CLX. THE ESTIMATION OF GLUCOSE IN PHYSIOLOGICAL FLUIDS BY THE REDUCTION OF COPPER SALTS.

BY WILLIAM WHITE TAYLOR.

From the Department of Physiology, University of Edinburgh.

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THE importance of the estimation of glucose in physiological fluids does not need to be emphasised; that there are difficulties in connection with the estimation is proved by the many variations and modifications of the copper reduction method which have been introduced from time to time¹.

It is a commonplace among teachers of physiological chemistry that a normal urine, to which glucose has been added, cannot be titrated satisfactorily with Fehling's solution, the end-point being much more indefinite than with diabetic urine; and attempts, unsuccessful so far as I am aware, have been made to ascertain to what substances, either by their presence or absence, the difference is due. It occurred to me that there might not be the same difficulty with reagents of the type of Benedict or Folin-McEllroy, which give a white precipitate of cuprous thiocyanate, instead of red cuprous oxide.

On trial this was found to be the case, the end-point being easily observed, though the liquid is yellowish, and not colourless as with pure glucose solutions.

But when a known amount of glucose solution was added to normal urine (*i.e.* no trace of reduction of Folin-McEllroy qualitative reagent, no albumin, bile, or ketone bodies), the amount of glucose indicated by the titration against Folin-McEllroy quantitative reagent was always greater than the amount actually added. This discrepancy is not peculiar to the Folin-McEllroy reagent, for similar results are obtained with Benedict's reagent, and also with Fehling's standard reagent.

The following results are typical:

Reagent	5 cc. of 2 % glucose added to	
	20 cc. water	20 cc. urine
Folin-McEllroy (5 cc.)	6.80 cc.	4.95 cc.
	6.70 „ } (=0.370 %)	5.20 „ } (=0.485 %)
	6.75 „ }	5.15 „ }
Benedict (10 cc.)	5.40 „ (=0.370 %)	4.15 „ (=0.482 %)
Fehling (5 cc.)	5.8 „ (=0.43 %)	4.6 „ (=0.54 %)

¹ The principle and theoretical basis of the method and of the modifications recently introduced, are so well known that it is not necessary to give a review of them, nor of the literature relating to them.

The difference in the two values is over 30 %.

A further preliminary experiment was done to ascertain whether a similar effect was obtained with diabetic urine. The result was as follows:

5 cc. of Folin-McEllroy reagent.

(a) Diabetic urine		3.65 cc.	(= 0.685 %)
(b) ,, ,, (10 cc. + 1 cc. of 2 % glucose)		2.90 cc.	(= 0.862 %)

The calculated value for (b) is 0.804 %.

That the divergence is not constant, but depends on the amount of urine added to a glucose solution is shown by the figures given below:

5 cc. of a 2.5 % solution of glucose were made up with the specified amounts of urine and water to a total volume of 25 cc. and titrated against 5 cc. of Folin-McEllroy reagent.

Urine	Water	Titration	Glucose (%)
—	To 25 cc.	4.92	0.508
1 cc.	"	4.35	0.575
2 "	"	3.97	0.630
To 25 "	—	3.65	0.685

The final increase is 35 % which agrees with the 31 % increase previously found, considering that different samples of urine were used in the two experiments.

Of the various known constituents of normal urine, uric acid and creatinine appeared most likely to be the cause of these differences; for, though they, either alone or together, may not be capable of oxidation by the copper reagent used, it is quite possible that when glucose is being oxidised in their presence, they also undergo oxidation. It is also possible that these or other constituents of the urine enter into combination with part of the copper, forming a complex which is not so readily reduced as the original copper complex.

The Effect of Uric Acid.

In the experiments the results of which are given below, the solutions used were 0.5 % uric acid, dissolved by the aid of lithium carbonate, and 0.5 % glucose. The stated amount of uric acid was added to 5 cc. of Folin-McEllroy reagent and the titration carried out in the usual manner. The table gives a summary of the results obtained.

5 cc. of Folin-McEllroy reagent		
Uric acid (0.5 %)	Glucose (0.5 %)	Decrease in cc. for 0.1 cc. uric acid
—	4.98 cc.	—
0.2 cc.	4.90 "	0.040
0.3 "	4.85 "	0.043
0.4 "	4.78 "	0.050
0.6 "	4.66 "	0.054
0.8 "	4.59 "	0.049
1.0 "	4.48 "	0.050
1.5 "	4.29 "	0.046
2.0 "	4.15 "	0.042

The figures in the third column are obtained by subtraction of the corresponding titration values from that of glucose alone, and division of this difference by the number of 0.1 cc. added. They show that the effect produced is practically proportional to the amount of uric acid added.

The Effect of Creatinine.

The solutions used in the following determinations were 0.320 % creatinine, 0.51 % glucose, the procedure being the same as with uric acid.

The next table gives a summary of the results.

5 cc. of Folin-McEllroy reagent		
Creatinine (0.320 %)	Glucose (0.51 %)	Decrease in cc. for 0.1 cc. creatinine
—	4.86 cc.	—
0.05 cc.	4.84 „	0.04
0.10 „	4.66 „	0.20
0.15 „	4.55 „	0.21
0.20 „	4.58 „	0.14
0.25 „	4.55 „	0.12
0.50 „	4.60 „	0.05
1.0 „	4.45 „	0.04

One difference between the uric acid and the creatinine results is at once apparent. With the latter the figures in column 3 are not even approximately constant; in fact after 0.15 cc. there is practically no change in the titration value until 1 cc. has been added.

Another difference, equally apparent when carrying out the estimations is, that creatinine, even in small amounts, retards the reduction to such an extent that it is not easy to complete the titration within the prescribed eight minutes. Only when almost the whole of the glucose required in any given case, as found by a preliminary titration, is added at the beginning, and the solution then kept boiling for at least six minutes (instead of the usual four minutes) is it possible to finish in the time, and obtain concordant results.

The colour of the reagent is distinctly changed to a greenish blue on addition of the creatinine, and the final change is from a greenish tint to colourless.

The Effect of Urea and Ammonium Salts.

When a brief account of the preliminary observations on this subject was presented at the International Physiological Congress, Professor Emil Werner suggested that it would be interesting to know whether ammonium salts were also oxidised along with the glucose by the copper reagent. For he had found in his investigation [Werner, 1922] of the decomposition of urea by alkaline hypobromite, that when glucose is added to the urea, as recommended by Méhu [1879] the volume of gas evolved is increased, although the formation of cyanate is not prevented, and is in fact increased; the increase in volume of gas is due to the production of carbon monoxide from the glucose, although

none is evolved when glucose alone is added to alkaline hypobromite. Further, when ammonium chloride, to which glucose is added, is acted on by alkaline hypobromite, the volume of gas evolved progressively diminishes with increasing amounts of glucose, though it contains carbon monoxide, while the amount of cyanate progressively increases. This point will be referred to later.

As a matter of fact, it has been found that the presence of ammonium salt has no marked effect on the estimation of glucose by means of the Folin-McEllroy reagent and the same applies in the case of urea.

The Effect of Creatinine + Uric Acid.

Since urea and ammonium salts were found to have no appreciable effect, it remained to ascertain how far the presence of both creatinine and uric acid affected the titration. The results are given below.

<i>Creatinine + Uric Acid.</i>				
5 cc. of Folin-McEllroy reagent				
Creatinine (0.320 %)	Uric acid (0.5 %)	Glucose (0.5 %)	Difference	
			Obs.	Calc.
—	—	4.94	—	—
0.2 cc.	—	4.66	0.28	—
—	0.8 cc.	4.64	0.30	—
0.2 "	0.8 "	4.60	0.34	0.58
0.2 "	0.8 "	4.58	0.36	
0.1 "	—	4.72	0.22	—
0.1 "	0.8 "	4.60	0.34	0.52

It is evident that the effect is not additive, although it is greater than that of either alone. The retardation of reduction was very marked; indeed it was almost impossible to complete the titration in less than 10–12 minutes, and this may affect the end-point of the reaction. Of the duplicate estimations given above, the first took eleven minutes, the second between eight and nine minutes, as almost the whole amount was added at once.

DISCUSSION OF RESULTS.

The experiments described above have established that when glucose is oxidised in urine, or in the presence of uric acid, or creatinine, by means of alkaline copper reagents, the reducing action is greater than when the same amount of glucose in water is treated with the same reagent.

Regarding the mechanism of the reaction, three possible explanations suggest themselves:

(1) Coupled reaction: *i.e.* the copper reagent which does not appreciably oxidise the uric acid or creatinine when alone, does so when glucose is oxidised in their presence. Many such coupled reactions are known.

(2) The glucose is oxidised in a different manner or to a different stage in the presence of these substances, which themselves do not undergo oxidation.

(3) The substances enter into combination with the copper reagent, forming complexes which resist reduction.

Which of these explanations is the most probable, the evidence available at present does not enable one to decide. In favour of (3) one may point to the colour change when creatinine is added, and to the marked retardation of the reduction. There is also the fact that after the addition of a small amount of it, subsequent increase in the amount produces little or no further change. It should be borne in mind that all the copper reagents in use contain fairly stable complexes, and that whether tartrate, citrate, phosphates, or carbonates are used, reduction takes place quite readily.

Many experiments were done on the retarding effect of these substances on Folin-McEllroy qualitative reagent, the amount of glucose being added which alone just produced a yellow precipitate in three minutes in a boiling water bath. Both creatinine and uric acid delayed the first change in colour, but the order of apparent maximum precipitation was not the same at different intervals of time.

According to Werner [1922] no carbon monoxide is evolved when glucose is acted on by alkaline hypobromite (at least not in a reasonable period of time), whereas when ammonium chloride is present, considerable amounts of carbon monoxide are evolved along with the nitrogen. According to my experiments, ammonium sulphate has a negligible effect; and besides, unlike Werner's case, here it is the glucose which is the active reducer, as ammonium salt alone is not appreciably oxidised by the copper reagent.

It was thought that some direct evidence of a coupled reaction might be obtained in the case of ammonium salt. As Werner had found that cyanate is always produced when ammonia is acted upon by alkaline hypobromite in the presence of glucose, he suggested that cyanate would probably be found in my experiments with ammonium salts. But though Werner's copper-pyridine-chloroform test for cyanate is very delicate, I failed to detect any cyanate in reductions of Folin-McEllroy qualitative reagent (which contains no thiocyanate) by glucose in presence of ammonium sulphate. Further experiments on a larger scale are necessary.

A further point requires consideration: in the type of glucose estimation investigated a fixed amount of copper reagent is completely reduced by gradual addition of the reducing solution. And it is clearly established that it is inadmissible to calculate glucose from the titration value, when uric acid and creatinine are also present. It is a matter of importance to know whether the same applies to methods in which an excess of copper reagent is treated with variable amounts of glucose.

Some preliminary experiments have been made on Folin's micro-method, and these indicate that creatinine and uric acid have a distinct effect, as was indeed to be expected. For, in the first place, the prescribed time of immersion in boiling water is *exactly* six minutes for both the standard and the unknown. It is soon found that if the time is increased, the reduction is also increased, *e.g.* identical solutions (Folin standards) heated for six, nine and twelve minutes gave colorimeter readings of 20, 19.2, 18.2. When a small amount of

creatinine is added, the retardation predominates and the glucose estimation is correspondingly low, when the time of heating is six minutes; while, if heated for ten minutes the increased reduction comes in, and the estimated glucose is higher, *e.g.*

	6 min.	10 min.
Folin standard	20.0 mm.	17.7 mm. (6 min. $S=20$)
„ + 0.1 cc. Creatinine	22.6 „	16.3 „

Further work on this and other methods in which excess of copper reagent is used are in progress.

SUMMARY.

1. A glucose solution diluted with normal urine has a greater reducing power on Folin-McEllroy copper reagent (also on Benedict and Fehling) than when diluted with water. The difference may amount to over 30 %.
2. Creatinine and uric acid (and to a practically negligible degree ammonium salt) produce the same effect. Urea has no action.
3. Creatinine causes a marked retardation of the reduction.
4. There are indications that the methods of glucose estimation, in which excess of copper reagent is used are similarly affected by uric acid and creatinine.

REFERENCES.

- Méhu (1879). *Compt. Rend. Acad. Sci.* **89**, 175.
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