THE MECHANISM OF ALCOHOLIC FERMENTATION

Thesis

by

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THE MECHANISM OF ALCOHOLIC FERMENTATION

Introduction

The alcoholic fermentation of sugar containing materials is one of the oldest biochemical processes of which we have historical record. On the other hand, only in recent years have we acquired much evidence as to the mechanism by which alcohol and carbon dioxide are formed from the sugar. Although Pasteur' proved conclusively that fermentation is closely associated with the life of certain organisms, yet it was not until Buchner[2] showed that fermentation may take place in the absence of living cells that the importance of organic catalysts was realized. It was these researches of Buchner which made possible the intelligent investigation of this important mechanism.

In the several years which have passed since this memorable discovery, an enormous amount of work has been done in the field and many data have accumulated. Many theories have also been advanced to explain the confusing results which have been obtained, but objections have been raised to all that have so far been presented. On reviewing these theories, however, it seemed possible by a slight modification of some of them to account for a large proportion of the available facts in a modified theory. Since the modified theory suggests the advisability of a number of experimental investigations, it would seem of value to give an account of it at the present time, together with an outline of possible modes of experimental attack and a statement of the meagre results already obtained.

The development of the new theory is made more intelligible by first discussing the older theories most intimately involved, and by then attempting to show how they may perhaps be correlated. In discussing those theories which have been advanced it is of course necessary to include a summary of the evidence on which they are based; but no attempt will be made to include information other than that which is directly involved. It is hoped that no omissions of unfavorable facts have been made. The following discussion will be confined to the process of fermentation as accomplished by enzyme systems, and will not touch upon the complex question of the mode of action of the living organisms.

Discussion

One of the most important contributions to the understanding of the fermentation process was made by Neuberg who found that on adding sulfites[3] to fermenting mixtures, the production of alcohol was diminished, the amount of glycerol was greatly increased, and acetaldehyde in large quantities was "fixed".as the bisulfite addition compound. He thus modified the usual reaction, represented by equation

(1) $C_6H_{12}O_6 = 2CO_2 + 2C_2H_5OH$

to one which was closely represented by

(2) $C_6H_{12}O_6 + NaHSO_3 = CH_2OH.CHOH.CH_2OH + CO_2 + CH_3CHO.NaHSO_3$

When the sulfite was replaced by sodium hydroxide[4] the acetaldehyde became replaced by alcohol and sodium acetate in equivalent amounts according to the equation

(3) $2C_6H_{12}O_6 + NaOH = 2CH_2OH.CHOH.CH_2OH + 2CO_2 + C_2H_5OH + CH_3COCNa$

This behavior he explained by assuming that acetaldehyde was first formed, and then converted to alcohol and acid, according to the reaction:

$$(4) \quad 2CH_3CHO = CH_3CH_2OH + CH_3COONa$$

making the primary reaction in each case

$$(5) \quad C_6H_{12}O_6 = CH_2OH.CHOH.CH_2OH + CO_2 + CH_3CHO.$$

Now it has long been known[5] that one of the most active yeast enzymes, namely carboxylase, possesses the specific property of converting α-ketonic acids to aldehydes by the removal of carbon dioxide. Pyruvic acid, for example, is decomposed by it into acetaldehyde and carbon dioxide according to the equation

$$(6) \quad CH_3COCOOH = CH_3CHO + CO_2.$$
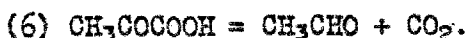
If, therefore, pyruvic acid were an intermediate in the fermentation process, it would be easy to explain the equimolal formation of aldehyde and carbon dioxide as given in equation (5), by assuming them both to be derived from pyruvic acid. In substantiation of the view that pyruvic acid is actually an intermediate in the process, it has been found[6] that not only is carboxylase an invariable constituent of all fermenting complexes, but that the latter are able to ferment pyruvic acid as rapidly as glucose.[7]

The above, however, does not indicate why the removal of the acetaldehyde produces such changes. If the reactions be studied it will be noted that the appearance of pyruvic acid (or of aldehyde and carbon dioxide) is always accompanied by the appearance of an equi-molal amount of glycerol; that is, the basic reaction may be represented

$$(7) \quad C_6H_{12}O_6 = CH_2OH.CHOH.CH_2OH + CH_3CO.COOH.$$

Neuberg[8], therefore, assumed them to be simultaneously produced from a
common precursor by a process of auto-oxidation and reduction, as
indicated in equation 8.

(8) $2(C_3H_6O_3)* = CH_3CO.COOH + CH_2OH.CHOH.CH_2OH$
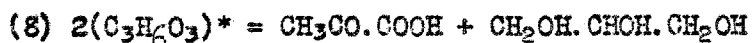
The system was then completed by adding a reaction whereby the aldehyde
is used to oxidize the precursor to pyruvic acid and is reduced itself
to alcohol.

(9) $(C_3H_6O_3) + CH_3CHO = CH_3.CO.COOH + CH_3CH_2OH.$

The removal of the acetaldehyde by fixation methods prevents (9) and re-
quires that all the preformed precursor be disposed of by (8) giving rise
to glycerol and pyruvic acid. The acid is then converted to aldehyde and
carbon dioxide by the agency of the carboxylase (equation 6), and the
aldehyde becomes fixed by the added reagents.

In the absence of the fixing agents (8) proceeds only until a certain
small concentration of aldehyde is attained; from this point the pre-
cursor is disposed of exclusively by (9). (This assumes a higher specific
rate for (9) than for (8).) It is to be noted that this mechanism also
accounts for the invariable presence of glycerol in fermenting mixtures.

While the mechanism outlined above affords a very satisfactory
explanation of many points, yet it is not complete in that it gives no
explanation of the formation of the reactive precursor. Neuberg was led
to suggest that this precursor was methyl glyoxal[8], perhaps reacting in the

*This is a different nomenclature from that adopted by Neuberg, and
is intended to represent an intermediate of the empirical formula $C_3H_6O_3$
or one capable of being converted by hydration or dehydration to compounds
of that formula. Thus $C_3H_8O_4$ or $C_3H_4O_2$ are not excluded.

enol form, $CH_2=COH.CHO$. The methyl glyoxal was assumed to be formed from its aldol, which in turn was supposed to result by the dehydration of the hexose. These were represented by him

(10) $C_6H_{12}O_6 = C_6H_8O_4 + 2H_2O$

and   (11) $C_6H_8O_4 = 2CH_3.CO.CHO$ (or $CH_2 = COH.CHO$)
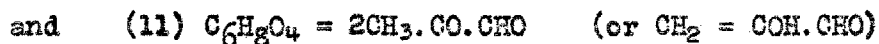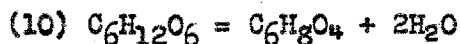
However, as methyl glyoxal[9] is only slowly fermented to alcohol and carbon dioxide by enzyme preparations, this substance can hardly be the true intermediate in the process; for it would seem apparent that the real intermediate must undergo reaction at least as rapidly as the parent substance. It is not justifiable, however, to exclude the possibility that the enol form of methyl glyoxal or some other modification may constitute the reactive precursor.

Harden has approached the problem from a different view-point, and has with his co-workers shown that the process of carbohydrate fermentation is not one involving only organic materials, but that inorganic phosphates are intimately concerned. By a long series of studies[10] they appear to have demonstrated conclusively that, if inorganic phosphate be added to a fermenting mixture, an excess of carbon dioxide (over that formed without the addition) is evolved in direct proportion to the amount of added phosphate. They have also shown that the phosphate enters into an organic combination which they have isolated and which, after considerable debate, has been accepted as being a hexose diphosphoric ester, $C_6H_{10}O_4(R_2PO_4)_2$.[*] By studying the relative amounts of the substance involved, Harden[11] has found that the reaction is represented by the equation

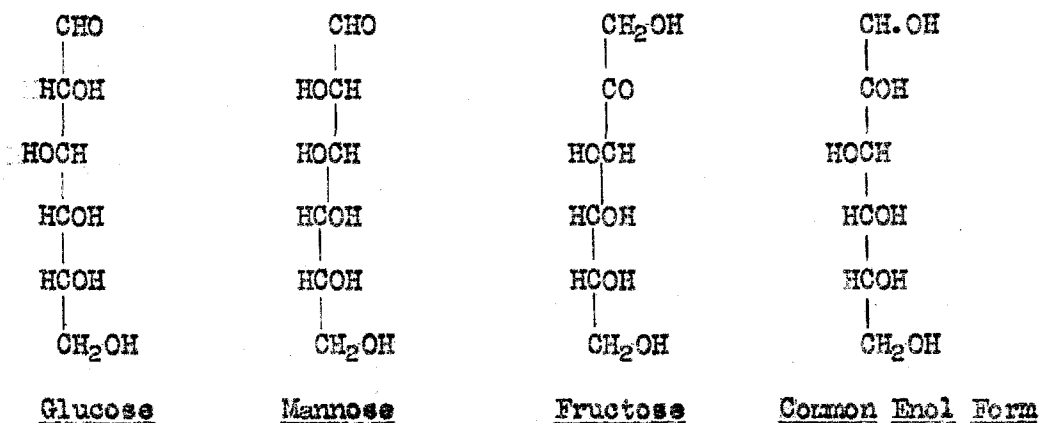(12) $2C_6H_{12}O_6 + 2R_2HPO_4 = C_6H_{10}O_4(R_2PO_4)_2 + 2CO_2 + 2C_2H_5OH + 2H_2O$.
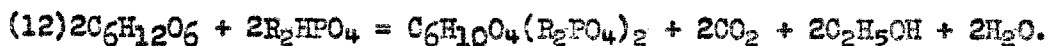
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[*]R is used to represent either Na or H.

He was also able to establish the fact that the diphosphate thus formed is hydrolyzed back to a hexose[12] (equation 13) by the agency of an enzyme which is present in the yeast preparations

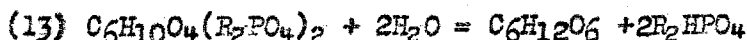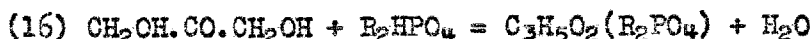(13) $C_6H_{10}O_4(R_2PO_4)_2 + 2H_2O = C_6H_{12}O_6 + 2R_2HPO_4$
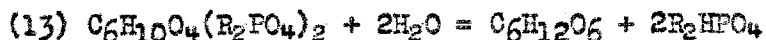
Additional work[13] has shown that identical diphosphates are formed whether glucose, fructose, or mannose be the hexose used. This has been interpreted by Harden[14] as showing that either the three hexoses are changed to their common enolic form (see below) previous to fermentation, or that only the half of the molecule which is common to all three is employed for the formation of the diphosphate, the other half undergoing decomposition to form alcohol and carbon dioxide. Of the two possibilities he was inclined to favor the latter. A representation of the three sugars may aid in visualizing the above arguments.

CHO	CHO	CH_2OH	CH.OH
HCOH	HOCH	CO	COH
HOCH	HOCH	HOCH	HOCH
HCOH	HCOH	HCOH	HCOH
HCOH	HCOH	HCOH	HCOH
CH_2OH	CH_2OH	CH_2OH	CH_2OH
Glucose	Mannose	Fructose	Common Enol Form

Lebedev, who has also investigated the phosphate question, has presented the view[15] that the hexose deomposes to dihydroxy acetone and glyceric aldehyde. According to his theory the latter forms a phosphate and recondenses, while the former undergoes fermentation.
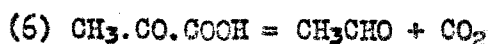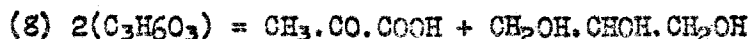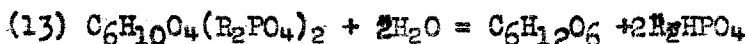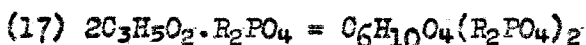
In equation form this beomces:

(14) $C_6H_{12}O_6 = CH_2OH.CHOH.CHO + CH_2OH.CO.CH_2OH$

(15) $CH_2OH.CHOH.CHO = CH_3CH_2OH + CO_2$

(16) $CH_2OH.CO.CH_2OH + R_2HPO_4 = C_3H_5O_2(R_2PO_4) + H_2O$

(17) $2C_3H_5O_2(R_2PO_4) = C_6H_{10}O_4(R_2PO_4)_2$

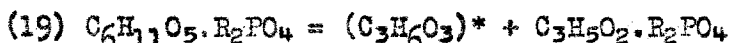(13) $C_6H_{10}O_4(R_2PO_4)_2 + 2H_2O = C_6H_{12}O_6 + 2R_2HPO_4$

Harden[16] has objected to this scheme on the ground that the phosphate plays an apparently minor part, while actually no fermentation whatever occurs in total absence of phosphate. He also points out that, if this scheme were correct, then glyceric aldehyde should undergo rapid fermentation, whereas it has been found that glyceraldehyde is almost unfermentable.[22]

New Theory

The new idea which it is desired to present is that a hexose monophosphate is first formed, and is then split by enzyme action into two dissimilar portions, one being a very reactive form of a triose and the other a triose phosphate. The triose phosphate is assumed to polymerize to the stable hexose diphosphate of Harden, while the reactive triose is the "enol" of Neuberg.

If we combine these new steps with others selected from the theories just given, the complete mechanism in equation form will read as follows:

(18) $C_6H_{12}O_6 + R_2HPO_4 = C_6H_{11}O_5.R_2PO_4 + H_2O$

(19) $C_6H_{11}O_5.R_2PO_4 = (C_3H_6O_3)* + C_3H_5O_2.R_2PO_4$

(17) $2C_3H_5O_2.R_2PO_4 = C_6H_{10}O_4(R_2PO_4)_2$

(13) $C_6H_{10}O_4(R_2PO_4)_2 + 2H_2O = C_6H_{12}O_6 + 2R_2HPO_4$

(8) $2(C_3H_6O_3) = CH_3.CO.COOH + CH_2OH.CHOH.CH_2OH$

(6) $CH_3.CO.COOH = CH_3CHO + CO_2$

(9) $(C_3H_6O_3) + CH_3CHO = CH_3.CO.COOH + CH_3CH_2OH$
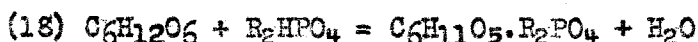
*This symbol has been described in the footnote, page 4.

It is to be noted that, if we omit equation (7), which represents a temporary initial reaction, and sum the remaining equations, then we arrive at the ordinary equation of fermentation (1). This represents the condition which obtains when the reactions have gone to completion, and no intermediate products remain. In the earlier stages of the process, the specific rate for (13) being low, the diphosphate accumulates, giving rise to the condition expressed by Harden in his equation of fermentation (12).

Further consideration shows that, if the specific rate for (19) is not too high as compared with that for (18), then during the earlier fermentation when the concentrations of sugar and phosphate are very large, monophosphate should appear, disappearing again as the sugar or inorganic phosphate become exhausted. The facts of the case are exactly in accord with this view, for it has been shown by Euler[17] that at the beginning of fermentation, esterification of inorganic phosphate exceeds carbon dioxide production, the two becoming equal as the reaction proceeds. While these facts are easily explained by the new hypotheses, they do not appear to have been included by any of the older theories.

Another quite similar observation, also due to Euler,[18] is that a peculiar yeast extract which he prepared was able to produce esterification of phosphate without any evolution of carbon dioxide. If through some abnormality of the enzyme system (18) were to proceed and some later step fail, just such a condition would result. Euler[19] did not isolate the organic phosphate in the above instance, but it would be predicted as being largely the mono-phosphate ester.

The final observation relating to this point, and probably the most important of all, is found in the recent work of Robison[20] on the isolation of a monophosphate ester. By adding inorganic phosphate in such amount as to maintain a maximum fermentation, and by then isolating the organic phosphates Robison was able to prepare a very fermentable mono-phosphate. It is to be noted that these are just the conditions which would be expected to lead to the greatest accumulation of the mono-phosphate. While the work is incomplete in that the ester was not shown to be a single mono-phosphate, yet the isolation would appear to be strong confirmation of the assumptions which have been made. It is particularly interesting that the initial rate of fermentation of this new ester is approximately as great as that for glucose and phosphate mixtures; exactly as would be required by the theory. That the initial rate soon diminishes would tend to confirm Robison's opinion that he was dealing with mixtures.

The other phase of the fermentation question, in which the modified theory appears to be particularly successful, is that pertaining to the fermentability of various intermediates which have been suggested. Of the four isomers which have been extensively examined in the past, namely lactic acid[21] ($CH_3CHOH.COOH$), methyl glyoxal[9] ($CH_3.CO.CHO$), glyceraldehyde[22] ($CH_2OH.CHOH.CHO$), and dihydroxy acetone[23] ($CH_2OH.CO.CH_2OH$) only the last is fermented with appreciable rapidity. As the present theory requires that some reactive isomer be the true intermediate, the non-fermentability of the first three substances can be explained by assuming that each is changed to the reactive form only very slowly. However, as the theory also requires that at least one of the possible phosphate esters of the

empirical formula $C_3H_5O_2 \cdot R_2PO_4$ be rapidly condensed to hexose diphosphate, it is also possible to account for the ready fermentability of the dihydroxy acetone. The explanation would be that the dihydroxy acetone undergoes easy conversion to a phosphate ester which is identical with that normally formed during fermentation, and which, therefore, enters the cycle by condensing to hexose diphosphate (equation 17). The appearance of the same diphosphate during the fermentation of dihydroxy acetone or of the hexoses[24] is interesting in view of this explanation. The fact that the other suggested substances do not undergo similar conversions may well depend upon their inability to form a condensable triose mono-phosphate.

In summarizing the proposed theory it may be said that it appears to unite much of the various theories which have been previously presented into a comprehensive whole. Harden's results become more obvious, while an origin of Neuberg's active precursor is provided. Explanations are provided for the known facts concerning the varying fermentabilities of certain intermediates which have been proposed in the past. Robison's researches on a hexose mono-phosphate are incorporated, as well as the peculiar results of Euler on esterification of phosphate without corresponding evolution of carbon dioxide. It seems possible that further application of the theory will be useful in the understanding of other peculiar results which have been obtained, particularly in the case of the mode of action of co-zymase.

Experimental Program

If the new theory which has been proposed actually represents the course of the fermentation process, certain experimental results would be expected. These may be briefly enumerated and then discussed.

It should be possible:

1. To demonstrate and perhaps to isolate a highly fermentable hexose mono-phosphate. The specific rate for its fermentation should be at least as great as that for its parent hexose. It is of the greatest importance to know whether the same monophosphate results from all fermentable hexoses.

2. To demonstrate at least traces of a triose phosphate during fermentations.

3. To isolate or synthesize a triose phosphate which is rapidly converted by the yeast enzymes to a diphosphate, identical with the hexose diphosphate which normally appears.

4. To demonstrate traces of the reactive or fermentable isomer of the formula $(C_3H_6O_3)$.

5. To isolate or more probably synthesize this reactive isomer and to show that it rapidly undergoes the changes postulated by Neuberg, when added to the proper enzyme systems.

It may be expected that, if the fermentable triose is as reactive, as has been assumed, then (4) and (5) above will be extremely difficult. While they are perhaps necessary for a conclusive proof of the theory, yet it seems hardly advisable to attempt them initially.

Also, while (1) is not only quite fundamental and much more probable
of success than the former pair, it can not be undertaken at the present
time, as Robison[20] has expressed his intention of continuing his investiga-
tions along these lines.

There remain (2) and (3) and these are intimately related. The most
promising initial investigation appears to be to attempt the synthesis
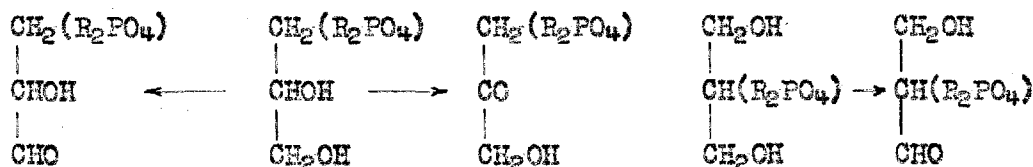of a triose phosphate which undergoes rapid condensation to hexose di-
phosphate, and then from a study of its properties to demonstrate it as a
normal constituent of enzymic fermentations. As we have no a-priori
grounds for postulating a particular triose phosphate, it becomes necessary
to synthesize them all. Of the three possible triose phosphates, namely
glyceraldehyde, α-phosphate, glyceraldehyde β-phosphate, and dihydroxy
acetone phosphate, only the last appears in the literature. This was
prepared[25] by reacting ethyl metaphosphate with dihydroxy acetone, and was
reported to reduce Fehlings solution after preliminary boiling with acids,
and to form an osazone melting at 143°, which still contained phosphorus.
The method would appear to be of general applicability, thus we may expect

$$
\begin{array}{ccccc}
CH_2(R_2PO_4) & CH_2OH & CH_2OH & CH_2OH & CH_2(R_2PO_4) \\
| & | & | & | & | \\
CHOH & \longleftarrow \ CHOH \longrightarrow & CH(R_2PO_4) & CO \longrightarrow & CO \\
| & | & | & | & | \\
CHO & CHO & CHO & CH_2OH & CH_2OH
\end{array}
$$
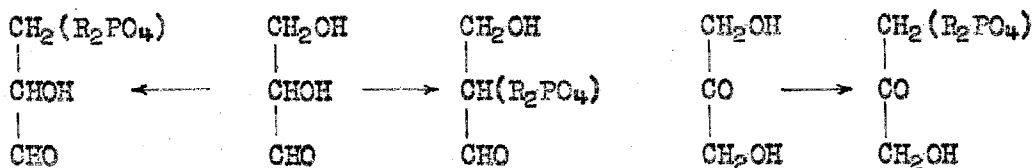
A second and perhaps more useful method would be the oxidation of the
known glycerophosphates.

$$
\begin{array}{ccccc}
CH_2(R_2PO_4) & CH_2(R_2PO_4) & CH_2(R_2PO_4) & CH_2OH & CH_2OH \\
| & | & | & | & | \\
CHOH & \longleftarrow \ CHOH \longrightarrow & CO & CH(R_2PO_4) \rightarrow & CH(R_2PO_4) \\
| & | & | & | & | \\
CHO & CH_2OH & CH_2OH & CH_2OH & CHO
\end{array}
$$

In the above the possibility of ring compounds has been excluded. Should it be found that no one of the three compounds above possesses the predicted properties, then it might be advisable to attempt the synthesis of such complex substances as

$$CH_2 = CR_2PO_4 - CHO \qquad or \qquad CH_2R_2PO_4 - CH - CHOH \!\!\! > O$$
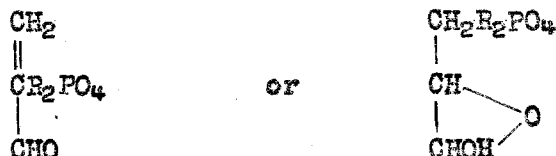
but this need not be considered further at this time.

While the synthesis and investigation of the triose phosphate is perhaps the most obvious method of approach, yet it should not be forgotten that the other suggested problems are equally important, and should be attempted when possible.

Experimental

The work thus far has been devoted to attempts to oxidize the two known glycerophosphates to aldehyde and ketone. The two isomeric glycerophosphates have been shown to give quite different disodium salts, one being nicely crystalline, and the other hygroscopic and very difficulty crystallized. Syntheses starting from allyl alcohol in one case[26] and from $\alpha\alpha$-dichlorhydrin in the other[27] have proved the crystalline isomer to be the β-phosphate and the other to be the α-phosphate.

From Eastman a solid purified salt was secured. Crystallization from concentrated solutions gave a large yield of a beautifully crystalline material which was undoubtedly the β-glycerophosphate.

Monsanto Chemical Company supplied a 50% syrup of disodium phosphate which was claimed to be largely the α form. On evaporating to a very thick syrup and cooling for several days with frequent stirring, only a small proportion of crystalline material separated indicating only a small amount of the β form. Extraction of the highly viscous residual mass with several lots of absolute alcohol with subsequent evaporation of the alcoholic extract gave only a small residue, indicating very little free glycerol. Evaporation of some of the original solution for some time on the water-bath showed a solid content of about 50% making the solution approximately $2\frac{1}{2}$ molal.

β -glycerophosphate. - Great difficulty has been experienced in the attempted oxidation of the β isomer. In the first place, it can be oxidized only with the utmost difficulty and is not even comparable in this respect with glycerol. Moreover, in all the many attempts so far tried, even when the oxidizing agent was completely reduced, no aldehyde remained at the end of the experiment. Even when much less than theoretical amounts of oxidants were used, the same results were obtained. This is presumably due to the fact that very strong oxidizing agents have been found necessary to initiate any oxidation at all, and therefore probably oxidize the aldehyde first formed to acid.

A typical run with bromine and varying amounts of sodium carbonate at different temperatures may be given as an illustration. The carbonate and bromine were added in amounts to correspond with the following equations:

(a) $C_3H_7O_2 \cdot PO_4Na_2 + Br_2 = C_3H_5O_2PO_4H_2 + 2NaBr$

(b) $C_3H_7O_2 \cdot PO_4Na_2 + Br_2 + \frac{1}{3}Na_2CO_3 = C_3H_5O_2 \cdot PO_4HNa + \frac{1}{3}CO_2 + \frac{1}{3}H_2O + 2NaBr$

(c) $C_3H_7O_2 \cdot PO_4Na_2 + Br_2 + Na_2CO_3 = C_3H_5O_2PO_4Na_2 + CO_2 + H_2O + 2NaBr$
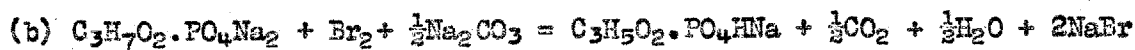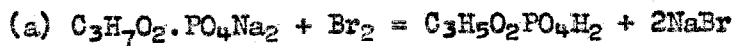
Tests were made with the typical aldehyde-ketone reagents: Fehlings solution in the cold (F_c) and heated (F_h), Schiffs reagent (S), and alkaline permanganate (K). The method used in reporting is: -, test negative; ± questionable; + faintly positive; ++ positive; +++ strongly positive; ++++ very strong test.

					F_c	F_h	S	K
(a)	No Na_2CO_3	100°,	30 min.		-	-	++	±
(a)	"	"	45°, 18 hrs.		-	-	+++	-
(a)	"	"	20°	"	-	-	+	-
(b)	$\frac{1}{3}$	"	"	"	-	-	+	±
(c)	1	"	"	"	-	-	±	±

It will be noted that even in the first mixture which became acid during the reaction, no reducible substance was formed. The positive results found with the Schiffs reagent merely point to some residual oxidizing agent.

As oxidants chromic anhydride in glacial acetic acid and chromates with sulfuric acid were tried, as well as those reagents which have been particularly recommended for the partial oxidation of glycerol. In this latter group were bromine[28], hypobromites[29], hydrogen peroxide in the presence of ferrous salts[30] and nitric acid[31] of varying strengths. In no case were more than traces of aldehydes ever indicated, although a number of different concentrations and temperatures were tried.

Apparently it will be necessary to attempt this reaction not only in acid solution and at a low temperature, but also, if possible, in the presence of some reagent which will unite with the aldehyde as the latter forms and thus remove it from the sphere of action.

α-glycerophosphate. - The oxidation of the α-isomer has proved somewhat more successful, though as yet the desired product has not been isolated. After trying several of the above-mentioned reagents, it was found that best results were apparently secured by using bromine as the oxidizing agent*. While alkali carbonates added to the bromine mixture appear to speed up the reaction, the amount of reducing substance produced is somewhat decreased, so their use has been abandoned. As now performed the 50% glycerol phosphate solution is mixed with an equal volume of water and the theoretical amount of bromine is added. Small batches are used, as better results are obtained for some unknown reason. The mixture is allowed to stand with frequent shaking at room temperature until the bromine disappears. On testing such a batch the following results are usually found (using the nomenclature described above): F_c, ++; F_h, +++; S, + or ++; K, +++. On applying Denigés color tests[32] which are supposed to be specific for dihydroxyacetone, the test is ordinarily ++ or +++.

It remains to isolate the substance to which these tests are due. As the original and final substances are almost identical, it is not to be expected that the isolation will be simple. On adding absolute alcohol it is possible to separate an oil which shows all the above tests, but this

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*Grimbert and Bailly[33] used bromine water in their work on the structure of the glycerophosphates. They made no attempt to isolate the product of the oxidation, but merely proved that the mixture gave the usual aldehyde-ketone tests and Denigés test for dihydroxy acetone.

fails to accomplish much, as the original glycerophosphate is likewise precipitated. Barium and calcium salts likewise precipitate both compounds, and no salts have been found which differentiate between the two. Because of the phosphate group, the substances can not be distilled, although this might serve to remove any phosphate-free substances which may have been formed in the reaction.

Fractional crystallization appears most hopeful, as the α-glycerophosphate is practically non-crystallizable. On one occasion by evaporating the reacted mixture to a small bulk and cooling to about 10° a crystalline salt was isolated. This decomposed on heating, giving an odor like that of burnt sugar (unlike the original glycerophosphate), and left an inorganic residue. It gave a +++ test with alkaline permanganate and reduced Fehlings solution slowly on boiling. After being first boiled with acid, it reduced Fehlings solution rapidly and in the cold. On the other hand it showed no Schiffs reaction, gave negative results with Denigés dihydroxy acetone tests, and in the one experiment which was tried, formed no phenyl hydrozin derivative. Only a small amount of the substance was avilable and subsequent attempts to prepare it have thus far been without avail.

The work on both the α and β isomers is being continued.

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