# ON THE ACTION OF PHOSPHATE UPON HEXOSES. I. THE FORMATION OF ACETOL FROM GLUCOSE IN ACIDIC SOLUTION OF POTASSIUM PHOSPHATE.

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Since it was made most probable that in yeast fermentation as well as many other biological processes, zymohexose molecule splits primarily into two $C_3$-fragments, a considerable interest has been centred in the study of the chemical decomposition of this type. The chemical formation of $C_3$-compounds—lactic acid, methylglyoxal or acetol—from sugars has been demonstrated by several investigators⁽¹⁾. Almost all of these demonstrations have been accomplished by the action of various alkaline reagents upon sugars. These experiments, however, seem to the present writer an inadequate explanation of the chemism of biochemical $C_3$-compounds formation which may proceed rather in an acidic medium, for example alcoholic fermentation in which optimum *p*H is 6.2–6.6 for the cell-free yeast preparations⁽²⁾. From such a point of view, it is desirable to realize chemically an analogous decomposition of hexose in an acidic medium. It is well known, however, that hexoses are generally stable in acidic medium⁽³⁾, and there are so far only two investigators who have demonstrated the formation of $C_3$-compound (methylglyoxal) from hexoses in acidic media: namely Cameron⁽⁴⁾, by the action of aromatic amines in dilute acetic acid; and Bernhauer⁽⁵⁾, by the action of hydrogen peroxide in dilute sulphuric acid in the presence of ferrous sulphate.

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(1) A. Emmerling and G. Loges, *Ber.*, **6** (1873), 23; **16** (1883), 837; M. Nencki and N. Sieber, *J. prakt. Chem.*, (2) **24** (1881), 298; H. Kiliani, *Ber.*, **15** (1882), 136, 699, 701; G. Pinkus, *ibid.*, **31** ( 1898), 31; W. Löb, *Biochem. Z.*, **12** (1908) 78; J. U. Nef, *Ann.*, **335** (1904), 326; **357** (1907), 294; **376** (1910), 1; A. Windaus and F. Knoop, *Ber.*, **38** (1910), 1166; H. D. Dakin and H. W. Dudley, *J. Biol. Chem.*, **15** (1913), 127; C. Neuberg, W. Oertel and B. Rewald, *Biochem. Z.*, **55** (1913), 495; **71** (1915), 144; A. Fernbach and M. Schoen, *Compt. rend.*, **158** (1914), 976; J. Meisenheimer, *Ber.*, **41** (1918), 1009; M. Oppenheimer, *Biochem. Z.*, **45** (1912), 134; O. Baudisch, *ibid.*, **84** (1918), 279; F. Fischler, *Z. physiol. Chem.*, **157** (1926), 1; **165** (1927), 53; K. Bernhauer and coworkers, *Biochem. Z.*, **212** (1929), 443; **219** (1930), 232.

(2) K. Myrbäck, "Homogene Katalyse," II. (1931), p. 114.

(3) F. Fischler (*loc. cit.*) and K. Bernhauer (*Biochem. Z.*, **210** (1929), 186) observed that glucose does not undergo any decomposition into $C_3$-compounds even on boiling with dilute mineral acids.

(4) C. N. Cameron, *J. Am. Chem. Soc.*, **49** (1927), 1789.

(5) K. Bernhauer, *Biochem. Z.*, **230** (1931), 484.

It has been also observed that even in acidic medium, glucose is instable(6) and fructose is autoxydizable(7) if alkali phosphate is coexistent. It is also well known that inorganic phosphates play an important part in the carbohydrate metabolism and are even indispensable in yeast fermentation(8). It is now generally admitted that a rôle of the phosphate intervenes in the stage of primary fission of hexose molecule into $C_3$-fragments(9).

Taking these facts into consideration, the present writer has investigated the action of acidic solution of potassium phosphate on glucose and begun with distillation of their mixture, hoping to get some fragments of glucose molecule in the distillate. By such a distillation method, methylglyoxal has been occasionally proved to be formed from hexoses by the action of some alkaline reagents, e.g., sodium carbonate, sodium sulphite(10), and sodium phosphate(11).

**Acetol.** After adjusting *p*H to 6.6–6.8, a mixture of glucose and about 40% solution of potassium phosphate was subjected to distillation, the volume of the content of the distilling flask being kept constant by addition of water in small portions. The distillate was almost neutral to litmus paper and gave a marked iodoform reaction and reduced Fehling's solution even in the cold. With phenylhydrazine it produced a yellow, and with *p*-nitrophenylhydrazine a scarlet, flocculent precipitate. These precipitates were recrystallized and identified with bisphenyl- and bis-*p*-nitrophenyl-hydrazones of methylglyoxal. In another experiment with a mixture of *p*H 6.2–6.3 the same result was obtained, except that the distillate was faintly acidic.

At first consideration of these results, it was thought that the distillates contained methylglyoxal, and that the glucose underwent by such treatment decomposition analogous to that engendered by alkaline reagents. These hydrazine derivatives, however, can be formed, of course, either from methylglyoxal itself, or from acetol or from lactic aldehyde(12). For deciding the true origin of the derivatives, a distillate from the mixture of *p*H 6.2–6.3 was treated with semicarbazide hydrochloride and

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(6) L. J. Henderson, *J. Biol. Chem.*, **10** (1911), 3.

(7) O. Warburg and M. Yabusoe, *Biochem. Z.*, **146** (1924), 380.

(8) K. Myrbäck, *Z. physiol. Chem.*, **177** (1929), 158.

(9) A. J. Kluyver and A. P. Struyk, *Naturwiss.*, **14** (1926), 883; O. Meyerhof, *ibid.*, **14** (1926), 1175; H. v. Euler, "Biokatalysatoren," (1930), p. 12.

(10) F. Fischler, *loc. cit.*

(11) H. D. Dakin and H. W. Dudley, *loc. cit.*

(12) A. Wohl, *Ber.*, **41** (1908), 3599.

sodium acetate, and acetolsemicarbazone was obtained as the main reaction product instead of bis-semicarbazone of methylglyoxal. In addition to the acetolsemicarbazone, the reaction product, however, contained always a small quantity of some substance difficultly soluble in hot water. By careful fractional crystallization from hot water, it was separated into hydrazodicarbonamide(13), diacetyl-bis-semicarbazone, and methylglyoxal-bis-semicarbazone. Up to this point, the yields of the latter two were far inferior to that of acetolsemicarbazone. The tests for other probable volatile decomposition products in the distillate, such as formaldehyde, acetaldehyde, acetone, levulinic acid, and furfurol derivatives, were all negative, the distillates if existent being very dilute.

In a series of experiments operated at *p*H 7.0–7.1, 6.6–6.7, 5.9–6.0, and 4.9–5.0, similar results were obtained, and at *p*H 6.3–6.5, an appreciable quantity of acetol was isolated from the distillate.

**Estimation of Acetol.** It is now desirable to know the yield of acetol from glucose under such conditions. The distillation was continued until the distillate gave no more iodoform reaction or only faintly, and the content of acetol was estimated in the total distillate. For the estimation of acetol, Fischler's iodometry(14) for methylglyoxal was applied, assuming

$$CH_3COCH_2OH+5I_2+9KOH = CHI_3+C_2O_4K_2+7KI+7H_2O$$

instead of

$$CH_3COCHO+4I_2+7KOH = CHI_3+C_2O_4K_2+5KI+5H_2O\ .$$

The results of experiments at various *p*H are tabulated in Table 1.

Table 1. Yields of acetol.
(5g. glucose+50c.c. 40% phosph. solution.)

| *p*H. | Acetol in g. | % on glucose. |
|---|---|---|
| 7.0–7.1 | 0.23 | 4.6 |
| 6.5–6.7 | 0.24 | 4.8 |
| 6.3–6.5 | 0.20 | 4.0 |
| 5.9–6.0 | 0.21 | 4.2 |

In the region of *p*H studied, the yields were almost alike: 4–5%(15) of glucose used, i.e., 10–12 mol per cent., assuming one molecule glucose gives one molecule acetol. Since, as already mentioned, the distillate is contaminated with some other iodine-consuming substances than acetol, though in a small quantity, the values in the table are naturally approximate.

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(13) A condensation product of semicarbazide itself (C. Neuberg, *Biochem. Z.*, **191** (1927), 478.).

(14) F. Fischler and R. Boettner, *Z. analyt. Chem.*, **74** (1928), 28.

(15) In a similar yield, Fischler (*loc. cit.*) got methylglyoxal from glucose by distillation with dilute sodium carbonate solution and neutral sodium sulphite.

**Influence of *p*H.** Although the *p*H of the distilling mixture scarcely influenced the yield of acetol, as described above, it had a great effect upon the rate of its formation in the distillate. To compare the rates, the distillations of the mixtures of various *p*H were carried out under similar conditions and at as constant a rate (200 c.c. distillate in 1.5 hrs.) as possible, and the acetol contents were successively estimated in each 200 c.c. of the distillate. The experimental results are summarized in Fig. 1, which is prepared by plotting the acetol content (mg.) against time (hour).

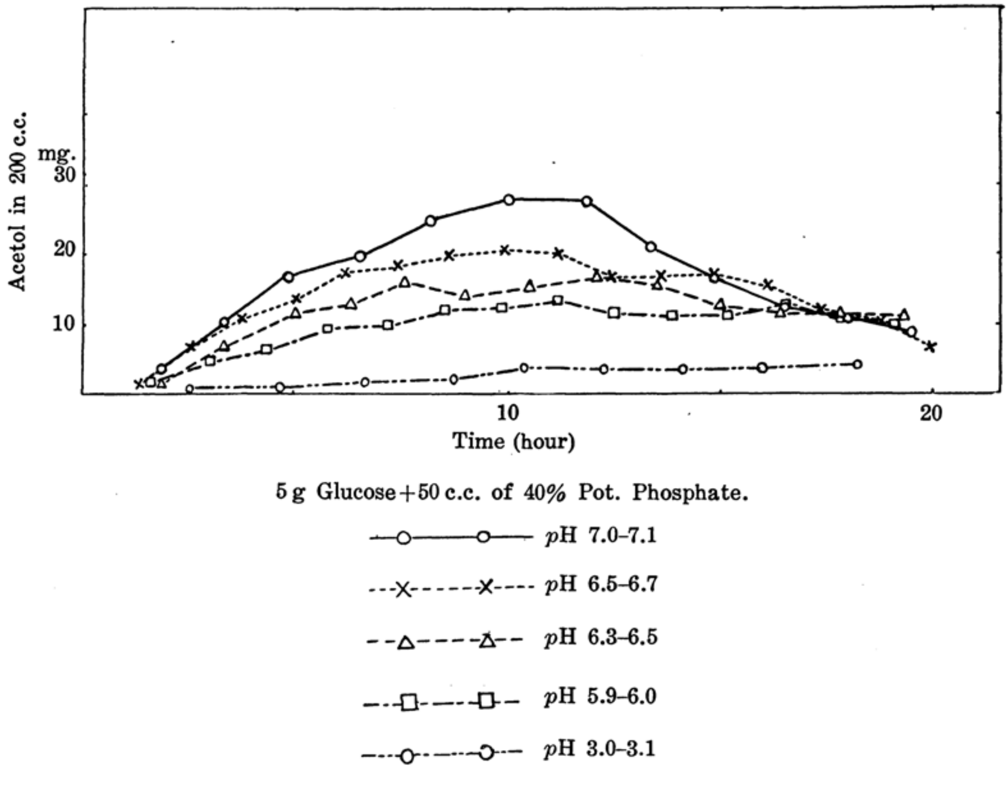
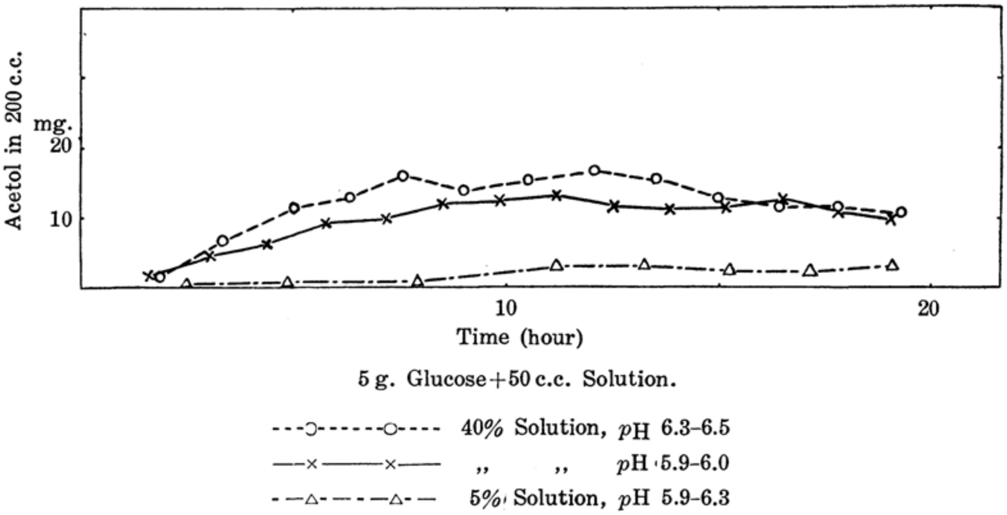


Fig. 1.

The maxima of curves in Fig. 1, showing the maximum rates, descend parallel with *p*H values, viz., 28 mg. for *p*H 7.0–7.1; 17 mg. for *p*H 6.3–6.5; 5 mg. for *p*H 3.0–3.1, etc. It is also obvious that the lower in *p*H the distilling mixture, the more the distillation of acetol is prolonged. Hence we may understand the fact that the yield of acetol was almost independent of the *p*H value of the mixture.

**Influence of Concentration of the Phosphate.** Glucose alone in water or in dilute mineral acids(16) gives no distillate which shows the iodoform reaction. Glucose in 5% phosphate solution (*p*H 5.9–6.3), however, yielded an iodoform-giving distillate. But the iodine-consuming capacity (calc. for acetol) of the distillate was far inferior to that with the corresponding 40% solutions. As shown in Fig. 2, which was plotted in the same principle as Fig. 1, the maximum rate of acetol formation is only 3 mg. for 5% solution whereas it is 17 mg. or 13 mg. for 40% solution.



5 g. Glucose+50 c.c. Solution.

40% Solution, *p*H 6.3–6.5
,, ,, *p*H 5.9–6.0
5% Solution, *p*H 5.9–6.3

Fig. 2.

## Experimental Part.

**Bis-phenylhydrazone and Bis-*p*-nitrophenylhydrazone of Methylglyoxal.** Glucose (Merck) (10 g.) was dissolved in 100 c.c. of about 40% potassium phosphate solution which was prepared by mixing(17) 18 g. anhydrous $KH_2PO_4$, 22 g. anhydrous $K_2HPO_4$ (Merck), and 60 g. water. After adjusting *p*H of the mixture to 6.6–6.8 with aqueous potassium hydroxide, it was subjected to distillation, keeping the level of the flask-content as constant as possible by adding, drop by drop, distilled water. On continuing the distillation, the flask-content became yellow and then brown in colour, and a dark brown tarry matter appeared after about 10 hours from the beginning. The

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(16) F. Fischler, *loc. cit.;* K. Bernhauer, *loc. cit.*

(17) For the further experiments at other *p*H, this ratio of mixing was modified and the minute adjustment of *p*H was made by aqueous phosphoric acid and aqueous potassium hydroxide.

distillate was almost neutral to litmus paper, gave a marked iodoform reaction, and reduced Fehling's solution in the cold. The latter two reactions, however, faded gradually in the course of distillation. On heating about 2 litres of the distillate with phenylhydrazine and acetic acid on a boiling water bath for six hours, a yellow flocculent precipitate was isolated, but the quantity was scant. By repeating the same experiment three times more, about 1 g. of the precipitate was obtained. Recrystallization from aqueous ethyl alcohol gave a light yellow crystalline substance melting at 145–147° in major quantity (0.5 g.), and a less soluble and higher melting substance in minor quantity. The analysis, and the determination of the molecular weight, of the former indicate it is methylglyoxal-phenylosazone. (Found: C, 71.6, 71.1; H, 6.1, 6.3; N, 21.9, 21.8; M, 246.6. Calc. for $C_{15}H_{16}N_4$: C, 71.4; H, 6.3; N, 22.2%; M, 252.2.)

On warming 9 liters of the distillate (about 3 g. as methylglyoxal)(18) from an experiment with 50 g. glucose, on a boiling water bath for four hours with 17 g. *p*-nitrophenylhydrazine (in 30% aqueous acetic acid), a dark red precipitate was obtained. After washed with hot alcohol, it was fractionated by crystallization from nitrobenzene into a substance in deep scarlet needles (m.p. 296–297°, yield 3.5 g.) and a similar appearing but lower melting substance (m.p. 275–278°, yield 1 g.). The former substance (Found: C, 52.8, 52.7; H, 4.4, 4.3; N, 24.4, 24.2. Calc. for methylglyoxal-bis-*p*-nitrophenylhydrazone $C_{15}H_{14}O_4N_4$: C, 52.6; H, 4.1; N, 24.6%) gave the characteristic colour reaction(19) for methylglyoxal-bis-*p*-nitrophenylhydrazone on warming with alcoholic alkali, i.e., a beautiful deep blue colouration which changes slowly to purple then to violet and finally to dull brown. The latter (m.p. 275–278°) gave also similar analytical results and the same colour reaction, and hence was concluded to be the same osazone with a little impurity.

In these experiments the distillation residue became more acidic than the starting mixture, e.g., *p*H shifted from 6.6–6.8 to 6.2–6.3. When a distillation was started with a mixture of *p*H 6.2–6.3, it was observed that almost no change of *p*H took place throughout the distillation, and the distillate gave the same osazones.

**Acetolsemicarbazone, Diacetyl- and Methylglyoxal-bis-semicarbazone.** The distillate (about 9 litres, 3.1 g. as acetol) prepared from a mixture (*p*H 6.2–6.4) of 200 g. glucose and 2 litres of 40% potassium phosphate solution, was warmed with 23 g. (5 mols) of semicarbazide hydrochloride and 28 g. of sodium acetate on a boiling water bath for half an hour. The reaction product was evaporated(20) to about 200 c.c. under a reduced pressure and in the current of carbon dioxide. The solution was filtered from a little quantity (0.17 g.) of a yellowish white precipitate, which melted over 265°. On further concentration of the filtrate under similar conditions, a faintly brown crystalline deposit amounting to 2.7 g. was obtained, and it melted at about 195°. These two solid substances were fractionated by crystallization from hot water into the following fractions:

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(18) It was estimated by Fischler's iodometry. *Z. analyt. Chem.*, **74** (1928), 28. See below.

(19) H. D. Dakin and H. W. Dudley, *loc. cit.*

(20) During the course of evaporation, some iodine-consuming substance was distilled and it was estimated 0.5 g. as acetol. It is, however, not sure at present whether it is actually acetol or not.

| | m.p. | g. |
|---|---|---|
| I. | 275–277° | 0.04 |
| II. | 263–265° | 0.01 |
| III. | 257–258° | 0.01 |
| IV. | 236–239° | trace |
| V. | 198–199° | 1.75 |

Fraction (I) melted at 279–280° after a recrystallization from warm acetic acid and this purified sample showed no lowering in melting point when mixed with diacetyl-bis-semicarbazone (Found: C, 35.7; H, 5.6. Calc. for $C_6H_{12}O_2N_6$: C, 36.0; H, 6.0%). Fraction (II) was recrystallized from boiling water in slightly yellow crystalline powder, m.p. 267–268° (Found: N, 45.5. Calc. for $C_5H_{10}O_2N_6$: N, 45.2%)(21), and its melting point was not depressed on admixing with methylglyoxal-bis-semicarbazone(22). On recrystallization of fraction (III) from hot water, the melting point showed no change. It was identified with hydrazodicarbonamide(23) (a condensation product of semicarbazide itself) by a mixed melting-point determination and an analysis (Found: N, 47.7. Calc. for $C_2H_6O_2N_4$: N, 47.5%.). Fraction (IV) was not further studied. After decolourized with animal charcoal, the hot water solution of fraction (V) was allowed to cool. The substance crystallized out in colourless, glistening, flat needles (about 1.5 g.) which melted at 199–200° alone or with an authentic sample of acetolsemicarbazone(24). (Found: C, 36.5, 36.4; H, 7.3, 7.2; N, 31.7, 31.8. Calc. for $C_4H_9O_2N_3$: C, 36.6; H, 6.9; N, 32.0%.)

Several other similar experiments were performed with similar results. In the experiments at other *p*H, e.g., 7.0–7.1, 6.6–6.8, 5.9–6.0, 4.8–5.0(25), the same acetolsemicarbazone was actually obtained as the main product. From 4.3 litres of the distillate (0.7 g. acetol) in an experiment at *p*H 6.2–6.3, 0.6 g. of acetolsemicarbazone (in a fairly pure stage, m.p. 196–198°) and 0.05 g. of a difficultly soluble substance was able to be obtained. The yield of acetolsemicarbazone from the distillate was theoretical (61%), if the acetol (0.15 g.)(26) that escaped the reaction with semicarbazide was taken into account.

**Acetol.** The distillate (8 litres, about 3.2 g. acetol). from an experiment at *p*H 6.2–6.3, was concentrated under a reduced pressure to about 600 c.c. (1.4 g. acetol). About 1 kg. of anhydrous sodium sulphate was added in small portions to the concentrated solution and the resulting solid mass was crushed in a mortar. The powdered mass was extracted three times with two litres of ether each time. The

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(21) The pure sample was only few mg. and it was microanalysed with the sample from other similar experiments.

(22) The authentic sample was prepared from aqueous methylglyoxal (C. Neuberg and E. Hoffmann, *Biochem. Z.*, **224** (1930), 491.). When a raw product (m.p. 258–260°) was recrystallized from a large volume of boiling water, it melted at 267–268°.

(23) C. Neuberg, *Biochem. Z.*, **191** (1927), 474.

(24) J. U. Nef, *Ann.*, **335** (1904), 253, 259.

(25) In the course of distillation at *p*H 6.9–7.1 and 6.6–6.8, an adequate quantity of dilute potassium carbonate solution was added from time to time the distilling mixture in order to keep the *p*H as constant as possible. No change in *p*H was observed throughout the distillation at *p*H 5.9–6.0 and 4.8–5.0.

(26) See the foot note (20).

total ethereal extract (about 5 litres) was dried over anhydrous sodium sulphate and then evaporated. The residue was fractionally distilled under a reduced pressure. The main fraction, 0.7 g. of an oil (b.p. 96–98°/159 mm., $d_{25}^{25}$ 1.059, $n_D^{25}$ 1.415) had a characteristic odour, reduced markedly Fehling's solution in the cold, and reacted easily with semicarbazide, giving acetolsemicarbazone.

**Estimation of Acetol in the Distillate.** In order to get some comparable values of the yields and the rates of formation of acetol in the distillation experiments at various *p*H, all the distillations were carried out in the same apparatus and at as constant a rate as possible. Forty per cent. potassium phosphate solution (50 c.c.) of a certain *p*H was distilled at the rate of 200 c.c. in 90 minutes, adding little by little a solution of 5 g. glucose in 1000 c.c. water and afterward fresh water to make the volume of the distilling flask-content constant. During the distillation, whenever the *p*H of the starting solution was higher than 6.2, it was necessary to add from time to time some aqueous potassium carbonate in order to keep the *p*H constant. The distillation was continued until the iodoform reaction became very faint. It took 25 to 34 hours. In each 200 c.c. of the distillate, the amount of acetol was estimated in the following way. To 10 c.c. of the distillate were added 10 c.c. N/10 iodine solution and 10 c.c. of 10% potassium hydroxide. After the mixture had been left for half an hour at room temperature, it was acidified with 8 c.c. of 15% hydrochloric acid and then titrated back with N/10 sodium thiosulphate solution. One c.c. of N/10 iodine solution is equivalent to 0.73 mg. of acetol, assuming the foregoing equation.

The experiments were made with the solutions of various *p*H, e.g., 7.0–7.1, 6.5–6.7, 6.3–6.5, 5.9–6.0, and 3.0–3.1. Yields of acetol and rates of its distillation in these experiments are summarized in Table 1 and Fig. 1.

As an example, one of experimental records is cited in Table 2.

Table 2. 5 g. glucose, *p*H 6.3–6.5, Distil. temp. 105–107°.

| Time (min.) for distilling 200 c.c. | c.c. of 0.0993 N -iodine solution for 10 c.c. distillate. | Acetol (mg.) in 200 c.c. distillate. |
|---|---|---|
| 110 | 0.10 | 1.5 |
| 90 | 0.45 | 6.6 |
| 100 | 0.78 | 11.5 |
| 80 | 0.88 | 12.9 |
| 75 | 1.08 | 15.9 |
| 85 | 0.95 | 13.9 |
| 90 | 1.03 | 15.1 |
| 95 | 1.14 | 16.8 |
| 85 | 1.05 | 15.4 |
| 90 | 0.87 | 12.7 |
| 85 | 0.80 | 11.7 |
| 85 | 0.78 | 11.5 |
| 90 | 0.74 | 10.9 |
| 85 | 0.78 | 11.5 |
| 85 | 0.52 | 7.6 |
| 85 | 0.42 | 6.2 |
| 90 | 0.39 | 5.7 |
| 75 | 0.34 | 5.0 |
| 70 | 0.31 | 4.6 |
| 75 | 0.19 | 2.8 |
| 75 | 0.18 | 2.7 |
| Total 1800 min. (30 hrs.) | | 202.5 mg. |

Distillate . . . . . . 4200 c.c.
10% $K_2CO_3$ solution to keep *p*H 6.3-6.5. . . . 7 c.c.
Yield . . . . . . . . . 4% on glucose

In order to determine the influence of concentration of potassium phosphate solution, the distillations of 5 g. glucose in 5% and 40% solutions of this salt were investigated under a similar precaution as in the foregoing experiments. The results are plotted in Fig. 2.

## Summary.

On steam distillation of a mixture of glucose and about 40% potassium phosphate solution of *p*H from 7.0 to 5.0, an appreciable quantity (4–5% of glucose used) of acetol and a little quantity of diacetyl- and methylglyoxal are found in the distillate. The more concentrated and the nearer to neutrality the phosphate solution is, the more favourable for the formation of acetol.

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