DISODIUM PHOSPHATE AS A CATALYST FOR THE QUANTITATIVE OXIDATION OF GLUCOSE TO CARBON DIOXIDE WITH HYDROGEN PEROXIDE.

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The experiments described in this paper represent a confirmation and extension of part of Löb's observations on the influence of phosphates on oxidative glycolysis. By the experiments herein described it is proved that disodium phosphate catalyzes the quantitative oxidation of glucose to carbon dioxide by hydrogen peroxide. Additional experiments on the influence of the carbonates of sodium and other compounds are included and a partial interpretation of the results is offered.

Previous experiments on the influence of phosphates on the oxidation of butyric acid[1] with hydrogen peroxide were being extended by further experiments when it was realized that if the results of Löb and his coworkers,[2,3] on the influence of phosphates on glucose oxidation with peroxide, could be demonstrated by an adequate method the results would help clarify the influence of phosphates on butyric acid oxidation and have considerable interest in other ways. The results of some earlier work on the oxidation of glucose[4] indicated that probably the amount of oxidation observed by Löb could be exactly determined by the method suggested by those data. This was confirmed.

[1] Witzemann, E. J., *J. Biol. Chem.*, 1918, xxxv, 83.

[2] Löb, W., and Pulvermacher, G., *Biochem. Z.*, 1910, xxix, 316. Löb, W., and Gutmann, S., *Biochem Z.*, 1912, xlvi, 288. Beysel, W., and Löb, W., *Biochem. Z.*, 1915, lxviii, 368.

[3] Löb, W., *Biochem. Z.*, 1911, xxxii, 43.

[4] Witzemann, E. J., *J. Am. Chem. Soc.*, 1916, xxxviii, 150.

The statements under consideration as given in Löb's summary[3] are quite definite and are in part as follows:

"(1) In salt-free sugar solutions hydrogen peroxide produces only a vanishingly small amount of oxidative glycolysis.

(2) The glycolysis is markedly increased by raising the hydroxyl ion concentration.

(3) With the small OH ion concentrations in solutions having the alkalinity of blood, which is only slightly different from that of water, glycolysis is very slight if it is not accelerated by phosphates.

(4) The phosphate ions accelerate the glycolysis by the OH ions; the most favorable OH ion concentration within the limits tested lies at pH 8.302 to 7.070. At pH $\lesseqgtr$ about 5.600 there is no longer a perceptible OH ion effect exceeding that of pure water, even in the presence of phosphate ions.

(5) The acceleration of the glycolysis increases with constant OH ion concentration with increase in the absolute amount of phosphate added."

An examination of the experimental data, however, leaves one in doubt as to whether Löb really measured the oxidative glycolysis. In fact Michaelis and Rona[5] were not convinced by Löb's data and interpreted his observations differently. Obviously his determination of optical rotation and the reduction of Fehling solution by the glucose solutions before and after oxidation was not a determination of the absolute amount of oxidation. Consequently the term "oxidative glycolysis," which he uses to describe these phenomena, might include two processes.

(*a*) Destruction of glucose by oxidation at the expense of oxygen from the hydrogen peroxide used. This is what Löb meant.

(*b*) Destruction of glucose by intramolecular rearrangement under the influence of alkali. This kind of chemical change is what Michaelis and Rona appear to think Löb really saw at least in part.

If a neutral phosphate system such as Löb used, which is known to be a constituent of many living organisms, has any considerable effect upon oxidation the scope and nature of the effects should be known. The possible importance of such facts biologically for instance, when considered in relation to the well known indispensible relationships between phosphates and much normal cellular oxidation, is too obvious to require further comment.

[5] Michaelis, L., and Rona, P., *Biochem. Z.*, 1912, xlvii, 447.

The data described in this paper are sufficiently definite to give a new interest to the many facts already in hand in this field and to serve as a definite point of reference in the further study of these questions.

The results described here have a general interest in another way also. In the interpretation of the action of alkaline substances on sugars two points of view are recognized. According to the one the known effects of alkaline substances on sugars are due essentially to the hydroxyl ions. The other older view recognizes that the undissociated molecules and other ions may also aid or produce other effects than those of the so called hydroxyl ion effects. Without reviewing this problem any further in this paper it may simply be stated that the data herein presented offer varied and interesting support for the latter view.

EXPERIMENTAL.

Considering the importance of Löb's claims from several points of view it seemed highly important to determine accurately how much oxidation actually took place in his experiments with phosphates. This, it was thought, could be done, by applying the results of the author's previous study of the complete oxidation of glucose with potassium permanganate[4] to the analysis of the results obtained by Löb's experiments.

1. Methods of Analysis.—Previous experiments on the oxidation of glucose showed that in alkaline solution it is quantitatively oxidized to carbon dioxide and oxalic acid with potassium permanganate. The oxalic acid in turn is quantitatively oxidized to carbon dioxide by permanganate in sulfuric acid solutions. The plan was therefore as follows:

1. Oxidize glucose with hydrogen peroxide in the presence of phosphates just as Löb did.

2. After the expiration of the proper time interval add excess manganese dioxide to decompose unchanged hydrogen peroxide.

3. After decomposition is complete filter off the manganese dioxide, washing the filter and the original flask thoroughly.

4. Add excess sodium hydroxide solution.

5. Add an accurately known amount but excess of a strong accurately standardized solution of potassium permanganate

(about 3 gm. per 100 cc.). Heat this mixture to boiling and set aside over night after covering the top of the hot flask.

6. Add excess concentrated sulfuric acid.

7. Add an accurately known amount but excess of an accurately standardized solution (about 6 gm. per 100 cc.) of oxalic acid.

8. Dilute the clear colorless solution to a convenient definite volume and using an aliquot portion titrate back the excess oxalic acid with dilute potassium permanganate solution (0.1 N).

9. Calculate the total permanganate required for complete oxidation of the solution in No. 8, add the permanganate added in the beginning, and substract the permanganate equivalent of the oxalic acid used. The result is the amount of permanganate utilized by the glucose or other incompletely oxidized compounds present and may easily be calculated to its glucose equivalent.

In order to test the accuracy of the above method a solution of pure glucose containing 10 gm. per liter was prepared. 20 cc. of this solution, containing 0.200 gm. of glucose, 15 cc. of 35 per cent sodium hydroxide solution, and 75 cc. (= 2.028 gm.) of potassium permanganate solution were heated to boiling. After standing over night excess concentrated sulfuric acid was added and then 50 cc. (≡ 1.519 gm. of $KMnO_4$) of an oxalic acid solution. This colorless solution was diluted to 500 cc. in a graduated flask. 25 cc. portions were titrated back with 0.0996 N potassium permanganate. 5.70 cc. were required. $5.70 \times 20 \times 0.003146 = 0.359$ gm. of $KMnO_4$ required for the excess oxalic acid that was added.

2.028	gm.	$KMnO_4$ originally added.
0.359	"	$KMnO_4$ required for excess oxalic acid.
2.387	"	$KMnO_4$ used (total).
1.519	"	$KMnO_4$ equivalent of oxalic acid added.
0.868	"	$KMnO_4$ reduced by the glucose.

Since 2.40 molecules of $KMnO_4$ are required to oxidize 1 molecule of glucose to carbon dioxide the equation

$$758.4 : 180 = 0.868 : x$$
$$x = 0.206 \text{ gm. glucose}$$

gives the amount of glucose originally present.

Another oxidation made at the same time gave 0.198 gm. of glucose.

These were the results obtained with the first pair of oxidations tried and give fairly the maximum analytical error as demonstrated by subsequent experience. The results show that the method will be satisfactory provided the amount of oxidation observed exceeds the experimental error of 2 or 3 per cent.[6]

Experiments with the Phosphates of Sodium.

2. Repetition of Löb's Experiments.—Having established the fact that it is possible to determine glucose quantitatively in the proposed way the author repeated and analyzed by the method described above a number of the experiments carried out by Löb.

In Table I the results obtained in five oxidations carried out at room temperature for just 1 week are given. The results are calculated as though the oxygen required by the unoxidized compounds in the solution was all consumed by unchanged glucose. This is almost certainly not entirely true but since the incompletely oxidized compounds are possibly a complex mixture, difficult to analyze,[7] it seemed permissible and correct for the purposes of comparison to calculate the permanganate consumed to glucose. The results show that the influence of the phosphates

[6] A similar method was developed by Greifenhagen and coworkers (Greifenhagen, W., König, J., and Scholl, A., *Biochem. Z.*, 1911, xxxv, 169), and was found sufficiently accurate in use by Levene and Meyer (Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 265). These results were discovered after the completion of my own work.

[7] On the basis of Löb's earlier work (Löb, W., *Biochem. Z.*, 1908, xii, 78, 466; 1909, xvii, 132. Löb, W., and Pulvermacher, G., *Biochem. Z.*, 1909, xvii, 343. Löb, W., *Biochem. Z.*, 1909, xx, 516; xxii, 103; 1910, xxiii, 10; xxvi, 231), but specifically on the basis of a later statement (Löb, W., *Biochem. Z.*, 1915, lxviii, 368) it might be concluded that the incompletely oxidized compounds are formic and polyhydroxy acids arising from formaldehyde and pentoses. Tests on solutions from complete oxidations known to reduce permanganate equivalent to 0.02 to 0.04 gm. glucose in 75 cc. gave distinct tests for sugar with Haines' or Fehling's solution. Since this is near the limits of sensitiveness of these reagents, it appears that no large proportion of intermediate oxidation products (between hexose and CO_2) can be present. This appears to conform with the observations of Smolka (Smolka, A., *Sitzungsb. Math. Natur. Akad. Wiss.*, 1887, xcv, pt. ii, 5) on the oxidation of glucose with insufficient neutral permanganate, who recovered only final oxidation products (HCO_2H, $H_2C_2O_4$, and CO_2) and the calculated amount of unchanged glucose.

is progressively greater with increasing concentration, but that it is not a linear function of the concentration since the relative acceleration diminishes with increasing phosphate concentration.

The results in Table II were obtained under exactly the same conditions as those in Table I except that the solutions were kept 98 hours (4 days, 2 hours) in an incubator at 37°C.

TABLE I.

Glucose + H_2O_2 + Phosphates at Room Temperature.

20 cc. glucose solution (0.200 gm.) + 20 cc. 3 per cent H_2O_2 in total volume of 75 cc.

No.	0.33 M Na_2HPO_4.	0.33 M NaH_2PO_4.	H_2O	Reaction.	Glucose recovered.	Glucose oxidized.
	cc.	*cc.*	*cc.*	*pH*	*gm.*	*per cent*
1	0.0	0.0	35	7.07	0.2006	0.00
2	1.6	0.4	33	7.347	0.1863	6.85
3	6.4	1.6	27	7.347	0.1210	39.50
4	16.0	4.0	15	7.347	0.0658	67.10
5	25.6	6.4	3	7.347	0.0442	77.90

TABLE II.

Glucose + H_2O_2 + Phosphates at 37°.

20 cc. glucose solution (0.200 gm.) + 20 cc. 3 per cent H_2O_2 in total volume of 75 cc.

No.	0.33 M Na_2HPO_4.	0.33 M NaH_2PO_4.	H_2O	Reaction.	Glucose recovered.	Glucose oxidized.
	cc.	*cc.*	*cc.*	*pH*	*gm.*	*per cent*
1	0.0	0.0	35	7.07	0.186	6.50
2	1.6	0.4	33	7.347	0.1163	41.85
3	6.4	1.6	27	7.347	0.019	90.50*
4	16.0	4.0	15	7.347	0.034	83.00
5	25.6	6.4	3	7.347	0.035	82.50

* In the experiments at 37° it was generally observed that oxidation was less complete in No. 5 than in Nos. 3 or 4. Special experiments to interpret this apparent anomaly have not been done but the effect appears to be due to the fact that the velocity of oxygen activation by the Na_2HPO_4 is greater than the velocity of oxygen consumption and consequently the excess active oxygen is lost as such from the reaction mixture. This interpretation is so far supported by facts given in this paper and by others not mentioned.

3. Fate of the Glucose.—The results given above prove quite conclusively that the glucose is oxidized. Of the large number of compounds into which it could conceivably be converted without oxidation only a small number are not completely oxidizable to carbon dioxide by permanganate in acid or alkaline solution.[8] Nevertheless it seemed necessary to demonstrate actually that carbon dioxide was an important product of this oxidation.

All the experiments on carbon dioxide recovery were done with mixtures corresponding to No. 5 in Tables I and II. In determining the CO_2 the oxidation mixture was placed in a round bottom flask attached to a reflux condenser and arranged so that CO_2-free air could be bubbled through the mixture and then passed through wash bottles containing clear barium hydroxide solution.[9] On warming the flask nearly all the CO_2 was driven over. Excess dilute sulfuric acid was finally added and the mixture heated to boiling.

A. 75 cc. of such a solution, which had been kept in the incubator until all peroxide was gone and in which oxidation was nearly complete, gave in the CO_2 apparatus 0.13 gm. of barium carbonate or about 10 per cent of the calculated CO_2 yield. The rest of the CO_2 had been lost into the air.

B. 75 cc. of such a solution after 10 days at room temperature gave 0.69 gm. of $BaCO_3$, equivalent to 0.154 gm. of CO_2 or a 52.6 per cent yield of CO_2. The solution, to which excess sulfuric acid had been added while in the CO_2 apparatus, was alkalinized with sodium hydroxide, treated with MnO_2 to remove unchanged peroxide, filtered, and treated as usual with permanganate. The permanganate consumed was equivalent to 0.0749 gm. of glucose or 37.5 per cent recovered. $52.6 + 37.5 = 90.1$ per cent of the 0.200 gm. of glucose used recovered in this way.

Results similar to this were obtained under the same conditions a number of times.

[8] It was not until the experiments described above had been completed that it was suspected that oxidation to CO_2 was nearly quantitative. Löb expressed the opinion that formic and polyhydroxy acids are the main products and there was no reason to doubt this until the small amount of permanganate required to complete the oxidation suggested that the oxidation might already be largely completed to CO_2.

[9] Evans, W. L., and Witzemann, E. J., *J. Am. Chem. Soc.*, 1912, xxxiv, 1086.

C. In order to obtain a more complete conversion into CO_2 and a good recovery the oxidation was set up in the incubator. Two strong round bottom 300 cc. flasks, one of which contained barium hydroxide solution and the other the glucose oxidation mixture, were connected by a glass tube having two right angle bends in rubber stoppers. The stoppers were wired in and then covered over with molten paraffin. The whole was placed in the incubator at 37°C. and agitated a few moments every day for a week. It was then taken out and allowed to stand at room temperature several days with occasional agitation. The oxidation mixture gave 0.28 gm. of $BaCO_3$ in the CO_2 apparatus. The attached $Ba(OH)_2$ flask gave 0.83 gm. of $BaCO_3$. This corresponds to 0.2474 gm. of CO_2 altogether or an 84.4 per cent yield of CO_2. The oxidation mixture treated as in (B) reduced permanganate equivalent to 0.0195 gm. of glucose or 9.8 per cent of the glucose used. $84.4 + 9.8 = 94.2$ per cent of the glucose recovered in this way.

On the basis of these results there can be no doubt that the glucose unaccounted for by the permanganate consumed is really oxidized to CO_2.

In developing the above proof that practically quantitative oxidation to CO_2 is obtained several other facts were observed.

1. The carbon dioxide formed is freely and easily lost from the solution during the oxidation even at the room temperature. In this the oxidation resembles vital oxidation in which the carbon dioxide is spontaneously lost during respiration. As much as two-thirds or more of the carbon dioxide obtained is evolved and absorbed by barium hydroxide in a closed apparatus at 37°C.

Ordinary alkaline oxidation systems, although they undergo changes in many ways similar to those occurring in living organisms, differ in that the CO_2 formed is bound and held in the system as carbonate or bicarbonate. This easy formation and loss of CO_2 is probably the most important physical characteristic of a vital oxidation system. It is not yet certain to what extent the phosphate systems can carry out the other functions belonging to alkaline systems, that are so important in the non-oxidative transformations of sugar in organisms, but indications are not lacking that they can also aid in some of these changes under suitable conditions.

2. The solutions in which all hydrogen peroxide had disappeared and which contained material oxidizable by permanganate equivalent to only 0.02 to 0.04 gm. of glucose in 75 cc. (*i.e.*, 0.027 to 0.053 per cent) reduced Fehling solution distinctly. Since this is close to the limits of sensitiveness of this test with pure glucose it is clear that most of the glucose attacked had been completely burned to carbon dioxide, and that no appreciable quantity of intermediate products such as polyhydroxy acids could be present.

4. Influence of Additional Glucose and Peroxide.—On the basis of the results in the preceding section it was of considerable interest to know whether the same phosphate mixture would repeatedly catalyze the oxidation of glucose. In other words whether the products of the reaction in any way "poison" the catalyst. If CO_2 is the sole final product and if it is evolved as was shown above, the phosphate mixture should serve repeatedly in this oxidation just as it is known to do in the fermentation of glucose.[10]

Experiment 5, Table II, was set up in the incubator. After 3 days it was free from peroxide. 0.20 gm. of glucose and 20 cc. of 3 per cent peroxide were again added. After 1 week in the incubator the peroxide had again disappeared. The same materials were again added. After another week this was repeated. On determining the permanganate consumed in the usual way it was found to correspond to 0.0831 gm. of glucose. Since 0.80 gm. of glucose had been used this corresponds to 10.4 per cent of the glucose used, which is about what is recovered from a single experiment of this kind.

These results show that the functional activity of the disodium phosphate is not impaired in the catalysis. Since this does not occur it is clear that the disodium salt is not changed into monosodium phosphate by the carbonic acid, nor any other acid intermediate oxidation product, to any marked extent. If sodium bicarbonate were formed in this way the oxidation would be retarded or stopped in the typical way in which this compound acts (*cf.* Section 7).

[10] Harden, A., and Young, W. J., *J. Chem. Soc.*, 1905, xxi, 189; *Proc. Roy. Soc. London, Series B*, 1906, lxxvii, 405; 1908, lxxx, 299; 1909, lxxxi, 336. Young, W. J., *Proc. Roy. Soc. London, Series B*, 1909, lxxxi, 529. Harden, A., and Young, W. J., *Biochem. Z.*, 1911, xxxii, 173. Young, W. J., *Biochem. Z.*, 1911, xxxii, 177.

5. Influence of Changing the Ratio of the Phosphates.—The results in Table III constitute a repetition of part of Löb's experiments (Table XI)[3] on the influence of a change in the ratio of the two phosphates. All the experiments were set up in 250 cc. Florence flasks and kept in the incubator for 45 hours before analyzing.

TABLE III.

25 cc. (0.25 gm.) glucose + 25 cc. H_2O_2 + 20 cc. salt solution + 5 cc. water at 37°.

No.	0.33 M Na_2HPO_4.	0.33 M NaH_2PO_4.	H_2O	Reaction.	Glucose recovered.	Glucose oxidized.
	cc.	*cc.*	*cc.*	*pH*	*gm.*	*per cent*
1	0	0	25	7.07	0.2407	3.7
2	16	4	5	7.347	0.1992	20.3
3	10	10	5	6.813	0.2068	17.3
4	4	16	5	6.239	0.2316	7.4
5	2	18	5	5.910	0.2342	6.3

TABLE IV.*

20 cc. glucose (0.200 gm.) + 20 cc. 3 per cent H_2O_2 at 37° for 10 days.

No.	0.33 M Na_2HPO_4.	0.33 M NaH_2PO_4.	H_2O	Glucose recovered.	Glucose recovered after 2 days.
	cc.	*cc.*	*cc.*	*gm.*	*gm.*
1	25.6	6.4	59	0.0242	0.0387
2	25.6	32.0	34	0.0180	0.0271
3	25.6	64.0	2	0.0166	

* All the experiments in this table have a total volume of 131 cc. The reaction of No. 1 is distinctly alkaline to litmus paper while that of No. 3 is distinctly acid. Accordingly pH passes from a point on the alkaline side (about 7.347) to a point decidedly on the opposite or acid side of neutrality.

The results show a diminishing velocity of glucose oxidation as the ratio of monosodium phosphate used increases or as the ratio of disodium phosphate decreases.

From these experiments alone it might be concluded that the OH ion is significant in this oxidation but results given in the next paragraph do not confirm this idea.

When the ratio of the two phosphates is changed by changing the amount of monosodium phosphate but keeping the disodium phosphate constant in amount different results are obtained.

The results in Table IV show that in the presence of a constant amount of disodium phosphate increasing amounts of monosodium phosphate do not retard the oxidation of glucose. In fact the presence of the monosodium phosphate seems to facilitate the completion of the glucose oxidation in spite of the fact that relatively No. 3 is comparable with No. 4 in Table III as far as the proportion of the two phosphates is concerned. Exactly the same result was obtained with Nos. 1 and 2 when they were allowed to react only 2 days. No velocity experiments have been made to determine whether the excess NaH_2PO_4 retards the oxidation as it does the evolution of O_2 from H_2O_2 but the results as given indicate that it does not.

It seems clear that if Löb had done these experiments, as well as some others described below, he would have found it impossible to ascribe so much influence to the OH ions in this catalysis, as he did.

6. Influence of Time on the Oxidation.—In order to follow the glucose oxidation from day to day a large experiment containing

TABLE V.

Glucose.

		gm.
	At beginning contained	0.200
(*a*)	After 24 hrs. "	0.1869
(*b*)	" 48 " "	0.1611
(*c*)	" 72 " "	0.1551*
(*d*)	" 96 " "	0.1212
(*e*)	" 120 " "	0.1173*
(*f*)	" 168 " "	0.0823

* When the results described in this table are plotted the two values marked with the asterisk lie considerably outside the curve. This is due to the fact that undecomposed hydrogen peroxide was still present when the potassium permanganate was added. Thus when two solutions containing exactly the same amount of glucose but one of which also contained 5 cc. of 3 per cent hydrogen peroxide were analyzed, without decomposing the peroxide, the former was found to contain 0.1802 gm. of glucose by the complete oxidation method. The other containing the peroxide apparently contained 0.2165 gm. of glucose when calculated on the basis of the oxygen consumed. This difference is due to the well known fact that hydrogen peroxide reduces permanganate with the evolution of oxygen.

The results are given in this form in order to illustrate this error.

six times as much material as No. 2 in Table II was set up and placed in the incubator at 37°. 75 cc. of this solution (corresponding to 0.200 gm. of glucose) were taken out for analysis at definite intervals as indicated in Table V.

Experiments with the Carbonates of Sodium.

The preceding results clearly confirm Löb's claim that in the presence of phosphate mixture glucose is oxidized by hydrogen peroxide. Since he failed to observe appreciable amounts of oxidation when he used the other common reaction regulator mixtures it seemed unnecessary to test these again for the present. It did seem advisable, however, to make some experiments with the carbonates of sodium for several obvious reasons.

7. Influence of Sodium Carbonate-Bicarbonate.—If the phosphates do not exercise a catalytic effect in this oxidation and the effect observed is due to OH ions then an equimolecular amount of sodium carbonate and bicarbonate should have fully as much effect. That this is not true was definitely established by the following experiment in which 2.43 gm. of $Na_2CO_3.10H_2O$, 0.72 gm. of $NaHCO_3$, 35 cc. of distilled water, 20 cc. (0.200 gm.) of glucose solution, and 20 cc. of 3 per cent H_2O_2 were kept 4 days at 37°. Upon analysis the peroxide was found to have been completely decomposed and equivalent of 0.1902 gm. of glucose was recovered; *i.e.*, 5 per cent was apparently oxidized as against 80 per cent oxidized with the corresponding phosphate mixture.

In the above experiment the two carbonates were used in the same molecular amounts and proportion as the two phosphates in Experiments 5 in Tables I and II. The solution therefore contained at least the same amount of available alkali but had a somewhat higher OH ion concentration than the phosphate mixture referred to. If OH ion concentration and available alkali are the controlling factors in these oxidations this experiment should have shown as much or more oxidation than was obtained with the phosphate mixture.

8. Influence of H_2CO_3 and $NaHCO_3$.—The results in Table V suggest that the velocity of decomposition of sodium bicarbonate, possibly produced in the oxidation, may be a factor in determining the velocity of oxidation. The following three experiments were

done in order to test the influence of this condition. The experiments were set up in similar 250 cc. flasks and kept in the incubator for 24 hours at 37° after which they were analyzed in the usual manner.

(1) 32.0 cc. of 0.33 M NaH_2PO_4 solution + 1.22 gm. of $Na_2CO_3.10H_2O$. This mixture effervesced in the cold. It was heated to boiling to expel CO_2, cooled, and the following were added:

20 cc. (0.200 gm.) of glucose solution, 20 cc. of 3 per cent H_2O_2, 3 cc. of distilled water. 0.0761 gm. of glucose was recovered.

(2) Components the same as in (1).

All ingredients were mixed except the peroxide be
the $Na_2CO_3.10H_2O$ in order to prevent the loss of C
as possible. 0.1906 gm. of glucose was recovered.

(3) 25.6 cc. of 0.33 M Na_2HPO_4 solution.
6.4 " " 0.33 " NaH_2PO_4 "
20.0 " (0.200 gm.) of glucose solution.
20.0 " of 3 per cent H_2O_2.
3.0 " " distilled water.

0.0774 gm. of glucose was recovered.

On the basis of the conditions of the experiments the results of (1) and (3) were expected to be identical because the reaction mixtures as used were identical. As a matter of fact the amount of glucose recovered was nearly the same in (1) and (3). It was expected that the oxidation in (2) would be somewhat slower. In fact only 5 per cent of the glucose was oxidized in (2) as compared with over 60 per cent in the others. This indicates that not only do H_2CO_3 and $NaHCO_3$ not catalyze the oxidation of glucose with hydrogen peroxide but that they actually retard it.

The interpretation of the influence of the sodium carbonate added in (2) has not been fully established as yet. There are several factors to be considered, three of which are as follows: (*a*) Na_2CO_3 may under the conditions in (2) not react completely to give only Na_2HPO_4 and H_2CO_3; (*b*) if so, any Na_2CO_3 or $NaHCO_3$ remaining would rapidly catalytically decompose the H_2O_2; (*c*) the presence of CO_2 to the point of supersaturation may retard the activation or dissociation of H_2O_2.

9. Influence of Sodium Carbonate.—The following experiments were done in order to determine what influence sodium carbonate exercises on the action of disodium phosphate.

(1) 32.0 cc. of 0.33 M Na_2HPO_4 solution.
3.0 " " water.
20.0 " " glucose solution (0.200 gm.).
20.0 " " 3 per cent hydrogen peroxide.
0.0311 gm. of glucose was recovered.

(2) Same as in (1) with 0.61 gm. of $Na_2CO_3.10H_2O$.
0.1741 gm. of glucose was recovered.

(3) Same as (1) with 1.22 gm. of $Na_2CO_3.10H_2O$.
0.1737 gm. of glucose was recovered.

The solutions were kept in the incubator at 37°C. for 45 hours and on analysis the amounts of unchanged glucose given were found. All hydrogen peroxide present had been decomposed.

The results show that sodium carbonate exercises a strongly negative influence on this oxidation in spite of the fact that the OH ion concentration is higher in (2) and (3) and the available alkali in (3) is twice what it was in Experiments 5, Tables I and II.

This negative influence on the final result of the oxidation may be due simply to the fact that the velocity of decomposition of peroxide by Na_2CO_3 is many times greater than that by Na_2HPO_4 and that the glucose oxidation induced by Na_2CO_3 itself is relatively small in comparison.

The above observations on the influence of the carbonates of sodium permit us to conclude that, whatever the mechanism of CO_2 formation in this oxidation may be, carbonates of sodium are not intermediate stages in the process of CO_2 liberation.

10. Influence of Sodium Hydroxide.—The following three experiments were done in order to determine the influence of sodium hydroxide on the effect of the phosphate mixture.

(1) 25.6 cc. of 0.33 M Na_2HPO_4 solution + 6.4 cc. of 0.33 M NaH_2PO_4 + 20 cc. (0.200 gm.) of glucose solution + 20 cc. of 3 per cent H_2O_2 + 3 cc. of water.

(2) The same as (1) except that one-half the water was replaced with 1.5 cc. (0.0857 gm.) of NaOH solution.

(3) The same as (1) except that all the water was replaced with 3 cc. (0.1714 gm.) of NaOH solution.

After 50½ hours at 37°C. the solutions contained no unchanged peroxide. They were analyzed and found to reduce $KMnO_4$ corresponding to glucose as follows:

(1) 0.0088 gm. of glucose.
(2) 0.0140 " " "
(3) 0.0618 " " "

The sodium hydroxide has a perceptible but not a large retarding effect which is interpreted tentatively in the light of other experiments as due simply to its effect in increasing the decomposition of the hydrogen peroxide. In this respect its activity is not so great as that of the carbonates which coincides with its smaller retarding effect on the action of the phosphate mixture.

11. Partial Interpretation of the Influence of Disodium Phosphate.—Since there are three compounds actively concerned in this oxidation reaction and since glucose and peroxide alone do not react appreciably there remain three possible ways of interpreting the reaction on the basis of the formation of molecular complexes, which are so frequently found to underlie catalytic phenomena.

(1) Na_2HPO_4 and H_2O_2 may give an unstable complex which in turn reacts to oxidize glucose.

(2) Na_2HPO_4 and glucose may form a hexose phosphate which is more sensitive to H_2O_2 than free glucose.

(3) The three compounds may form a single complex the instability of which gives rise to the oxidation.

(1) and (2) are readily capable of being tested experimentally by known methods. (3) could conceivably take place in several ways none of which appears to be readily capable of experimental confirmation.

The Na_2HPO_4-H_2O_2 Complex.—That such a complex may be formed is suggested by the experiments of Petrenko on H_2O_2 derivatives of Na_3PO_4.[11] A perphosphate of Na_2HPO_4 is unknown. Moreover perphosphoric acid is apparently unknown.[12] However, pyrophosphoric acid gives a peracid with H_2O_2, stronger

[11] Petrenko, G., *J. russ. phys.-chem. Ges.*, 1902, xxxiv, 204, 391; *Chem. Zentr.*, 1902, i, 1263; ii, 95. *Cf.* also, Gemlin-Kraut, Handbuch der anorganische Chemie, Heidelberg, 7th edition, 1906, i, pt. 1, 146.

[12] Price, T. S., Per-acids and their salts, New York, 1912, 77.

than Caro's acid and which oxidizes Mn to $KMnO_4$ and its sodium salt $Na_4P_2O_7$ gives a stable persalt with 3 per cent H_2O_2.[13]

There is therefore some basis in fact, even in this little studied field, for the idea that Na_2HPO_4 may form an unstable perphosphate as is suggested in the succeeding paragraphs.

Decomposition of Hydrogen Peroxide by the Phosphate Mixture.—Various experiments were done on the influence of Na_2HPO_4 on hydrogen peroxide although it is definitely stated[14] that it is without influence on peroxide. My own experiments, which will not be described here, show that it does decompose hydrogen peroxide and that the presence of an equimolecular amount of NaH_2PO_4 retards but does not stop the decomposition.

TABLE VI.

0.1 N $KMnO_4$ Consumed by 5 Cc. of the Mixture.

No.	23.5 hrs.	47.2 hrs.	96.2 hrs.
	cc.	*cc.*	*cc.*
1	22.70	22.62	22.45
2	22.10	21.06	18.45
3	19.35	14.53	2.75
4	17.06	11.65	4.31
5	15.72	9.86	4.08

The only experiments to be described here represent a repetition of the glucose oxidations at 37° in Table II in which the glucose was omitted and in which the peroxide content was determined at intervals during 96 hours. The results given in Table VI indicate the amount of peroxide remaining, at the various intervals, in terms of cc. of 0.1 N $KMnO_4$ consumed by 5 cc. of the mixture.

The phosphate mixtures and the hydrogen peroxide were warmed for 24 hours at 37°C. before being mixed in order to prevent a lag which is otherwise observed during the first 24 hours.

[13] Schenck, R., Vorländer, F., and Dux, W., *Z. angew. Chem.*, 1914, xxvii, pt. 1, 291.

[14] Gemelin-Kraut,[11] p. 137.

The data show a progressively increasing decomposing effect with increasing phosphate content although the mixture is neither appreciably acid nor alkaline.[15]

In conclusion it may be stated that there are clear indications that Na_2HPO_4-H_2O_2 may form an unstable complex, but as yet there is no satisfactory evidence.

The Na_2HPO_4-Glucose Complex.—Harden and Young, von Lebedew, and others[10,16] showed that, in the yeast fermentation of glucose, Na_2HPO_4 combines with glucose to form a hexose phosphate ester. The presence of this complex was demonstrated in part by the fact that much of the phosphate was no longer precipitated with "magnesia mixture." Other hexose phosphoric esters have been obtained by chemical methods[17] but the laboratory preparation of von Lebedew,[16] and the commercial manufacture[18] of hexose phosphate ester are carried out only in the presence of growing yeast. It therefore seemed necessary to determine experimentally whether such a complex is formed in aqueous solutions of glucose and the phosphate mixture alone.

A solution corresponding to No. 5 in Table II except that it contained 20 cc. of water instead of the peroxide solution was kept 3 weeks in the incubator at 37°C. At the end of this time the glucose content was found by reduction methods to be unchanged. After standing 3 weeks more in the laboratory the

[15] It should be noted that the interpretation of this decomposition of H_2O_2 in this case cannot be attributed to the OH ion, since the solution has about the OH ion concentration of water, which is without influence. It is interesting to note in this connection that Schenck, Vorländer, and Dux[13] found that $Na_4P_2O_7$ solutions, which are so alkaline as to feel "soapy," actually stabilize H_2O_2 by forming a stable perpyrophosphate. Moreover, it was found in experiments which will not be given here that the presence of $Na_4P_2O_7$ with Na_2HPO_4 retards or prevents the oxidation of glucose with H_2O_2, but not by decomposing the H_2O_2 as with $NaHCO_3$ or Na_2CO_3.

[16] von Lebedew, A. V., *Biochem. Z.*, 1910, xxviii, 213; 1911, xxxvi, 248. Embden, G., and Laquer, F., *Z. physiol. Chem.*, 1914–15, xciii, 94. Embden, G., Griesbach, W., and Laquer, F., *Z. physiol. Chem.*, 1914–15, xciii, 124.

[17] *Cf.* foot-note, Meyer, V., and Jacobson, P., Lehrbuch der organischen Chemie, Leipsic, 2nd edition, 1902, ii, pt. 2, 927.

[18] *Cf.* for instance Bayer and Company, German Patent 292,817, February 26, 1915; *Chem. Abstr.*, 1917, xi, 1519.

phosphate was precipitated with "magnesia mixture" and weighed as the pyrophosphate. A solution of the phosphates alone made up to the same volume was similarly precipitated at the same time. The two precipitates after ignition showed the same weight, within a small fraction of 1 per cent, which shows that hexose phosphate ester was not formed to any significant extent.

Similar solutions containing glucose and the phosphate mixture were kept under observation in the polariscope in comparison with glucose solutions without phosphates. In 4 days there was no measurable change in optical rotation in either solution.

These data, taken with the absence of positive data in the literature, seem to prove that a hexose phosphate ester such as was found by Harden and Young is not formed under these conditions and consequently has no part in bringing about this oxidation of glucose. If some other type of complex is formed its presence was not demonstrated by these methods.

Influence of Time on the Glucose-Phosphate-Peroxide Reaction.—When it was found that the rate of peroxide decomposition has a definite relation to the phosphate concentration it was of interest to learn what relation the rate of glucose oxidation bears to the rate of peroxide decomposition. The results given in Table VII are typical for the rate of glucose oxidation as obtained by compiling experimental results obtained in conditions like those used for Table II.

In order to test this more fully experiments like Nos. 1 to 5 in Table II were set up having a total volume of 150 cc. and which were placed in the incubator at 37°. The phosphate-glucose mixture and the peroxide were warmed separately for 24 hours before mixing to eliminate what appeared to be a temperature lag in the curves. 10 cc. were removed and analyzed at definite intervals and the results were calculated and recorded in Table VIII on the basis of 75 cc. and thus correspond to the results in Table VII. The materials used in Table VII were not warmed before mixing which accounts for the difference in the slope of the curves when the data are plotted.

The results in both series are substantially the same and show that the rate of glucose disappearance in the presence of the phosphate mixture is appreciably faster than the rate of H_2O_2 disappearance in the absence of glucose (Table VI).

The above constitutes a partial experimental interpretation of the catalytic influence of disodium phosphate on the oxidation of glucose with hydrogen peroxide. The results clearly suggest that the glucose is really oxidized by an unstable disodium perphosphate, formed by the action of peroxide on disodium phosphate.

TABLE VII.

Glucose Remaining from 0.200 Gm. Used.

No.	50 hrs.	97 hrs.
	gm.	*gm.*
1		0.1897
2		0.1843
3		0.1249
4	0.1037	0.0135
5	0.0088	

TABLE VIII.

Glucose Remaining from 0.1902 Gm. Used.

No.	26.5 hrs.	49.2 hrs.
	gm.	*gm.*
1	0.1702	0.1455
2	0.1362	0.1074*
3	0.0489	
4	0.0195	
5	0.0273	

* The behavior of No. 2 is quite variable. Sometimes oxidation is as slow as in No. 1 without phosphates and sometimes it is nearly as fast as in No. 3, but more frequently it is about as given in these results.

12. Is a Glucose-Phosphate Solution Oxidized by Air?—Having shown that the disodium phosphate plays a specific rôle in this catalysis the question arises as to whether the use of peroxide is necessary. A few experiments were done in order to determine whether air could be used instead of peroxide. It is well known that caustic alkalies catalyze the oxidation of sugars by air, with the formation of more or less CO_2 depending on the conditions of the experiment. In the absence of definite data it was possible that the phosphate mixture might play the rôle of caustic

alkali. A mixture like No. 5 in Table II was placed in a wash bottle. A rapid air stream was bubbled through it for 48 hours during 6 days. The permanganate required by 10 cc. was determined at the beginning and at the end of the experiment and showed that no perceptible oxidation had taken place. This shows that disodium phosphate does not act like alkali in this respect, but rather conforms to the rôle of a true peroxidase.[19]

SUMMARY.

1. The work of Löb on the accelerative effect of phosphate mixtures on the oxidation of glucose with hydrogen peroxide was repeated and confirmed.

2. The confirmation consisted in proving by an adequate method that the destruction of glucose, conceded by all in this case, is oxidation.

3. It was shown that glucose may be quantitatively oxidized to CO_2 with hydrogen peroxide in the presence of the phosphate mixture. This fact it appears was not suspected by Löb, and increases the importance of his observations considerably.

4. The results as a whole show that although optimal OH ion concentration is possibly necessary it is less important than

[19] *Cf.* Bach, A., in Oppenheimer, C., Handbuch der Biochemie des Menschen und der Tiere, Jena, 1st edition, 1913, suppl., 160.

Inorganic compounds known to play the rôle of peroxidase in *in vitro* oxidations have usually been, as Bach states, metallic salts of the heavy metals such as iron and manganese. The synthetic peroxidases of Trillat (Trillat, M. A., *Compt. rend. Acad.*, 1904, cxxxviii, 274), of Dony-Hénault (Dony-Hénault, O., *Bull. acad. roy. belg.*, 1908, 105), etc., prepared from manganese were prepared to resemble and imitate what it was thought are the essential properties of an oxidizing enzyme. The peroxidase disodium phosphate differs from these inorganic peroxidases in that the peroxidase property is dependent on the phosphate part of the molecule. Other sodium compounds do not exhibit the same effect. On the other hand dipotassium phosphate, as was shown by experiments not yet published, has the same effect. That the remaining alkali and alkaline earth dibasic phosphates may act in the same way seems likely.

From this point of view then these results are of considerable interest because we have a compound playing the rôle of peroxidase, in which the non-metallic part of the molecule carries the characteristic property. In this respect it seems likely that it resembles the biological peroxidases more closely than the heavy metal derivative peroxidases do.

Löb's experiments and interpretation would indicate, when the phosphate mixture is used, and that the optimal limits, if they exist, are wider than he states.

In fact it seems more accurate to refrain from emphasizing the segregated OH ion in interpreting the reaction and simply state that the effect is specifically related to the presence of disodium phosphate under suitable conditions.

5. The amount of disodium phosphate used is the most significant factor in determining the reaction. Little or much monosodium phosphate was used with a constant amount of disodium phosphate without producing a marked negative effect on the reaction.

6. The phosphate mixture may be used repeatedly at 37° for the oxidation of additional amounts of glucose owing to the fact that the product (CO_2) is evolved from the reaction mixture during the process of oxidation. Disodium phosphate accordingly plays the rôle of a typical catalyst.

7. Consequently disodium phosphate functioning in the manner described in this paper is the only chemical substance known to be generally necessary to the life of organisms, that is known to catalyze the quantitative oxidation of glucose to carbon dioxide.

8. That compounds like hexose phosphate ester are the intermediates involved in the acceleration of oxidation described in this paper seems almost certain at first, in the light of the results of Harden and Young,[10] of von Lebedew,[16] and of Embden, Griesbach, and Laquer.[16] The attempts so far made to establish the formation of such a compound failed to demonstrate its formation under these conditions.

9. On the other hand a close parallelism between the rate of spontaneous decomposition of peroxide and the rate of glucose oxidation in the same solutions was established. This together with other facts developed gives experimental basis for the idea that the oxidation really depends upon the intermediate formation of a highly reactive perphosphate.

10. In producing this accelerating effect upon glucose oxidation disodium phosphate does not play the rôle of both oxygenase and peroxidase, as some inorganic compounds do, but acts only as a peroxidase. It is unable to activate atmospheric oxygen to any appreciable extent.

11. That the phosphate catalysis does not depend alone on unlimited capacity to decompose peroxide is clearly shown by the fact that the hydroxide and carbonates of sodium, which are much more effective in decomposing peroxide, diminish the glucose oxidation roughly in proportion to their increased ability to decompose peroxide.

12. Glucose is not oxidized by hydrogen peroxide in solutions containing $NaHCO_3$ and Na_2CO_3 when these are used in the same molecular concentrations as the two phosphates in the phosphate mixture.

13. These results together with those with sodium hydroxide show that available alkali, contrary to what was observed in the permanganate oxidation of glucose,[4, 20] is without appreciable influence on the oxidation of glucose with hydrogen peroxide.

[20] Witzemann, E. J., *J. Am. Chem. Soc.*, 1917, xxxix, 2657.