[CONTRIBUTION FROM THE COASTAL LABORATORY OF THE CARNEGIE INSTITUTION OF WASHINGTON]

THE OXIDATION OF CARBOHYDRATES WITH AIR

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Although the naturally occurring hexose sugars are easily oxidized in the plant and animal organism, these compounds in aqueous solution are remarkably stable toward atmospheric oxygen. Similarly, oxidation with pure hydrogen peroxide is exceedingly slow, while small quantities of either iron salts, caustic alkalies, or disodium phosphate greatly catalyze the reaction.[1] In a strongly alkaline solution glucose is oxidized by air, but these conditions have only remote physiological significance, because it has not been possible to oxidize glucose with air in solutions of hydroxyl-ion concentrations approximating those found in the living organism, even in the presence of iron salts or phosphates.

The ideal catalyst for the oxidation of sugar with air would be one which is reduced by the sugar and again oxidized by atmospheric oxygen, the two reactions progressing at approximately the same rate. For practical purposes it is immaterial whether the oscillation is between simpler conditions of higher and lower electronic charges (thus $Fe^{\cdot\cdot} \rightleftarrows F^{\cdot\cdot\cdot}$) or between an oxide and its peroxide.

For several years the author has tried out a large number of catalysts both homogeneous and heterogeneous in an effort to meet these conditions, but in the great majority of cases in which there was any oxidation at all, the oxidation proceeded very slowly or the catalyst rapidly ran down. Increasing the reduction potential of the sugar by means of alkali almost universally resulted in an increased rate of oxidation but, for the reasons already stated, it was desired to avoid these conditions of alkalinity.

Apparatus

The oxidation experiments were carried out in 300cc. Kjeldahl flasks, each closed with a 2-hole rubber stopper. The air passed through a glass tube reaching to the bottom of the flask and bent back so that the last centimeter formed a short leg of a small U-tube. To this end was sealed a fine-mesh platinum gauze to break the air into small bubbles. Before passing into the flask the air was freed from carbon dioxide by passing over soda lime. From the flask the air passed through a small water trap, then through a Meyer 10-bulb tube filled with 0.1 *N* barium hydroxide solution. The air was drawn through the apparatus by means of a small rotary pump. In order to maintain a regular air-stream each flask was provided with a separate pump. The carbon dioxide absorbed in the barium hydroxide solution was determined electrometrically.[2] The oxidations were carried out in a thermostat at 38°.

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[1] (a) Cross, Bevan and Smith, *J. Chem. Soc.*, **73**, 463 (1898). (b) Morrell and Crofts, *ibid.*, **75**, 786 (1899); **77**, 1219 (1900); **81**, 666 (1902); **83**, 1284 (1903); **87**, 280 (1906). (c) Spoehr, *Am. Chem. J.*, **43**, 248 (1910). (d) Glattfeld, *ibid.*, **50**, 135 (1913). (e) Nef, *Ann.*, **403**, 242 (1914). (f) Witzemann, *J. Biol. Chem.*, **45**, 1 (1920).

[2] Spoehr and McGee, *Carnegie Inst. Pub.*, **325**, 28 (1923).

Methylene Blue as a Catalyst

The reduction of methylene blue by alkaline glucose solutions has been known for a long time[3] and has been used as the principle of a method of sugar analysis. The property of methylene blue in the presence of palladium of oxidizing a variety of organic compounds in the absence of oxygen has served as the basis of Wieland's dehydrogenation theory of vital oxidation.[4]

The author has found that methylene blue is reduced by glucose solutions in the presence of disodium phosphate. When air is drawn through such a colorless solution, the blue color again appears and carbon dioxide is formed. In view of the fact that glucose is not oxidized by air in solutions of disodium phosphate it was at first thought that methylene blue would serve as a valuable catalyst for the study of the oxidation of sugar with air. The action of methylene blue and a number of other dyes was studied. In the course of the investigation, however, a much better catalyst was found and the results with methylene blue are therefore given here only in brief summary. The rate of decolorization of methylene blue (0.00005 M) is directly proportional to the concentration of disodium phosphate (ranging from 0.025 to 0.75 M). The decolorization is also proportional to the concentration of the glucose in 0.05 to 0.5 M solution; lower concentrations of glucose reduce relatively more slowly. Levulose reduces five to eight times as rapidly as glucose, depending upon the concentration of methylene blue and of disodium phosphate.

Through a solution of 3 g. of glucose in 150 cc. of water, containing 17 g. of disodium phosphate dodecahydrate ($Na_2HPO_4.12H_2O$) and 0.1 g. of methylene blue, a continuous stream of air was drawn. The carbon dioxide formed was determined every 24 hours. Thus in 168 hours 0.0310 g. of carbon dioxide was evolved.

That small quantities of iron and of manganese play a very important role in the oxidative processes in the living organism as well as in the autooxidation of organic and inorganic substances *in vitro* has repeatedly been emphasized.[5] The oxidation of glucose with air and methylene blue is also greatly accelerated by small quantities of iron. However, the difficulty is encountered that in the presence of disodium phosphate the iron is precipitated as an insoluble phosphate. This difficulty was first circumvented by using the "soluble ferric phosphate" which is ferric

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[3] Ihl, *Z. anal. Chem.*, **29**, 386 (1890). Herzfeld, *Deut. Zuckerind.*, **13**, 234 (1886). Neuman-Wender, *Z. anal. Chem.*, **33**, 118 (1894). Fröhlich, *Zentr. inn. Med.*, **19**, 89 (1898). Kuster and Woker, *Arch. ges. Med.*, **155**, 92 (1913).

[4] Wieland, *Ber.*, **46**, 3327 (1913); 47, 2085 (1914); **54**, 2353 (1921); **55**, 3639 (1922). Warburg, *Biochem. Z.*, **142**, 518 (1923).

[5] Warburg, *Z. physiol. Chem.*, **92**, 231 (1914); *Biochem Z.*, **119**, 134 (1921); **136**, 266 (1923). Sakuma, *ibid.*, **142**, 68 (1923). Abegg and Auerbach, "Handbuch der Anorganischen Chemie," vol. IV, part 2, pp. 635, 691.

phosphate dissolved in sodium citrate but does not give the usual test for the ferric ion. The addition of 0.01 g. of "soluble ferric phosphate" to a glucose mixture as described above, increases the rate of carbon dioxide formation threefold.

In the repeated reduction and oxidation of methylene blue in the oxidation experiments with air, the dye is affected so that its activity is constantly decreased. This is presumably due to the fact that in the oxidation of the leuco methylene blue to the colored compound, hydrogen peroxide is formed which acts on the methylene blue. There is no appreciable difference in the rate of oxidation of glucose when the sulfate of methylene blue is used instead of the chloride. Methylene blue alone catalyzes the oxidation of glucose with air only in alkaline solution; disodium phosphate is sufficiently alkaline for this purpose. A diminution of the hydroxyl-ion concentration, such as may be produced by the addition of glycine, greatly reduces the rate of oxidation.

When sodium ferro-pyrophosphate was added to a glucose-sodium phosphate mixture containing methylene blue, the rate of carbon dioxide formation was increased more than twenty times. It was soon found, however, that sodium ferro-phosphate catalyzes the oxidation of glucose with air even without methylene blue. The oxidation experiments were therefore continued, using sodium ferro-pyrophosphate as the oxidation catalyst without methylene blue.

Sodium Ferro-Pyrophosphate as a Catalyst

The pyrophosphates of iron are soluble in a solution of sodium pyrophosphate. There are thus formed salts in which the iron is present as a complex ion. These salts have been investigated by Pascal,[6] who gave to the sodium ferro-pyrophosphate the formula $Na_8Fe_2(P_2O_7)_3$. This substance is a strong reducing agent, liberating the free metal from salts of gold, silver, mercury and copper. When ferrous sulfate is added to sodium pyrophosphate there is first formed a precipitate of ferrous pyrophosphate which, on the addition of more sodium pyrophosphate, dissolves to form a clear, light green solution. Such a solution gives none of the ordinary tests for the iron ion, nor does it give a precipitate with alkalies.

The mono- and dibasic sodium or potassium phosphates can be added to the sodium ferro-pyrophosphate solution so as to obtain solutions of various hydrogen-ion concentrations, thus producing even slightly acid solutions. Strong acids, however, break down the complex salt.

Such a mixture of sodium ferro-pyrophosphate with the phosphate mixture is light green when alkaline, and colorless when neutral or slightly acid. On the addition of a solution of hexose sugar or of sucrose it turns dark, and finally appears black. A thin layer of such a solution is dark

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[6] Pascal, *Ann. chim.*, [8] **16,** 386 (1909).

claret in color. The rate of coloration depends upon the temperature; at ordinary temperature it requires about 30 minutes; on heating, the color develops rapidly. This is apparently a colloidal solution, but the colored particles cannot be separated by dialysis. When a rapid stream of air is drawn through the black solution it again becomes colorless or yellow. When the air stream is slow the solution becomes brown, but when the air stream is very slow the color does not change perceptibly.

When the sugar solution is added to the iron-phosphate mixture (both solutions having been previously boiled to expel dissolved oxygen) in an atmosphere of hydrogen, there is a fleeting coloration which disappears again even at 90°. A small quantity of air rapidly produces the dark coloration which then cannot be reduced by hydrogen. Unfortunately, it has not been possible thus far to establish the composition of the black material.

When air is drawn through a solution containing sodium ferro-pyrophosphate, phosphate mixture and glucose, carbon dioxide is formed. The rate of carbon dioxide formation at first rises rapidly and then slowly diminishes. In Fig. 1 is plotted the rate of formation of carbon dioxide from a solution of 150 cc. of water, 6.7 g. of sodium pyrophosphate decahydrate ($Na_4P_2O_7.10H_2O$), 1 g. of ferrous sulfate heptahydrate, 17 g. of disodium phosphate dodecahydrate and 3 g. of glucose, yielding, in 456 hours at 38°, 0.8356 g. of carbon dioxide, or 19.0% of complete oxidation.

In a similar experiment *without* iron only an exceedingly small quantity of carbon dioxide was formed. The amounts of carbon dioxide formed during 24 hours were below the degree of accuracy of the method of carbon dioxide determination employed. The great difficulty of obtaining chemicals absolutely free from traces of iron is well known, so that the slight oxidation may have been due to this cause.

The effect of varying the amounts of iron on the rate of oxidation of glucose is shown in Table I. The carbon dioxide determinations were made in periods of 24 hours for 264 hours.

TABLE I

CARBON DIOXIDE FORMED BY THE OXIDATION OF GLUCOSE WITH AIR, USING VARIOUS AMOUNTS OF IRON, AT 38°

In each experiment 6.7 g. of $Na_4P_2O_7.10H_2O$, 17 g. of $Na_2HPO_4.12H_2O$, 3 g. of glucose and 150 cc. of H_2O were used.

$FeSO_4.7H_2O$	0.5	1.0	2.8
CO_2 found g.	0.4876	0.6916	0.6578

Similar experiments were carried out in which manganous sulfate was used in place of ferrous sulfate, but the amount of carbon dioxide was about 0.1% of that obtained with iron.

When ferric sulfate is used, which according to Pascal[7] forms sodium

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[7] Ref. 6, p. 363.

ferri-pyrophosphate, $Na_6Fe_2(P_2O_7)_3$, the amount of carbon dioxide is less than with the ferrous salt. Two solutions, each containing 150 cc. of water, 6.7 g. of sodium pyrophosphate, 17 g. of disodium phosphate, 3 g. of glucose and the one, 1 g. of ferrous sulfate, the other the molecular equivalent of the iron, 1.01 g. of ferric sulfate ($Fe_2(SO_4)_3.9H_2O$). Air was drawn through these solutions for 144 hours. The carbon dioxide formed in the case of the ferrous sulfate totaled 0.5600 g. and in that of the ferric sulfate, 0.5392 g.

Pascal calls attention to the similarity between sodium ferro-pyrophosphate and potassium ferrocyanide. Attempts to obtain an oxidation of glucose in the presence of disodium phosphate by means of air and potassium ferrocyanide were, however, fruitless.

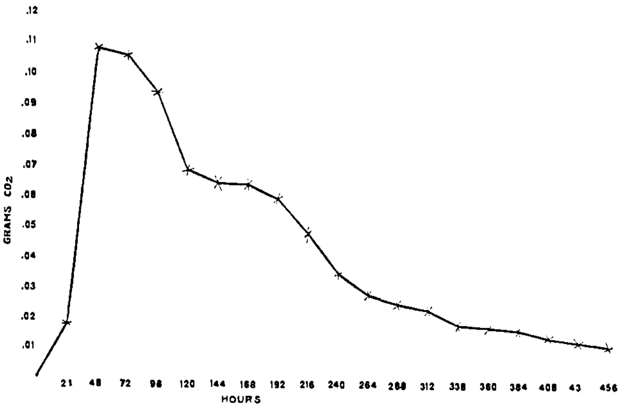

Fig. 1.—The rate of carbon dioxide formation in the oxidation of glucose with air and sodium ferro-pyrophosphate at 38°.

From the curve, Fig. 1, of the rate of carbon dioxide formation from glucose it is evident that this decreases with time. It was thought that this was due to oxidation of sodium ferro-pyrophosphate by air to the corresponding ferric salt with consequent diminished oxidation. In order to test this, a stream of air was drawn through a solution containing sodium ferro-pyrophosphate and disodium hydrogen phosphate in the same proportions as the experiment recorded in Fig. 1 but containing no glucose. At intervals, 150 cc. of this solution was withdrawn and 3 g. of glucose added; air was then drawn through this solution at 38° and the carbon dioxide determined every 24 hours as usual. The ability of the sodium ferrophosphate to catalyze the oxidation of glucose by air is not

TABLE II

THE EFFECT OF PREVIOUS AERATION ON THE CATALYST

Hrs. of passage of air through $Na_8Fe_2(P_2O_7)_3$ sol...	0	24	96	576
CO_2 formed in 120 hrs. after adding 3 g. of glucose, g.	0.3940	0.3904	0.3690	0.4684

diminished by previously drawing air through such a solution, as becomes evident from the data in Table II.

The Effect of Various Amounts of Phosphates

Witzemann, in oxidizing glucose with hydrogen peroxide and disodium phosphate, concludes that "the amount of disodium phosphate used is the most significant factor in determining the reaction." A similar situation exists in the oxidation of glucose with air, methylene blue and sodium ferro-pyrophosphate. Thus four solutions, each containing 150 cc. of water, 3 g. of glucose, 4 g. of sodium pyrophosphate, 0.5 g. of ferrous sulfate, 0.1 g. of methylene blue and various amounts of disodium phosphate, were oxidized with air at 38° for 216 hours. The amount of carbon dioxide formed was determined every 24 hours. The total amount of carbon dioxide thus formed is given in Table III, together with the respective concentration of disodium phosphate in each solution.

TABLE III

THE EFFECT OF DISODIUM PHOSPHATE ON THE OXIDATION OF GLUCOSE BY AIR

Molal concentration of disodium phosphate.......	0	0.1	0.166	0.333
CO_2 formed in 216 hours, g......................	0.2747	0.3808	0.4290	0.5002

From these experiments it might appear that the hydroxyl-ion concentration is the significant factor in the rate of oxidation. That this is not the case becomes apparent from the following experiment in which various amounts of potassium dihydrogen phosphate were added to the mixture to adjust the hydrogen-ion concentration of the solution. It was not feasible to determine the value of the hydrogen-ion concentration of the solution accurately in the presence of iron and methylene blue. The values were therefore determined electrometrically of the solutions containing only glucose, sodium pyrophosphate, disodium phosphate and various amounts of potassium dihydrogen phosphate. Thus, five solutions, each containing 150 cc. of water, 3 g. of glucose, 6.7 g. of sodium pyrophosphate (0.1 *M*), 0.5 g. of ferrous sulfate, 0.1 g. of methylene blue,[8] 17 g. of disodium phosphate and the respective amounts of monopotassium phosphate gave on oxidation in the same manner the following amounts of carbon dioxide in 216 hours at 38°.

TABLE IV

THE EFFECT OF HYDROGEN-ION CONCENTRATION ON THE OXIDATION OF GLUCOSE

Molal concentration KH_2PO_4............	0	0.1	0.125	0.166	0.25
*P*H.................................	8.8	7.4	7.2	7.0	6.8
CO_2 in 216 hours, g....................	0.4690	0.5618	0.6662	0.5756	0.5344

It is therefore apparent that the highest rate of oxidation does not cor-

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[8] It was necessary to use a different lot of methylene blue from that used in the previous experiment.

respond to the greatest alkalinity, but rather is attained in a very slightly alkaline solution.

The Oxidation of Different Carbohydrates

That some carbohydrates are more easily oxidized than others is a familiar fact. Among the hexoses levulose has been found to be the most easily oxidized. This is also the case under the conditions here discussed. On the other hand, *d*-glucose has often been described as the most stable hexose, which is also indicated by the present experiments.

Not only are the reducing sugars oxidized by air in the presence of sodium ferro-pyrophosphate and disodium phosphate, but non-reducing sugars such as sucrose and polyhydric alcohols which do not reduce Fehling's solution are also easily oxidized. The oxidation of sucrose takes place in alkaline solution (*P*H 8.8) as well as in neutral or slightly acid (*P*H 6.8) solution. It was thought that this might be due to a slow hydrolysis of sucrose into glucose and levulose under these conditions. No indications of an inversion could, however, be detected. Moreover, the disaccharide, trehalose, which is hydrolyzed with difficulty, is also oxidized. In Table V are given the results of oxidation of various substances with air in 150 cc. of water, and the quantities of carbon dioxide formed in 120 hours at 38° are shown. In each case 6.7 g. of sodium pyrophosphate (0.1 *M*) and 1 g. of ferrous sulfate were used.

Table V

The Oxidation of Carbohydrates and Related Substances by Air in the Presence of Sodium Ferro-pyrophosphate and Alkali Phosphates

0.125 *M* KH_2PO_4 was used in every experiment except the second with sucrose; 0.333 *M* Na_2HPO_4 was used in all experiments.

Substance	G.	CO₂ in 120 hours G.	Substance	G.	CO₂ in 120 hours G.
d-Glucose	3	0.4874	Sucrose	5.7	0.5134
Levulose	3	.6556	Trehalose	5.7	.2024
d-Mannose	3	.5286	Glycerol	1.5	.4752
d-Galactose	3	.5461	Mannitol	3.0	.0362
Sucrose	5.7	.4462			

The Effect of the Phosphate Mixture on Glucose

In Fig. 1 is given the rate of carbon dioxide formation in the oxidation of glucose, showing that this is at first relatively low, rising rapidly to a maximum during the first 48 hours of the experiment. This curve is characteristic of the oxidation of glucose by this method under a variety of conditions. The rate of oxidation of levulose exhibits a similar curve with the exception that the initial rate is very much higher than that of glucose. Witzemann observed[9] a similar lag in the initial rate of oxidation of glucose with hydrogen peroxide and disodium phosphate.

[9] Ref. 1 f, p. 18.

Nef[10] stated that with 0.25–2 equivalents of sodium carbonate, solutions of the hexoses undergo mutual transformation, so that starting with any of the following sugars there is finally obtained an equilibrium mixture of glucose, levulose, mannose and pseudo-levulose. Under these conditions of alkalinity, according to Nef, there is no splitting of the olefin-dienols nor any saccharinic acid formation. A splitting of the hexose molecule occurs only with higher concentration of alkali. Now these sodium carbonate solutions show a hydrogen-ion concentration of *P*H 11–12, and the solutions of sodium pyrophosphate and disodium phosphate about *P*H 9. On the basis alone of the hydroxyl-ion concentration it would not be expected that there would be any splitting of the hexose. It remained to be established whether the phosphate mixture causes saccharinic acid formation and especially whether under these conditions any transformation of the glucose into levulose occurs. If this were the case, in view of the fact that levulose is so much more easily oxidized than glucose, the explanation for the initial lag in the rate of oxidation of glucose might lie in the formation of levulose from glucose under the influence of the phosphate mixture.

Accordingly a solution containing 600 cc. of water, 26.8 g. of sodium pyrophosphate (0.1 *M*), 68 g. of disodium phosphate (0.33 *M*) and 12 g. of glucose was kept in a thermostat at 38°. At definite periods, portions of this solution were withdrawn which were: (1) titrated with 0.1 *N* hydrochloric acid (using methyl orange as indicator) to determine whether any saccharinic acid had formed, (2) analyzed by means of the Cajori iodine method and the optical rotation determined to establish whether any levulose had been formed,[11] and (3) after the addition of 0.5 g. of ferrous sulfate to 150 cc. of the sugar-phosphate mixture, oxidized with air in the manner described. The results of these experiments are summarized in Table VI. The amount of acid necessary to neutralize the solution shows a small decrease, indicating probably slight saccharinic acid formation. Iodine oxidizes only the aldoses and does not affect the ketoses. From the decreasing amount of iodine required it may be concluded that the

TABLE VI

EFFECT OF DISODIUM PHOSPHATE AND SODIUM PYROPHOSPHATE ON GLUCOSE

Time Hours	0.1 *N* HCl required for 100 cc. Cc.	0.1 *N* iodine required for 100 cc. Cc.	Optical rotation in 1cm. tube Degrees	CO_2 formed in 120 hours G.
0	456.2	191	+0.89	....
24	455.0	188	+ .81	0.2742
48	455.0	184	+ .80	....
72	452.4	180	+ .78	.2810
144	...	174	+ .69	.3860

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[10] Ref. 1 e, p. 225.

[11] *J. Biol. Chem.*, **54**, 617 (1922).

quantity of glucose has decreased with presumably an increase in the ketose sugars. Similarly, the optical rotation of the solution also decreased with time, which also indicates a conversion of glucose to levulose. Finally, the oxidation experiments show that the longer the glucose-phosphate mixture has stood, the higher is the rate of oxidation. In view of the fact that levulose is more easily oxidized than glucose, this is what could be expected if a partial transformation of glucose into levulose and the other sugar took place, as has been shown to occur in weak alkaline solution.

While the results in Table VI indicate the influence of disodium phosphate on the rate of oxidation of glucose and bear on the general problem of the effect of phosphates on metabolism, definite conclusions cannot be drawn until the laborious investigations of the influence of phosphates on the mutual transformations of the hexose sugars under various conditions are completed. The oxidation of glucose and the other carbohydrates with air under these conditions results in a complex mixture of products. The difficulties of a quantitative determination of these products are increased by the fact that the products are all further oxidized by air, though at a lower rate than glucose. A discussion of the theory of the mechanism of the reaction, especially as it affects the oxidation theories of Wieland and of Warburg, is withheld for the future.

Summary

Glucose and other hexoses are oxidized by air in solutions containing disodium phosphate and methylene blue with the formation of carbon dioxide. This reaction is greatly accelerated by small quantities of iron salts. Sodium ferro-pyrophosphate is a more active catalyst than methylene blue in causing the oxidation of carbohydrates with air. Not only are the hexose sugars thus oxidized but also sucrose, trehalose, mannitol and glycerol. There is some evidence that glucose in a solution containing disodium phosphate undergoes transformation into levulose.

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