

FACTS AND INTERPRETATIONS IN THE MECHANISM OF ALCOHOLIC FERMENTATION¹

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"It is the great beauty of our science, that advancement in it, whether in a degree great or small, instead of exhausting the subject of research, opens the doors to further and more abundant knowledge, overflowing with beauty and utility."—FARADAY.

I. INTRODUCTION

The study of the biochemistry of alcoholic fermentation has displayed great variations in its development up to the present time. The changing knowledge applies not only to the research on the stoichiometric course of the reaction, but also to the fundamental considerations under which individual investigators attacked and pursued the complex question.

The following considerations of Turpin (168) were entertained already one hundred years ago:

¹ This article is an extension of a paper presented at the Third International Congress of Microbiology, held in New York, September, 1939. For the earlier status of the subject *cf.* F. F. Nord: *Chem. Rev.* **3**, 41 (1926).

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“Que par fermentation on doit entendre: association composée d'eau, de corps vivants se nourrissant et se développant par absorption, de l'une des parties du sucre, et en isolant, soit l'alcool, soit l'acide acétique; action toute physiologique qui commence et finit avec l'existence des infusoires végétaux ou animaux qui la déterminent, et dont la vie ne cesse que par l'épuisement totale de la matière saccharine et nutritive. C'est alors que mourants d'inanition et ne pouvant plus se soutenir dans l'épaisseur ou à la surface du liquide, on les voit se précipiter les uns sur les autres et s'entasser au fond du vase sous forme de lie mucilagineuse, de sédiment ou de levure.”

The discussion to be presented does not require that a comprehensive account of the historical evolution of this fascinating subject be given. We shall, however, attempt neither to omit nor to diminish such difficulties as have resulted to a certain degree from the practical application of cell-free fermentation, discovered by Buchner (15) at the turn of the century.

Alcoholic fermentation by the action of undamaged cells has been studied during recent years by use of the enzyme systems of: (a) common top and bottom yeasts, (b) various fusaria, (c) *Thermobacterium mobile* Lindner, and (d) *Zyмосarcina ventriculi*. Of these systems, the action range of the last two mentioned has been only “felt out.” We know, however, that the thermobacterium (69, 79, 110) forms from sugar equimolecular quantities of carbon dioxide (about 45 per cent) and alcohol (about 41 per cent), also some lactic acid (about 6 per cent); and further, that the last mentioned fermentation (154), discovered by Goodsir about one hundred years ago, is predominantly of alcoholic nature, producing small quantities of hydrogen (0.5 per cent), acetic acid (6.6 per cent), acetylmethylcarbinol (1.7 per cent), and formic acid (0.8 per cent). The hydrogen comprises about one-fifth of the total volume of carbon dioxide evolved. These latter systems, as well as the yeast *Torulopsis pulcherrima* (136), will not be considered in the subsequent presentation.

The first enlightenment as to the course of the enzymatic breakdown of carbohydrates comes from two statements, valid until now: In the course of his important metabolism researches, Magnus-Levy in 1902 gave expression to the opinion that acetaldehyde was formed as a breakdown product of carbohydrate, and in 1910 O. Neubauer (105), in the course of his distinguished work on the fermentation of amino acids, announced this pioneer discovery:

“Weiter ist zu schliessen, dass die hier als Zwischenprodukt auftretende *Brenztraubensäure* durch gärende Hefe unter Reduktion zu *Kohlensäure und Alkohol* zersetzt wird, d. h. mit anderen Worten, dass sie leicht *vergärbar* sein muss. Eigens angestellte Versuche, die noch nicht völlig abgeschlossen sind, haben die Richtigkeit dieses Schlusses bestätigt. Damit ist nun ohne weiteres der Gedanke gegeben, die

Brenztraubensäure könnte ein *Zwischenprodukt bei der alkoholischen Gärung des Zuckers* sein;"

As a matter of fact, Fernbach and Schoen (37) in 1913 identified pyruvic acid in the presence of calcium carbonate in fermentation mashes containing living yeast cells, and isolated it in pure form (as the calcium salt) in quantity equal to 1.23 per cent of the original sugar.

About eight years later Neuberg, together with von Grab (47), confirmed this after he had in the first place rejected it, just as he had rejected the fermentation of pyruvic acid itself. Consequently, in 1919 he induced Kerb (66) to publish the following statement: "Brenztraubensäure, die schon auf Grund des Alkoholertrages nicht in nennenswerter Menge gebildet sein konnte, war auch *nicht* spurenweise qualitativ *nachzuweisen*."

Because of Neubauer's discovery, the year 1910 is the experimental turning point from which our present knowledge on the course of enzymatic carbohydrate breakdown has developed.

II. CONCEPTS OF THE PHASE SEQUENCE OF ALCOHOLIC FERMENTATION

In alcoholic fermentation nature shows us a process which, in the case of the action of yeast, because of its optional anaerobic mode of life, will not lead to the oxidation end products, but, in contrast to alcoholic fermentation by fusaria (see later), ends with the formation of alcohol, an incompletely degraded product.

It is therefore not surprising that until now, about one hundred and thirty years after Gay-Lussac (42) set forth his basic equation

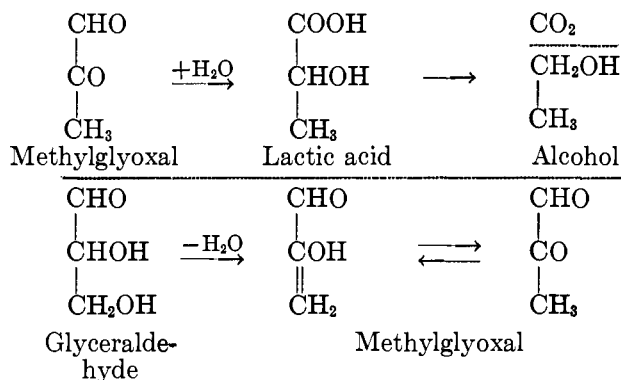
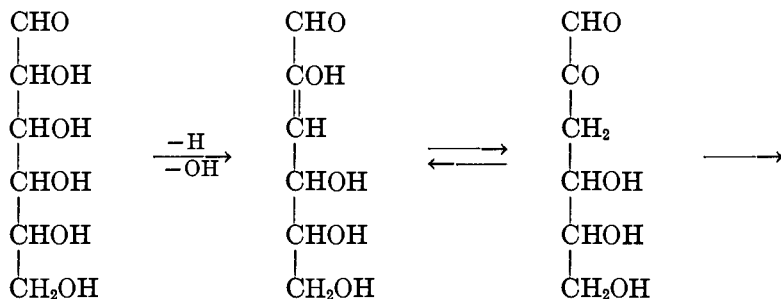


almost every natural scientist who occupied himself with studies of fermentation believed that he must establish his own theory as to the reaction mechanism.

The rapid development, which received its impetus from Neubauer's discovery, exempts us, however, from discussing individually in this review the many visions put on paper. They were more or less offsprings from the circle of thought of the great epoch of preparative chemistry. They were in ignorance of much which has become for us common knowledge of the experimentally proven mechanisms of enzyme action, and intimated that the breakdown product of sugar must be a smaller molecule than the fermentation substrate and must be easily split by the enzyme system of yeast.

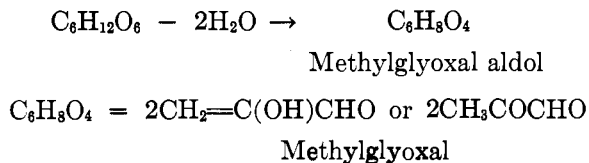
The Wohl-Neubauer-Neuberg-Kerb reaction scheme has longest

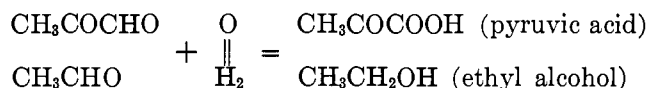
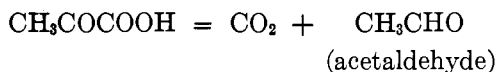
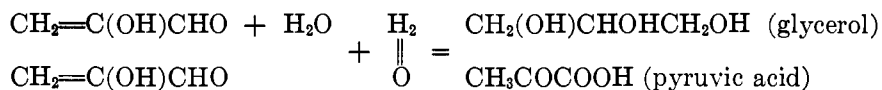
withstood the stormy developments. Its central thought is based on the fact that a molecule of water can easily be split from an hydroxyl compound.



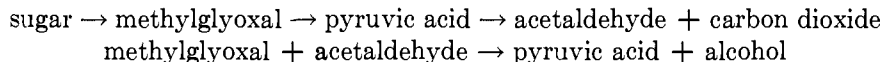
The six-carbon compound which will undergo further decomposition is, in this reaction scheme, methylglyoxal glyceraldehyde aldol, which, owing to easy hydrolysis, gives rise to two three-carbon split products, namely, methylglyoxal and glyceraldehyde. This possibility was considered in view of the findings of Pinkus (135) (production of methylglyoxal by heating glucose in alkaline medium) and of Wohl (181) (formation of methylglyoxal from glyceraldehyde itself).

On the basis of Neubauer's observation and the assumption of Magnus-Levy, Neuberg and Kerb (109) set up a remodelled reaction scheme in which the hypothetical methylglyoxal glyceraldehyde aldol of the Wohl scheme was replaced by the methylglyoxal aldol.

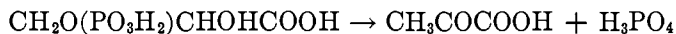




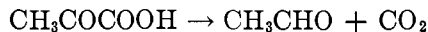
The presupposition of this sequence of phases was that the methylglyoxal would belong to that group of compounds which, in the course of the rearrangements taking place on the sugar molecule, are capable of taking over the rôle of a structurally intelligible *primary* biological split product. In addition, it was supposed to serve as one of the two different aldehydes which may undergo the mixed Cannizzaro reaction. In the course of this oxidation-reduction² the methylglyoxal should lead to the formation of pyruvic acid which, under the influence of carboxylase, is split into acetaldehyde and carbon dioxide. The acetaldehyde would then be reduced to ethyl alcohol and at the same time the methylglyoxal would be oxidized to pyruvic acid, thus continuing the cycle:



The discovery of phosphoglyceric acid (113) among the products of the action of yeast juices or muscle extracts on carbohydrate, to which Embden (30) was the first to attach significance, led to observations and considerations which seemed at the time appropriate to bring nearer an understanding of the later phases of sugar dissimilation in the living cell as well. The essential feature of the new scheme was the fact that the methylglyoxal disappears therefrom and that the pyruvic acid, which, as before, without doubt represents an intermediate product in sugar decomposition, is formed from the phosphoglyceric acid.



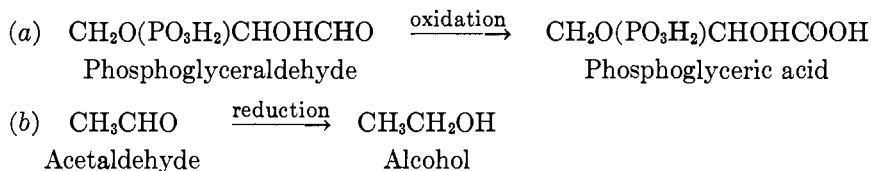
As the next step, we see that the pyruvic acid is split by the action of carboxylase into acetaldehyde and carbon dioxide:



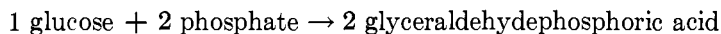
² Lavoisier, on page 101 of his *Traité élémentaire de chimie* (Paris, 1864), wrote: "Les effet de la fermentation vineuse se réduisent donc à séparer en deux portions le sucre, qui est un oxyde, à oxygéner l'une aux dépens de l'autre pour former l'acide carbonique a désoxygéner l'autre en faveur de la première pour en former une substance combustible qui est l'alcool; en sorte que, s'il était possible de recombinaer ces deux substances, l'alcool et l'acide carbonique, on reformerait du sucre."

In the phase of oxidation-reduction a further change, already indicated above, enters the scheme. As dismutation partner of the acetaldehyde, there enters in place of the methylglyoxal a triosephosphoric acid which has been synthesized by Fischer and Baer (40; *cf.* also 98) and the *d*-form of which is the readily fermentable (155; *cf.* also 171) 3-glyceraldehydemonophosphoric acid.

The oxidation-reduction phase may be represented as follows:

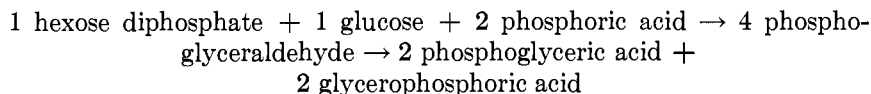


and furnishes the aforementioned phosphoglyceric acid, the mother substance of pyruvic acid. The cycle then will continue as long as the Fischer-Baer phosphoglyceric acid is present. It can be imagined that it is formed by the reaction:



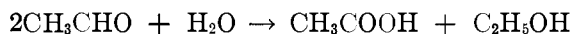
The reaction once started continues as long as sugar and phosphate are present,—i.e., formation of glyceraldehyde, its oxidation to phosphoglyceric acid (with simultaneous reduction of acetaldehyde to alcohol), transformation of the latter to pyruvic acid, and finally to acetaldehyde and carbon dioxide,—and is ended by reduction of aldehyde to alcohol.

Apparently, the fulfillment of a presupposition is indispensable,—a trace of hexose diphosphate must be present before the reaction can begin. The hexose diphosphate is, however, not consumed, but acts as a catalyst. This has been referred to as the “stationary” condition. This phase of the reaction scheme was supposed to be initiated by the “Angärung,” the onset of fermentation, in the course of which the required concentrations of reacting substances are built up. Here, again, the hexose diphosphate is required (and consumed), so that one molecule of it and one molecule of glucose plus two molecules of phosphoric acid form four molecules of phosphoglyceraldehyde:

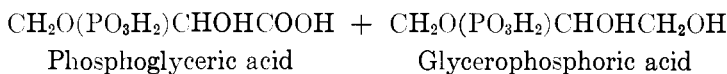


Since in this phase there is still no acetaldehyde formed which could take part in the oxidation-reduction of phosphoglyceraldehyde, it must be replaced by a second molecule of phosphoglyceraldehyde, of which one molecule is oxidized to phosphoglyceric acid and the other reduced to

glycerophosphoric acid, thus resembling the well-known transformation of acetaldehyde into acetic acid and ethyl alcohol.



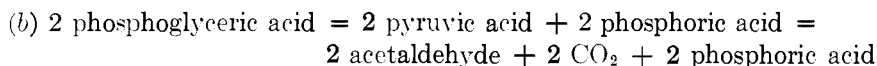
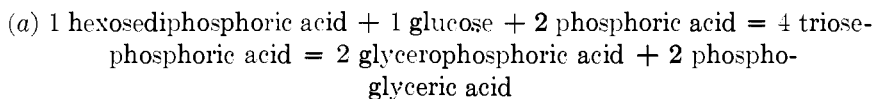
Phosphoglyceraldehyde



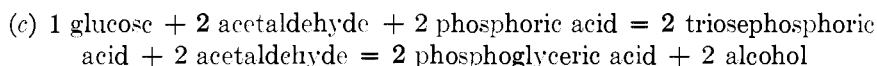
As soon as sufficient quantities of acetaldehyde are available, the reduction of phosphoglyceraldehyde slows down and the concentration of glycerophosphoric acid becomes slight. This was said to account for the appearance of slight traces of glycerol in the mash.

One of the various phase sequences which was formulated on this basis by Meyerhof (94),—but in the meantime also discarded,—is set up by the following reactions:

Onset of fermentation:



“Stationary” condition:



The greater part of the experiments serving as a basis for this scheme were conducted with the aid of juices in the presence of sodium fluoride. This inhibits phase *b* of the sequence, whereby the course is reduced to phase *a* in which a mixture of glycerophosphoric acids is formed. This acid appeared to Lohmann to be a difficultly hydrolyzable hexosephosphoric acid. Sodium fluoride does not inhibit the reduction of acetaldehyde or the oxidation of phosphoglyceraldehyde, and, in the presence of sodium fluoride, added acetaldehyde is further reduced, or, from glucose and phosphoric acid (in the presence of hexosediphosphoric acid) phosphoglyceric acid is produced as long as acetaldehyde is present.

III. BEHAVIOR AND PROPERTIES OF YEAST JUICES

It can be taken for granted that the methods for obtaining the different press and maceration juices are known. Until recently they were obtained chiefly from bottom yeasts. They form lyophilic colloidal dispersions,

which even after a storage period of 2 months at about -5°C . not only maintain their original activity, but show an increased rate of fermentation at the beginning of the proper experiments (124).

Comprehensive experiments have been conducted in an attempt to clarify this phenomenon. By the use of different lyophilic model-colloids the information was obtained that in different concentrations, under the influence of frost, the surface of the colloidal particles is either increased or diminished, and that the colloids are changed to such an extent that, among other things, they show measurable differences when exposed to the adsorption of various gases (120). It was this, among other evidence, which was seen as proof that freezing causes a disaggregation-aggregation of the particles. As a consequence of this change, exerted by physical means, it should be possible to bring about and observe an alteration in the

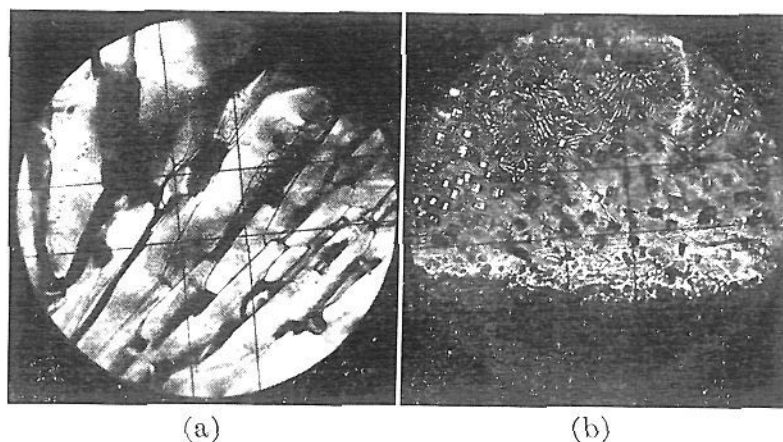


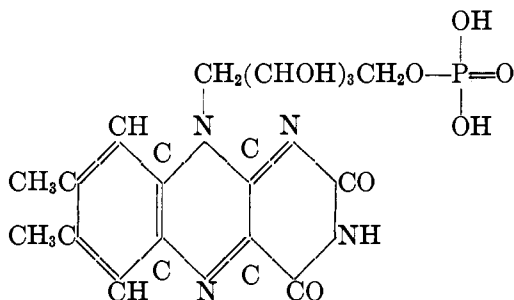
FIG. 1. (a) Disaggregation of a 1 per cent solution of egg albumin. Magnification $90\times$. Photographed at -14°C . (b) Aggregation of a 2 per cent solution of egg albumin. Magnification $90\times$. Photographed at -10°C .

particle radius. This change also appears, independently of the shape of the particle, in a difference in the rate of diffusion. Under the influence of frost, therefore, substances close to biological concentrations, in general, undergo a breaking up of the particles with simultaneous increase of their surface; at higher concentrations an increase in size, possibly to the point of coagulation, occurs with simultaneous decrease in surface area (see figure 1). Therewith the circle of proof is closed with regard to the later-mentioned dualistic or carrier theory of enzyme action, insofar as this applies to the colloidal-chemically acting part of the zymase system (117) and not to the structural-chemically acting part governing the manner and intensity of action as derived from the variability in the degree of dispersity.

The mechanism of the phenomenon also was cleared up recently (59, 60). If the activity of certain enzymes is explained by the carrier theory of

the mechanism of enzyme action through the common action of carrier and active group, then one must assume, in view of the aforementioned experimental findings, that in the case of a disaggregation a large colloidal particle breaks into two or more smaller particles, each of which, under all circumstances, is composed of carrier and active group. In other words, the crushed original colloidal particle is composed of several similarly constructed particles. A like conclusion follows from the aggregation process and, in connection with that, from the preceding experimental findings with so-called "protein-model bodies." Confirmation³ of these experiments has been found also in the case of peroxidase, tyrosinase (41), catalase (76), certain viruses (145), and fibrinogen (140).

Alongside this colloid-chemical proof for the carrier theory of enzyme action we find flavin ferment (containing no polysaccharide) as the first enzyme to be observed from which more could be learned about the linkage between carrier and active group, or the purely physicochemical mechanism of its activity. The total enzyme, the elementary analysis of which is given by Theorell (165) as 51.5 per cent carbon, 7.37 per cent hydrogen, 15.9 per cent nitrogen, 0.043 per cent phosphorus, and 1.0 per cent sulfur, consisting of the carrier protein with flavinphosphoric acid as the active group,⁴ can be obtained from Lebedew juice. The crystalline active group (165) of this "yellow ferment"⁶ is identical with a derivative of vitamin B₂, lactoflavinphosphoric acid, which has the constitution of a 6,7-dimethyl-9-*d*-ribitylisoalloxazine-5'-phosphoric acid,



The accompanying, natural carrier protein, fully capable of coupling, can be obtained by dialysis of a salt-free aqueous solution of the pure enzyme,

³ Cf. also H. B. Bull: *Z. physik. Chem.* **A161**, 192 (1932).

⁴ Regarding its synthesis see R. Kuhn, H. Rudy, and F. Weygand: *Ber.* **69**, 2034 (1936).

⁵ The method of Theorell (*Biochem. Z.* **278**, 236 (1935)) was meanwhile replaced by a simpler process of Warburg and Christian (*Biochem. Z.* **298**, 368 (1938)): namely, splitting by action of hydrochloric acid in ammonium sulfate solution. Protein and prosthetic group can be obtained in good yield within an hour. Compare also P. Karrer: *Ergeb. Vitamin- und Hormonforsch.* **2**, 381 (1939).

whereby the linkage between the flavinphosphoric acid and the protein may be reversibly broken. The carrier protein, of albumin character, has approximately the same isoelectric point and rate of electrophoretic migration as the complete enzyme. From measurement of the reaction velocity it may be concluded that the "yellow ferment" can neither react in a noteworthy measure with molecular oxygen nor, in the physiological cell metabolism, with oxidized cytochrome. Under these circumstances it can be doubted that the flavin enzyme is a means for the oxidation of dihydrocozymase in the cell. This might be the task of the newly discovered diaphorase (1, 26), the realm of action of which has still to be marked off in relation to the neoflavin (49) enzyme. It has recently been found to be identical with the flavoprotein of heart muscle (23).

The findings elucidated here represent the experimental proof of the concept first put in words by Mathews and Glenn (91) as to the dual theory of enzyme action:

"What we ordinarily call an enzyme, such as invertase, diastase, pepsin, etc., is a combination of a colloid with an active principle. The active principle is the enzyme itself and should of course be called the enzyme, but it has happened that the substances isolated as enzymes have been generally the combination of this active principle with the inert substance, colloidal in nature.

"The colloidal part of the molecule which is inert might with propriety be called the zymophore or ferment bearer, since in cells most of the enzymes are probably thus united or borne, but as this word has been used by Ehrlich to designate the active principle itself, we may call the colloid simply the carrier or bearer, and the active principle, the enzyme or kinase."

With regard to the colloid-chemical part of the proof, (see page 430) that function of the carrier must also be taken into account which, by changing the electronic configuration of the substrate by activation, can bring about a corresponding exchange of electrons with the pyridine system (see later).^{5a}

It is, however, time to call attention to the fact that in the further course of the discussion, a connection between mode of action and active group (for instance, of cocarboxylase) comes to the fore, which leaves doubts concerning its presence originally as an active group of specific proteins in the living cell.

Contrary to the general assumption that for the preparation of active maceration juice according to Lebedew (75) only dry preparations of bottom brewers' yeast are suitable, F. Lipmann (83) was able to show that from bakers' yeast, after suitable drying and upon addition of phosphate, a highly active juice can be obtained by extraction. The fermentation by bakers' yeast juice differs greatly from that by bottom yeast

^{5a} See also reference 171, especially p. 46.

juice. As long as there is present a great excess of inorganic phosphate, the fermentation follows the Harden-Young equation, where each mole of carbon dioxide formed corresponds to one mole of phosphate esterified.

If, however, the phosphate content of the bakers' yeast extract sinks to about 20 per cent of the original concentration, the fermentation now proceeds at only a slightly lower rate without simultaneous disappearance of the remaining phosphate, while with bottom-yeast extract the fermentation, after consumption of free inorganic phosphate, falls abruptly to a very slow rate. Therefore the fermentation here proceeds from an esterification phase to a phase without esterification, in which not the Harden-Young but the Gay-Lussac equation holds. In this case, the cell-free fermentation proceeds during this phase like fermentation in the living cell. Very noteworthy was the observation made, in the course of these experiments, of the significant rise in the CO_2/P quotient, i.e., the increasing recession of esterification in relation to the fermentation. The bakers' yeast extract has a yellowish color, which is largely due to the content of the "yellow ferment."

It seems suitable for the purpose to refer to this difference in the course of fermentation by the two closely related extracts in order to be able to emphasize the significance of the later constant fermentation rate, since, according to the recent reinvestigations (115) of older findings, it is known that with all juices studied hitherto, the "further" fermentation has already reached a diminished rate before esterification has ceased.

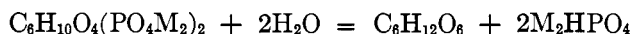
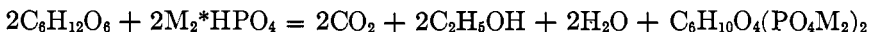
The experimental background of these observations is to be recognized in results first described by Wroblewski (184). He found that sodium phosphate increased the activity of pressed juice. Moreover, Iwanow (63) showed, in 1905, that living yeast, like other plants, transformed inorganic phosphate to organic phosphate.

Harden and Young (53), in the course of their researches, then observed that the rate of fermentation of a press juice is rapidly increased if, to the fermenting mash, in the presence of soluble phosphate, boiled extract is added. At the end of the fermentation no appreciable amounts of phosphate could be detected by the usual means. The investigators concluded from their findings that two sugar molecules are involved in fermentation. While one sugar molecule, together with two moles of phosphate, forms hexose diphosphate,⁶ another molecule forms alcohol and carbon dioxide. In the further course of the reaction the inorganic phosphate can be regenerated by the action of phosphatase on hexose diphosphate, and the fermentable hexose set free. Thus the cycle can begin

⁶ The hexose diphosphate when heated with oxalic acid in aqueous solution furnished a mixed ester which consisted, however, predominantly of a keto- or mono-ester, called also Neuberg ester (Biochem. Z. 88, 432 (1918)).

again. By kinetic measurements Harden and Young were, moreover, able to determine that the quantities of carbon dioxide and ethyl alcohol evolved were (within certain limits) proportional to the added phosphate.

The equations set forth by them read as follows:



* M = metal.

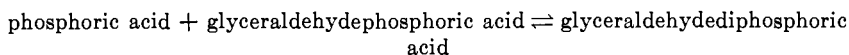
The fact that Harden and Robison (50) had found, besides the diphosphate, also a mixed monophosphate, later closely investigated by Robison (147) (the Robison ester) led Raymond (142) to interpret the original equations of Harden and Young in a different way.⁷

The true rôle of phosphates in the metabolism of living yeast is even now not clarified beyond doubt. It may, however, be regarded as certain that, even if yeast cells do break down carbohydrates by a detour of intermediary phosphorylation, this is not necessarily the only way in which the degradation is accomplished.

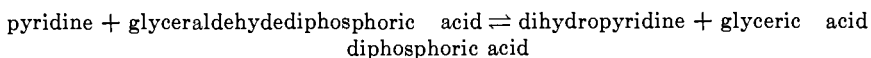
In contrast to Buchner's cell extract or the often-studied maceration juices, the frozen extract, first obtained by Dixon and Atkins (27), represents a system which, above all, has the advantage that it also contains the insoluble enzymes otherwise left behind in the cell fragments. Moreover, its preparation can be accomplished with no autolysis whatsoever and the extract can be evaporated to dryness without change in its enzymatic potentialities.

The first investigators to study this juice more closely were Tait and

⁷ Cf. F. F. Nord: Chem. Rev. **3**, 50 (1926). During the preparation of this paper there appeared a short preliminary communication by E. Negelein and H. Brömel (Biochem. Z. **301**, 135 (1939)), according to which the diphosphoglyceric acid (see page 427) isolated by them from Lebedew juice is removed by the action of various enzymes and coenzymes, whereby the total phosphate is transferred to the sugar. In the living yeast cell, half of the phosphate from the above acid is supposed to be transferred to the sugar and the other half is supposed to be freed:



In the presence of diphosphopyridine nucleotide and a specific carrier protein, the following sequence is supposed to occur:



At first glance, however, the presentation lacks the explanation required for the understanding of the mechanism of the indispensable intermediary reactions in the living cell. Concerning the properties of the acid, consult Biochem. Z. **303**, 132 (1939).

Fletcher (162), who determined the hydrogen-ion concentration of the frozen extract to be 6.2. This specification is in accord with the results

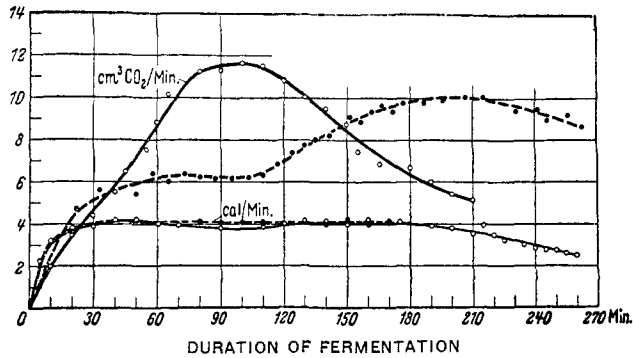


FIG. 2. Course of reaction using fresh living yeast (2 g. in 200 cc. of a 4.5 per cent solution of glucose). Experiment No. 1: ---- carbon dioxide evolved; — heat evolved. Experiment No. 2: — carbon dioxide evolved; ---- heat evolved.

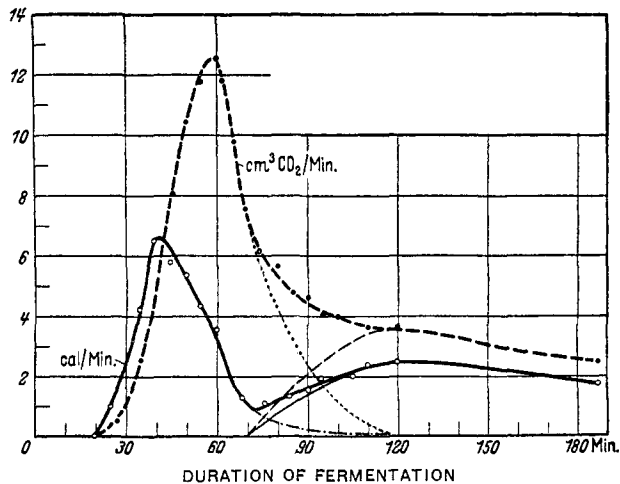


FIG. 3. Course of reaction using maceration juice (9 g. of glucose in 200 cc. of juice). - - - - - extrapolation of the fermentation curves of free sugar; ——— extrapolation of the fermentation curves of the products of phosphorylation.

of measurements by Mahdihassan (89) of the internal hydrogen-ion concentration of different living-cell systems.

Each finding which concerns the similarity or dissimilarity of the course of material or energetic conversions in the living cell, or by action of

enzyme preparations, is of importance for the understanding of the mechanism of enzyme action or the course of the phase sequence. The function of the cell consists in the liberation of energy. Energy given off in the form of heat is lost. Therefore the relation of two reaction products of alcoholic fermentation,—the carbon dioxide evolved and the energy liberated as heat,—were compared during fermentation by living yeast cells and by Lebedew extract (58) (see figures 2 and 3). It was thus noted that the heat of reaction in the course of fermentation changed continually. This meant that fermentation with living yeast does not proceed according to a fixed outlined scheme. The thermochemical course of fermentation with yeast maceration juice shows, in contrast, that here at least two different conversions occur: (a) the fermentation of free sugar in the presence of free phosphate (inhibited by phloridzin); and (b) the subsequent fermentation of the residual substrate in the absence of free phosphate (not inhibited by phloridzin). Neither reaction shows a resemblance in thermochemical respects to fermentation with living yeast.

The phosphorylation of sugar in maceration juice is accompanied by a heat of reaction of the order of magnitude of 22 cal. per millimole of phosphate esterified. This value corresponds to almost the total heat which is evolved during the decomposition, $\text{sugar} \rightarrow \text{alcohol} + \text{carbon dioxide}$: namely, found, 24 cal.; calculated, 28 cal.⁸ In general, the relation between the course of the actual reactions in the cell and those found individually in destroyed systems and united afterwards in a scheme may be considered as being the same as the relation between a quotient of differences and a differential quotient.

IV. DISCOVERY OF ZYMASE AND COZYMASE

The (previously mentioned) discovery of cell-free fermentation by Buchner and Hahn had far-reaching consequences. In the dispute between Liebig and Pasteur concerning the recognition of the essence of alcoholic fermentation, it signified that neither of the last named was right but that, at the time, a third came nearest to the truth. Traube had postulated, as early as 1858, that all of the fermentations brought about by living organisms were caused by enzymes secreted by the cells.

Buchner and Hahn first used their extracts in animal studies and noted that they changed considerably in a short time. To protect them against deterioration and loss of activity, they tried, without success, the usual chemically acting preservatives and therefore added sugar to them. This experiment became the first step in the study of cell-free fermentation. In the work mentioned Buchner was able to determine that the

⁸ Regarding the heat of decomposition for other conversions in the sphere of carbohydrate breakdown, compare L. Genevois: *Ann. fermentations* 2, 65 (1936).

cell-free, thermolabile yeast juice induced the fermentation of various monosaccharides and of maltose, and that this capability was not lost either by the action of chloroform, benzene, or sodium arsenate or by filtration, evaporation, or precipitation with alcohol.

From these facts, E. Buchner arrived at the following fundamental conclusions:

“Zunächst ist bewiesen, dass es zur Einleitung des Gärungsvorganges keines so komplizierten Apparates bedarf, wie ihn die Hefezelle vorstellt. Als Träger der Gärwirkung des Pressaftes ist vielmehr eine gelöste Substanz, zweifels ohne ein Eiweisskörper zu betrachten; derselbe soll als Zymase bezeichnet werden.”

If the extract thus obtained was filtered through a Chamberland candle, the subsequent fractions displayed gradually diminishing powers of fermentation until no activity at all was shown. The juice contained not only zymases but also digestive enzymes which split proteins down to the amino acids. Hydrocyanic acid, in contrast to its action on fusaria (see later), produced a complete but reversible suppression of the juice fermentation.

By addition of boiled yeast juice,—the so-called “Kochsaft,”—alcoholic fermentation, which is caused by a certain volume of pressed juice, is considerably increased. This observation was the starting point for another great discovery. Harden and Young (52) separated top-yeast juice into an inactive filtrate and an inactive residue by means of ultrafiltration (Martin). Buchner and Antoni (16) had obtained the same results at an earlier date by the dialysis of yeast juice. When the filtrate and residue, neither of which alone could produce fermentation, were again united, an alcoholic fermentation of sugar took place. The residue, filtered, washed, and freed of inorganic phosphate, consisted chiefly of glycogen, dextrans, and proteins, as well as other enzymes present in the juice. It would not ferment sugar. Harden and Young concluded from these experiments that the fermentation of sugar induced by yeast juice is dependent, aside from zymase, on the presence of a dialyzable system which, according to Tholin (166), is thermostable to about 80°C. According to a reference of Bertrand (13) they called the system coenzyme. von Euler and Myrbäck (32) proposed its present name, cozymase (see later).

Neuberg and von Euler (107) have specified the following nomenclature: By zymase is meant the total enzyme system involved in alcoholic fermentation free of activators. By holozymase is meant the enzyme system of fermentation consisting of zymase plus all the activators. By apozymase is meant the holozymase free of cozymase.

Some of the investigations of von Euler and Myrbäck (32) may be considered as preliminary studies toward the isolation of cozymase and the detection of its manner of action (see page 460).

V. KINETICS OF CELL FERMENTATION

If one is trying to investigate the physical and chemical suppositions which are suitable to account for the realization of a cell reaction, then it is obviously necessary, in order to simplify conditions, to use unicellular systems and to start from the following considerations: In order that a material change can take place in the cell it is necessary that the substrate pass into the reaction space, i.e., permeate through the cell membrane,

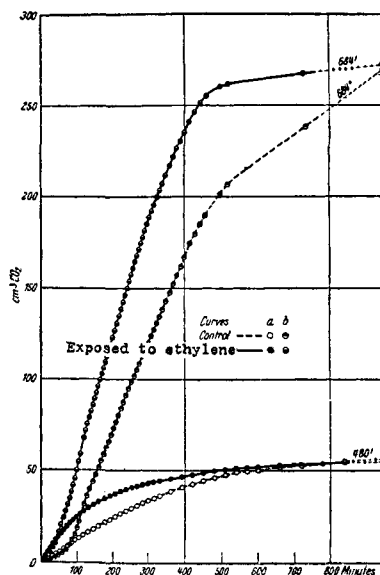


FIG. 4

and that, after entrance into the reaction space, it be chemically or enzymatically converted, whereby, in the latter case, the substrate molecule and catalyst must come into appropriate contact with one another.

In the course of extensive researches⁹ on the influence of cell perme-

⁹ See F. F. Nord: *Z. angew. Chem.* **42**, 1022 (1929); *Science* **79**, 159 (1934); *Food Manuf.* **9**, 55 (1934); *Ergeb. Enzymforsch.* **1**, 77 (1932). The proof that ethylene, acetylene, and related compounds can influence enzymatic reactions, on the one hand accelerating by increasing cell permeability, and on the other hand retarding through being reversibly taken up by the lyophilic colloid elements, is demonstrated in figure 4. In the illustration the lower pair of curves shows the course of carbon dioxide evolution in a fermentation by living top-yeast cells of a 2 per cent solution of pyruvic acid. The essentially steeper upper pair of curves illustrates the course of fermentation of sugar. The sugar fermentation was carried out with the same yeast which was used on the pyruvic acid, after being repeatedly washed and centrifuged.

ability¹⁰ on enzymatic reaction, the possibility of renewed study of the kinetics (126) of fermentation by live yeast presented itself.

The earlier results are founded on the hypothesis that one is dealing here with a reaction course the speed of which is proportional to the quantity of yeast, and which can be represented as a first-order reaction. In contrast to that, earlier studies have shown that the rate of fermentation is approximately independent of the glucose concentration within the range of 0.5 per cent to 10.0 per cent. Insufficient notice was taken of this contradiction. Here we are dealing not with a reaction course which is influenced only by the amount of enzyme surface, but with a breakdown of the sugar molecules which must first pass through a membrane. The aforementioned contradiction is adjusted by this concept. It was tested by the influence of different speeds of stirring on the reaction course.¹¹ By exerting this influence on the fermentation action it was established that under the prevailing experimental conditions the sedimentation of cells, which was considered as responsible for the inhibition of diffusion in the outer medium, could be compensated by a rotation speed of twenty turns per minute. Up to one hundred fifty turns per minute produces a proportionate rise in the speed of fermentation (see figure 5). Since the differences in diffusion are already compensated at a stirring speed of twenty turns per minute, this further acceleration of the reaction is due solely to the relative membrane motion in regard to the substrate solution or to the increase in membrane surface, whereby more sugar is allowed to permeate into the cell. By running fermenta-

¹⁰ According to the observations made by B. Luyet (Compt. rend. **204**, 1214, 1506 (1937); Compt. rend. soc. biol. **125**, 403 (1937)), the action of increased pressure or of heating is identical. Both destroy the permeability of yeast cells in the same way.

¹¹ The course of the rising branches of the curves submitted, especially the progressive shortening of the onset phase, and the observations on the behavior of a frozen zymase solution (compare also table 1 in the work of Nord and Franke (124) and *Ergebnisse der Enzymforschung* **1**, 79 (1932)) are in good agreement with the onset of the curves in figure 11 of an investigation by F. Lynen (*Ann.* **539**, 1 (1939)) on the behavior of frozen extracts. Lynen assumed that the progressive flattening of the upper parts of his fermentation curves could be traced back to the addition of increasing quantities of frozen yeast. We have, on the other hand, proven (a) that the initial ascent of the curves of fermentation and its consequences are governed by a physical effect and (b) that cryolysis in biological concentrations discloses a disaggregation of the carrier particles. In contradiction to the *conclusions* of Lynen, based on the chemistry of enzymes, there are to be found our *confirmed results* concerning the kinetics of cell fermentation and the increased activity caused by freezing of the carrier systems concerned. So far as the effect of freezing is concerned there is no question whether enzymes will be damaged through the action of low temperatures, since with the aid of cryolysis the proof of augmenting their activity was produced in the case of different enzymes.

tions with different initial concentrations of sugar, the conclusion was reached that the reaction showed no maximum rate at a concentration of 4 per cent—contrary to the findings of Slator (153)—but continued to rise beyond this point (see figure 6). At higher concentrations the per-

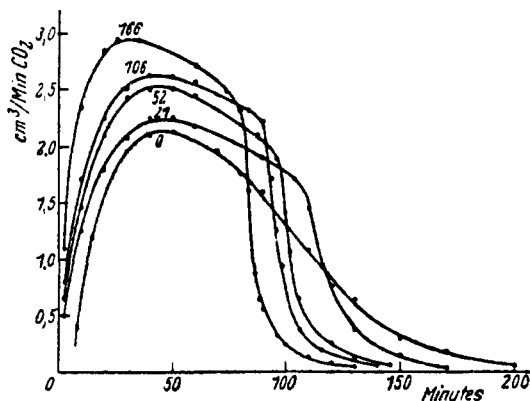


FIG. 5. Effect of stirring on the rate of fermentation

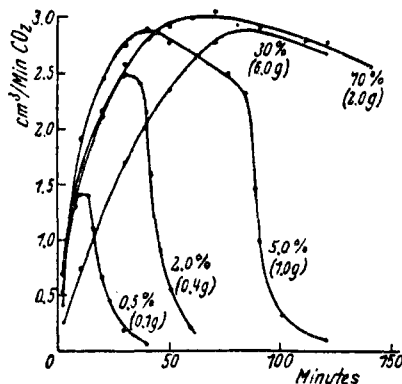


FIG. 6. Effect of concentration of sugar on the rate of fermentation

meability of the membrane is decreased. The graph showing the reaction course itself (time:rate) corresponds to the exponential function

$$-\frac{d\sigma}{dt} = \frac{\mu k}{k - \mu} S_0 (e^{-kt} - e^{-\mu t})$$

in contrast to the former consideration which was represented by a straight line. It is the solution of the differential equation

$$\frac{d^2c}{dt^2} + (\mu + k) \frac{dc}{dt} + \mu kc = 0$$

in which μ is a constant, c is the concentration of sugar in the cell, and k is the unimolecular reaction constant.

Similar experiments were later carried over to multiplying yeast cells. The aforementioned results were hereby confirmed (68, 61).

Medwedew (92), assuming that the energy of activation for alcoholic fermentation amounts to only 5000 cal., calculated, for a change of 500 cal. within a range of 10° , the high temperature coefficient of 3.0.

If one henceforth tries to study fermentation from the above explained and experimentally and theoretically founded standpoint, then it is possible to obtain fundamental knowledge and criteria for the interpretation of the biological and chemical reaction mechanism of fermentation. A difference worthy of notice is shown by comparison of the rate

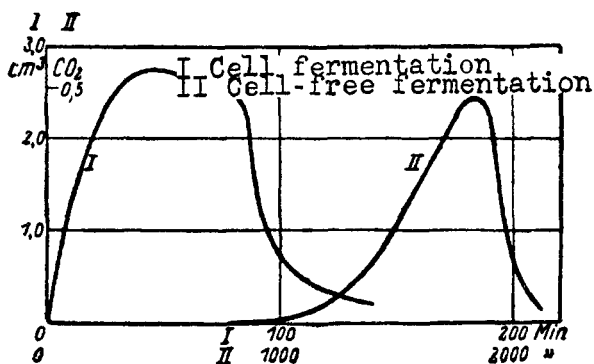


FIG. 7

curves of a fermentation with living cells which shows an acceleration at the onset of the reaction, and one which is brought about by cell-free juice (figure 7). Since the rate of substrate conversion on the enzyme surface, which doubtless is governed by a certain relation between the specificity of the carrier and the structure of the active group, or the molecular structure of the substance, is independent of the speed with which the mash is stirred, it has to be concluded from the course of figure 5 that the occurrence of enzymatic substrate conversions in the living cell is decisively influenced by the rate of diffusion. An effect of this kind on the reaction rate is impossible in a system of destroyed structure,^{11a} and therefore draws attention to the importance of the assumption that the dissimilation of phosphorylated basic substances or their derivatives in the living yeast cell may at least be paralleled by the direct (phosphorus-

^{11a} This is not contradicted by certain findings of Lipmann (Biochem. Z. 274, 414 (1934)), because his observation of an accelerated induction in a maceration juice shows the influence of an oxygen atmosphere as compared to one of nitrogen.

free) breakdown of a substrate molecule, just as in an embryonal tissue (103)¹², rabbit erythrocytes (127, 186), propionic acid bacteria (172, 178), as also with living fusaria (see later), or during the oxidation occurring in the cerebral cells (64). By controlled pH relations (see page 449), the so-called intermediate *products* were accordingly (in the first case) regarded solely as *phases* of transformation without having definite molecular structure in the usual sense. The fragments were, in the course of degradation, stabilized to a certain degree by adsorptive power, and thus their dislocation was regulated.

It appears that the above conclusions would be weakened on the ground of experiments of von Hevesy, Parnas, Ostern, and others¹³. These investigators prepared radioactive adenylic acid from radioactive phosphate, $\text{Na}_2\text{H}_{15}^{32}\text{PO}_4$, and adenosine, and added it to fermenting yeast. After the lapse of appropriate incubation, they could recover a "significant part" of the radioactive phosphate bound in the sugar phosphoric acids.

However, one of the collaborators, von Hevesy (56) himself, gave the following interpretation of the results thus far obtained:

"Although the investigation of the formation of labelled acid-soluble P compounds, both by *in vivo* and by *in vitro* experiments, supplies us with valuable information as to the formation of organic compounds of this type, the very appreciable speed with which some of them are resynthesised in the organism somewhat restricts the applicability of isotopic indicators in the study of their formation."

The expectations which were attached to the strength of evidence of the experiments carried out with the aid of ^{32}P , were consequently not fulfilled (99).

In accordance with our earlier statements, the conclusion is justified that induction bears no importance to cell fermentation. Consequently, there is no common ground, in this respect, to be found for a direct comparison of a fermentation caused by living cells with cell-free fermentation, where, even according to Meyerhof (95), the evolution of carbon dioxide is preceded in the same period by esterification.

¹² Compare, for example, the very foresighted statement of E. Grafe (Biochem. Zentr. **6**, 446 (1907)): "Diese Tatsache ist von höchstem Interesse, da in dieser Zeit der Grundstock für die Organe in Embryo gelegt ist. Es würde sich also dieser Zeitpunkt von dem an die Entwicklung allein durch Wachstum der nun differenzier-ten Gewebe vor sich geht, auch chemisch scharf . . . markieren." Cf. also G. von Hevesy, H. B. Levi, and O. H. Rebbe: Biochem. J. **32**, 2147 (1938).

¹³ Quoted from P. Ostern, T. Baranowski, and J. Terszakowec (Z. physiol. Chem. **251**, 261 (1938)).

VI. IS PHOSPHORYLATION ESSENTIAL TO ALCOHOLIC SUGAR BREAKDOWN IN THE CELL?

At the very outset of the task of outlining the breakdown of hexoses by the enzymes of yeast systems, a fundamental difficulty presented itself. Aside from the fact that, as was previously mentioned, no possibility exists of becoming acquainted with a definite course which would characterize the phase sequence of degradation, the initial phase of substrate mobilization seems obscure. Even today, after the lapse of twenty-five years, the relation is best expressed by the following statement (148);

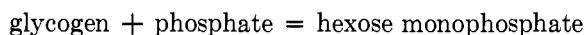
“Conceptions of the breakdown of glucose in which the process is depicted as though it proceeded from one fixed molecule to another, are fundamentally inadequate.”

A starting point for the forthcoming explanation (which was noted late) is to be found in a contribution of Somogyi (157), who ascertained that when a yeast suspension and a glucose solution are appropriately brought together in the presence of protein, no sugar can be found in the filtrate. His assumption that here we dealt with adsorption was strengthened by the establishment of the specificity of the phenomenon for fermentable sugars as opposed to the case of lactose, arabinose, etc., which are not adsorbed on the yeast cell surface under the same conditions, but can be quantitatively recovered. One can compare this observation, in the spirit of present terminology, with the spreading, deformation, or loosening of the linkages of the substrate molecule by the adsorptive power of enzymes present in or on the cell. This introduces a molecular structure which is called the fermentable sugar form or “transport form” (119)¹⁴ and which was thoroughly discussed, without interpreting it as a case of spatial isomerism in the classical sense of structural chemistry. On the basis of the above considerations (page 442), it is conceivable that a deformed molecule disintegrates in the cell into labile C₃ fragments without uniting with the phosphoric acid present, and essential for the building up of the body of yeast cells.

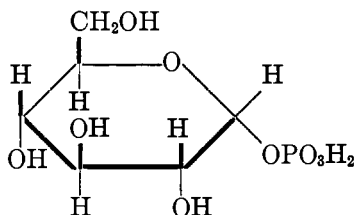
Willstätter and Rohdewald (180) have taken an opposite standpoint, after the prior work of Grüss (48), in support of Wertheimer (173), and they claim to have found 99 to 100 per cent of the sugar removed from solution in the form of a polyose. This value is for top yeast; about 50 per cent was claimed in the case of bottom yeast. The transformation of sugar to the glycogen complex, composed of glycogen, yeast gum, and a membrane polyose, “seems” to be followed by a glycogenolysis, which “probably” transforms it into a sugar form more capable of reaction

¹⁴ Wieland later made use of an analogous concept in connection with considerations of the formation of succinic acid in the enzymatic oxidation of acetic acid (Helv. Chim. Acta **15**, 521 (1932)).

(see above), which is subject to phosphorylation. The observation of Somogyi was criticized by Benedict (11) and also by Raymond and Blanco (143), although the enzymatic attackability (139) of glycogen, under the experimental conditions prevailing and corresponding to the protein content of yeast, varied. According to Parnas (131), glycogen, and according to Ostern (128), starch¹⁵ also, but not inulin, can be enzymatically split and transformed into hexose monophosphate according to the equation:



This process was justifiably called phosphorolysis (instead of hydrolysis) by Parnas and is supposed to be reversible (149a; cf. also 31). But Meyerhof recently induced Goda (45)¹⁶ to maintain that he had found "keine Anhaltspunkte für die Annahme von Willstätter und Rohdewald, wonach in der lebenden Hefe der Zucker nur auf dem Wege der Glycogensynthese vergärbar wäre." This utterance is especially noteworthy in view of the discovery by Cori, Colowick, and Cori (20, 20a)¹⁷ that glucopyranose-1-phosphate



is formed in washed muscle pulp in the presence of adenylic acid¹⁸ by incubation of glycogen with inorganic phosphate (and magnesium) and was supposed to be observed also in yeast juice fermentations (150, 121).

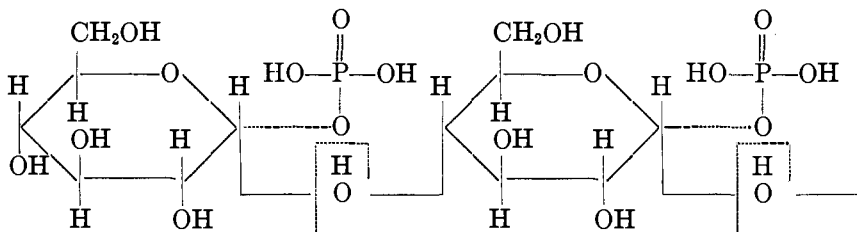
¹⁵ According to Sarzana and Cacioppo (*Biochim. terap. sper.* **25**, 359 (1938)), injected starch is utilized by animal tissues without previous transformation into glucose or glycogen.

¹⁶ Krueyk and Klingmüller (*Biochem. Z.* **300**, 343 (1939)) were also unable to show a synthesis of polysaccharide in yeasts of different ages. This is in agreement with recent findings of W. Kiessling (*Biochem. Z.* **302**, 70 (1939)), who also found that the primary step in the course of glucose fermentation does not involve formation of glycogen.

¹⁷ Concerning the preparation of the crystalline potassium salt of the Cori ester, compare W. Kiessling: *Biochem. Z.* **298**, 421 (1938).

¹⁸ In experiments undertaken by Bauer and von Euler (*Z. physiol. Chem.* **255**, 89 (1938)) serving to reexamine the observations of Kendal and Stickland (*Biochem. J.* **32**, 572, (1938)), there was no clear stand taken. The latter were found to be in good agreement with the finding of Parnas or of Cori and Cori, showing that during the course of phosphorylation of glycogen through muscle preparation the action of adenosinetriphosphoric acid is much superior to the action of adenylic acid.

The glycogen molecule was thus split, according to Cori and Cori, without water taking part in the reaction.



The diagram shows the entrance of inorganic phosphate at the maltose linkage.

This is again in harmony with the above-mentioned glycogen formation as a primary step of fermentation in the living cell, whereas the reversibility of the phosphorylisis of glycogen and the alleged separability of the phosphorylating system of glucose or glycogen stands at variance with it. The conversion of the Cori ester is, however, not of a uniform nature (151). Besides the 6-ester, there originate also substances giving the same color reaction with iodine which is characteristic for glycogen. By contact with soluble muscle ferments this non-reducing Cori ester is transformed to glucopyranose-6-phosphate. The transformation is greatly accelerated by magnesium ions (19), proceeds instantaneously, and seems not to be an equilibrium reaction. From the glucopyranose-6-phosphate is formed,—by the action of phosphohexokinase (163) (oxoisomerase of Lohmann),—fructofuranose-6-phosphoric acid, the Embden ester (hexosemonophosphoric acid), which is an equilibrium mixture consisting of Robison and Neuberg esters in the ratio of 85 per cent aldose to 15 per cent ketose.

In this connection it is important to record the mobility of opinion concerning the interpretation of the author's own experiments. In 1936 A. Schäffner (149) wrote:

“Die katalytische Reaktionsweise des organisch gebundenen Phosphates lässt die weitgehende Schlussfolgerung über die Entbehrlichkeit des Phosphates bei der alkoholischen Gärung u. E. vorderhand als nicht berechtigt erscheinen.”

In 1938, on the contrary, in the previously mentioned work (150) he expressed himself as follows on the question of the appearance of glycogen at the onset of fermentation:

“Die Vergärung von Glucose mittels zellfreien Hefeauszügen geht—mindestens zu einem grossen Teil—nicht über Glykogen. Die Feststellung, dass in zellfreien Extrakten ein anderer Weg zu Hexosemonophosphat führt als in der lebenden Zelle, rührt an das Problem, wie weit überhaupt von Ergebnissen, die mit zellfreiem Material erreicht werden, auf Vorgänge in der Zelle geschlossen werden darf. Wenn auch die Schlüsse mit aller Vorsicht gezogen werden müssen, so würde es u. E. zu

weit gehen, den Reaktionen, die *in vitro* festgestellt werden können, jeden biologischen Sinn abzuspochen. Es ist so gut wie ausgeschlossen, dass bei der Isolierung aus der lebenden Zelle ein ganz neuer Apparat von Enzymen auftritt. Viel wahrscheinlicher ist es, dass dabei ein Enzym verloren geht oder dass die Konzentrationsverhältnisse der Enzyme geändert werden. So darf man auch in unserem Fall annehmen, dass alle Phosphorylierungsmechanismen, die *in vitro* nachgewiesen werden konnten, auch in der lebenden Zelle eine Rolle zu spielen haben, dass aber entweder die Verkettung der Phosphorylierungsmechanismen eine besonders geartete ist, die wir nicht kennen und die in zellfreien Extrakten gestört ist, oder dass die lebende Zelle die verschiedenen ihr zur Verfügung stehenden Wege der Phosphorylierung einschlagen kann, je nach dem Bedürfnis und der Zweckmässigkeit."

Wieland, Claren, and Wille (175) have reported that bottom yeast undergoes no functional damage as a result of the aerobic removal of its nutrients. Such a yeast, which can be obtained by shaking an aqueous suspension for 15 to 20 hr. in an oxygen atmosphere, is, after this procedure, completely intact morphologically and physiologically. It is especially suited to the study of transformations having reaction rates which are insignificant as compared to the representative reactions of yeast which has not been pretreated. This "impoverished" yeast, in the conversion of alcohol, behaves in an entirely different manner from yeast which has not been pretreated, with which, according to Meyerhof, a carbohydrate resynthesis in the ratio: one part of alcohol \rightarrow two to five parts of carbohydrate is supposed to occur. In the presence of the "impoverished" yeast, on the other hand, of fourteen parts of alcohol, eleven are oxidized, fat is formed from two parts, and only one part is converted into carbohydrate (177). Top yeast, when shaken under oxygen, gives up after 4 to 6 hr. the greater part of its dissimilable contents (176)¹⁹. So far as the author knows, no juices have as yet been prepared from this "impoverished" yeast. It is also necessary to determine how this yeast or the preparations obtained therefrom behave with reference to esterification. What would be the first phase of dissimilation in the case of a yeast which is fermentatively active but free from polyoses?

An investigation of this question becomes especially desirable in view of the report of Cori, Schmidt, and Cori (22) that the appearance of glucose-1-phosphoric acid is a consequence of a reversible enzymatic equilibrium. Adenylic acid is supposed to act as a coenzyme in both esterification and hydrolysis. The polysaccharide synthesized in the reaction is assumed not to be identical with glycogen, and gives a blue color with iodine which, by the way, was observed and described by Bernard (12).

The findings of Macfarlane (87; *cf.* also 78), who comments on her own

¹⁹ According to P. Liang (*Ann.* 521, 216 (1936)), living (also "impoverished") yeast cells almost completely lose their power of fermentation when exposed to freezing at very low temperatures.

experiments as follows: "The coincident changes in total, labile, and organic (acid-soluble) phosphate could not be consistently related to the fermentation process," are without significance for the understanding of the initiating phase in living-cell fermentation.

Neuberg (106) was able to demonstrate a phosphorylation in living yeasts, with the exception that in his experiments it was not a carbohydrate but a mono-ester which served as substrate.

The fermentation of disaccharides has also a particular interest in this connection, since no conclusion has yet been reached concerning the course of degradation and the sequence of the phases in the fermentation of these important carbohydrates by yeast. Since only a few observations concerning the rôle of phosphorylation have been made, it is still undecided whether phosphorylation has to be included in the sequence, and, if so, at what stage.

As an assumption for the fermentability of disaccharides, there was formerly required, in general, the presence of specific carbohydrases in the enzyme system of the corresponding organism. Even recently, Armstrong and Armstrong (4), for example, express themselves as follows on this question: "maltose is fermented only by those yeasts which contain maltase and then not until hydrolysis has been brought about by the enzyme," and later "only those yeasts which contain lactase are capable of fermenting milk-sugar"; then they say with respect to cane sugar, "cane sugar is fermented by yeasts only after previous inversion with the invertase of yeast."

Through the researches of Willstätter with Oppenheimer (179) on lactose, and with Steibelt (160) on maltose the question of the possibility of the direct fermentation of bioses was investigated. They assumed the existence of particular zymases for maltose and lactose in yeast which ferment the above-mentioned composite sugars without requiring a preliminary split to monoses. Sobotka and Holzman (156) believed that they were able to confirm these experiments by the use of a particular American yeast which respire as well as ferments and grows at a pH of 2.5, and supposed, in consequence, the existence of a particular maltozymase. They assumed, like Hvistendahl (62), that a disaccharide phosphate (similar to the isolated hexose esters) existed as an intermediate phase in the direct course of biose fermentation. This assumption was, however, disproved experimentally by Baba (9). Wright (183), by investigating the degradation of lactose by *Streptococcus thermophilus*, also recently arrived at the conclusion that disaccharides can undergo direct fermentation. Leibowitz and Hestrin (77), in experiments on the fermentation of α -methylglucoside, report the same results *cf.* 151a. Living *Fusarium lini* Bolley is capable of direct fermentation of maltose without phosphorylation (123).

There remains only the discussion of the question of the fermentability of cellular or of added trehalose by living yeast, the investigation of which was carried out by many workers. One of these investigators, Myrbäck (102), had to interpret his results in a contradictory way. In 1936 he believed that he had proved a measurable carbon dioxide evolution, lying beyond the limits of experimental error, with top as well as bottom yeast. A repetition of the experimental work compelled him in the years 1937-1939 to take the opposite stand.

Investigations conducted in the author's laboratory (127a) indicate that added trehalose, in the course of its fermentation by living *Fusarium lini* Bolley, is not necessarily esterified by added inorganic phosphate nor is its dissimilation dependent upon preliminary hydrolysis, inasmuch as its rate of fermentation exceeds that of glucose.

VII. FUNDAMENTAL CONSIDERATIONS

It was the everlasting contribution of Buchner and Hahn, by their discovery of cell-free fermentation, that they not only settled the dispute between Liebig and Pasteur, whose views were so happily supplemented by the unitary theory of enzyme action of Traube,²⁰ but that they also left to posterity a means and a method which have proved indispensable, although not decisive, for the investigation of the sequence of carbohydrate degradation and the enzymology of the corresponding systems. In the course of later investigations a mass of observations and results have been obtained which were fundamentally determined in the case of cell-free fermentation from two viewpoints, and in the case of living-cell fermentation from a third viewpoint. In the first case, a disturbance of the ratio of the various components and an injury to the total enzyme system is automatically caused, whereby an accumulation and even a stabilization of the intermediate products can arise (Iwanow (1905); Harden and Young

²⁰ The crystallized systems of Sumner, Northrop, Anson, Dounce, and others can at present be regarded as an experimental confirmation of this theory. However, it seems questionable to wish to support the homogeneity of these crystals by the measurements of Svedberg (K. G. Stern: *Enzymologia* **5**, 191 (1938)). Apart from the contrary findings which were obtained by means of cryolysis (*cf.*, for example, O. M. von Ranke-Abonyi and F. F. Nord: *Kolloid-Z.* **58**, 198 (1932)), it must be unequivocally concluded from the discussions of Bawden and Pirie or of K. M. Smith (*Nature* **142**, 842-3 (1938)), that purified tobacco mosaic virus undergoes an aggregation, i.e., a change of particle radius when centrifuged. Independently, R. W. G. Wyckoff (*Cold Spring Harbor Symposia Quant. Biol.* **6**, 365 (1938)) expressed himself on the question of "molecular weight" as follows: "I do not think that we should talk much about the molecular weights of the macromolecules until all the necessary factors have been determined." *Cf.* R. W. G. Wyckoff: *Ergeb. Enzymforsch.* **8**, 4 (1939).

(1906)). On the other hand, by selective poisoning of parts of the enzyme system a tremendous quantity of factual material has been furnished which will certainly not easily lose its statistical value. In living-cell fermentation there occurred, because of the introduction of a reagent not akin to the whole system, the removal of a supposed intermediary product whereby the same was excluded from the reaction sequence. This method, arising from the demands of war, originated with Connstein and Lüdecke (18)²¹ and consisted in the application of an earlier observation of Dumas (29), who had established in 1874 the possibility of influencing alcoholic sugar degradation by the presence of alkali sulfites.

All these methods can, under favorable conditions, lead to the isolation of compounds, or their derivatives, which in the normal course of events would not appear. It is, however, to be emphasized that the removal of an intermediary product, as well as the selective poisoning or injury to the enzymatic system, leaves open the possibility of disturbing other phases along with the actual change in the normal sequence.

Important objections to the unlimited application of the reaction sequence found in cell-free fermentations and other preparations (due to their chaotic state, in contrast to the living structures) to the realm of action of enzyme systems in undamaged cells are to be found in the field of biophysical chemistry. First, the degree of dispersion (121, 125; *cf.* also 122) of the carrier of dualistic enzyme systems (*loc. cit.*) can change under the influence of various factors. Over and above this is the question whether portions of a sequence of phases in a cell-free or artificial enzyme system constitute a mirrored image of the actions of an enzyme system of living cells. In considering an answer to this question, account must be taken of the important findings of molecular anatomy, which show that the disperse particles of the various protoplasmic substances of cells have widely differing pH values. In other words, it is possible in the same cell for acid as well as alkaline particles to exist side by side *without* immediately neutralizing or precipitating each other (158; *cf.* also 167). Therefore, according to Spek (159),

“bei einem in gesetzmässiger Weise lokal zwischen 5.0 and 8.0 (oder darüber hinaus) variierenden pH schon viel eher auch *sie* (die Enzyme) *an bestimmten Orten in der Zelle zu höchster Aktivität gesteigert, an andern völlig wirkungslos werden können.* Die Frage der Lokalisation der Fermente und die Beschaffenheit ihrer kollidalen Träger wird jetzt von neuer Seite her sehr aktuell.”²² (See also page 442.)

²¹ On the basis of the reports of Dr. Alonzo Taylor, the American Under-Secretary of Agriculture, who was in Germany in the summer of 1916, this procedure had already been tested in in the United States in the beginning of 1917, in order to determine whether it was suitable for technical use.

²² Italics inserted by author.

From the latest advances in our knowledge of the surfaces within the cells (36),²³ we know that many of them possess particular properties and that the correct understanding of reactions stands in the closest connection with the properties of colloidal structure. Here physical and organic chemistry meet. No doubt, by an experiment carried out *in vitro* this intracellular arrangement of things is disturbed.

Finally, according to the measurements of Potter (137; *cf.* also 138), during the fermentation of sugar by the living cell 8 coulombs of electrical energy are liberated. This liberation is independent of the pH of the medium as long as the efficiency of the organism is not injured.

There arises now the occasion to indicate the possibility that the reactions of a cell-free fermentation constitute only fragments of the total reaction mechanism. Besides these, the details of the energy conversions, such as growth, energy transport,²⁴ and energy transformation, are of at least the same significance for the reactions of the living organisms as the stoichiometrically perceptible processes. Nevertheless, the clarification of the sequence of phases which is effected by the enzyme systems separated from the cells is a meritorious work. However, the attempt to interpret the action of living-cell systems only with the aid of the clarification mentioned before has a justification which borders on speculation.²⁵

A further important experimental basis for this conception lies in the mode of action of the amino acid oxidase discovered by Krebs (73), the prosthetic group of which was isolated by Warburg and Christian (170), and the carrier of which was isolated by Negelein and Brömel (104). The amino acid oxidase occurring in tissue slices of kidney and liver is capable of oxidatively deaminating *l*- or *d*-amino acids. If, however, the tissue is injured by grinding, by drying, by octyl alcohol, or by hydrocyanic acid, then only the "unnatural" amino acids are attacked while the natural amino acids are no longer deaminated.

The living cell is thought of in this relation as an "organization." If the interpretation and presentation of a living system with the help of a chemical and physical terminology be regarded as the ultimate goal of biochemistry, then it must be clear that thereby the attempt is made to

²³ With regard to the properties of the outer surfaces of the cell, *cf.* E. N. Harvey and J. F. Danielli: *Biol. Rev.* **13**, 319 (1938).

²⁴ Even Meyerhof (*Handbuch der Physik*, Vol. 11, p. 249 (1926)) once expressed himself on these questions as follows: "Schliesslich sei noch einer wichtigen Bedeutung der freiwilligen Stoffwechselreaktionen, insbesondere der Oxydation, gedacht, nämlich die *Energie* für unfreiwillige chemische Vorgänge, insbesondere 'Synthesen', zu liefern." Compare also L. Algera: *Rec. trav. botan. néerland* **29**, 37 (1932).

²⁵ About ten years ago Hill concluded his address dedicated to the memory of Mond with the following statement: "It is dangerous to speculate too far, but it is foolish not to speculate at all."

penetrate into a field wherein chemical reactions are bound in a complexity which was not envisioned in the development of classical chemistry. The chemical (and still less, the stoichiometric) manner of expression does not possess a means of giving an exhaustive expression of this more comprehensive realm. Nevertheless, by the indication of this difference the view should not be taken that the one is of itself more important than the other. We must here remind ourselves of an earlier statement of Kögl (71), "... dass das Zustandekommen eines normalen Gewebes letzten Endes grossere Rätsel birgt als jenes eines chaotischen Zellgefüges."

Therefore, another still unsolved problem remains,—to find the way which will enable us to draw from the overlapping of the effects of Buchner's preparations and of the living cells, completely valid conclusions concerning the total activity of the latter.

How sharp the dispute of opinion is in the evaluation of the probative strength of the experimental material at hand may be impressively illustrated by a repetition of the following evaluation, written by Meyerhof in 1937 (96) in defense of his hypothesis:

"Ob es in bestimmten Zellarten anaerobe Wege des Kohlenhydratabbaues gibt, die nicht über die hier beschriebenen phosphorylierten Intermediärprodukte führen, bleibe dahingestellt. Beweise dafür liegen nicht vor."

A year later Fink and Krebs (38) expressed themselves as follows on the same question:

"Diese Gleichung (namely, that of Gay-Lussac) gestattet uns, mit Sicherheit die höchstmöglichen *Ausbeuten* an Alkohol und Kohlensäure aus Zucker anzugeben, wenn auch der *Mechanismus* dieser chemischen Umwandlung heute noch seiner endgültigen Lösung, namentlich in bezug auf die vitale Gärung harrt."

How slightly justified are the categorical explanations of Meyerhof, can, furthermore, be seen from a communication of Deuticke and Hollmann (25), who, on the basis of their experiments, draw the conclusion that, in contrast to the proceedings in the structurally destroyed, enzymatically incomplete organ, the carbohydrate degradation in the *intact* muscle proceeds less through the Harden and Young ester than through hexose monophosphate.

Moreover Shorr and Barker (151b) stated recently: "Disruption of cell structure may result in the liberation of highly reactive carbohydrate systems no longer under control by the intact cellular organisation. This fact should be borne in mind when interpreting data derived from tissue mince and extending them by analogy to the living cell."

Another staunch exponent of the conformity of the course of reaction in

the case of yeast juices and living yeast cells is Macfarlane (88), who lately even admits,

"Yet there are a number of differences between the cell and the cell-free extract which may be summarized as follows: fresh yeast does not ferment added glycogen or hexosediphosphate; the rate of fermentation of sugar is not increased by inorganic P, cozymase, or arsenate; there is no stoichiometric relationship between the CO₂ evolved and the actual decrease in inorganic P."

It is furthermore to be considered that a number of native proteins have been crystallized. In contrast, however, no crystallized *denatured* proteins have thus far become known. If we attempt to bring this conclusion into agreement with the clarification, just recently begun (on the basis of the work of Mirsky and Anson (3; also 185 and 93), for example) of the processes in the case of various types of denaturation of proteins, brought about in several ways, then the fact is not to be overlooked that, at least in the case of the known crystallized enzymes, regarded as unitary systems, the decrease in activity can be proven to run parallel to the increased denaturation. If this observation be transferred to the carrier proteins of dualistic enzyme systems, then it would be conceivable that in the enzyme systems (e.g., in maceration juices and others), in consequence of the possible dissolution or loosening of the various types of valence bindings of the carrier proteins during the preparation, a change in the specificity of the latter occurs which is capable of causing an extensive influence on the activities, or of eliminating parts of the total system. For these reasons, also, it is not conclusive if, from the enzymatic behavior of structurally destroyed systems, which can be more or less incomplete, forceful conclusions as to the qualitative actions of the parent systems within the living cells are drawn.²⁶

VIII. MECHANISM OF FERMENTATION BY FUSARIA

The findings and their explanations selectively reported in section V are so contradictory that it seems impossible to reduce them at present in a constructive sense to a common denominator. In order, therefore, to be justified in drawing at least analogous conclusions, an attempt had to be made to bring forward for comparison a *living-cell* system or its metabolism, whose stoichiometrically conceivable effects are comparable to those of yeast. This is found in a group of polycellular fungi, the fusaria: *Fusarium lini* Bolley, *Fusarium oxysporum*, and *Fusarium graminearum*

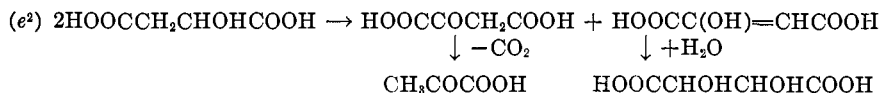
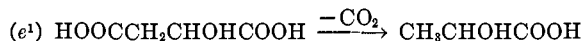
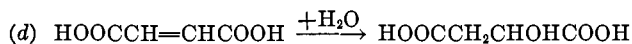
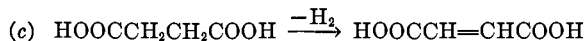
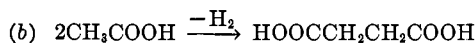
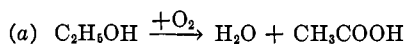
²⁶ Compare also in this connection F. Hofmeister, *Die chemische Organisation der Zelle*, p. 8 (Braunschweig, 1901): ". . . so wenig der Biochemiker durch chemische Analyse einer zertrümmerten Taschenuhr deren regelmässigen Gang erklären könne, ebensowenig sei von der chemischen Untersuchung des toten und zertrümmerten Protoplasmas eine Aufklärung über dessen Lebenserscheinungen zu erwarten."

Schwabe (*Gibberella saubinettii*), whose biology (182) has been very fruitfully studied. Concerning the biochemical effects of their enzyme systems only loose stoichiometric conclusions were known up to a few years ago.²⁷

Fusaria cause a genuine alcoholic fermentation of hexoses as well as of pentoses. The ratio between carbon dioxide and ethyl alcohol in the case of the fermentation of hexoses is in almost complete agreement with that of a typical yeast fermentation. The ratio of carbon in the alcohol to the carbon in the carbon dioxide is 1 to 1 in the case of pentoses, in contrast to the ratio of 2 to 1 if the fungus grows on glucose. Accordingly, they possess zymases, phosphatases, and phosphatases, and an aeroglucosedehydrase and aeropentosedehydrase. They have a very powerful dehydrase system which enables them, for example, to utilize ethyl alcohol or polyvinyl alcohol as the only source of carbon and to dehydrogenate these with the formation of carbon dioxide.²⁸ They fer-

²⁷ A presentation by F. F. Nord and collaborators of the actual status of our knowledge of the mechanism of alcoholic fermentation and the other enzymatic effects of fusaria is to be found in *Ergebnisse der Enzymforschung*, Vol. 8 (1939), in the *Biochemische Zeitschrift* (1936-38), in the *Berichte der deutschen chemischen Gesellschaft* (1938), and in *Chemiker-Zeitung* (1938). Reports on the physiology of individual fusaria have been made by Y. Tochinai (*J. Coll. Agr. Hokkaido Imp. Univ.* **14**, 171 (1926); *Ann. Phytopath. Soc. (Japan)* **1**, part 3 (1920); *Trans. Sapporo Nat. Hist. Soc.* **8**, 1 (1920)), by H. H. Hochapfel (*Zentr. Bakt. Parasitenk.* II **64**, 174 (1925)), in the dissertation of G. Luz or H. Grossmann (Zürich, 1934), and by S. Medvedeva (*Compt. rend. acad. sci. (U.R.S.S.)* **15**, 503 (1937)).

²⁸ The complete phase sequence for the dehydrogenation of alcohols by means of *Fusarium lini* Bolley up to the stage of carbon dioxide can at present be formulated as follows:



In accordance with considerations presented in the literature (R. Sonderhoff: *Ergeb. Enzymforsch.* **3**, 163 (1934); K. Bernhauer: *Ergeb. Enzymforsch.* **3**, 216 (1934)) and undisputed experimental evidence, there first takes place a dehydrogenation of the succinic acid by way of fumaric acid to malic acid. The malic acid itself represents the branching point for the further course of reaction. It may lead (a) after de-

ment dextrans as well as disaccharides, of which, for example, maltose can be fermented without phosphorylation, directly and indirectly. They have a very powerful catalase system; their cell multiplication is promoted by hydrocyanic acid, and their dehydrases are not inhibited by this reagent. In comparison to *B. coli* or to yeast, the quantity of cellular phosphorus donors (adenosinetriphosphoric acid or muscle adenylic acid) present therein is small.

In contrast to yeast, these systems, however, are advantageous in that, since their cellular substrates are apparently not so easily capable of mobilization, the stoichiometrically conceivable part of the course of the reaction is slow and correspondingly more extended than in the case of the sequence in living yeasts. This afforded a possibility of obtaining an insight into the first phases of the dissimilation of the carbohydrates, which phases in the case of the undamaged yeast cells forced the adoption of the usual detours. In contrast to the conclusions of Meyerhof in the case of alcoholic fermentation by means of yeast juices (*cf.* page 442), it was shown with living fusaria, as well as with dried preparations, that the disappearance of inorganic²⁹ phosphorus during the fermentation of hexoses begins, on the average, 2 to 5 days after the onset of the fermentation and *after* the evolved carbon dioxide is obtained. At appropriate phosphate concentrations ribose and arabinose, as well as xylose, can be phosphorylated in the later course of the dissimilation. Added organic phosphorus donors have, in alcoholic fermentation with fusaria, only the function of cell regenerators. An accumulation of such donors could indeed be established in the micelle, but under normal conditions it was without essential influence on the kinetics of carbohydrate degradation. The hastening of the enzymatic reaction sequences, established under special conditions after addition of adenylic acid, was not necessarily caused by a phosphate transfer to the substrate, *since it was also observed*

carboxylation to lactic acid, and (b) after dehydrogenation as keto oxalacetic acid to pyruvic acid, and as an enol compound to tartaric acid.

Besides the identification and isolation of acetic, succinic, and malic acids in the course of the dehydrogenation of alcohols by means of the enzyme system of fusaria, lactic acid could also be qualitatively, and tartaric acid quantitatively, found as products of stabilization. There is, therefore, a restricted agreement with the findings of Chrzaszcz and Tiukow (*Biochem. Z.* **229**, 355 (1930)). But in order to avoid a disarrangement of the enzymatic course of the reaction and an unphysiological accumulation of transient products, we did not avail ourselves of the possibility of disturbing the reaction cycle (compare F. F. Nord: *Naturwissenschaften* **24**, 763 (1936)). Meanwhile a rapid and accurate analysis for the quantitative estimation of small amounts of succinic acid has been developed in this laboratory (45a).

²⁹ In these experiments special care was taken to avoid the presence of sodium fluoride as an impurity. *Cf.* A. Harden: *Nature* **134**, 101 (1934).

in a corresponding manner if alcohol was the substrate.³⁰ Accordingly, there are cell systems in which, during the course of alcoholic sugar decomposition, the degradation does not set in with phosphorylation. Correspondingly, there exists a fundamental agreement with the mechanism of carbohydrate metabolism in the case of brain tissue, of tumors (5, 44; *cf.*, to the contrary, 97), of chicken embryos (*loc. cit.*), and in the case of kojic acid formation by *Aspergillus tamarii* Kita (46) in which the phosphorus-free degradation is supreme. The phosphorylating degradation arises there also, as with fusaria (here also in the case of pentoses), only much later and to a quantitatively insignificant extent. The cellular physiological and evolutionary mechanical causes of this agreement might, however, be different for the systems mentioned. Whether the phosphorus-free course of degradation leads through a form of methylglyoxal can, at the moment, not be answered. Let it be noted here, however, that *Fusarium lini* Bolley is capable of using hydroxymethylglyoxal (62a, 126a), in the monomeric as well as in the trimeric form, as a source of carbon (118).

The present status of these researches gives further clues to the view that the application of experiments, correct in themselves, which were carried out with the help of cell-free enzyme systems, to the sequences of reaction of living cells is permissible only with the generally omitted limitation that they constitute, at best, only a part of the totality of reactions proceeding codependently in the living cell. Hence it is questionable whether all known "cell-free" reactions can demand a place in the reaction sequence in the living cell.³¹

However, using the wet crushing mill of Booth and Green (13a), an active cell-free juice was prepared from *Fusarium lini* Bolley which, thus far, seems to furnish an enzyme system which does not suffer the usual distortions and deficiencies of maceration juices obtained from yeast (180a).

In no case should the fact be overlooked that fusaria thus far constitute the only systems in which, in contrast to yeast, it has been possible in the latter course of the alcoholic fermentation with their *living* cells to establish a phosphorylation of the added hexoses or pentoses.

The discovery of the aeroglucosedehydrase and aeropentosedehydrase stands in good agreement with the supposition, already expressed by Boysen-Jensen (14), that various organisms can oxidize sugars. We have established the appearance of the corresponding acids. A splitting of the carbon chain, therefore, did not occur. It is interesting to see, and ought to be remembered, that the carbohydrate dissimilation can occur under

³⁰ This assumption is valid as long as the enzymatic formation of ethyl phosphates is not established.

³¹ Compare also the discussion of the papers of Cori or Goddard in the Cold Spring Harbor Symposia on Quantitative Biology, Vol. 7 (1940).

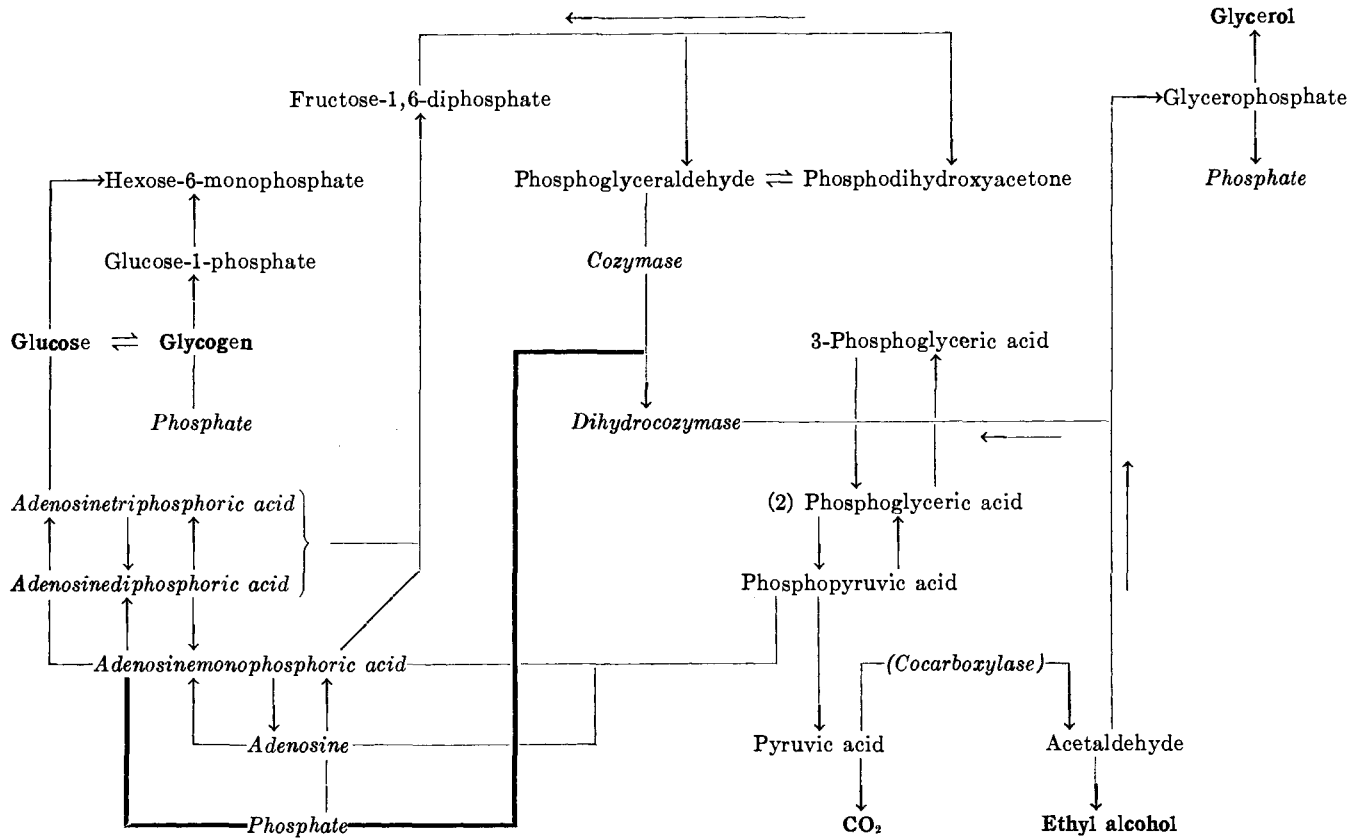
the influence of fusaria in three ways: namely, (a) by oxidation, (b) by splitting of carbon chains, and (c) by the detour of phosphorylation.

IX. PROCESSES IN THE COURSE OF CONVERSIONS WITHIN THE C₃ SERIES

If now the attempt is undertaken to present diagrammatically the pretended sequence of phases of alcoholic fermentation, starting from the previously mentioned stage of hexosemonophosphoric acid (*cf.* page 434), then we must keep in mind that here, as well as in the living yeast, 3-phosphoglyceric acid (114, 129; *cf.* also 9, 141, 168a) has a key position. Phosphoglyceric acid is formed after the previously mentioned hexosemonophosphoric acid has been converted into a di-ester, wherein adenylypyrophosphoric acid serves as a phosphate donor for the formation of the diphosphate. Phosphoglyceraldehyde or phosphodihydroxyacetone is formed in the presence of aldolase from the fructose-1,6-diphosphate. In the presence of cozymase (see page 460), phosphate, and adenosine-diphosphoric acid, there arises from the phosphoglyceraldehyde the above-mentioned 3-phosphoglyceric acid (\rightleftharpoons 2-phosphoglyceric acid), adenosinetriphosphoric acid, and dihydrocozymase. From the 2-phosphoglyceric acid there further arises phosphopyruvic acid, which with adenosine gives adenylic acid and pyruvic acid.³² The pyruvic acid is split by carboxylase into acetaldehyde and carbon dioxide, and the acetaldehyde is stabilized into ethyl alcohol, whereby the cozymase is regenerated from the dihydrocozymase. According to a proposal by Parnas (132), these steps can be summarized in the sequential picture of alcoholic fermentation given in table 1.

³² The conversion of phosphoglyceric acid to pyruvic acid can, according to Neuberger and Kobel (*Biochem. Z.* **272**, 459 (1934)) also be accomplished in a purely chemical way by removal of water, using potassium pyrosulfate. The fermentability of pyruvic acid found by O. Neubauer (*loc. cit.*) was originally denied by C. Neuberger and A. Hildesheimer (*Biochem. Z.* **31**, 173 (1911)). They wrote at the time: "Aus diesen Versuchen folgt, dass die freie Brenztraubensäure *nicht*, wohl aber ihre löslichen Alkali- und Erdalkalisalze mit Hefe 'gären'". This observation might, according to present knowledge, be applied to added acid (present in the keto-form). From measurements of adsorption spectra by Henri and Fromageot (*Bull. soc. chim.* [4] **37**, 852 (1925)), it is known that pyruvic acid *in vivo* occurs only in the easily fermentable enol-form. *Cf.* polarigraphic measurements of Müller and Baumberger (*J. Am. Chem. Soc.* **61**, 594 (1939)) and Zambotti and Ferrante (*Boll. soc. ital. biol. sper.* **14**, 372 (1939)). This observation could also be supported by the isolation of the so-called glucic acid, $\text{CHOH}=\text{CHCOOH}$, and also by its preparation (Nelson and Browne: *J. Am. Chem. Soc.* **51**, 830 (1929)). The latter arises from the action of weak calcium hydroxide on dilute solutions of glucose at 70°C. with the exclusion of air and greedily absorbs atmospheric oxygen with strong evolution of heat. According to Browne (*Science* **77**, 223 (1933)), the strong heat effect observed in hay fermentation can also possibly be traced to the oxidation of similar intermediate compounds.

TABLE 1
Phase diagram of alcoholic fermentation



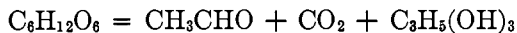
Further details of the decomposition into the phosphorus-containing C_3 bodies and of the further decomposition of these C_3 compounds needs no special treatment. However, before the discussion of the biochemically and enzymologically most interesting parts of the reactions or of the sequences—namely, the mechanism of the hydrogenation and dehydrogenation with the help of cozymases, and the description of the action of carboxylases or cocarboxylases—is begun, the disproportionation of the C_3 bodies, already indicated on page 427, within the frame of the classical equation of Gay-Lussac,



should be elucidated. Müller-Thurgau and Osterwalder (100) observed that sulfurous acid added to fermenting sugar solutions reacts immediately with the acetaldehyde (133a) present in the mash. It is clear that this compound is to be regarded as the acetaldehyde sulfurous acid of Ripper (146),³³ the sodium salt of which has been known since the time of Bunte (17). In the course of comprehensive investigations on the hydrolysis of bound sulfurous acid, Kerp (67; *cf.* also 72) later established that the dissociation constant of acetaldehyde sodium sulfite (2.84×10^{-6}) bears a ratio to that of the corresponding glucose compound (311×10^{-8}) of approximately 1:90,000. On this basis were built up the experiments of Connstein and Lüdecke (18), which later found their biochemical interpretation in the light of the extensive investigations of Neuberg, Reinfurth, and Hirsch (112; *cf.* also 43). The above-mentioned acetaldehyde bisulfite compound, $CH_3CHOHOSO_2Na$, which can easily be split by hot soda solution with the reformation of acetaldehyde, accumulates in the mashes and can be separated. The corresponding hexose compound is, in the presence of water, practically completely dissociated. The relations can be reviewed by the following equation:



The process in a salt-free solution is to be expressed by the following equation:



The ratio between glycerol and acetaldehyde is constant at any given instant.

If the fermentation be carried out in the presence of simple alkali salts which do not unite with acetaldehyde, only traces of the latter are found. In this case, the Cannizzaro dismutation sets in between two molecules of

³³ Compare, for the estimation of acetaldehyde, E. Parkinson and E. C. Wagner: *Ind. Eng. Chem., Anal. Ed.* **6**, 433 (1934).

acetaldehyde and not between a molecule of acetaldehyde and a molecule of triosephosphoric acid. The acetaldehyde is found indeed in molecular proportions but as acetic acid or as ethyl alcohol.



Two moles of glycerol correspond to each mole of acetic acid. That the last-named process, the "mixed" Cannizzaro reaction, if occurring between two different aldehydes, has, nevertheless, a fundamental significance could be shown by model experiments (116; 24) and *in vitro* (171). On the above basis, Neuberg and von Grab (47) were able to sustain the results of Fernbach and Schoen (37) by realizing, through the addition of β -naphthylamine to a fermenting cell-free sugar solution, the Döbner synthesis (28) of α -methyl- β -naphthocinchonic acid and therewith isolating the transient pyruvic acid.³⁴



Using certain crystalline proteins in conjunction with dihydropyridine nucleotide, a triose or triosephosphate, it was demonstrated by Negelein and Broemel (104) that it is the protein of the reducing "Gärungsferment" which catalyzes the formation of glycerol.

By diminishing the enzyme concentration and simultaneously the concentration of the coenzymes by the introduction of a substance causing plasmolysis (toluene) (111), it was possible with top and bottom yeasts to change the alcoholic sugar dissimilation into a lactic acid fermentation (8). This was carried out with the aid of glutathione at a certain stage of the fermentation.

These observations, while in agreement with the findings of Aubel and

³⁴ A review of fifty different methods of determining pyruvic acid is given in a work of Wendel (J. Biol. Chem. **94**, 717 (1932)). A method which helps in determining 2γ in 10 cc. (corresponding to a dilution of 1:5,000,000) of blood pyruvate with an experimental error of 1.5 per cent was reported by G. D. Lu (Biochem. J. **33**, 249 (1939)). Cf. also Fromageot and Desnuelle: Biochem. Z. **279**, 174 (1935)). Here also attention may be drawn to the results of the investigation by C. V. Smyth (J. Biol. Chem. **125**, 635 (1938)) of the utilization of pyruvic acid by bakers' yeast. A remarkably high respiratory quotient was observed. The oxygen consumption and the loss of carbon dioxide were not equimolecular and did not correspond to the equation laid down for the decomposition. On the other hand, the acid caused no increase in the fermentation of reserve carbohydrate. All signs pointed to the appearance of strongly reducing substances, such as fats or fatty acids, which are indispensable as sources of energy. Without giving results on the fats or fatty acids formed, it could be concluded from the petroleum ether extract that the yeast under aerobic conditions can take up two to four times as much pyruvic acid as under similar anaerobic conditions. Concerning the composition of yeast fats compare G. Weiss: Biochem. Z. **243**, 269 (1931).

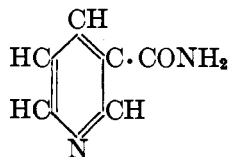
Simon (6), do not, so far as the question whether methylglyoxal should be considered as a primary transformation product in a fermentation by living cells, possess conclusive force since, according to Lohmann (85), the ketone-aldehyde mutase requires glutathione as a coenzyme. Enzyme systems containing the zymase complex which, however, are free from glutathione are not capable of attacking added methylglyoxal.

It is difficult not to see in these equations and findings a realization of the far-seeing statement of Pasteur (133):

“Aujourd’hui, il faut comprendre, au contraire, que l’équation d’une fermentation est essentiellement variable avec les conditions dans lesquelles elle s’accomplit, et que la recherche de cette équation est un problème aussi compliqué que celui de la nutrition chez un être vivant.”

X. COZYMASE AND ITS MODE OF ACTION

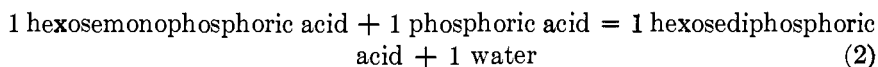
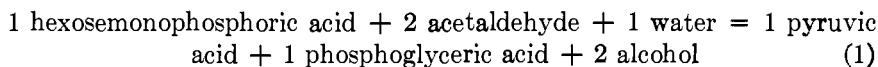
As has already been shown, the first oxido-reduction processes in the sequence given in table 1 set in after the splitting of the hexose diphosphate. Here is visible the influence of a dehydrogenating enzyme which also participates in the formation of lactic acid from pyruvic acid as well as in the formation of alcohol from acetaldehyde. In its action the system has been known since the discovery of the erstwhile coenzyme, the present cozymase system, by Harden and Young (51). The Harden-Young system consists of three components: (1) the phosphorylating coenzyme (cophosphorylase), which is an adenine nucleotide, and is either adenosinetriphosphoric acid (Lohmann) or diadenosinepentaphosphoric acid (Ostern); (2) cozymase (codehydrase I) which, according to the work of von Euler and Myrbäck (101) was regarded up to 1933 as an adenine mononucleotide. The molecular weight was given by von Euler, in agreement with the molecular weight of adenylic acid, as 350. The preparations gave on hydrolysis a content of 28 per cent adenine (168b). (3) The third constituent (Warburg and Christian) is a dinucleotide of molecular weight of approximately 800 (codehydrase II) and contains adenine as a purine base (approximately 17 per cent), the amide of nicotinic acid as a pyridine base (approximately 16 per cent),



which occurs therein in a quaternary linkage, and 13 per cent phosphorus. The elementary analysis (169) gives the empirical formula $\text{C}_{22}\text{H}_{32}\text{O}_{19}\text{N}_7\text{P}_3$,

which corresponds to 1 mole of adenine, 1 mole of pentose,³⁵ 1 mole of hexose, 3 moles of phosphoric acid, and 1 mole of nicotinic acid amide, minus 6 moles of water. Its active group is represented by the pyridine nucleus. By the reversible change pyridine \rightleftharpoons dihydropyridine, this coenzyme transports hydrogen. On the basis of this discovery of the chemical mode of action of the last-named coenzyme, as well as the report of the analysis of nicotinic acid amide, the constitution of von Euler's cozymase could also be reinvestigated. The cozymase for this test was again obtained by von Euler from yeast and by Warburg from horse blood cells. In both cases we are dealing with hydrogen-transporting coenzymes,—“pyridine nucleotides,”—of which von Euler's compound, on the basis of the original determination of constitution by Warburg, is to be regarded as a *diphosphopyridine nucleotide* and Warburg's as a *triphosphopyridine nucleotide*.³⁶ The close relationship of both coenzymes also made their mutual conversion possible, since codehydrase II on dephosphorylation can be converted into codehydrase I, and codehydrase I can be converted enzymatically and chemically into codehydrase II (*cf.* page 462). The redox potential of the diphospho system appears to lie in the vicinity of -0.26 volt (9a).

The fermentation test (“Gärttest”) serves for the quantitative determination of the catalytic action of triphosphopyridine nucleotide. The substrate of the fermentation test consists of (1) hexosemonophosphoric acid, (2) phosphoric acid, and (3) acetaldehyde. In the test, the concomitant course of the following reactions can be weighed:



Here we are obviously concerned with a coupled reaction in which hexosemonophosphoric acid can be replaced by glucose or fructose. Hence no fermentation arises. If the hexosemonophosphoric acid be replaced by hexosediphosphoric acid, the fermentation becomes about ten times slower.

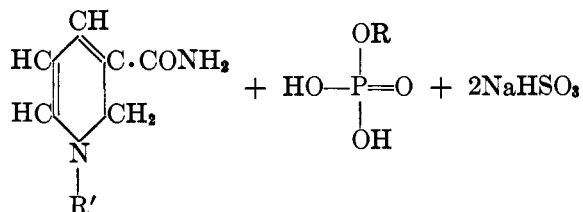
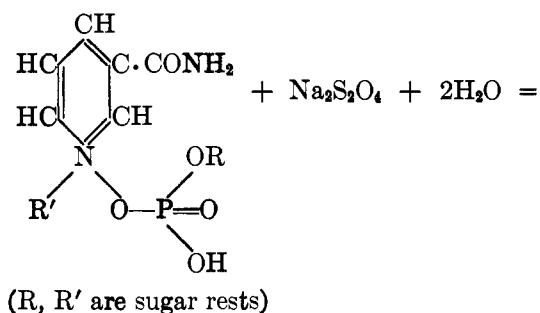
For the colorimetric determination (giving the order of magnitude) of nicotinic acid amide (in a cozymase preparation also), Karrer and Keller (65) employed a method which has as its basis the known reaction of

³⁵ The amount of pentose does not seem to be established beyond doubt. For the determination of small amounts of pentoses, *cf.* W. Mejsbaum (*Z. physiol. Chem.* **258**, 117 (1939)).

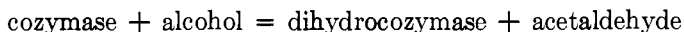
³⁶ Regarding nomenclature, see the proposal of F. G. Fischer (*Ergeb. Enzymforsch.* **8**, 187 (1939)).

pyridinium compounds with 2,4-dinitrochlorobenzene to give pyridinium salts. These are split by alkali to yellowish-red derivatives, $C_{11}H_9O_5N_3$, of glutaconaldehyde. The intensity of color of the solution obtained is measured by a "Stufen" photometer. A colorimetric method which serves to determine nicotinic acid and nicotinic acid amide in the presence of the cohydrazes I and II with a limit of error of about 20 per cent was given by Euler and coworkers (34). Bandier and Hald (10) give an exact colorimetric determination of nicotinic acid which is based on the reaction of König (70). Cyanogen bromide at 70–80°C. is used, and the appearance of a color reaction with *p*-methylaminophenol is the basis of the determination. This method is to be preferred to both previously mentioned procedures. Dihydrocozymase is oxidized by the flavin enzyme, but not by oxygen or methylene blue.

Cozymase, which under physiological conditions behaves like a zwitterion anion (54), is reversibly reduced by hydrosulfite through the semi-quinone stage (55), according to the following equation:



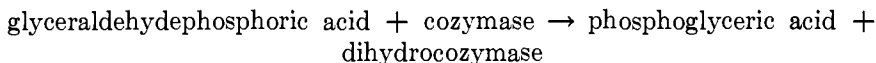
The mechanism shown proves that cozymase³⁷ acts as a hydrogen carrier somewhat in the sense of the equation



The transport of the hydrogen follows only in union with a specific protein from yeast which has the rôle of mediating the oxido-reduction between

³⁷ So far as its preparation is concerned, compare P. Ohlmeyer: *Biochem. Z.* **297**, 66 (1938).

the hydrogenated codehydrases on the one hand and the yellow prosthetic groups on the other. In alcoholic fermentation, the loss of hydrogen follows through the donor system of the triosephosphoric acid which simultaneously becomes phosphoglyceric acid:



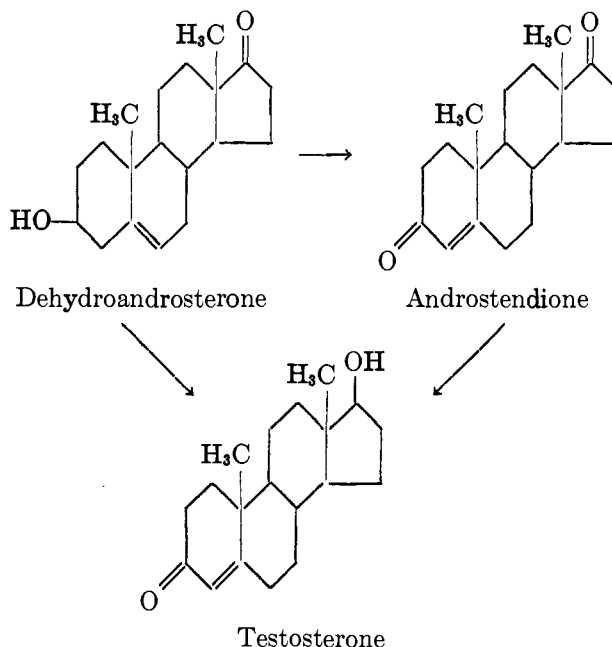
If, during the fermentation process a non-cellular hydrogen acceptor enters the arena in order to compete with acetaldehyde, there is present an example of the reaction known since the time of the discovery of the phytochemical hydrogenations by Lintner and von Liebig (80) and continued through the investigations of Neuberg,³⁸ Nord, Fischer, and Mamoli and Ercoli (90; *cf.* also 174).

An interesting contribution to the interpretation of the mechanism of these processes has been offered by the work of Fischer and Eysenbach (39). According to this work, the known hydrogen migration, which first reduces codehydrases, finally leads to the hydrogenation of ethylene linkages if the pH dependence of this reaction be considered. It is further important that the saturation of ethylenic linkages can also be carried out by enzyme solutions obtained from killed yeast. Here it is of interest that the addition of the unsaturated alcohol to the fermenting yeast juices carries with it a cessation of carbon dioxide evolution. A normally proceeding alcoholic fermentation does not, therefore, constitute a premise for the continuation of hydrogenation. If the fermentation be poisoned by fluoride ions, the speed of hydrogenation of an unsaturated alcohol is not changed by the removal of acetaldehyde as a concurrent acceptor. On the contrary, it was repeatedly observed that the reduction of cinnamaldehyde to cinnamic alcohol as well as the subsequent hydrogenation to saturated bodies was inhibited by the presence of fluoride. Fischer concludes therefrom that several reactions are capable of furnishing the hydrogen for the hydrogenation.

By union of biochemical hydrogenations and dehydrogenations, biochemical syntheses can be accomplished in a purely enzymatic manner. For instance, when dehydroandrosterone was subjected to the action of dehydrogenating bacteria and the reaction mixture was then added to fermenting yeast, the synthesis of the testicular hormone, testosterone, was accomplished. It is, therefore, conceivable that dehydroandrosterone

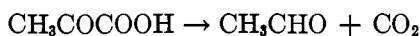
³⁸ The discussion of "carboligase" is omitted as, according to the findings of Dirscherl and Schöllig (*Z. physiol. Chem.* **252**, 71 (1938)), it does not exist. *Cf.* also B. Tanko and L. Munk: *Z. physiol. Chem.* **262**, 144 (1939).

constitutes an intermediate stage in the formation of testosterone in the organism.

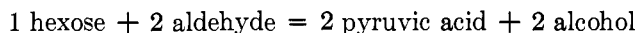


XI. CARBOXYLASE AND COCARBOXYLASE

In 1910 Neubauer discovered the fermentability of pyruvic acid. The process corresponds to the equation



Although Neuberg (108) at first rejected³² the finding of the fermentability of pyruvic acid, he later adopted the conception that pyruvic acid is an intermediary product of enzymatic carbohydrate degradation. According to his opinion, still regarded as correct, every zymase contains a carboxylase which splits carbon dioxide from pyruvic acid. If, according to Pasteur's hypothesis of *internal* respiration, the sugar is oxidized by oxidants which arise during the oxidation, e.g., acetaldehyde, then pyruvic acid arises from the sugar, while the acid in turn is hydrogenated to alcohol, corresponding to the equation:



From this equation, in the presence of carboxylase, pyruvic acid and acetaldehyde are eliminated.

obtained in the course of his work compounds which showed extensive carboxylase-like activity and contained the amino group as an active group.

XII. APPLICATION OF ISOTOPES IN THE BIOCHEMISTRY OF ALCOHOLIC FERMENTATION

(a) Extensive experiments on the fermentation of sugar in heavy water were performed by O. Reitz (144) in consequence of the preliminary measurements of the kinetics of the alcoholic fermentation of sucrose and *d*-glucose by E. Pacsu (130). It could thereby be shown that the inclusion of deuterium into the methyl group takes place to the extent of about 33 per cent, which means that only one deuterium atom is included therein. The main quantity of the hydrogen included in the alcohol lies in the CH₂OH group. Fermentation alcohol formed in pure deuterium oxide would, accordingly, have the formula CH₂DCD₂OD. Independently of the conceptions that can be formed on the transitions involved herein, the decarboxylation of pyruvic acid leads to the conclusion that all the hydrogen atoms on the hydroxylated carbon atom of the alcohol become heavy.

The dependence of the velocity of fermentation on concentration shows that deuterium oxide reacts about half as fast as water in alcoholic fermentation.

(b) In the investigation of the uptake and loss of active phosphorus from nutrient solutions, the conclusion was reached with yeast that evidently no exchange of individual phosphorus atoms can occur between the cell and the medium. In the yeast, accordingly, phosphorus must be bound in an unexchangeable form, e.g., as adenylyphosphoric acid (57).

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