XXXV. THE EFFECT OF GLUTATHIONE ON THE DETERMINATION OF BLOOD-SUGAR.

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IN an earlier paper [Herbert and Groen, 1929] it was shown that the discrepancies between the results of blood-sugar methods were due to non-glucose reducing substances present mainly in corpuscles. It appeared that ferric hydroxide filtrates and zinc hydroxide filtrates contained little or none of the non-glucose material, and that therefore the original MacLean method and the original Hagedorn-Jensen method gave figures close to the true sugar value. The non-sugar material was shown to be present in tungstic acid filtrates, and to have a marked effect on all the analytical methods applied to tungstic acid filtrates except that of Benedict [1928]; the Hagedorn-Jensen method, applied to tungstic acid filtrates, was affected most of all. It was suggested that the principal non-glucose substance responsible was glutathione.

In the present investigation the effect of pure reduced glutathione on blood-sugar methods has been studied. The work falls into two sections; (1) a study of the effect of glutathione on the analytical methods, when no treatment corresponding to protein precipitation is used, and (2) a study of the behaviour of glutathione in various methods of protein precipitation, using mixtures of plasma and glutathione.

Two different samples of glutathione were employed, both of which were received from Sir Frederick Hopkins's laboratories. The first, received in December 1928, was a deliquescent substance, the solutions of which had a smell resembling that of mint. The second sample was received in July 1929, and consisted of the pure tripeptide of glycine, glutamic acid, and cysteine, prepared by Hopkins's new method [1929].

The following blood-sugar methods have been used.

- (1) The Folin-Wu method [1920].
- (2) The Shaffer-Hartmann method as modified by Somogyi [1926].
- (3) The Hagedorn-Jensen method [1923].
- (4) The Benedict method [1928].

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THE EFFECT OF PURE REDUCED GLUTATHIONE ON THE FERRICYANIDE
REAGENT AND THE VARIOUS COPPER REAGENTS.

In this section of the work no treatment corresponding to protein precipitation was used; aqueous solutions containing glucose and glutathione were analysed by the various methods. For each method the quantities of glucose and glutathione taken were those which might be expected to be present when blood is analysed, assuming for the moment that all the blood-glutathione reaches the filtrate, and remembering that the amount of filtrate taken corresponds to 0.5 cc. blood in the Shaffer-Hartmann method, 0.2 cc. blood in the Folin-Wu and Benedict methods, and 0.1 cc. in the Hagedorn-Jensen method. In these analyses the amount of glucose was that corresponding to a blood-sugar of 100 mg. per 100 cc., and the amounts of glutathione

Fig. 1.

those corresponding to 36 to 150 mg. glutathione per 100 cc. blood. In this way, the effect of the glutathione was determined as an increment above the true sugar, and the conditions were comparable with those obtaining in blood analyses, except for the omission of the protein-precipitation methods. The volume of solution was in each method the same as in blood analyses. Results are given in Tables I and II. Fig. 1 gives the results in Table II in graphic form. We have tabulated only our results with the pure tripeptide, in the presence of an amount of glucose corresponding to a blood-sugar figure of 100 mg. per 100 cc. The earlier sample of glutathione gave similar figures.

In other experiments the amount of glucose varied from 50 to 100 mg. per 100 cc., and the glucose equivalent of glutathione was the same. In the complete absence of glucose, however, the reduction due to glutathione was

not in all methods the same as in the presence of glucose; this will be discussed separately for each method.

No special precautions were taken to prevent oxidation of the solutions by air, but in spite of this the results were, upon the whole, constant.

Table I. *Reducing action of glutathione in the presence of glucose.*
No treatment corresponding to protein precipitation.

Method	Glutathione taken, mg.	Reduction due to glutathione expressed as	
		cc. N/200 thiosulphate	Glucose mg.
Hagedorn-Jensen	0.06	0.190	0.033
	0.09	0.290	0.051
	0.12	0.375	0.066
	0.15	0.475	0.084
Shaffer-Hartmann	0.18	0.55	0.065
	0.30	1.05	0.115
	0.45	1.55	0.175
	0.60	2.10	0.235
Folin-Wu	0.12	—	0.026
	0.18	—	0.036
	0.30	—	0.060
	0.48	—	0.090

Table II. *Glucose equivalents of various concentrations of glutathione (determined in the presence of glucose).*

Glutathione, mg. per 100 cc.	Glucose equivalent of glutathione, mg. per 100 cc.			
	Hagedorn-Jensen	Shaffer-Hartmann	Folin-Wu	Benedict [1928]
36	—	13	—	—
60	33	23	13	0
90	51	35	18	0
120	66	47	—	0
150	84	—	30	0
240	—	—	45	—

The Hagedorn-Jensen method.

In the presence of glucose, the ratio of the amount of glutathione present to its glucose equivalent is 100 : 56, and this ratio is constant for the range covered by the analyses. (Tables I and II.)

In the absence of glucose, the glucose equivalent of glutathione is a little higher, viz.:

0.050 mg. G. SH $\equiv$ 0.039 mg. glucose,

0.100 mg. G. SH $\equiv$ 0.070 mg. glucose.

The relation of glutathione to thiosulphate (determined in the presence of glucose) is 0.1 mg. G. SH $\equiv$ 0.32 cc. N/200 thiosulphate. This is greater than the theoretical quantity required solely for the oxidation of the sulphydryl group. On the basis of the tripeptide formula, in the oxidation of the —SH to the —SS— form, one molecule of glutathione (m.w. 307) corresponds to one atom of hydrogen, i.e. 0.1 mg. G. SH corresponds to 0.065 cc. N/200 thiosulphate. Therefore there must be some change other than the oxidation of

glutathione from the —SH to the —SS— form; this would be expected when such an unstable compound is boiled in alkaline solution in the presence of an oxidising agent.

Glutathione has some reducing action on the Hagedorn-Jensen reagents at room temperature. If glutathione is added to the ferricyanide, and the iodide-zinc-sulphate-chloride solution and acetic acid are added at once, a variable reduction occurs, which is less than that observed when glutathione is heated with the ferricyanide, but greater than that theoretically required for the change from the —SH to the —SS— form. On the other hand, if the three reagents are mixed and the glutathione is added to the acid mixture, the reduction approaches the theoretical figure for the oxidation of the sulphydryl group.

Shaffer-Hartmann (Somogyi) method.

In the presence of glucose the ratio of glutathione to its glucose equivalent is 100 : 39, and this ratio is constant for the range covered by the analyses (Tables I and II).

In comparing the effects produced by glutathione in the presence and in the absence of glucose, it is necessary to consider a peculiarity in Somogyi's table representing the relation of glucose to thiosulphate for this method [Somogyi, 1926]. In this table, whereas throughout the major part of the range the relation of glucose to thiosulphate is constant (1 cc. *N*/200 thiosulphate is equivalent to 0.110 mg. glucose), in the lowest region of the table this relation does not hold, and 1 cc. *N*/200 thiosulphate is equivalent to 0.205 mg. glucose. When the reducing action of glutathione is determined in the presence of glucose, the glucose value corresponding to the glutathione is read as an increment in the upper part of the table; when the glutathione is analysed alone the lowest part of the table is used. It is found that the reduction attributed to glutathione, when expressed as glucose, remains almost the same in the absence as in the presence of glucose, although the thiosulphate corresponding to the glutathione is not the same, viz.:

in the presence of glucose

0.5 mg. G. SH $\equiv$ 1.75 cc. *N*/200 $\equiv$ 0.195 mg. glucose;

in the absence of glucose

0.5 mg. G. SH $\equiv$ 0.85 cc. *N*/200 $\equiv$ 0.185 mg. glucose.

For our purpose it is most convenient to assume, as we have done in calculating these figures, that in the mixture the reduction value of glucose remains the same, and the reduction value of glutathione is increased; but the actual mechanism of the reduction is unknown. When, in the Shaffer-Hartmann method, all the reduction values are expressed as glucose, the effect is obscured.

The relation of glutathione to thiosulphate (determined in the presence of glucose) is very similar in the Shaffer-Hartmann and Hagedorn-Jensen methods; viz. in the Shaffer-Hartmann method, 0.60 mg. G. SH is equivalent to 2.10 cc.

$N/200$ thiosulphate, and in the Hagedorn-Jensen method, 0.06 mg. G. SH is equivalent to 0.19 cc. $N/200$ thiosulphate.

Glutathione reduces the reagent at room temperature. To determine this reduction the glutathione and the copper reagent were mixed at room temperature, acidified at once and allowed to stand for 2 to 5 minutes; the mixture was then titrated in the usual way, when it was found that:

0.50 mg. G. SH $\equiv$ 0.36 cc. $N/200$ thiosulphate,

1.00 mg. G. SH $\equiv$ 0.76 cc. $N/200$ thiosulphate.

The requirement for the change from the —SH to the —SS— form is 1 mg. G. SH $\equiv$ 0.65 cc. $N/200$ thiosulphate. The oxidation of the sulphydryl group by iodine may therefore account for the reaction which occurs at room temperature, but there must be an additional reaction when the glutathione is heated with the copper reagent, as in the orthodox method of estimation.

The Folin-Wu method.

In the presence of glucose the ratio of glutathione to its glucose equivalent is 100 : 20, and this relation is constant for the range covered by the analyses (Tables I and II). The effect in the absence of glucose is the same (in this case the glucose standard is necessarily more dilute than for determinations in the presence of glucose). The presence of glutathione or of its oxidation products does not seem to cause any alteration in the blue colour, or to cause it to fade. The colours were matched as quickly as possible after dilution.

At room temperature glutathione reduces the copper reagent, as was previously noted by Herbert and Groen [1929]. This reduction in the cold is much less than that which occurs when glutathione is heated with the copper reagent.

The Benedict [1928] method.

In the presence of glucose, glutathione produces no measurable effect. In the absence of glucose, a very slight reduction of the reagent occurs (as indicated by the colour developed on addition of the phosphomolybdate), but with the amounts of glutathione handled, this was too small to be estimated.

The boiling-time was in all cases 10 minutes. There was a tendency for the colour to fade more rapidly in the glutathione mixtures than in the standard glucose solution, but no fading was observed within 10 minutes after dilution, during which time the readings were completed.

The results obtained with aqueous solutions show that the glucose equivalents of glutathione are in the approximate ratio 3 : 2 : 1 : 0 in the Hagedorn-Jensen, Shaffer-Hartmann, Folin-Wu, and Benedict methods respectively. Therefore when any blood filtrate which contains glutathione is analysed by these methods these effects will be observed. In considering actual blood analyses it is essential to know how the glutathione behaves at the stage of protein precipitation; this question will next be considered.

THE BEHAVIOUR OF PURE REDUCED GLUTATHIONE IN THE VARIOUS METHODS OF PROTEIN PRECIPITATION.

Mixtures of plasma and glutathione were submitted to the various processes for protein precipitation, and the filtrates were analysed by the different methods¹. Since plasma contains no glutathione, the total quantity of glutathione in the mixture was known, and the results can legitimately be compared with those obtained with simple solutions (Table II and Fig. 1).

Tungstic acid filtrates. For all four methods the analysis of tungstic acid filtrates from mixtures of plasma and glutathione gave the same results as had been obtained on the corresponding aqueous solutions (glucose and glutathione). Table II and Fig. 1 may be applied to tungstic acid filtrates. We therefore conclude that the whole of the glutathione passes into the tungstic acid filtrates. As would be expected, the filtrates show a strongly positive nitroprusside reaction.

Zinc hydroxide filtrates. Two methods of preparing zinc hydroxide filtrates were employed: (1) the technique described by Hagedorn and Jensen for use with their ferricyanide method [1923], and (2) the technique of Somogyi [1929, 1, 2] which may be used in association with various analytical methods.

In the Hagedorn-Jensen precipitation method the blood is diluted 1 in 61 and is heated with the zinc hydroxide in a boiling water-bath before filtration; the relative quantities of zinc sulphate and sodium hydroxide are such that the filtrates are slightly acid (p_H 6.8). In Somogyi's precipitation technique the blood is laked in a solution of zinc sulphate and sulphuric acid, sodium hydroxide is added, and the mixture is filtered; the whole process is carried out at room temperature; the dilution of the blood is 1 in 10; and there is a slight excess of sodium hydroxide, so that the filtrate is slightly alkaline (p_H 7.4). Mixtures of plasma and glutathione have been submitted to both these processes, and the filtrates analysed by the ferricyanide method.

When the Hagedorn-Jensen precipitation technique was followed, the added glutathione caused a slight increment above the plasma-sugar figure, though this effect was much less than that observed with the tungstic acid precipitation. The figures obtained were:

60 mg. glutathione per 100 cc.	≡	10 mg. glucose per 100 cc.
90 " " " 100 "	≡	19 " " " 100 "
120 " " " 100 "	≡	33 " " " 100 "

Evidently a small proportion of the added glutathione passed into these filtrates. With the larger quantities of glutathione, the filtrates showed a faintly positive nitroprusside reaction.

¹ In using the ferricyanide method for filtrates other than the original zinc filtrates of Hagedorn and Jensen, we took an amount of filtrate corresponding to 0.1 cc. plasma, and diluted this to 12 cc. before heating with the alkaline ferricyanide. The conditions were therefore strictly comparable with those obtaining in the original method, in which the total amount of filtrate corresponds to 0.1 cc. of blood or plasma, and the volume of filtrate and washings is approximately 12 cc.

When Somogyi's precipitation method was used the added glutathione caused no increment above the plasma-sugar figure, and the filtrate showed no nitroprusside reaction. Therefore no glutathione passes into filtrates made by Somogyi's technique.

It was found that the difference in the behaviour of glutathione in the two methods of zinc hydroxide precipitation was due to the difference in reaction of the protein precipitation mixtures. If the amount of alkali in the Hagedorn-Jensen technique was increased (keeping other conditions the same) so that a slightly alkaline filtrate was obtained, no glutathione passed into the filtrate, as indicated by the figures given on analysis by the ferricyanide method. On the other hand, if the amount of alkali in Somogyi's technique was diminished, so as to produce a neutral filtrate, a part of the glutathione passed into the filtrate, as indicated by the effect on the ferricyanide reagent, and by the fact that a positive nitroprusside reaction was obtained.

From the above observations we can predict the effect which would be produced by a given amount of glutathione in blood when various analytical methods are applied to the various blood-filtrates. It is known that reduced glutathione is present in blood, and occurs only in the corpuscles [Hunter and Eagles, 1927; Holden, 1925; Thomson and Voegtlin, 1926; Uyei, 1926; Hopkins, 1929]. The exact quantity in human blood is not known; the figure given by Hunter and Eagles—40 to 50 mg. per 100 cc. whole blood—is based on the intensity of the nitroprusside reaction in tungstic acid filtrates, and may be taken as approximately correct if it may be assumed that glutathione is the only sulphhydryl compound of which any appreciable amount is present in blood.

If there are 50 mg. reduced glutathione per 100 cc. of whole blood, and if this is the only non-glucose substance capable of producing an appreciable effect on blood-sugar methods, we should expect the following results when the various methods are applied to whole blood: on tungstic acid filtrates, the Hagedorn-Jensen figure would be higher than the true sugar value by 28 mg. per 100 cc.; the Shaffer-Hartmann figure would be too high by 19 mg. per 100 cc.; the Folin-Wu figure too high by 10 mg. per 100 cc. The Benedict method alone would give a true sugar figure for tungstic acid filtrates. All four analytical methods applied to the zinc hydroxide filtrates made by Somogyi's technique would give true sugar values. The original Hagedorn-Jensen method (*i.e.* the Hagedorn-Jensen precipitation technique together with the ferricyanide method of analysis) would give slightly high figures (about 9 mg. per 100 cc. above the true sugar value).

The comparison between these predicted effects and the results observed in blood analyses is given in the following paper.

SUMMARY.

The effect of aqueous solutions of reduced glutathione upon the various oxidising reagents employed in blood-sugar methods has been determined. The greatest effect (expressed in terms of glucose) is produced in the ferricyanide reduction; next in order come the Shaffer-Hartmann (Somogyi) copper reduction, and the Folin-Wu copper reduction. The copper reagent of Benedict is unaffected by glutathione.

In the tungstic acid precipitation, applied to mixtures of plasma and glutathione, all the added glutathione passes into the filtrates. In Somogyi's zinc hydroxide precipitation no glutathione passes into the filtrates. The zinc hydroxide precipitation of Hagedorn and Jensen allows a portion of the added glutathione to pass into the filtrates.

The blood-sugar determinations will be unaffected by glutathione (1) when any analytical method is applied to a filtrate containing no glutathione (*e.g.* Somogyi's zinc filtrate), or (2) when the analytical method employed is unaffected by glutathione, whatever the protein precipitation method (*e.g.* Benedict's method applied to any blood filtrate).

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