PRESENT STATUS OF THE PROBLEM OF SUGAR FERMENTATION

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Modern research on the alcoholic destruction of sugar began in 1897 with the discovery of E. Buchner that the process is of enzymatic nature. In 1905, Harden and Young found that the zymase of Buchner consisted of a thermolabile ferment fraction, which was later designated as "apo-zymase" by Neuberg and v. Euler, and of a thermostable ultrafiltrable factor, "co-zymase," the presence of which is necessary for the fermentation of sugar. Later it was recognized that phosphate was necessary for alcoholic fermentation. This circumstance led in 1910 to the drawing up of Harden's fermentation equation:

\[ 2C_6H_{12}O_6 + 2PO_4HR_2 = C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O + 2CO_2 + 2C_2H_5OH \]

It states, that one molecule of sugar always forms an ester and that a second molecule of sugar decomposes to ethyl alcohol and carbon dioxide. No consideration, however, was given to the mechanism of the formation of \( CO_2 \) and \( C_2H_5OH \) out of \( C_6H_{12}O_6 \), for neither alcohol nor carbonic acid is preformed in the sugar molecule in any form whatsoever. The first success in the search for intermediary products was Neuberg's discovery in 1910 that pyruvic acid is broken down into carbon dioxide and acetaldehyde according to the equation:

\[ \text{CH}_3\cdot\text{CO}\cdot\text{COOH} = \text{CO}_2 + \text{CH}_3\cdot\text{CHO} \]

by all yeasts that ferment hexoses as well as by the enzyme preparations (carboxylase) prepared from the former.

We have in pyruvic acid, which is fermented in the enolic form (CH$_2$:COH·COOH) according to Neuberg and Weinmann (1928), therefore, a compound that directly produces one end-product, CO$_2$, of alcoholic fermentation by a simple biochemical splitting-out process which stands in contrast to the difficult decomposition of the $\alpha$ keto-acid, and in addition acetaldehyde which serves as a direct precursor of the second end-product. In 1913 Neuberg as well as Dakin and Dudley discovered the biochemical transmutation of methyl glyoxal (pyruvic acid aldehyde), i.e., its oxido-reductive transformation into lactic acid. In the same year Neuberg's fermentation scheme was formulated:

(α) C$_6$H$_{12}$O$_6$ = 2H$_2$O + CH$_2$:C(OH)·CHO (methylglyoxal)

(β) CH$_2$:C(OH)·CHO H$_2$ + H$_2$O = CH$_2$(OH) = CH(OH) = CH$_2$(OH) (glycerine)

+ CH$_2$:C(OH)·COH (pyruvic acid)

(λ) CH$_3$:CO·COOH = CO$_2$ + CH$_3$:COH (acetaldehyde)

(δ) CH$_3$:CO·COH O CH$_3$:CO·COOH (pyruvic acid)

+ CH$_3$:COH II$_2$ = CH$_3$:CH$_2$OH (ethyl alcohol)

Up to the first publication cited the bases of the formulae were chiefly of a theoretical nature. Later, all of the important statements of the scheme were experimentally proved. The process begins with a reactive form of sugar, am-hexose; that is, broken down into two molecules of a substance with three carbon atoms, which substance easily forms methyl-glyoxal by giving up water (α). This yields glycerine and pyruvic acid (β). Through the action of carboxylase the pyruvic acid decomposes to acetaldehyde and carbon dioxide (γ). Finally, out of methyl-glyoxal and acetaldehyde, through a mixed reaction, pyruvic acid along with ethyl alcohol is formed (δ). Methyl-glyoxal is always formed from the am-hexose, and CO$_2$ + acetaldehyde are continually produced from pyruvic acid. Accordingly, after the formation of but one molecule of glycerine—which is in fact a constant

\footnote{Alloiomorph.}
fermentation by-product—all the remaining reactions in the cycle can take place. The important point in this fermentation paradigm is the repeatedly occurring oxido-reductions, in the course of which the oxygen of water oxidizes the molecule, and the hydrogen reduces another molecule. The appearance of trioses was purposely not taken into consideration.3 Should they ever be found to be intermediate products, their place in the scheme is an obvious one, as has been set forth in both of the monographs mentioned.

The different stages in the breaking-down of hexoses by yeast were verified by appropriate experiments. The carboxylatic cleavage of pyruvic acid had been realised in 1910, as has been already mentioned. The separate intermediate products were successfully isolated in practice, partly by the fixation of an intermediate form by a fixing agent (fixation procedure), partly by the variation of the hydrogen ion concentration of the medium, and partly through alteration of the proportion of substrate to complex enzyme material, or to the activating-apparatus of the latter, making allowance for circumstances (ferment-inhibiting procedure).

Neuberg and Reinfurth in 1916 and 1918 established the intermediate nature of the aldehyde by the sulfite fixation procedure (second type of fermentation) through which up to 80 per cent of the theoretically possible amount of acetaldehyde was fixed in the form of a bisulfite compound. At the same time glycerine is formed by reduction equivalent to the product of oxidation, acetaldehyde, according to the equation of the second type of fermentation:

\[ C_6H_{12}O_6 = CH_3\cdotCHO + CO_2 + C_3H_8O_3 \]

The same authors have established in special experiments that the pyruvate-sulfite-complex undergoes an easier carboxylatic cleavage than pyruvic acid itself. In 1920 Neuberg and Reinfurth brought about the fixation of the intermediately formed acetaldehyde also by means of Dimedon (dimethylhydroresor-

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cinol), and later Neuberg, Kobel, and Tychowski (1927–28) as well as Klein and Fuchs (1929) used acid-hydrazids, which react slowly with hexose and at the same time are only slightly poisonous, as fixing agents for the acetaldehyde.

In an alkaline medium a change of the intermediately formed acetaldehyde to ethyl alcohol and acetic acid takes place (third type of fermentation, Neuberg and Hirsch, 1919). Glycerine is again formed as a reduction product equivalent to this biological formation of the oxidation product of acetaldehyde, according to the equation of the third type of fermentation:

\[ 2C_6H_{12}O_6 + H_2O = 2C_2H_5OH + 2CO_2 + C_2H_4O_3 + CH_3·COOH \]

Neuberg and Kobel confirmed the cleavage of sugar into two compounds containing 3 carbon atoms only in 1928, when they attained an accumulation of methyl-glyoxal as a result of the action of the autolytic product of yeast on hexose-di-phosphoric acid salt and isolated this keto-aldehyde as bis-2,4-di-nitrophenylhydrazone. They designated this transformation:

\[ C_6H_{12}O_6 = 2CH_3·CO·COH + 2H_2O \]

as the fifth type of fermentation. The methods were later considerably simplified and improved. The change of sugar to methyl-glyoxal is brought about by the action of component ferments of the complex apo-zymase, which effect a decomposition without the coöperation of co-zymase, and which remain active for years in dry yeast. The method which leads to the accumulation of methyl-glyoxal is also a ferment-inhibiting procedure. The keto-aldehyde is collected when apo-zymase that is free from, or poor in co-ferment attacks hexose-di-phosphate. Since these agents are abundantly present in apo-zymase, one can also proceed by using very small quantities of normal yeast in the presence of a plasmo-lytic substance such as, for example, toluol or brom-benzole and many other plasmo-lytic agents. The co-ferment is also sufficiently inactivated by that means—at least the keto-aldehyde formed remains, since co-ferment is necessary for its further assimilation. Auhagen and Neuberg have only lately demonstrated that the methyl-glyoxal formed as an intermediate is converted into
lactic acid, in the presence of glutathion, the co-ferment of lactic acid fermentation. This switching of the alcoholic destruction of sugar to lactic acid fermentation is supported by the preceding appearance of methyl-glyoxal.

Since the co-ferment must also be put out of operation in order that the accumulation of 3-carbon-atom bodies may occur, it is natural that the realization of the fifth type of fermentation was not successful when normal hexose was used as a starting material. We know, that the processes of the “first attack” occur in the presence of co-ferment phosphate-esterification, and that the form of hexose capable of reacting, am-hexose, is first created thereby. One can proceed, therefore, only with such an am-form of sugar, as is presented by am-fructose-1,6-di-phosphoric acid, when the dividing in two of hexose without the assistance of co-ferment is aimed at. Methyl-glyoxal accumulated in the same way through the action of numerous other cells, of rarer yeasts, of fungi, of bacteria, and of tissues of higher plants and animals could also be isolated by Neuberg and Scheuer out of the reaction mixtures as a dioxime as well as in the form of angular 3-methylnapththopyracine.

Up to now methyl-glyoxal from the alcoholic destruction of sugar by yeast has been obtained with a yield of 85 per cent, and from the bacterial lactic acid fermentation up to 100 per cent of the theoretical.

The intermediate formation of pyruvic acid, which was provided for in the fermentation scheme drawn up by us, was made fully certain in 1929 by Neuberg and Kobel. Indeed a few earlier references on the presence of pyruvic acid in fermentation experiments exist. The identification itself was often indirectly accomplished, the yield was always inconsiderable, and indeed in most cases not to be distinguished from an oxidative formation. The pure anaerobic decay of hexose to pyruvic acid and glycerine was realised by Neuberg and Kobel to 100 per cent of the theoretical. The process corresponds to the equation:

$$\text{C}_6\text{H}_12\text{O}_6 = \text{CH}_3\cdot\text{CO}\cdot\text{COOH} + \text{CH}_2\text{OH}\cdot\text{CHOH}\cdot\text{CH}_2\text{OH},$$

and was designated by the authors as the fourth type of fermentation. Under particular conditions hexose-di-phosphate as well as
free sugar was broken down into two C₃-bodies. With the use of hexose-di-phosphate as a substrate the experimental procedure was similar to that which permitted the isolation of methyl-glyoxal. It differs from the latter procedure only in that a little larger quantity of enzyme is required for the formation of pyruvic acid. The formation of pyruvic acid out of zymo-di-phosphate takes place very rapidly by the action of juice from macerated yeast or of fresh yeast, in the presence of toluol or other plasmolytic agents.

When the substrate does not consist of phosphorylated sugar, a displacement of the hydrogen ion concentration towards the alkaline side by addition of trimagnesiumdiphosphate, disodium-phosphate, or magnesium oxide, leads to a pH between 7 and 8 at the beginning. The pH at the end lies then between 5 and 7, while the pH at the termination of normal fermentation with living yeast is around 3.1. In yeast-juice experiments an introductory pH of about 6.9 is necessary for glycerine-pyruvic-acid cleavage. The possibility of production of pyruvate is present in that the action of carboxylase is inhibited with the H ion concentration displaced toward the alkaline side (Hägglund). The breaking down of the ester of hexose-di-phosphoric acid is connected with a dephosphorylation, and, on using free sugar as a substrate, first of all an esterification takes place and then, with the giving up of water, the formation of pyruvic acid occurs.

By the action of dried yeast on a mixture of glucose, acetaldehyde, hexose-di-phosphate and inorganic phosphate in the presence of sodium fluoride Nilsson observed in 1930 the formation of a phospho-glyceric acid that he considered identical to the glyceric-acid-mono-phosphoric acid, synthesized by Neuberg, Weinmann, and Vogt in 1928. Neuberg and Kobel simplified the biochemical preparation of phosphoglycemic acid by showing that neither dried yeast nor already formed hexose-di-phosphate was necessary for its production. They demonstrated, in addition, its formation out of sugars of very different sorts (hexoses, glyceraldehyde-phosphate, disaccharides). They also showed that the acetaldehyde can be replaced by other aldehydes, and that the acid of the 3-carbon-atom series mentioned is formed in the presence of toluol
by action of maceration juices as well as of fresh yeasts, and established that it changes over to pyruvic acid just as hexose-di-phosphoric acid does and under the same conditions. Finally, under conditions optimum for the decomposition of pyruvic acid by carboxylase, they accomplished the fermentation of simple glyceric acid and also of its twofold phosphorylated ester. If one now supposes that phospho-glyceric acid which is only obtainable under abnormal conditions—i.e., in the presence of a hydrogen acceptor and by fluoride poisoning—actually appears also in the course of normal decomposition, then it is easy to insert it in the Neuberg fermentation scheme mentioned at the beginning. It then appears as a phosphorylated precursor of pyruvic acid, which is fixed by the ionic poisoning of the medium. If desmolysis, the rupturing of the carbon chain, sets in on phosphorylated am-hexose, then the phosphoglyceric acid + glycerine-phosphoric acid or phosphoglyceric acid + glycerine, as the case may be, are to be inserted before the pyruvic acid + glycerine, or making allowance for circumstances, logically before the end-product of the second and third type of fermentation.

After Fischer and Baer had synthesised in 1932 glyceraldehyde-3-mono-phosphoric acid and Smythe and Gerischer had established that the dextrorotatory components ferment with yeast with about the same velocity as zymo-hexoses, the possibility that the glyceraldehyde-phosphoric acid forms an intermediate product in the alcoholic decomposition of sugar was taken into consideration. Neuberg and Kobel recently showed that in the presence of sodium fluoride its transmutation by yeast takes place without splitting-out phosphate and yields phospho-glyceric acid. All of these findings, however, are no proof of the real nature of this compound as an intermediate product. Let the cases of oxalacetic acid and of d-Mannononose be recalled, which in spite of fermentability are not to be considered as intermediate forms. As long as one has not succeeded in isolating phosphoglyceraldehyde out of ferment mixtures, perhaps calorimetric measurements may throw light on the possibility of an intermediate formation of glyceraldehyde-phosphoric acid. For the free glyceraldehyde, which it is true could only be used in dimolecular form for the
determination of the heat of combustion, no basis is presented, to warrant the conception of its rôle as an intermediate product according to the energetic measurements of Neuberg, Hofmann and Kobel. The isomer, dihydroxyacetone, is excluded as such for energetic reasons, as well as according to the biochemical findings of Neuberg and Kobel and of Iwasaki who established in 1928 that in the fermentation of dihydroxyacetone first of all hexose-phosphoric acid ester forms and the decomposition occurs only after condensation to hexose sets in.

The existence of a triose-phosphoric acid ester was already taken into consideration years ago. In 1907 Iwanoff declared that the phosphoric acid ester isolated by him by fermentation contained triose-mono-phosphoric acid. In the same year Wohl developed the conception that glyceraldehyde appears as the first decomposition product of sugar by means of an aldol-depolymerisation. With loss of water it then changes over to methylglyoxal. In 1927 Kluyver and Struyk considered a decomposition of phosphorylated sugar, namely of hexose-mono-phosphate to glyceraldehyde and glyceraldehyde-phosphoric acid, for which process they supposed a phosphorylation in the 2-position. They strengthened this hypothesis with the circumstance that in distillation of the fermentation mixture with sulfuric acid according to the method of Neuberg, Färber, Levite and Schwenk, methyl glyoxal can be demonstrated to a small extent. As a matter of fact Neuberg and Kobel were able to determine with certainty the condition required for the production of triose-phosphate. It was only necessary to diminish the quantity of enzyme still more than is necessary for the production of methyl glyoxal. They then obtained with the same agents (i.e., yeasts, lactic acid bacilli and parts of green plants) large quantities—30 per cent and more—of esterified triose. Nilsson supposes that the decomposition of a hexose-mono-phosphoric acid commences with the equilibrium between a 6-position phosphorylated form of enolic character (see Neuberg, 1913) and the two stable esters of hexose-mono-phosphoric acid, fructose-6-phosphoric acid (Neuberg's ester) and glucose-6-phosphoric acid (Robison's ester) respectively, and
that with the assistance of co-enzyme it is broken down to glyceraldehyde-3-phosphoric acid according to the equation:

\[
\begin{align*}
\text{CHOH} & = \text{C(OH)} - \text{CHOH} - \text{CH}_2\text{O} - \text{PO}_3\text{H}_2 \\
& = \text{C}_3\text{H}_6\text{O}_3 + \text{CHO} - \text{CHOH} - \text{CH}_2\text{O} - \text{PO}_3\text{H}_2.
\end{align*}
\]

None of these three substances was isolated. The support for this scheme was only the finding of the author that in the presence of fluoride, which according to the investigation of Meyerhof (1927) and Lipmann (1928) inhibits fermentation and prevents the break-down of phosphoric acid ester, as well as in the presence at the same time of acetaldehyde and hexose-di-phosphate, a substance is formed which he declared to be identical with phosphoglyceric acid, Nilsson has established that in the presence of the ionic poisoning phenomenon mentioned acetaldehyde is reduced to alcohol and he thence concludes that it is a mixed reaction between acetaldehyde and glyceraldehyde-phosphoric acid to ethanol and phosphoglyceric acid. According to this view, however, glyceric acid is no normal intermediate product of desmolyses, but a fixation product like, for example, the acetaldehyde in the fixation process which makes use of sulfite. He accepts glyceraldehyde as a real intermediate product (see also Embden and Meyerhof and associates). Normally this is thought by Lebedew to arise from hexose-di-phosphate and to be condensed again to the latter, while the phosphorus-free compound \(\text{C}_3\text{H}_6\text{O}_3\), the empirical composition of which corresponds to that of a hydrate of methyl glyoxal, is decomposed in known manner to alcohol and carbon-dioxide. According to this interpretation the fermentation equation of Harden would be fulfilled, which equation provides for a parallelism between the esterification to hexose-di-phosphate and the evolution of \(\text{CO}_2\). Support for the intermediate appearance of glyceraldehyde-phosphoric acid and its condensation to hexose-di-phosphate is seen by the Swedish author in the fact that galactose in spite of its difference in structure produces the same hexose-di-phosphoric acid as the zymohexoses properly speaking. This finding does not, however, permit of its being accepted as a fully valid argument in as much
as we know since the investigations of Neuberg and Leibowitz (1927) as well as Veibel (1931) that the diverse sugar esters of phosphoric acid change by a biochemical enolization extraordinarily easily from one to the other in the fermentation mixture, and that several of the esters are always simultaneously present. In addition, the well-known findings of Nef (1914) indicate that a transition from the galactose series to the glucose series also may take place chemically. In the triose-phosphates the energetic relations may be differently arranged than in the free triose, consequently, the possibility may exist that methyl-glyoxal has a precursor in phosphotriose, just as pyruvic acid has in phosphoglyceric acid. New findings suggest, it is true, that genuine glyceraldehyde-3-phosphoric acid does not at all produce methyl-glyoxal under conditions under which we isolated methyl-glyoxal in large quantities from hexose-di-phosphate with practically all types of cells. Indeed, phosphoglyceraldehyde gives rise to methylglyoxal upon heating, just as the free trioses do (Spoehr, Cameron, Neuberg and Collatz), when it is boiled in the presence of hydrochloric 2,4-di-nitrophenylhydrazine. With our experimental procedure alone—precipitation in the cold with the reagent and retention for one or two hours at room temperature—only traces of methylglyoxal-bis-hydrazone are formed at the most from glyceraldehyde-3-phosphoric acid. Hydrazones containing phosphorus, or free from phosphorus, making allowance for circumstances, are formed that are not derivatives of methyl glyoxal. Also under the influence of the enzymatic component complex of yeast, designated by us provisionally glycolase, phosphoglyceraldehyde gives rise to no methyl-glyoxal. The methyl-glyoxal we obtained biochemically from hexose-di-phosphate is not merely an artificial product formed from glyceraldehyde-phosphoric acid during the isolating procedure. The finding of Auhagen and Neuberg referred to also supports this view. The question must remain open, as to whether the course taken during the decomposition proceeds at all beyond phosphoglyceraldehyde. The phosphoglyceric acid formation observed under the effect of fluoride can not be applied in this respect without consideration. Attention is indeed to be paid, however, to the isomer di-hydroxy-
acetone-phosphoric acid, since combined triose has been demonstrated with certainty to be among the products of glycolytic reaction (see foregoing pages). The formation of phosphoglyceric acid out of phosphorylated di-hydroxy-acetone would then assume a biological isomerisation, such as has been observed by Neuberg and associates in the case of the transformation of fructose-phosphate into glucose-phosphate. Di-hydroxy-acetone-phosphoric acid might then become methyl-glyoxal, analogous to the formation of pyruvic acid from glycemic-phosphoric-acid, and could be inserted in the fermentation scheme as a veritable intermediate product.