XXXV. THE ESTIMATION OF GLUCOSE IN BIOLOGICAL MATERIAL.

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THE methods of estimation of glucose in general use in biological research are all based on its power of reducing various substances in alkaline solution. Amongst others the following compounds have been employed: copper salts, sodium picrate, potassium ferricyanide, methylene blue, indigotetrasulphonic acid and dinitrosalicylic acid. Additional variations have been introduced by different methods of estimating the amount of substance reduced and by different compositions of the various solutions used.

Recent years have witnessed the development of methods of estimation of glucose which are applicable to very small amounts or to a particular medium, *e.g.* blood or urine.

Two tests have usually been employed in order to determine the fitness of a given method for a given piece of physiological research. Firstly, a demonstration that certain other biological substances whose presence is suspected, *e.g.* acetone or β -hydroxybutyric acid, do not, in a pure aqueous solution, reduce the substance used to oxidise the glucose. Secondly, the correct recovery of known amounts of glucose added to the biological material for which the method is devised. If these criteria are satisfied, the original reducing power of the material is calculated as, and attributed to, its glucose content. The standard of reference employed is usually a solution containing solely a known weight of the purest glucose obtainable.

For clinical purposes any standard method probably gives reliable and valuable information when used by an experienced worker. For research work, however, it would seem highly desirable to gain some knowledge that would either confirm or modify the above assumption in a quantitative manner.

Comparative research on different methods of estimation of glucose when applied to the same biological fluid has not hitherto yielded very encouraging results.

De Wesselow [1919] found that the Lewis-Benedict picric acid method yielded results 30–50 % higher than those obtained by MacLean's method, due in the main to unknown substances in the corpuscles. Folin and Wu [1919] stated that their method yielded lower results than the Lewis-Benedict

method. Höst and Hatlehol [1920] compared four methods over a large number of normal bloods and found that Bang's [1918] copper method and that of Hagedorn and Jensen [1923] agreed fairly well, as did Folin and Wu's method with the Myers-Bailey [1916] modification of the Lewis-Benedict method, but that the two latter frequently yielded decidedly higher values than did the two former. They add: "It is uncertain whether any of the blood-sugar methods hitherto used give only the amount of glucose." The fact [Wishart, 1920; Macleod, 1921] that direct determinations of the amounts of glucose present in whole blood, plasma, and corpuscles, do not tally with the haematocrit determinations is, in reality, a serious criticism against the validity of the methods employed, because it implies that whatever be the nature of the various reducing agents present, the effect of each depends in some degree on the varying concentrations of the others or possibly of some of the non-reducing substances present. The discovery of insulin and the subsequent researches on hypoglycaemia have brought the question of glucose estimation into prominence.

Winter and Smith [1924] stated that in hypoglycaemic convulsions following an injection of insulin the blood of rabbits is frequently practically free from glucose although their blood sugar, as estimated by Bang's method, is about 0.04 %. The writer has not been able to observe so complete an absence of glucose from the blood but, as will be shown below, the concentration of glucose under such circumstances may be very considerably below the amount estimated to be present by certain micro-methods. According to Macleod [1924] the level of blood sugar at which convulsions occur depends on the previous feeding and the quantity of insulin injected.

The problem of the nature, concentration and physiological behaviour of the reducing substances in blood other than glucose is probably of considerable importance.

The absence of close agreement between the standard methods for estimating glucose in biological fluids suggests perhaps that the proportion of other reducing substances to glucose in blood may be by no means insignificant, and that their concentration may vary considerably in different physiological or pathological conditions. It is by no means certain that the same group of substances is always involved.

As a preliminary study, an investigation was made into one aspect of the problem of glucose estimation which has been practically entirely neglected, namely the effect of the presence in the reducing fluid of other biological substances with glucose on the reducing power of the latter.

Taylor [1924] showed that the presence of creatinine or of uric acid interfered with the estimation of glucose by Folin and McEllroy's [1918] direct method.

This method was not a very convenient one for research on such fluids as concentrated blood-filtrates and the effect of such substances as uric acid, in the absence of glucose, cannot be determined.

Accordingly, the two methods chosen for investigation were the Wood-Ost method [Wood and Berry, 1904], modified as to quantities of the reagents by Cole [1920], and the Hagedorn and Jensen method.

The Wood-Ost method has the following advantages.

The degree of alkalinity is less than in those methods using alkaline hydroxides. There is no organic polyhydroxy-substance used. The amount of cuprous oxide is estimated directly after removal by filtration and hence the possible complication of the other biological substances interfering with the estimation of the unreduced cupric salts is avoided. The sole object of consideration was the reducing power of glucose.

The Hagedorn and Jensen method was chosen because of the need for a micro-method. No tartrate or citrate is used in this reagent since potassium ferricyanide replaces the copper sulphate.

EXPERIMENTAL DETAILS.

The experiments consisted for the most part in determining the reducing power by means of the two methods of aqueous solutions containing known amounts of glucose and of the various added substances. The glucose used was a specimen of Kahlbaum's "Dextrose" and its purity was estimated to be 99.6 %. The other organic chemicals were the purest obtainable and were carefully recrystallised.

The concentrations of uric acid and of creatinine employed were admittedly considerable in order to demonstrate their effects as clearly as possible. The concentrations of amino-acids are not unreasonable in comparison with the amount of glucose present. The amino-acid nitrogen of a protein-free blood filtrate varies from 6–10 mg. per 100 cc. of blood. This corresponds to a total concentration of amino-acids of 40–70 mg. per 100 cc. of blood, *i.e.* from one-half to two-thirds of the estimated concentration of reducing carbohydrates.

The following are examples of the results obtained:

A. *Wood-Ost method; values in mg. in the 10 cc. of fluid used.*

(1)	Glucose taken	Uric acid	"Glucose" found	Calculated
	None	17.7	4.8	—
	"	35.4	9.3	—
	"	53.0	15.2	—
	52.0	0.7	52.2	52.2
	52.0	2.8	52.7	52.8
	52.0	7.0	53.7	53.8
(2)		Creatinine		
	None	50.5	16.1	—
	50.8	0.81	51.5	51.1
	50.8	2.02	51.5	51.4
	54.4	4.04	55.4	55.7
	50.8	8.1	53.7	53.4
	50.8	20.2	57.7	57.2
(3)		Urea		
	59.6	100.0	59.1	—

(4)	Glucose taken	Glycine	"Glucose" found	Calculated
	None	20.0	None	—
	54.5	4.0	58.5	—
	54.5	8.0	62.3	—
	54.5	20.0	67.8	—
(5)		Tyrosine		
	None	50.0	None	—
	59.6	8.0	67.0	—
	59.6	20.0	71.7	—
(6)		Cystine		
	None	94.5	3.4	—
	50.7	1.9	52.7	50.8
	50.7	3.8	54.5	50.8
	50.7	7.6	54.5	51.0
(7)		Glutaminic acid		
	None	90.0	None	—
	50.6	None	50.6	—
	50.6	0.9	51.1	—
	50.6	1.8	51.8	—
	50.6	3.6	53.6	—
	50.6	9.0	54.1	—
(8)		Cystine, glutaminic acid		
	52.0	3.8 7.2	58.3	52.1

B. Hagedorn and Jensen method. All figures given are mg. per 100 cc.

(1)	Glucose taken	Uric acid	"Glucose" found	Calculated
	None	100	53	—
	"	500	264	—
	112	50	143	138
(2)		Creatinine		
	None	100	67	—
	"	500	282	—
	167	100	226	234
(3)		Glycine		
	167	None	167	—
	None	100	None	—
	167	100	167	—
(4)		Cystine		
	109	None	110	—
	None	100	44	—
	109	10	119	113
	109	100	132	153
(5)		Glutaminic acid		
	109	None	108	—
	109	50	108	—
	109	100	109	—
	164	100	164	—
	167	100	167	—

With regard to the Wood-Ost method, the following points may be noted. In the case of uric acid, a considerable amount of copper urate was precipitated which caused some delay in filtering, but the liberated uric acid reacts only very slightly with potassium permanganate in the cold. The solutions remained blue during the reduction. Creatinine, as was pointed out by Taylor [1924], has a most remarkable power of delaying the precipitation of the red

cuprous oxide; whether due to combination with it or with the glucose appears to be uncertain. The solutions containing the larger amounts of creatinine and glucose acquired the opaque yellowish-green appearance which is seen when urine containing glucose is heated with Fehling's solution. This colour did not occur in the absence of glucose. The amount of cupric salt reduced by mixtures of glucose and either of the above substances is approximately that calculated from their separate reducing powers. Urea had a slight depressing effect, presumably due to the ammonia liberated by partial hydrolysis.

In the case of the mixtures of glucose with amino-acids, the amount of copper salt reduced was decidedly greater than that reduced by the glucose alone, even when the amino-acid alone had no reducing power. If, as in the case of cystine, a small amount of copper was reduced by the amino-acid alone, the amount reduced by the mixture of it with glucose was still greater than that calculated from the sum of their two separate reducing powers. In the presence of cystine the rate of reduction appeared to be greatly increased and the solution became somewhat greenish in colour but remained perfectly transparent. In the case of the other amino-acids, the appearance of the solution was similar to that when glucose alone was used.

With the Hagedorn and Jensen method no coupled oxidation of amino-acids could be detected. The reducing power of mixtures of glucose with either cystine, uric acid or creatinine was equal to that calculated on the assumption that each substance reduced the ferricyanide independently of the other.

In order to show, if possible, the actual decomposition of a stable amino-acid in the presence of glucose by an alkaline copper solution, 1 g. of tyrosine was heated with 5 g. of glucose and 300 cc. of Benedict's qualitative copper solution. After cooling, the liquid was extracted with ether. The ethereal extract was freed from ether by distillation and the solid remaining after removal of the ether was dissolved in a few cc. of water and carefully filtered. The filtrate gave strong Millon's and xanthoproteic reactions but was free from amino-nitrogen. This indicated that a portion of the tyrosine had been decomposed.

In order to observe the measure of agreement between the Wood-Ost and the Hagedorn and Jensen methods on some blood filtrates, the following technique was employed. The proteins of blood were precipitated with tungstic acid; the precipitate was filtered off and well washed with cold water. The united filtrate and washings were adjusted to p_H 6.5 and concentrated under diminished pressure to a small bulk. Alcohol was added to make the concentration 75 % and the precipitate of sodium sulphate, etc., filtered off and washed with 75 % alcohol. The alcohol was removed under diminished pressure and the residue dissolved in a small volume of water and filtered. In this filtrate the reducing power was estimated by the two methods. The results appear below. The examples of rabbit's blood were part of a joint experiment with Dr H. D. Kay and Mr J. T. Irving. The writer desires to acknowledge their courtesy in this matter.

Source of protein-free filtrate	"Glucose" mg. per 100 cc.	
	Wood-Ost	H. and J.
1. Ox blood	280	250
2. Ox corpuscles	3	19
3. Sheep corpuscles	5	30
4. Rabbit's blood	900	1090
5. Rabbit's blood (convulsed)	320	600

In Nos. 4 and 5 the samples were suitably diluted before estimation.

The high values obtained are due to considerable concentration. Dr Kay isolated specimens of phenylglucosazone from No. 4 and No. 5.

The considerable divergences in the case of the blood of rabbits convulsed with insulin indicate that in this case at least unknown reducing substances are present in significant concentrations.

DISCUSSION OF RESULTS.

The facts recorded in this paper have two main bearings on research on carbohydrates.

Firstly, they indicate the need of testing any proposed method of analysis on mixtures of pure substances as similar as possible in composition to the mixtures it is intended to analyse in the course of the research, as well as on as many individual substances as possible.

Secondly, they emphasise the fundamental lack of specificity inherent in the methods at present available for the estimation of carbohydrates. Statements, for instance, that a given method "does not estimate substances in the blood filtrate other than reducing sugars" are probably in need of experimental confirmation. Similar considerations apply to Harned's [1925] search for the "absolute value" of the concentration of glucose in blood.

The apparent absence of amino-nitrogen from his blood-filtrates may have been due to the presence therein of sodium nitrate, arising from the decomposition of the mercuric nitrate, which would interfere with the Kjeldahl method of estimation.

The recently published micro-method of Benedict [1925] is interesting in its apparently greater specificity and the use of sodium bisulphite to increase the amount of cuprous oxide reduced by a given amount of glucose. Since, however, glucose is now shown to be capable of inducing coupled oxidations, an explanation of the beneficent effect of the former substance on these lines would seem to be at least as probable as one based on any power of combining with portions of the glucose molecule under the conditions obtaining during the reduction of the copper sulphate, and hence protecting them from the action of the alkali.

All conclusions concerning the nature of the reducing carbohydrate in blood or other biological fluids based on the ratio of its polarimetric estimation to that based on copper reduction methods may be viewed with reserve.

The presence of glutathione [Holden, 1925] in blood-filtrates lowers the rotation and increases the reducing power towards many copper reagents. The problem of its estimation in these filtrates is not easy of solution. Tunnicliffe's [1925] method is not suitable owing to the slowness of filtration from the precipitated proteins. By reduction with zinc dust and acid followed by filtration and the use of Tunnicliffe's method on a blood filtrate the writer estimated it to be present to at least 0.02 % in the original blood. This would be quite sufficient to alter appreciably the reducing power of the glucose present, in addition to lowering the rotation. The estimation of glucose based on the quantitative formation of glucosazone and its subsequent titration with titanous chloride as proposed by Knecht and Hibbert [1924] is, from the point of view of specificity, most attractive, but after a prolonged trial the writer was unable to place sufficient reliance on it for purposes of research. In spite of the utmost precautions in connection with the purity of all the substances used, the results obtained were frequently far too high, owing apparently to the oxidation of a portion of the phenylhydrazine during the formation of the osazone, which oxidised some of the titanous chloride. The method was originally worked with a specially purified foreign brand of phenylhydrazine which is not available in England, but it is doubtful if that is the whole explanation of the difficulty.

SUMMARY.

If glucose is estimated by a copper reduction method in a fluid containing amino-acids the value obtained may be too high, even though the amino-acids present do not alone reduce the copper solution. The error may amount to 10-15 % and appears to be due to an oxidation of the amino-acids coupled to that of the glucose.

The Hagedorn and Jensen method is unaffected by the presence of most amino-acids in moderate concentration.

If the Wood-Ost and the Hagedorn and Jensen methods be applied to the concentrated filtrate from the blood of a rabbit in hypoglycaemic convulsions following the injection of insulin, there is a great divergence between the amounts of glucose estimated to be present, due to unknown reducing substances. These methods agree more closely on normal blood-filtrates.

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