OXIDATION OF GLUCOSE BY IODINE IN THE PRESENCE OF INSULIN.

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1. Purpose of this Investigation.

This investigation was undertaken with the purpose of determining whether insulin, alone or in the presence of certain animal fluids, has any influence upon glucose *in vitro*. The establishment of such an influence might have much significance in relation both to the study of carbohydrate metabolism and to the development of methods of assaying insulin.

For this purpose it seemed desirable to use a property for determining the glucose content which is highly specific for this substance and would not be likely to be shown in at all the same degree by products that might result from a change occurring under the influence of the insulin. The rate of oxidation of glucose ($C_6H_{12}O_6$) to gluconic acid ($C_6H_{12}O_7$) by iodine in nearly neutral solution seemed to be an especially suitable property; preliminary experiments on the oxidation of glucose, mannose, fructose, and sucrose by iodine having confirmed the results of previous investigators that the rates are widely different for these different compounds. A series of experiments was therefore made on the comparative rates of this oxidation, using in one case a pure glucose solution, and in other cases glucose solutions which had been previously treated with aqueous insulin extract, alone or mixed with liver extract, blood serum, or oxalated blood.

This investigation forms part of a series of researches on the chemical nature and behavior of insulin undertaken in this laboratory under the general direction of Prof. A. A. Noyes, to whom we desire to express our thanks for advice as to the work and for his aid in the preparation of it for publication. This investigation was

assisted on the financial side from the funds which have been made available by Dr. Bertnard Smith for the general prosecution of insulin researches in this laboratory. We are also indebted to Dr. Smith, and to his associate Dr. Howard West, for cordial cooperation and assistance on the biological sides of this investigation; also to Mr. Albert L. Raymond of this laboratory for many valuable suggestions.

2. Previous Researches.

Various investigators have already studied the reaction between sugars and iodine, with reference to the development of methods of analysis. Thus, Romijn[1] early showed that glucose is quantitatively oxidized by iodine in alkaline solution to gluconic acid. He found that aldoses, in general, are oxidized under these conditions, while ketoses remain unchanged; and he devised a method of sugar analysis based on this principle. As the results obtained with potassium or sodium hydroxide were rather irregular, he substituted sodium borate for the alkali. The reaction, although slow, proved to be quantitative in this weakly alkaline solution. Bougault[2] substituted sodium carbonate for the borate; and this gave a much more rapid oxidation and led to a satisfactory method of analysis. Willstätter and Schudel[3] found that the reaction proceeded smoothly to completion when 0.1 N solutions of iodine and sodium hydroxide in the proportion of 2:3 were used. Colin and Liévin[4] modified Bougault's procedure by using disodium hydrogen phosphate in the place of the carbonate. Cajori[5] has recently published an article in which he gives a method of separately determining glucose, fructose, sucrose, and maltose, in mixtures; this method being based on the different behaviors of these sugars towards iodine and towards cupric hydroxide.

The effect of insulin on glucose has been studied by Winter and Smith.[6] These investigators made a series of experiments

[1] Romijn, G., *Z. anal. Chem.*, 1897, xxxvi, 349.

[2] Bougault, J., *J. pharm. et chim.*, 1917, xvi, series 7, 97; *Compt. rend. Acad.*, 1917, clxiv, 1008.

[3] Willstätter, R., and Schudel, G., *Ber. chem. Ges.*, 1918, li, 780.

[4] Colin, H., and Liévin, O., *Bull. Soc. chim.*, 1918, xxiii, series 4, 403.

[5] Cajori, F. A., *J. Biol. Chem.*, 1922, liv, 617.

[6] Winter, L. B., and Smith, W., *Brit. Med. J.*, 1923, i, 12; *cf. J. Physiol.*, 1922–23, lvii, 100.

upon the change in optical rotation produced in glucose and fructose solutions by the addition of small amounts of insulin and liver extracts. They thought they detected, in the case of both sugars, an appreciable change, which reached a maximum in 2 to 4 days and was greatly increased by the addition of phosphate solution. They suggested that the function of insulin might be the activation of the enzyme (presumably present in the liver) which is responsible for the transformation of ordinary α β-glucose into the γ form. The γ sugars are known to be very reactive chemically: in summarizing their properties, Hewitt[7] notes their great activity toward oxidizing agents and their marked instability in the presence of acid and alkali.

3. Experimental Procedure and Materials.

The oxidations were carried out in a nearly neutral solution with an excess of iodine, so as to insure a nearly complete conversion of glucose into gluconic acid in a convenient period of time. To give the proper acidity, solutions 0.3 molal in $NaHCO_3$ or 0.15 molal in Na_2HPO_4 were used. In these solutions the reaction is practically complete at room temperature in $2\frac{1}{2}$ hours. About five times the theoretical amount of iodine was employed. The oxidations were carried on simultaneously in 100 cc. rubber stoppered conical flasks. Since the runs compared were always parallel, it was not thought necessary to maintain a fixed temperature. The temperature, however, did not vary over $\frac{1}{2}$°C. throughout the entire work; and not more than a few tenths of a degree during a given set of oxidations.

The samples of sugars used were obtained as follows: sucrose from the U. S. Bureau of Standards; fructose, from Merck; and glucose and mannose, from Kahlbaum. Fresh solutions of the glucose were made up from time to time.

4. Rates of Oxidation of Glucose, Mannose, Fructose, and Sucrose.

The chemical equation expressing the oxidation of glucose or mannose by iodine is as follows:

$$C_6H_{12}O_6 + I_2 + H_2O = C_6H_{12}O_7 + 2HI$$

[7] Hewitt, J. A., *Brit. Med. J.*, 1923, i, 590.

The reaction rate at any moment should therefore be proportional to the prevailing concentrations both of the sugar and of the iodine, provided the temperature remains constant, and the hydrogen ion concentration, which has a large effect on the rate, also remains unchanged. That is, representing by A and B the initial concentrations of the sugar and iodine, respectively, by x the fraction of the sugar transformed at any time t, by $\frac{dx}{dt}$ the rate at which this fraction increases with the time, and by k the specific reaction rate at the given temperature and hydrogen ion concentration, the rate should be expressed by the equation:

$$A\frac{dx}{dt} = k\,(A - Ax)\,(B - Ax); \text{ or } \frac{dx}{dt} = k\,(1 - x)\left(\frac{B}{A} - x\right)$$

This equation yields on integration the following expression for the specific reaction rate.

$$k = \frac{0.4343}{t\,(B - A)} \log_{10} \frac{\left(\frac{B}{A}\right) - x}{\left(\frac{B}{A}\right)(1 - x)}$$

Reaction mixtures were made up in the case of each of these sugars by mixing the following solutions: 10.0 cc. of 0.1 per cent sugar solution; 10.0 cc. of 0.025 molal I_2 in 0.06 molal KI; 15.0 cc. of 1 molal $NaHCO_3$ or of 0.5 molal Na_2HPO_4; and 15.0 cc. of water. The mixtures were, therefore, initially 0.00111 molal in monosaccharide, 0.00500 molal in I_2, and either 0.3 molal in $NaHCO_3$ or 0.15 molal in Na_2HPO_4. Seven such mixtures of each sugar were placed in 100 cc. rubber stoppered conical flasks, and kept at the room temperature of 22–23°. From time to time one of the mixtures was removed, and 5 cc. of 6 N H_2SO_4 were added, whereby the reaction was stopped; the decrease in the free iodine content was then determined by titrating it with a standard $Na_2S_2O_3$ solution, which was 0.0200 molal in the $NaHCO_3$ experiments and 0.0189 molal in the Na_2HPO_4 experiments.

The results with glucose and mannose are given in Table I. In the columns headed 100 x are given the percentage of glucose oxidized as calculated from the decrease in iodine content; and in those headed k, the values of the specific reaction rate calculated by the above equation. The values given for the oxidation in the

$NaHCO_3$ solutions are the average of the three similar runs; those in the Na_2HPO_4 solutions represent one set of determinations.

It will be seen from Table I that, as would be expected for a bimolecular reaction taking place under constant catalytic conditions and temperature, the values of the specific reaction rate k are fairly constant for both glucose and mannose in the presence of the hydrocarbonate. In the presence of the hydrophosphate, however, the values of k show a progressive decrease.

Similar runs with fructose and sucrose showed an iodine consumption at the end of 160 minutes equivalent to only 6 per cent oxidation of the sugar.

TABLE I.

Rate of Oxidation of Glucose and Mannose by Iodine at 22–23°.

Time.	Glucose with $NaHCO_3$.		Glucose with Na_2HPO_4.		Mannose with $NaHCO_3$.		Mannose with Na_2HPO_4.	
	$100\,x$	k	$100\,x$	k	$100\,x$	k	$100\,x$	k
min.								
5	29.5	14.6	44.8	19.2	12.3	5.28	19.4	8.8
10	50.0	14.7	56.1	17.7	22.5	5.24	27.9	6.7
15	65.0	15.7	63.9	14.5	31.5	5.23	31.2	5.1
20	74.2	15.0	70.6	13.7	38.5	5.13	38.9	5.1
40	92.1	13.1	84.6	10.7	59.0	4.85	52.1	3.9
80	98.7		93.5		77.8	4.23	68.0	3.1
160	100.5		100.4		92.5		81.7	

These results conform with those of earlier investigators in that the aldoses are oxidized, while the ketose and biose remain practically unchanged. The small effect which is noted in the fructose and sucrose oxidations may well be due to experimental error. It is evident that even mannose and glucose differ greatly from each other in their rates of oxidation in both the hydrocarbonate and hydrophosphate solutions. The difference between the rates of glucose, mannose, and fructose should therefore be marked enough to indicate any transformation of glucose into isomeric hexoses which might occur in the presence of insulin.

5. Oxidation of Glucose in the Presence of Insulin.

The insulin used throughout the work was prepared by the process described by Collip[8] and was further purified by precipitation

[8] Collip, J. B., *Tr. Roy. Soc. Canada*, 1922, xvi, series 3, section v.

from aqueous solution by changing the hydrogen ion concentration. The precipitate was washed with ether and dried over calcium chloride. A 0.1 per cent solution of the dried substance was made up by dissolving it in approximately 0.001 N hydrochloric acid. Previous tests showed that the insulin was stable in this solution. The activity of this insulin was tested on rabbits several times: 0.3 cc. per kilo weight of rabbit lowered the blood sugar of a normal rabbit from 110 to 45 mg. per 100 cc. in 1 hour, and the convulsive dose was between 0.3 and 0.4 cc. The insulin was also found to retain most of its activity on standing for 1 hour in solutions with an alkalinity similar to that of the oxidation mixtures. In all the calculations, the iodine taken up by the insulin

TABLE II.

Oxidation of Glucose by Iodine in Presence of Insulin.

Time.	Solution 0.30 molal in $NaHCO_3$.			Solution 0.15 molal in Na_2HPO_4.		
	Without insulin.	With insulin.	Difference.	Without insulin.	With insulin.	Difference.
min.						
5	25.3	23.4	+1.9	34.0	34.9	−0.9
10	43.7	40.7	+3.0	49.3	47.8	+1.5
15	52.6	53.8	−1.2	57.9	57.2	+0.7
20	61.5	60.7	−0.8	64.9	64.2	+0.7
40	80.6	80.2	+0.4	79.5	79.7	−0.2
80	95.7	96.3	−0.6	92.2	91.6	+0.6
160	101.9	101.2	+0.7	96.2	96.8	−0.6

introduced into the reaction mixture was subtracted from the total iodine consumed.

The method of procedure was exactly the same as that described above. The reaction mixtures were made up just as in the experiments on the oxidation of the sugars alone; except that there were always run side by side duplicate mixtures differing only in the respect that one contained in the 50 cc. volume 1 cc. of 0.1 per cent insulin solution, and the other contained no insulin. The temperature was 22-23°.

Table II contains the results of these experiments. The numbers in the first column show the time elapsed after the mixing of the solutions; those in the other columns denote the percentage of glucose transformed.

It is evident from these results that insulin alone has no appreciable effect upon the oxidation of glucose by transforming it into either a more or less chemically reactive form. This is true even though a considerable excess of insulin was present over that required for the oxidation of the 10 mg. of glucose; thus the quantity was sufficient to lower the blood sugar of a 3 kilo rabbit 50 mg. per 100 cc. of blood in 1 hour.

6. Oxidation of Glucose in the Presence of Liver Extract and Insulin.

Both alcoholic and aqueous liver extracts were used in these experiments. The method of procedure was the same as that followed in previous experiments with the exception that the reaction mixtures were allowed to stand for 1 hour with the liver extract and insulin, before the addition of the iodine. A temperature of 22-24° was maintained by immersing the solutions in a water bath. As before, one-half of the solutions contained 1 cc. of 0.1 per cent insulin solution (in place of 1 cc. of water), in addition to the other ingredients, which were as follows: 10.0 cc. of 0.1 per cent glucose solution, 15.0 cc. of a solution 1.0 molal in $NaHCO_3$ or 0.5 molal in Na_2HPO_4, 2.0 cc. of alcoholic or aqueous liver extract, 12.0 cc. of distilled water, and 10.0 cc. of a solution 0.05 N in I_2 and 0.06 N in KI.

The alcoholic liver extract was prepared in the following manner. Fresh beef liver was ground and intimately mixed with an equal volume of 95 per cent alcohol. The juice was pressed out of the mass, diluted to 4 volumes, and filtered. The filtered solution was used in the oxidations.

The aqueous liver extract was prepared as follows: Equal volumes of fresh beef liver and 0.9 per cent sodium chloride solution were ground together thoroughly with sand in a mortar. The juice was then pressed out, diluted to 4 volumes, and filtered.

Table III contains the results of the experiments in which the alcoholic extract was used; and Table IV those in which the aqueous extract was used. The figures in the first column show the length of time of the oxidations; those in the other columns show the cubic centimeters of 0.025 molal I_2 consumed in the various reaction mixtures. The second pair of experiments in each table was made with half the quantity of the liver extract.

These experiments show that glucose is unaffected by insulin in the presence of alcoholic or aqueous liver extract.

TABLE III.

Oxidation of Glucose by Iodine in Presence of Alcoholic Liver Extract and Insulin.

Time.	Solution 0.30 molal in $NaHCO_3$.			Solution 0.15 molal in Na_2HPO_4.		
	Without insulin.	With insulin.	Difference.	Without insulin.	With insulin.	Difference.
min.						
5	1.63	1.69	−0.06	1.50	1.50	0.00
10	2.37	2.38	−0.01	2.09	2.08	+0.01
15	2.86	2.83	+0.03	2.36	2.38	−0.02
20	3.48	3.44	+0.04	2.75	2.71	+0.04
40	4.23	4.14	+0.09	3.36	3.37	−0.01
80	5.34	5.29	+0.05	4.14	4.12	+0.02
160	6.23	6.17	+0.06	4.93	4.84	+0.09
5	1.75	1.81	−0.06	1.46	1.45	+0.01
10	2.46	2.45	+0.01	1.91	1.90	+0.01
15	3.01	2.97	+0.04	2.23	2.20	+0.03
20	3.26	3.23	+0.03	2.43	2.42	+0.01
40	4.34	4.33	+0.01	2.97	2.94	+0.03
80	5.21	5.21	0.00	3.71	3.69	+0.02
160	5.41	5.41	0.00	4.46	4.39	+0.07

TABLE IV.

Oxidation of Glucose by Iodine in Presence of Aqueous Liver Extract and Insulin.

Time.	Solution 0.30 molal in $NaHCO_3$.			Solution 0.15 molal in Na_2HPO_4.		
	Without insulin.	With insulin.	Difference.	Without insulin.	With insulin.	Difference.
min.						
5	3.96	3.93	+0.03	3.51	3.49	+0.02
10	4.98	4.98	0.00	4.10	4.09	+0.01
15	5.35			4.40	4.38	+0.02
20	5.67	5.66	+0.01	4.74	4.72	+0.02
40	6.44	6.43	+0.01	5.46	5.27	+0.19
80	7.07	7.09	−0.02	6.25	6.24	+0.01
160	7.39	7.45	−0.06	7.02	7.01	+0.01
5	2.26	2.25	+0.01	1.52	1.49	+0.03
10	2.71	2.72	−0.01	1.93	1.90	+0.03
15	3.10	3.10	0.00	2.26	2.23	+0.03
20	3.47	3.48	−0.01	2.55	2.53	+0.02
40	4.00	4.02	−0.02	2.89	2.88	+0.01
80	4.61	4.64	−0.03	3.24	3.24	0.00
160	4.88	4.92	−0.04	3.59	3.60	−0.01

7. Oxidation of Glucose in the Presence of Insulin and Blood.

In order to make the work more complete, the effect of insulin on the oxidation of glucose in the presence of the blood serum and oxalated blood was studied. As in the other experiments, the results consistently indicated no change in the glucose on the addition of insulin.

The method of procedure was exactly the same as that followed in the work with liver extract. The reaction mixtures were changed only by substituting blood or blood serum for the liver extract.

Table V contains the results of the set of experiments in which 0.2 cc. of oxalated blood was added to each of the reaction mixtures. The temperature was 23.2–23.4°

TABLE V.

Oxidation of Glucose in Presence of Oxalated Blood and Insulin.

Time.	Solution 0.30 molal in $NaHCO_3$.			Solution 0.15 molal in Na_2HPO_4.		
	Without insulin.	With insulin.	Difference.	Without insulin.	With insulin.	Difference.
min.						
5	2.92	2.91	+0.01	2.51	2.48	+0.03
10	3.48	3.48	0.00	2.94	2.92	+0.02
15	3.83	3.89	−0.06	3.24	3.22	+0.02
20	4.07	4.12	−0.05	3.44	3.42	+0.02
40	4.75	4.77	−0.02	4.01	4.00	+0.01
80	5.20	5.23	−0.03	4.60	4.57	+0.03
160	5.64	5.64	0.00	5.03	5.05	−0.02

SUMMARY.

It was first shown by this investigation that, in confirmation of the results of others, the rate of oxidation of various sugars by iodine in solutions of $NaHCO_3$ or Na_2HPO_4 varies greatly with the nature of the sugar; thus mannose was oxidized only about one-third as fast as glucose, and fructose and sucrose were scarcely oxidized at all, under the conditions of the experiments. This indicated that a study of the relative rates of oxidation of glucose before and after treatment of it with insulin would furnish a sensitive means of determining whether any of the glucose had been transformed by it into any other substance, even into a stereomeric hexose.

Strictly comparable experiments were therefore made with glucose alone, with mixtures of it with insulin, with insulin and liver extract, or with insulin and blood serum or oxalated blood. In no case was any difference detected in the rate at which the iodine is consumed. This shows that no appreciable reaction takes place between glucose and insulin even in the presence of the animal fluids mentioned. It indicates, therefore, that the metabolic process must be more complicated in character; also that there is little promise of developing a method of assay for insulin on the basis of its action on glucose in glass.