicotinamide nucleotide inactivation in tissue and in yeast and blood cells was made recently by Handler and Klein (54), who found that the disintegration of animal tissues releases a heat-labile system which rapidly destroys the biological activity of the nicotinamide nucleotides. By grinding the tissues with sand and washing repeatedly with saline, suspensions of broken cells were obtained which showed strong inactivating power for these compounds. The enzyme so obtained is insoluble in water and salt solutions at *p*H 7.4, having a distinct *p*H value for optimum activity (see Fig. 4). All activity is destroyed in ten minutes by incubation at 70° C. Broken cell preparations from the liver,

kidney, leg muscle and brain of rats, dogs and rabbits inactivated the codehydrogenases present in these tissues to such an extent that only 10–20% remained after 30 minutes; after 120 minutes, no coenzyme was left. If the coenzymes were added to these tissue preparations in amounts corresponding approximately to the biological concentration, rapid destruc-

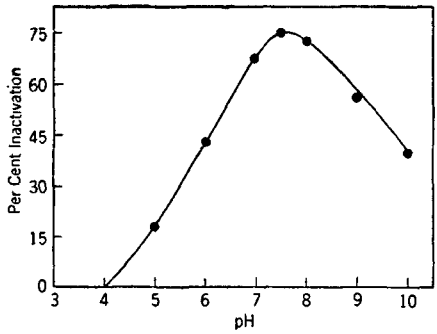
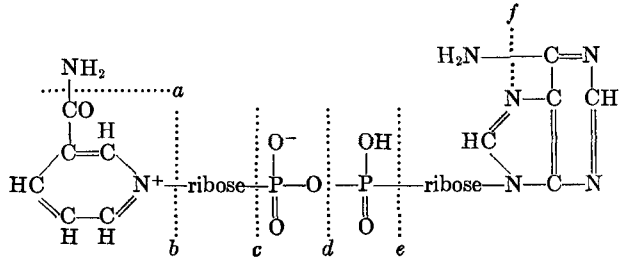


Fig. 4.—The effect of *p*H on the enzymatic inactivation of cozymase (54).

tion occurred, being fastest in the brain suspensions, which destroyed 100% of the added coenzyme I in five minutes. The high activity of these enzyme preparations permitted an investigation of the nature of the split products. Codehydrogenase I was treated with a washed preparation of broken cells from rabbit brain. When all coenzyme was inactivated, 97% of the nico-



SCHEME 7

VARIOUS POSSIBILITIES FOR THE SPLITTING OF CODEHYDROGENASE I

tinamide originally present in the nucleotide was found in a free state, while inorganic phosphate was entirely absent. Since considerable quantities of phosphate are liberated from adenylic acid and adenosine diphosphate under analogous conditions, it may be assumed that splitting does not occur at positions, *c*, *d* and *e* in the accompanying formula (54); and it

appears that the immediate cause of inactivation of the nicotinamide nucleotides by animal tissues *in vitro* may be attributed to the presence of an enzyme capable of splitting nicotinamide from these substances.

Mann and Quastel observed earlier that nicotinamide added to minced tissue in high concentrations inhibits the inactivation of codehydrogenase I. This is a rather specific effect as other closely related pyridine derivatives do not prevent the destruction of cozymase (94). They concluded that an equilibrium exists between the coenzyme, the pyridine base and other possible structural units. Handler and Klein (54) point out, however, that this is rather doubtful. They incubated samples of 40 gamma of codehydrogenase I with rabbit brain preparation. After 15 minutes, when more than half of the nucleotide was destroyed, 80 milligrams of nicotinamide was added and the incubation continued in individual tests at *p*H 3, 4, 5, 6, 7, 7.5 and 8 for one hour. No increase in pyridine nucleotides was produced by the addition of nicotinamide. It was also shown that nicotinamide does not exert its function by inactivating the cozymase-splitting enzymes. The biological significance of the coenzyme-protecting effect of the nicotinic acid is still doubtful. The high specificity of this compound would seem to be in its favor, but it is required in a concentration many hundred times higher than that encountered in any tissue.

The great majority of the investigations on enzymes governing synthesis and decomposition of the codehydrogenases have been carried out *in vivo* or with intact cells. It will be an arduous task to isolate and study them all in detail, and the work has barely begun. Nevertheless, we know now as much about the genesis and fate of the nicotinamide coenzymes as we do about those of any other coenzyme or similar compound.

Bibliography

1. Ackermann, D., *Z. Biol.*, **56**, 17 (1912); **59**, 18 (1912).
2. Adler, E., and Calvett, F., *Svenska Vet. Akad. Arkiv Kemi*, **B12**, 32 (1936).
3. Adler, E., Elliott, S., and Elliot, L., *Enzymologia*, **8**, 80 (1940).
4. Adler, E., von Euler, H., Günther, G., and Plass, M., *Biochem. J.*, **33**, 1028 (1939).
5. Adler, E., von Euler, H., and Hughes, W., *Z. physiol. Chem.*, **252**, 1 (1938).
6. Adler, E., and Günther, G., *ibid.*, **253**, 143 (1938).
7. Adler, R., and Sreenivasaya, M., *ibid.*, **249**, 24 (1937).
8. Anfinsen, C. B., *J. Biol. Chem.*, **152**, 285 (1944).
9. Axelrod, A. E., and Elvehjem, C. A., *ibid.*, **131**, 77 (1939).
10. Axelrod, A. E., Elvehjem, C. A., and Spies, T. D., *ibid.*, **138**, 667 (1941).
11. Axelrod, A. E., Gordon, E. S., and Elvehjem, C. A., *Am. J. Med. Sci.*, **199**, 697 (1940).
12. Baer, E., and Fischer, H. O. L., *J. Biol. Chem.*, **143**, 563 (1942); **150**, 213 (1943).

13. Barnes, F. W., and Schoenheimer, R., *J. Biol. Chem.*, **151,** 123 (1943).
14. Bernheim, F., and von Felsöványi, A., *Science,* **91,** 76 (1940).
15. Bovarnick, M. R., *J. Biol. Chem.*, **148,** 151 (1943); **149,** 301 (1943); **151,** 467 (1943); **153,** 1 (1944).
16. Bredereck, H., Berger, E., and Ehrenberg, J., *Ber.*, **73,** 269 (1940).
17. Bücher, T., *Naturwissenschaften,* **30,** 756 (1942).
18. Buchner, E., and Klatte, F., *Biochem. Z.*, **8,** 520 (1908).
19. Chain, E., *Biochem. J.*, **33,** 407 (1939).
20. Cohen, P. P., Hekhuis, L. G., and Sober, E. K., *Cancer Research,* **2,** 405 (1942).
21. Corran, H. S., Green, D. E., and Straub, F. B., *Biochem. J.*, **33,** 793 (1939).
22. Das, N. B., and von Euler, H., *Nature,* **141,** 604 (1938).
23. Decker, E., and Kauffmann, A. J., *J. prakt. Chem.*, **84,** 432 (1911).
24. Dixon, M., and Lutwak-Mann, C., *Biochem. J.*, **31,** 1347 (1937).
25. Dixon, M., and Zerfas, L. G., *ibid.*, **34,** 371 (1940).
26. Ellinger, P., and Coulson, R. A., *Nature,* **152,** 383 (1943); *Biochem. J.*, **38,** 265 (1944).
26*a*. Coulson, R. A., Ellinger, P., and Holden, M., *Biochem. J.*, **38,** 150 (1944).
27. Elvehjem, C. A., and Teply, L. J., *Chem. Revs.*, **33,** 185 (1943).
28. von Euler, H., and Adler, E., *Enzymologia,* **7,** 21 (1939).
29. von Euler, H., Adler, E., Günther, G., and Elliot, L., *ibid.*, **6,** 337 (1939).
30. von Euler, H., and Günther, G., *Z. physiol. Chem.*, **243,** 1 (1936).
31. von Euler, H., Heiwinkel, H., and Schlenk, F., *ibid.*, **247,** IV (1937).
32. von Euler, H., Högberg, B., Karrer, P., Salomon, H., and Ruckstuhl, H., *Helv. Chim. Acta,* **27,** 382 (1944).
33. von Euler, H., Malmberg, M., and Günther, G., *Z. Krebsforsch.*, **45,** 425 (1937).
34. von Euler, H., and Myrbäck, K., *Arch. ges. Physiol. (Pflügers),* **219,** 521 (1925).
35. von Euler, H., and Myrbäck, K., *Z. physiol. Chem.*, **131,** 179 (1923).
36. von Euler, H., Myrbäck, K., and Brunius, E., *ibid.*, **177,** 237; **183,** 60 (1929).
37. von Euler, H., Schlenk, F., Heiwinkel, H., and Högberg, B., *ibid.*, **256,** 208 (1938).
38. Fischer, E., and Raske, K., *Ber.*, **43,** 1750 (1910).
39. Gale, E. F., *Bact. Revs.*, **7,** 139 (1943).
40. Gingrich, W. D., and Schlenk, F., *J. Bact.*, **47,** 535 (1944).
41. Govier, W. M., *Science,* **99,** 475 (1944).
42. Govier, W. M., *ibid.*, **100,** 156 (1944).
43. Green, D. E., Dewan, I. G., and Leloir, L. F., *Biochem. J.*, **31,** 934 (1937).
44. Green, D. E., Knox, W. E., and Stumpf, P. K., *J. Biol. Chem.*, **138,** 775 (1941).
45. Greenstein, J. P., *J. Natl. Cancer Inst.*, **3,** 419 (1943); *Advances in Enzymology,* Vol. III, Interscience, New York, 1943, p. 315.
46. Guggenheim, M., *Die biogenen Amine.* Karger, Basel and New York, 1940.
47. Gulland, J. M., and Story, L. F., *J. Chem. Soc.*, **1938,** 259.
48. Günther, G., *Svenska Vet. Akad. Arkiv Kemi,* **A12,** 23 (1938).
49. Haas, E., *Biochem. Z.*, **285,** 368 (1936).
49*a*. Haas, E., *J. Biol. Chem.*, **155,** 333 (1944).
50. Haas, E., Harrer, C. J., and Hogness, T. R., *ibid.*, **142,** 835 (1942).
51. Haas, E., Horecker, B. L., and Hogness, T. R., *ibid.*, **136,** 747 (1940); **143,** 341 (1942).
52. Handler, P., *ibid.*, **154,** 203 (1944).

53. Handler, P., and Dann, W. J., *J. Biol. Chem.*, **146,** 357 (1942).
54. Handler, P., and Klein, J. R., *ibid.*, **143,** 49 (1942).
55. Handler, P., and Kohn, H. I., *ibid.*, **150,** 447 (1943).
56. Harden, A., and Young, W. J., *Proc. Roy. Soc. London*, **B77,** 410 (1906); **B78,** 369 (1906).
57. Heiwinkel, H., *Svenska Vet. Akad. Arkiv Kemi*, **A13,** 19 (1939).
58. Hoagland, C. L., and Shank, R. E., *J. Clin. Investigation*, **21,** 622 (1942).
59. Hoagland, C. L., and Ward, S. M., *J. Biol. Chem.*, **146,** 115 (1942).
60. Hoagland, C. L., Ward, S. M., Gilder, H., and Shank, R. E., *J. Exptl. Med.*, **76,** 241 (1942).
61. Hoagland, C. L., Ward, S. M., and Shank, R. E., *J. Biol. Chem.*, **151,** 369 (1943).
62. Hogness, T. R., in *A Symposium on the Respiratory Enzymes.* Univ. Wisconsin Press, Madison, 1942, p. 134.
63. Hoppe-Seyler, F. A., *Z. physiol. Chem.*, **221,** 45 (1933); **222,** 105 (1933).
64. Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **142,** 401 (1942).
65. Huff, J. W., and Perlzweig, W. A., *ibid.*, **150,** 483 (1943).
66. Huff, J. W., Perlzweig, W. A., and Sharp, D. G., *ibid.*, **150,** 395 (1943).
67. Hutchens, J. O., Jandorf, B. J., and Hastings, A. B., *ibid.*, **138,** 321 (1941).
68. Jachimowicz, T., *Biochem. Z.*, **292,** 356 (1937).
69. Jandorf, B. J., *J. Biol. Chem.*, **150,** 89 (1943).
70. Jandorf, B. J., Klemperer, F. W., and Hastings, A. B., *ibid.*, **138,** 311 (1941).
71. Johnson, F. H., and Eyring, H., *J. Am. Chem. Soc.*, **66,** 848 (1944).
72. Karrer, P., Kahnt, F. W., Epstein, R., Jaffé, W., and Ishii, T., *Helv. Chim. Acta*, **21,** 223 (1938).
73. Karrer, P., Ringier, B. H., Büchi, J., Fritzsche, H., and Solmssen, U., *ibid.*, **20,** 55 (1937).
74. Karrer, P., Schwarzenbach, G., Benz, F., and Solmssen, U., *ibid.*, **19,** 811 (1936).
75. Kensler, C. J., Dexter, S. O., and Rhoads, C. P., *Cancer Research*, **2,** 1 (1942).
76. Kensler, C. J., Sugiura, K., and Rhoads, C. P., *Science*, **91,** 623 (1940).
77. Kensler, C. J., Young, N. F., and Rhoads, C. P., *J. Biol. Chem.*, **143,** 465 (1942).
78. Klein, G., and Linser, H., *Z. physiol. Chem.*, **209,** 75 (1932).
79. Klein, J. R., and Handler, P., *J. Biol. Chem.*, **144,** 537 (1942).
80. Kohn, H. I., *Am. J. Physiol.*, **129,** 398 (1940).
81. Kohn, H. I., *Biochem. J.*, **32,** 2075 (1938).
81*a*. Krehl, W. A. and Strong, F. M., *J. Biol. Chem.*, **156,** 1 (1944).
81*b*. Krehl, W. A., Elvehjem, C. A., and Strong, F. M., *ibid.*, **156,** 13 (1944).
82. Kubowitz, F., *Biochem. Z.*, **293,** 308 (1937).
83. Kubowitz, F., and Ott, P., *ibid.*, **314,** 94 (1943).
84. Kuhn, R., and Beinert, H., *Ber.*, **76,** 904 (1943).
85. Lang, K., *Z. physiol. Chem.*, **277,** 114 (1942).
86. Lennerstrand, Å., *Biochem. Z.*, **287,** 172 (1936); **289,** 104 (1936).
87. Lennerstrand, Å., *Svenska Vet. Akad. Arkiv. Kemi*, **B14,** 1 (1940).
88. Lennerstrand, Å., *ibid.*, **A14,** 16 (1941).
89. Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **121,** 131 (1937).
90. Lutwak-Mann, C., *Biochem. J.*, **32,** 1364 (1938).
91. Lwoff, A., and Lwoff, M., *Compt. rend.*, **203,** 520, 896 (1936).
92. Lwoff, A., and Lwoff, M., *Proc. Roy. Soc. London*, **B122,** 253, 360 (1937).

93. Lynen, F., and Franke, W., *Z. physiol. Chem.*, **270**, 271 (1941).
94. Mann, P. J. G., and Quastel, J. H., *Biochem. J.*, **35**, 502 (1941).
95. Meyerhof, O., *Ergeb. Physiol. exptl. Pharmakol.*, **39**, 10 (1936).
96. Meyerhof, O., *Z. physiol. Chem.*, **101**, 165; **102**, 1 (1918).
97. Meyerhof, O., and Junowicz-Kocholaty, R., *J. Biol. Chem.*, **149**, 71 (1943).
98. Meyerhof, O., Kiessling, W., and Schulz, W., *Biochem. Z.*, **292**, 25 (1937).
99. Michaelis, L., Schubert, M. P., and Granick, S., *J. Am. Chem. Soc.*, **61**, 1981 (1939).
100. Myrbäck, K., *Ergeb. Enzymforsch.*, **2**, 139 (1933).
101. Najjar, V. A., and Holt, L. E., Jr., *Science*, **93**, 20 (1941).
102. Najjar, V. A., Stein, H. J., Holt, L. E., Jr., and Kabler, C. V., *J. Clin. Investigation*, **21**, 263 (1942).
103. Najjar, V. A., White, V., and McNair Scott, D. B., *Bull. Johns Hopkins Hosp.*, **74**, 378 (1944).
104. Najjar, V. A., and Wood, R. W., *Proc. Soc. Exptl. Biol. Med.*, **44**, 386 (1940).
105. Negelein, E., and Brömel, H., *Biochem. Z.*, **303**, 231 (1939).
106. Negelein, E., and Gerischer, W., *ibid.*, **284**, 289 (1936).
107. Negelein, E., and Wulff, H. J., *ibid.*, **293**, 351 (1937).
108. Northrop, J. H., *Crystalline Enzymes*. Columbia Univ. Press, New York, 1939.
109. Ohlmeyer, P., *Biochem. Z.*, **287**, 212 (1936).
110. Parnas, J. K., *Nature*, **151**, 577 (1943).
111. Perlzweig, W. A., Bernheim, M. L. C., and Bernheim, F., *J. Biol. Chem.*, **150**, 401 (1943).
112. Pittman, M., and Fraser, H. F., *U. S. Pub. Health Repts.*, **55**, 915 (1940).
113. Plentl, A. A., and Schoenheimer, R., *J. Biol. Chem.*, **153**, 203 (1944).
114. Potter, V. R., in *Advances in Enzymology*, Vol. IV. Interscience, New York, 1944, p. 201.
115. Potter, V. R., *Cancer Research*, **2**, 688 (1942).
116. Rubinstein, D., and Shekun, L., *Nature*, **143**, 1064 (1939).
117. Sarett, H. P., *J. Biol. Chem.*, **150**, 159 (1943).
118. Sarett, H. P., *J. Nutrition*, **23**, 35 (1942).
119. Sarett, H. P., Huff, J. W., and Perlzweig, W. A., *ibid.*, **23**, 23 (1942).
120. Saunders, F., Dorfman, A., and Koser, S. A., *J. Biol. Chem.*, **138**, 69 (1941).
121. Schlenk, F., *Arch. Biochem.*, **3**, 93 (1943).
122. Schlenk, F., in *A Symposium on Respiratory Enzymes*. Univ. Wisconsin Press, Madison, 1942, p. 104.
123. Schlenk, F., *J. Biol. Chem.*, **146**, 619 (1942).
124. Schlenk, F., *unpublished experiments*.
125. Schlenk, F., and von Euler, H., *Naturwissenschaften*, **24**, 794 (1936).
126. Schlenk, F., and Günther, G., in E. Bamann and K. Myrbäck, *Methodik der Fermente*. Part 7, Thieme, Leipzig, 1940.
127. Schlenk, F., Hellström, H., and von Euler, H., *Ber.*, **71**, 1471 (1938).
128. Schlenk, F., Högberg, B., and Tingstam, S., *Svenska Vet. Akad. Arkiv Kemi*, **A13**, 11 (1939).
129. Schlenk, F., and Vowles, R. B., *ibid.*, **B13**, 19 (1940).
130. Schoenheimer, R., *The Dynamic State of Body Constituents*. Harvard Univ. Press, Cambridge, 1943.
131 Slotta, K. H., and Neisser, K., *Ber.*, **71**, 1987 (1938).

132. Stevenson, E. S., Dobriner, K., and Rhoads, C. P., *Cancer Research*, **2**, 160 (1942).
133. Straub, F. B., *Biochem. J.*, **33**, 787 (1939).
134. Straub, F. B., *Z. physiol. Chem.*, **275**, 63 (1942).
135. Sumner, J. B., in *Advances in Enzymology*, Vol. I. Interscience, New York, 1941, p. 163.
136. Swingle, K. F., Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, **145**, 581 (1942).
137. Theorell, H., *Naturwissenschaften*, **22**, 290 (1934).
138. Thunberg, T., *Ergeb. Enzymforschung*, **7**, 163 (1938).
139. Trager, W., *Physiol. Revs.*, **21**, 1 (1941).
140. Vilter, R. W., Vilter, S. P., and Spies, T. D , *J. Am. Med. Assoc.*, **112**, 420 (1939).
141. Warburg, O., *The Metabolism of Tumors*, translated by F. Dickens. R. Smith, New York, 1930.
142. Warburg, O., *Ergeb. Enzymforschung*, **7**, 210 (1938).
143. Warburg, O., and Christian, W., *Biochem. Z.*, **287**, 291 (1936).
144. Warburg, O., and Christian, W., *ibid.*, **292**, 287 (1937).
145. Warburg, O., and Christian, W., *ibid.*, **298**, 368 (1938).
146. Warburg, O., and Christian, W., *ibid.*, **303**, 40 (1939).
147. Warburg, O., and Christian, W., *ibid.*, **311**, 209 (1942); **314**, 149 (1943).
148. Warburg, O., Christian, W., and Griese, A., *ibid.*, **282**, 157 (1935).
149. Waters, W. A., *Trans. Faraday Soc.*, **39**, 140 (1943).
150. Williams, R. J., *Studies on the Vitamin Content of Tissues, I and II.* Univ. Texas Press, Austin, 1941, 1942.
151. Wirth, J. C., and Nord, F. F., *Arch. Biochem.*, **1**, 143 (1942).

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SOME ENZYME REACTIONS ON SULFUR COMPOUNDS

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Any adequate consideration of enzyme reactions on sulfur and on its compounds should include the reactions carried out by the so-called sulfur bacteria. According to some authors (39), these bacteria can be divided into five groups that differ in the extent and the manner in which they can handle sulfur. Considered together, these organisms are capable of carrying out the oxidation and reduction of inorganic sulfur throughout its valence range of -2 to $+6$. To the extent that these organisms are capable of utilizing the energy of the sulfur oxidations, it seems quite sure that the reactions are under enzymatic regulation by the organisms. However, the mere fact that a reaction occurs in the presence of an organism does not prove that the reaction is under enzymatic regulation, except in a very indirect way. For example, it is quite well established (21, 35*a*) that elementary sulfur can be readily reduced by sulfhydryl groups. Most living organisms contain sulfhydryl groups. It would seem to follow that any living organism capable of bringing sulfur into chemical contact with its sulfhydryl groups will be able to reduce elementary sulfur. Such a reaction need not be an enzyme reaction except that the formation of the sulfhydryl groups may be enzymatic. Similarly, hydrogen sulfide or its salts and some of the partially oxidized forms of sulfur are readily oxidized by oxygen in the presence of traces of metals (24) or hemin or hemoglobin (22). The fact that these compounds are oxidized in the presence of an organism does not indicate that the oxidation is necessarily catalyzed by an enzyme in the organism. Although the reactions themselves are well established, there is little information available, as far as the writer is aware, about the enzymes of sulfur bacteria.

In most plants, sulfur is usually supplied to the cells in the form of sulfate. Reduction of it is apparently carried out with ease. Again there is little information available about the enzymes involved. A recent review (44) covers what knowledge there is.

In the case of animals, sulfur is usually supplied in the form of organic compounds, although the food supply normally includes variable amounts of inorganic sulfur, mostly as the sulfate. Inorganic sulfate and organic sulfonates are the end products of much of the sulfur of metabolism. It is clear that in the balance no appreciable amount of sulfate is reduced in animal tissues (40) and the sulfur requirement cannot be met by sulfate (14), but the writer is not aware of any good evidence showing whether or not sulfate can be reduced at all by animal tissue.

Present information indicates that the three compounds—methionine, thiamine and biotin—can supply the sulfur requirements of the mammalian organism under normal conditions. Methionine contains its sulfur as a thio ether. Thiamine contains a thiazole ring and biotin contains a thiophene ring. The fact that these three compounds are required indicates that they are not interconvertible. Actually, a considerable amount of the sulfur requirement is supplied by the amino acids, cysteine and cystine, which contain their sulfur in the sulfhydryl and disulfide form, respectively. Thiamine may also exist to some extent in the sulfhydryl form, for the thiazole ring opens rather easily under some conditions, forming a sulfhydryl group. In the case of thiamine, not much detailed information is available concerning the changes which the sulfur undergoes in the body, but it is known (10) that thiamine is readily metabolized and that its sulfur appears in the urine as neutral sulfur and as inorganic sulfate. Almost nothing is known concerning the metabolism of biotin. In the cases of cysteine and methionine, some definite enzyme reactions involving them are known and a consideration of these reactions will be our main task.

The fact that methionine is an essential amino acid and cannot be replaced by cystine is well established (10, 43). The fact that cystine is not essential, at least under some conditions, and that it can be and is formed when methionine is supplied, has been shown by a number of different lines of evidence. Perhaps the most direct evidence that methionine sulfur can be converted to cystine sulfur is supplied by the work of Tarver and Schmidt (40). These authors fed radioactive sulfur in the form of methionine and recovered cystine containing radioactive sulfur. On the basis of present evidence, the conversion of methionine to cystine must be considered as nonreversible under the conditions tested. The fact that the methyl group of methionine can be removed and transferred to other compounds is well established (42). Apparently the transmethylation is reversible, for methyl groups supplied as choline were recovered in methionine (34). The enzymes concerned in this reaction have not been studied in detail to the author's knowledge.

Binkley, Anslow, and du Vigneaud (7) found that the thio ether, *ll*-S-(2-amino-2-carboxyethyl) homocysteine is split by liver with the formation of cysteine.* This result naturally suggests that such a compound may be an intermediate in the conversion of methionine to cystine. Binkley and du Vigneaud (8) later found that homocysteine plus serine, in the presence of liver extracts, produced cysteine. These authors did not find a production of cysteine when methionine was used in place of homocysteine, but this result is not a serious limitation because the demethylation of methionine is known to occur readily in the intact animal. Floyd and Medes (16) have reported a formation of cysteine from methionine by liver and kidney slices under aerobic conditions, but the amount of cysteine obtained was very small.

The fact that methionine sulfoxide has been found capable of replacing methionine in the diet (1) may indicate that the body is capable of reducing such oxidation products.

In common with many other sulfhydryl compounds, cysteine is readily oxidized to the disulfide stage, in this case, cystine. The reverse reaction, the reduction of cystine to cysteine, can also occur in tissues. Because of this interconversion, cysteine and cystine are usually considered to undergo the same reactions. An exception is observed in the case of cystinuria, for in such cases ingested cysteine is excreted in the urine as cystine while ingested cystine is oxidized and utilized (11). All exclusively peptide compounds of cysteine are capable of undergoing an oxidation to the disulfide stage (except insofar as steric factors prevent it) and of undergoing the reverse reduction. This reversible change appears to be an important factor in determining changes in the structure and activity of a number of proteins. To the extent that thiamin exists in the sulfhydryl form, it can also undergo oxidation to the disulfide. The oxidized form still retains its vitamin activity (46). The sulfur of cysteine can be oxidized beyond the disulfide stage, and cysteic acid is produced as end product. Some tissues contain an enzyme that decarboxylates cysteic acid, thus producing taurine (9, 27). Tarver and Schmidt (41) isolated taurine containing radioactive sulfur after feeding methionine containing this isotope.

It has long been known that the carbon of cysteine can be converted to glucose in the phlorizinized dog (13). The manner in which the sulfur is removed was not known. It has been recognized for some time that certain bacteria can form hydrogen sulfide from cysteine. The fact that mammalian tissues contain an enzyme which catalyzes such removal of hydrogen

* For more recent details, see F. Binkley, *J. Biol. Chem.*, **155**, 39 (1944).

sulfide was first reported in 1939 by Fromageot, Wookey, and Chaix (18). These authors observed the enzyme in dog liver and Fromageot and his coworkers have since published several papers dealing with it. The author first observed the same reaction during a study of the possible formation of ascorbic acid by rat tissues (35, 35*a*).

Fromageot and coworkers (19) came to the conclusion that the reaction involved was:

$$3 \text{ cysteine} \rightleftharpoons \text{cystine} + \text{hydrogen sulfide} + \text{alanine}$$

They emphasized that the enzyme was quite different from the enzyme in some bacteria which produces both hydrogen sulfide and ammonia from cysteine (15). The author's laboratory was not able to confirm the above equation, but it may be correct to some extent. There does seem to be a tendency to form some cystine and small amounts of alanine. It might be possible to obtain conditions favoring these products, but our results (35, 35*a*) are much more in accord with the following equation:

$$\underset{\text{Cysteine}}{CH_2SHCHNH_2COOH} \rightleftharpoons \underset{\text{Pyruvic acid}}{CH_3COCOOH} + H_2S + NH_3$$

TABLE I

CONVERSION OF CYSTEINE TO HYDROGEN SULFIDE, PYRUVIC ACID, AND AMMONIA IN MOLE $\times 10^{-6}$ (35*a*)

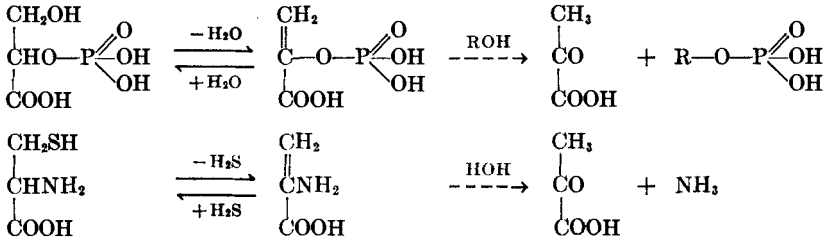
(2.0 ml. of chloroform-treated liver extract in phosphate buffer, *p*H 7.6; under nitrogen; temperature, 37° C.; time of reaction, 2 hrs.; cystine added, 20.0 $\times 10^{-6}$ mole)

H_2S produced	Pyruvic acid produced	Ammonia produced
8.6	5.2	9.5
11.0	4.9	10.1
8.0	5.9	8.9
9.7	4.4	6.0
9.1	7.6	7.6
9.7	4.9	5.3
4.1	2.3	2.4
7.2	4.7	4.1
6.7	6.5	...
7.5	4.0	...
8.4	5.1	...
7.0	3.7	...
9.7	5.0	...
5.5		7.3
11.4		15.1
3.6		3.5
Average 8.0	4.9	7.3
Average 2.6*	4.4	9.3

* Average of six aerobic experiments.

Table I shows some data in support of the second equation. It should be mentioned that the method used for the determination of pyruvic acid is known to give low results.

We were interested in knowing whether or not this formation of hydrogen sulfide could be reversed, so we allowed the reaction to occur in the presence of hydrogen sulfide containing radioactive sulfur (37). After a part of the cysteine had been converted, the reaction was stopped and the cysteine present was isolated. It was found to contain appreciable amounts of radioactive sulfur. We consider this result as proof that sulfide sulfur was converted to cysteine sulfur; and since we do not know any other method of accomplishing this conversion we will assume that the reaction that produces hydrogen sulfide was reversed. All our attempts to start with pyruvic acid, ammonia and hydrogen sulfide and demonstrate a formation of cysteine were unsuccessful. These findings naturally suggest a comparison with the formation of pyruvic acid from 2-phosphoglyceric acid (Scheme 1). In this case, the first reaction is the removal of water with the formation of phosphopyruvic acid, a reaction which is readily reversible.



SCHEME 1

The next step is the removal of phosphoric acid by a phosphate acceptor or by water with the production of pyruvic acid, a step which is not reversible. A difference between the two is that, in the phosphoglycerate case, the intermediate compound, phosphopyruvic acid, is stable enough to be worked with, while, in the cysteine case, the supposed intermediate, amino acrylic acid, is not stable. According to Chargaff and Sprinson (12) and Binkley (6), serine undergoes a similar change and is converted to pyruvic acid via amino acrylic acid. Thus, pyruvic acid is produced enzymatically from three relatively different compounds by what appear to be quite similar reactions. Such findings are in good agreement with the earlier work of Bergmann and coworkers (2–4) on the chemical behavior of such compounds. It has been suggested that the same enzyme may be acting on all three compounds (6, 12).

Some information is available on the partial purification (25, 26) and properties of the enzyme forming hydrogen sulfide. It is inhibited by cyanide (18, 35, 35*a*), arsenic trioxide (26) and such carbonyl reagents as phenylhydrazine, hydroxylamine and semicarbazide (26). The activity of the crude tissue preparations is not inhibited by sodium fluoride (18, 35*a*), but the purified enzyme is inhibited (6). Some other amino acids do not act as inhibitors, but other thiol compounds do (26). Magnesium apparently forms a component part of the enzyme (6, 12, 26) although it can be replaced by some other metals (6).

If homocysteine is used as substrate instead of cysteine, some hydrogen sulfide is produced. According to the author's measurements, considerably less hydrogen sulfide is produced from homocysteine than from cysteine and he would normally assume that the same enzyme acts on both substrates. However, Fromageot and Desnuelle (17) report that the ratio of the activity on the two compounds is quite different for different enzyme preparations, and conclude that they are acted upon by different enzymes. The other products resulting from the action on homocysteine have not been determined.

In view of the fact that hydrogen sulfide is very toxic for an intact animal, it seemed curious that tissues should contain an enzyme for producing it. Actually the bulk of the enzyme, at least in the rat, is located in the liver, although it is also present in some other tissues. The livers of different species vary considerably in their ability to produce hydrogen sulfide. Greenstein (20) has reported the interesting result, obtained by measuring ammonia formation, that, although normal liver of rats or mice energetically attacks cysteine, cystine and some of their peptides, hepatomas from these animals are devoid of such action. The fact that rat liver is rich in this cysteine enzyme naturally suggests a connection with the well-known toxicity of cysteine for young white rats under certain conditions. Since we could find no data on the effect of hydrogen sulfide on isolated tissues, we tested the effect of adding it to liver, kidney and brain preparations of the rat (36). The oxygen consumption of liver and kidney proved to be not at all sensitive to hydrogen sulfide. Even relatively large amounts were entirely removed and apparently without serious harm to the tissues. The sulfur of the added hydrogen sulfide could be recovered as sulfur, sulfate and polythionates. For brain, however, the picture was quite different. A concentration of 5×10^{-4} M hydrogen sulfide markedly inhibited the oxygen consumption. From these results it would seem that a local production of hydrogen sulfide might not be a serious matter provided it is detoxified before it reached the brain.

Mammalian tissues contain only very small amounts of free cysteine or cystine, but all contain appreciable amounts of glutathione. A peculiarity in the structure of glutathione is that the glutamic acid is linked by the γ-carboxyl group. As far as we know, the glutathione structure does not exist performed in protein but must be formed by some special enzyme or in some special manner. Our attention was recently directed to this problem in the following way. We had occasion to determine the total reducing material present in guinea-pig liver. To do so, we deproteinized with metaphosphoric acid and titrated with iodine. We were surprised to find (37) that the iodine titration increased rather rapidly as the time between the killing of the animal and the addition of metaphosphate increased. If this time was thirty minutes, the titration was about 100% greater than if the time was about two minutes. On checking into the matter, we found that much the same thing had been observed before and had been explained in different ways (23, 29, 33). We were struck by the fact that the character of

TABLE II

IODINE TITRATION OF GLUTATHIONE–CYSTEINE MIXTURES (TOTAL —SH CONSTANT) (28)

Ratio, glutathione–cysteine	5:0	4:1	3:2	2:3	1:4	0:5
Iodine reduced, ml	5.65	7.41	9.65	11.62	13.43	14.03

the end point changed with the increase in titration. Originally, it was sharp and definite, as is the end point with glutathione under such conditions. Later, it was much less sharp and definite and resembled that with cysteine. It seemed to us that the increased iodine titration might be best explained, as Bierich and Rosenbohm (5) had suggested, by a hydrolysis of glutathione and liberation of cysteine. The figures in Table II show how the iodine titration, under such conditions, changes when the total —SH is kept constant but the ratio of glutathione to cysteine changes.

In keeping with such an explanation was the fact that determinations by the Sullivan method (38) on successive preparations showed a definite increase in cystine paralleling the iodine titration (Table III). Also in keeping with the explanation were the facts that the nitrogen of the deproteinized extracts prepared at successive intervals and the total cystine, determined after hydrolysis, did not change during the time that the iodine titration did change. If more glutathione was added, an additional change in titration and in cysteine occurred. In these cases it was possible to isolate the cystine in good yield. From such findings we conclude that the glutathione was being hydrolyzed, and in amounts sufficient to account for the increased iodine titration.

From the fact that in the liver fixed as rapidly as possible the free cysteine is almost nil and the glutathione is quite appreciable (at least 2–3 mg.), it is apparent that the equation representing the hydrolysis is pushed far to the glutathione side. In contrast, in the liver preparations, the glutathione is spontaneously hydrolyzed and the equilibrium reached corresponds to a large degree of hydrolysis. It seems to follow that, in the

TABLE III

FREE CYSTINE AND IODINE TITRATION VALUES OF DEPROTEINIZED EXTRACTS OF GUINEA-PIG LIVER (28)

Determination	Time after removal of liver from animal, min.							
	0	10	15	20	30	40	50	60
Animal 1								
Iodine titration*	12.5	...	16.1	17.1	...	21.6	...	22.6
Colorimetric cystine**	0.19	...	0.40	0.58	...	0.89	...	1.00
Animal 2								
Iodine titration	13.3	15.2	...	19.4	...	24.1	...	25.4
Colorimetric cystine	0.18	0.36	...	0.71	...	0.98	...	1.08
Animal 3								
Iodine titration	13.1	15.4	...	21.3	...	26.3	...	26.3
Colorimetric cystine	0.17	0.35	...	0.74	...	0.90	...	0.87
Animal 4								
Iodine titration	7.0	...	...	8.5	10.0	10.5	11.3	11.4
Colorimetric cystine	0.16	...	...	0.53	0.68	0.78	0.80	0.80
Animal 5								
Iodine titration	14.6	17.9	...	20.6	23.1	25.0	...	25.6
Colorimetric cystine	0.31	0.56	...	0.75	0.93	0.93	...	0.87

* Expressed as ml. 0.001 *M* iodine per g. of tissue.
** Expressed as mg. cystine per g. of tissue.

intact animal, the synthesis of liver glutathione must be coupled with an energy-yielding reaction.

It is quite likely that the enzyme concerned in this glutathione hydrolysis is the same as the one described originally as antiglyoxalase (31) and studied in more detail later (32, 45). For some reason, guinea-pig liver is rich in the enzyme and rat liver is poor in it. According to our results it was rather difficult to extract the enzyme from the liver. Much of the activity re-

mained in the normally insoluble portion, but a solution with some activity could be obtained. The enzyme was found to have a rather alkaline *p*H optimum of 8.5–9.0. Dialysis caused a prompt inactivation. If the dialyzate was concentrated and re-added to the nondialyzable material, the activity returned. Similarly, a heated extract reactivated the dialyzed material; and, rather interestingly, an extract of rat liver that had only slight activity by itself readily activated the dialyzed enzyme from guinea-pig liver. Apparently rat liver contains the coenzyme but not much of the enzyme. The coenzyme is relatively stable: Irradiation with a mercury lamp for 2.5 hours did not inactivate it; heating for one hour at 100° C. in either 0.5 *N* acid or 1.0 *N* alkali caused only slight destruction. Wet ashing caused complete destruction. All of these results are included in Table IV.

TABLE IV

RESTORATION OF ACTIVITY TO DIALYZED ENZYME MATERIAL (28)

Material	Per cent activity
Original extract	100
dialyzed 20 hrs.	48
dialyzed 43 hrs.	39
dialyzed 89 hrs.	30
Preparation dialyzed 43 hours	
+ dialyzate to original concentration	87
+ equal volume heated extract	83
+ equal volume 1:5 rat liver homogenate	78
Preparation dialyzed 89 hours	
+ dialyzate to original concentration	87
+ dialyzate irradiated 2.5 hrs. at *p*H 8.4	96
+ dialyzate heated 1 hr. at 100° C. in 0.5 *M* HCl	78
+ dialyzate heated 1 hr. at 100° C. 1.0 *M* NaOH	74
+ dialyzate ashed	30

It is obvious that the organism makes some effort to keep glutathione intact, and one may wonder what advantages it offers over the constituent amino acids. Of course, it may play a role in the synthesis of protein, but not many data are available on that point. Only one specific function is known—that of coglyoxalase—and there is some doubt about how important a role the glyoxalase enzyme system plays. As far as the reducing properties are concerned, free cysteine contains the same reducing group, although the potentials are not the same (30). Of course, an obvious difference is that oxidized glutathione is more soluble than cystine, but another possible advantage is that gluthathione may be more stable than cysteine to the action of some other enzymes present in tissues. An ex-

ample of such an enzyme is the enzyme, discussed above, which produced hydrogen sulfide from cysteine, but not from glutathione.

The question of a name for the enzyme forming hydrogen sulfide is of some interest. Fromageot and coworkers gave the name "cysteinase" to the bacterial enzyme producing hydrogen sulfide and ammonia from cysteine, and "desulfurase" to the enzyme in mammalian tissue which, according to them, produces hydrogen sulfide but not ammonia. It is doubtful if these enzymes differ, at least in the manner suggested, and hence two names are possibly too many. "Cysteinase" is not a very descriptive term. The term "cysteine desulfurase" probably causes no confusion at the present time, but if the suggested mechanism of the reaction is correct the name would seem to be incorrect. It is at least theoretically possible to remove sulfur, as such, from cysteine, and an enzyme that could do so would properly be a desulfurase. However, the fact that hydrogen is removed along with the sulfur is rather an important one and, in the author's opinion, should be indicated in the name if possible. Calling an enzyme that removes hydrogen sulfide a desulfurase is analogous to calling an enzyme that removes water a deoxygenase. If we call the addition of water a hydration and an enzyme that removes water a dehydrase, then it would seem reasonable to call the addition of hydrogen sulfide a sulfhydration, and an enzyme that removes it a desulfhydrase. The enzyme producing hydrogen sulfide from cysteine would then be cysteine desulfhydrase.

Bibliography

1. Bennett, M. A., *Biochem. J.*, **33**, 1794 (1939).
2. Bergmann, M., and Delis, D., *Ann.*, **458**, 76 (1927).
3. Bergmann, M., and Grafe, K., *Z. physiol. Chem.*, **187**, 187 (1930).
4. Bergmann, M., Miekeley, A., and Kann, E., *ibid.*, **146**, 247 (1925).
5. Bierich, R., and Rosenbohm, A., *ibid.*, **231**, 39 (1935).
6. Binkley, F., *J. Biol. Chem.*, **150**, 261 (1943).
7. Binkley, F., Anslow, W. P., Jr., and du Vigneaud, V., *ibid.*, **143**, 559 (1942).
8. Binkley, F., and du Vigneaud, V., *ibid.*, **144**, 507 (1942).
9. Blaschko, H., *Biochem. J.*, **36**, 571 (1942).
10. Borsook, H., Buchman, E. R., Hatcher, J. B., Yost, D. M., and McMillan, E., *Proc. Natl. Acad. Sci. U. S.*, **26**, 412 (1940).
11. Brand, E., Cahill, G. F., and Harris, M. M., *J. Biol. Chem.*, **109**, 69 (1935).
12. Chargaff, E., and Sprinson, D. B., *ibid.*, **148**, 249 (1943); **151**, 273 (1943).
13. Dakin, H. D., *ibid.*, **14**, 321 (1913).
14. Daniels, H. L., and Rich, J. K., *ibid.*, **36**, 27 (1918).
15. Desnuelle, P., and Fromageot, C., *Enzymologia*, **6**, 80 (1940).
16. Floyd, N. F., and Medes, G., *Arch. Biochem.*, **2**, 135 (1943).
17. Fromageot, C., and Desnuelle, P., *Compt. rend.*, **214**, 647 (1942).
18. Fromageot, C., Wookey, E., and Chaix, P., *ibid.*, **209**, 1019 (1939).

19. Fromageot, C., Wookey, E., and Chaix, P., *Enzymologia*, **9**, 198 (1941).
20. Greenstein, J. P., *J. Natl. Cancer Inst.*, **3**, 491 (1943).
21. Guthrie, J. D., *Contrib. Boyce Thompson Inst.*, **9**, 223 (1938).
22. Haurowitz, F., *Enzymologia*, **10**, 141 (1941).
23. Hopkins, F. G., and Elliott, K. A. C., *Proc. Roy. Soc. London*, **B109**, 58 (1931).
24. Krebs, H. A., *Biochem. Z.*, **204**, 343 (1929); **209**, 32 (1929).
25. Laskowski, M., and Fromageot, C., *J. Biol. Chem.*, **140**, 663 (1941).
26. Lawrence, J. M., and Smythe, C. V., *Arch. Biochem.*, **2**, 225 (1943).
27. Medes, G., and Floyd, N. F., *Biochem. J.*, **36**, 836 (1943).
28. Neubeck, C. E., and Smythe, C. V., *Arch. Biochem.*, **4**, 435, 443 (1944).
29. Rosenbohm, A., *Biochem. Z.*, **289**, 279 (1937).
30. Ryklan, L. R., and Schmidt, C. L. A., *Univ. Calif. Pub. Physiol.*, **8**, 257 (1944).
31. Schroeder, E. F., Munro, M. P., and Weil, L., *J. Biol. Chem.*, **110**, 181 (1935).
32. Schroeder, E. F., and Woodward, G. E., *ibid.*, **120**, 209 (1937).
33. Schultze, M. O., Stotz, E., and King, C. G., *ibid.*, **122**, 395 (1938).
34. Simmonds, S., Cohn, M., Chandler, J. P., and du Vigneaud, V., *ibid.*, **149**, 519 (1943).
35. Smythe, C. V., *ibid.*, **140**, cxxiii (1941).
35*a*. Smythe, C. V., *ibid.*, **142**, 387 (1942).
36. Smythe, C. V., *Arch. Biochem.*, **2**, 259 (1943).
37. Smythe, C. V., and Halliday, D., *J. Biol. Chem.*, **144**, 237 (1942).
38. Sullivan, M. X., and Hess, W. C., *ibid.*, **116**, 231 (1936).
39. Stephenson, M., *Bacterial Metabolism*. 2nd ed., Longmans, Green, New York, 1939.
40. Tarver, H., and Schmidt, C. L. A., *J. Biol. Chem.*, **130**, 67 (1939).
41. Tarver, H., and Schmidt, C. L. A., *ibid.*, **146**, 69 (1942).
42. du Vigneaud, V., Cohn, M., Chandler, J. P., Schenck, J. R., and Simmonds, S., *ibid.*, **140**, 625 (1941).
43. Womack, N., and Rose, W. C., *ibid.*, **121**, 403 (1937); **141**, 375 (1941).
44. Wood, J. G., *Chronica Botanica*, **7**, 1 (1942).
45. Woodward, G. E., and Reinhart, F. E., *J. Biol. Chem.*, **145**, 471 (1942).
46. Zima, O., Ritsert, K., and Moll, Th., *Z. physiol. Chem.*, **267**, 210 (1941).

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