# CXXIX. THE DETERMINATION OF BLOOD-SUGAR.

## I. CRITICAL ANALYSIS OF THE REDUCTION OF ALKALINE COPPER REAGENTS BY GLUCOSE AND OTHER SUBSTANCES.

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Many and varied have been the methods employed for the determination of the sugar in the blood but so far there has been no really exact method evolved. The object of the present investigation is an attempt to evolve a method which would ultimately be satisfactory for the determination of the so-called "bound" sugar. It was first of all necessary to undertake a critical examination of the various methods in current use for the determination of the free sugar. Finally it was decided to limit this part of the work to an examination of the reduction of alkaline copper reagents by pure glucose and certain other substances found in blood.

All workers have expressed the blood-sugar in terms of glucose although at present there is much controversy as to whether the sugar of the blood exists as glucose, a mixture of glucose and other sugars, or as a more active form than the ordinary equilibrium mixture. For a method to be satisfactory the following criteria must be satisfied.

(1) Other substances present in a blood filtrate must not reduce the reagent and must not affect the reduction of the reagent by glucose.

(2) Glucose added to blood must be determined quantitatively.

The various methods so far employed to meet the first condition attempt:

(1) to precipitate as much of the nitrogenous compounds in the blood as possible. Reagents containing mercury and zinc salts have been used for this purpose [Bierry and Moquet, 1924; Harned, 1925; West, Scharles and Petersen, 1929; Somogyi, 1929];

(2) to add to the reagent to be reduced some substance or substances which will inhibit reduction so that within the time of heating only the sugar reduces the reagent [Benedict, 1927];

(3) to determine the total reducing power of a non-protein filtrate of blood, then to remove the sugar in the blood either by yeast fermentation

[Hiller, Linder and Van Slyke, 1925; Van Slyke and Hiller, 1926] or by glycolysis [Hiller, Linder and Van Slyke, 1925] and then to determine the so-called "residual" reduction value of the blood filtrate after this treatment. The difference between the total reducing value and the "residual" reduction value is assumed to represent the true blood-sugar.

All the chemicals used in this work were of A.R. standard—British Drug Houses, Ltd. The glucose was dried in an air-oven at 90° for 1 hour before being weighed out. It was found that a 1 % solution of glucose made up in saturated benzoic acid kept quite a considerable time. The various strengths of sodium thiosulphate were freshly prepared each day from a stock 0·1 *N* solution, which was tested from time to time against 0·1 *N* potassium dichromate and also 0·1 *N* potassium iodate. The keeping properties of the 0·1 *N* thiosulphate solution were found to be increased considerably by the addition of 2 g. potassium bicarbonate per litre.

Due allowance has been made for the ten-fold dilution of the constituents of blood which results from the deproteinisation according to the technique of Folin and Wu using sodium tungstate and sulphuric acid.

### The Shaffer and Hartmann method.

The Shaffer and Hartmann method [1920–21] was examined from three standpoints.

(1) Using pure glucose the reduction was carried out in air as described in the original paper.

(2) The reduction was carried out in an atmosphere of nitrogen.

(3) The reagent was made up in two separate solutions (*a*) containing copper sulphate, tartaric acid and sodium carbonate, (*b*) containing potassium iodide, iodate and oxalate. The reduction was carried out with solution A, solution B being added afterwards. This reaction was studied in an atmosphere of nitrogen and also in air.

The first line of investigation was carried out as follows. In an 8 × 1 inch test-tube 10 cc. of the glucose solution and 10 cc. of Shaffer-Hartmann reagent were mixed. This was placed in a boiling water-bath for 10 minutes and then cooled to about 40°. 10 cc. of *N* $H_2SO_4$ were added, the whole was well shaken for about 2 minutes and then titrated with 0·0125 *N* thiosulphate solution.

For the determination under (2) the test-tube containing the mixture of glucose and alkaline copper solutions was evacuated and then filled with a slow stream of nitrogen. This was repeated several times to remove air dissolved in the liquid, until the tube and fluid contained practically only nitrogen. The determination was carried out then as under (1).

For the determination under (3) the following solutions are required:

(*a*) Copper sulphate $CuSO_4$, $5H_2O$ ... ... 5 g.
Tartaric acid ... ... ... ... ... 7·5 g.
Sodium carbonate (anhydrous) ... ... 40 g.
} per litre.

(*b*) Sodium carbonate ... ... ... ... 1 g.
Potassium iodide ... ... ... ... 20 g.
Potassium iodate ... ... ... ... 1·4 g.
Potassium oxalate ... ... ... ... 36·8 g.
} per litre.

(*c*) $2N$ $H_2SO_4$

The technique in air was as follows. In an 8 × 1 inch test-tube 10 cc. of glucose solution and 10 cc. of solution A were mixed and put into a boiling water-bath for 10 minutes. The tube was then cooled and 5 cc. of solution B added, followed by 5 cc. of $2N$ $H_2SO_4$. The whole was then well shaken and titrated with $0{\cdot}0125N$ thiosulphate solution. It will be noted that the final concentrations are exactly the same as in the original method (1). The above procedure was repeated, the tube being filled with nitrogen as described under (2).

The results are expressed in terms of cc. $0{\cdot}0125N$ thiosulphate, being the difference in titration value of a blank using 10 cc. of distilled water and a determination using 10 cc. of the known glucose solution.

Table I.

| Concentration of glucose (mg. per 100 cc.) | Shaffer and Hartmann original reagent | | Modified solutions | |
|---|---|---|---|---|
| | Air | Nitrogen | Air | Nitrogen |
| 40 | 11·55 | 12·25 | 12·3 | 12·3 |
| 30 | 8·55 | 9·25 | 9·3 | 9·3 |
| 20 | 5·5 | 6·0 | 6·05 | 6·05 |
| 10 | 2·4 | 2·85 | 2·9 | 2·9 |
| 8 | 1·85 | 2·3 | — | — |
| 4 | 0·65 | 0·95 | — | — |

It is well known that glucose when heated with a solution of an alkaline carbonate is easily oxidised by the oxygen dissolved in the solution. In the Shaffer and Hartmann method the potassium salts in the reagent retard the reduction so that some of the glucose is oxidised by the dissolved oxygen before it is capable of reducing any copper. This explanation would account for the higher results obtained when the reduction is carried out in an atmosphere of nitrogen. It will be observed that when the potassium salts are not incorporated in the alkaline copper reagent but added after reduction the same results are obtained irrespective of whether the reduction is carried out in an atmosphere of air or nitrogen. This demonstrates that the amount of glucose removed by atmospheric oxygen prior to reduction of the copper is negligible under these conditions. These results also agree with the results obtained by the original Shaffer and Hartmann method carried out in an atmosphere of nitrogen.

It was decided to investigate the effects of varying the concentrations of the various items in the above alkaline copper reagent.

*The amount of copper reduced in relation to the concentration of copper sulphate, tartaric acid and sodium carbonate.*

Some substance must be incorporated in an alkaline copper solution which is capable of keeping the copper in solution. Benedict [1926] originally recommended a high concentration of sodium citrate, as he considered that such a reagent though not so sensitive to reduction as one containing a tartrate is nevertheless more specific for glucose. Later [1927] he dispensed with citrate and used a low concentration of tartrate, together with alanine and a high concentration of sodium nitrate. Folin and Wu [1919] and later Folin [1926] used a low concentration of tartrate—just sufficient to keep the copper in solution. These solutions containing a low concentration of tartrate are very sensitive to reduction and show very little auto-reduction when heated with distilled water in a boiling water-bath. Folin [1926] criticised Benedict's citrate reagent on the grounds that it is not sufficiently sensitive. On the other hand, Benedict criticises Folin's reagents as being too sensitive, in that they are reduced by such substances as formalin and chloroform. Folin [1929] criticises a modification of the Folin and Wu alkaline copper reagent by Somogyi and Kramer [1928] in respect of the high concentration of Rochelle salt in place of the low concentration of tartaric acid. He states that reagents having high concentrations of citrates and tartrates have high blanks thereby being unsuitable for colorimetric methods. Somogyi [1926] modified the Shaffer and Hartmann reagent by replacing part of the sodium carbonate by bicarbonate. He stated that a decrease in the alkalinity of the Shaffer and Hartmann reagent leads to an increase in the reduction—this decrease in alkalinity being obtained by replacing part of the sodium carbonate by bicarbonate. Somogyi appears to consider that alkalinity is the main factor, since two of his modifications of the Shaffer and Hartmann reagent which have been published both contain the same concentrations of carbonate and bicarbonate, but one contains 1·2 % Rochelle salt, the other 2·5 % Rochelle salt [cf. West, Scharles and Petersen, 1929]. Somogyi also uses the same concentrations of bicarbonate and carbonate in his modification of the alkaline ferricyanide reagent of Hagedorn and Jensen [1923]. It is of interest to note that in MacLean's [1916] and in the Wood and Ost alkaline copper reagents no tartrate or citrate is incorporated. Has the tartrate any effect on reduction other than simply holding the copper in solution? This was the first point to determine.

Reagents were prepared containing the same concentrations of copper sulphate and sodium carbonate but with different concentrations of tartrate. The results are shown in Tables II and III. In Table II the reagent contained:

| | | |
|---|---|---|
| Copper sulphate $CuSO_4, 5H_2O$ ... ... ... | 5 g. | per litre, |
| Sodium carbonate (anhydrous) ... ... ... | 40 g. | |

while in Table III it contained:

| | | |
|---|---|---|
| Copper sulphate $CuSO_4, 5H_2O$ ... ... ... | 10 g. | per litre. |
| Sodium carbonate (anhydrous) ... ... ... | 60 g. | |

The second point was to determine the influence of the concentration of sodium carbonate. The results are shown in Table IV. The influence of time of boiling is shown in Table V.

The amount of copper reduced was determined as follows. The reduction was carried out in a centrifuge tube. The cuprous oxide was removed by centrifuging and the supernatant fluid poured off. The cuprous oxide was then washed twice with distilled water in the centrifuge. To it was added a known amount of the Shaffer and Hartmann reagent but containing no copper sulphate, followed by the same volume of $N$ $H_2SO_4$. The whole was then stirred thoroughly. The excess iodine was titrated with a standard thiosulphate solution. It will be noted that the final concentrations of all the substances are the same as in the original Shaffer and Hartmann method with the exception of the copper sulphate.

The following technique was employed in preparing the alkaline copper solutions. The requisite amounts of tartaric acid and sodium carbonate were weighed out and dissolved together in distilled water. The solution was boiled to decompose any bicarbonate which might have been formed and was then cooled. To this was added the requisite amount of copper sulphate dissolved in distilled water and the whole made up to the required volume.

*Procedure.* Solutions required for determining the amount of reduced copper.

1. Iodide-iodate-oxalate solution.

| | | |
|---|---|---|
| Sodium carbonate (anhydrous) ... ... | 40 g. | per litre. |
| Tartaric acid ... ... ... ... ... | 7·5 g. | |
| Potassium iodide ... ... ... ... | 10 g. | |
| Potassium iodate ... ... ... ... | 0·8 g. | |
| Potassium oxalate ... ... ... ... | 18·4 g. | |

2. $N$ $H_2SO_4$.
3. 0·0125$N$ sodium thiosulphate.

In a 3 × 1 inch centrifuge tube 10 cc. glucose solution and 10 cc. of alkaline copper solution were mixed. The tube was then placed in a boiling water-bath for 15 minutes. It was then cooled and centrifuged. The supernatant fluid was removed and the cuprous oxide was washed twice with distilled water. 10 cc. of the iodide-iodate-oxalate solution were then added, followed by 10 cc. of $N$ $H_2SO_4$. The whole was stirred to dissolve the cuprous oxide and then titrated with 0·0125$N$ thiosulphate. A blank was performed under similar conditions using 10 cc. of distilled water. The difference between the blank determination and a determination using glucose was taken as a measure of the amount of copper reduced by the glucose.

## Table II.

Alkaline copper solutions containing:

| | | |
|---|---|---|
| Copper sulphate $CuSO_4, 5H_2O$ | ... ... | 5 g. |
| Tartaric acid ... ... ... | ... ... | 7·5–15 g. |
| Sodium carbonate (anhydrous) | ... ... | 40 g. |

per litre.

| Concentration of tartaric acid (g. per litre) | Amount of cuprous oxide formed by reduction with 10 cc. glucose solution in terms of cc. 0·0125 *N* thiosulphate: 10 mg. glucose per 100 cc. | 20 mg. glucose per 100 cc. | 30 mg. glucose per 100 cc. | 40 mg. glucose per 100 cc. |
|---|---|---|---|---|
| 7·5 | 3·0 | 6·05 | — | 12·3 |
| 8·0 | 3·1 | 6·05 | 9·6 | 12·3 |
| 9·0 | 3·3 | 6·6 | 9·6 | 12·7 |
| 9·5 | 3·3 | 6·6 | — | — |
| 10·0 | 3·3 | 6·6 | 9·9 | 12·8 |
| 12·0 | 3·3 | 6·6 | 9·9 | 13·1 |
| 15·0 | 3·3 | 6·6 | 9·9 | 13·1 |

## Table III.

Alkaline copper solutions containing:

| | | |
|---|---|---|
| Copper sulphate $CuSO_4, 5H_2O$ | ... ... | 10 g. |
| Tartaric acid ... ... ... | ... ... | 7·5–30 g. |
| Sodium carbonate (anhydrous) | ... ... | 60 g. |

per litre.

| Concentration of tartaric acid (g. per litre) | Amount of cuprous oxide formed by reduction with 10 cc. glucose solution in terms of cc. 0·0125 *N* thiosulphate: 2·5 mg. glucose per 100 cc. | 5·0 mg. glucose per 100 cc. | 10 mg. glucose per 100 cc. | 20 mg. glucose per 100 cc. | 40 mg. glucose per 100 cc. |
|---|---|---|---|---|---|
| 7·5 | — | — | 2·4 | 5·1 | 11·6 |
| 10·0 | — | — | 3·3 | 6·6 | 13·2 |
| 12·0 | — | — | 3·3 | 6·6 | 13·2 |
| 14·0 | — | — | 3·3 | 6·6 | 13·2 |
| 15·0 | 0·85 | 1·65 | 3·3 | 6·6 | 13·2 |
| 16·0 | — | — | 3·3 | 6·6 | 13·0 |
| 18·0 | — | — | 3·3 | 6·6 | 12·9 |
| 20·0 | — | — | 3·3 | 6·6 | 12·8 |
| 25·0 | — | — | 3·05 | 6·05 | 12·0 |
| 30·0 | — | — | 2·7 | 5·1 | 10·1 |

The effect of the concentrations of the sodium carbonate was then studied. The following series of solutions was prepared:

| | | |
|---|---|---|
| Copper sulphate $CuSO_4, 5H_2O$ | ... ... ... | 10 g. |
| Tartaric acid ... ... ... | ... ... | 15 g. |
| Sodium carbonate (anhydrous) | ... ... ... | 30–80 g. |

per litre.

## Table IV.

| Concentration of sodium carbonate (anhydrous) (g. per litre) | Amount of cuprous oxide formed by reduction with 10 cc. glucose solution in terms of cc. 0·0125 *N* thiosulphate: 2·5 mg. glucose per 100 cc. | 5 mg. glucose per 100 cc. | 10 mg. glucose per 100 cc. | 20 mg. glucose per 100 cc. | 30 mg. glucose per 100 cc. | 40 mg. glucose per 100 cc. |
|---|---|---|---|---|---|---|
| 30 | — | 1·55 | — | 5·75 | — | — |
| 40 | — | 1·65 | 3·3 | 6·6 | 9·9 | — |
| 60 | 0·85 | 1·65 | 3·3 | 6·6 | 9·9 | 13·2 |
| 80 | 0·85 | 1·65 | 3·3 | 6·6 | 9·9 | 13·2 |

The effect of time of boiling was studied, using the following alkaline copper solution:

| | | |
|---|---|---|
| Copper sulphate $CuSO_4, 5H_2O$ ... ... ... | 10 g. | per litre. |
| Tartaric acid ... ... ... ... ... | 15 g. | |
| Sodium carbonate (anhydrous) ... ... ... | 60 g. | |

Table V.

| Time of boiling (mins.) | Amount of cuprous oxide reduced by 10 cc. glucose solution in terms of cc. 0·0125 $N$ thiosulphate | |
|---|---|---|
| | 5 mg. glucose per 100 cc. | 20 mg. glucose per 100 cc. |
| 5 | — | 3·15 |
| 10 | 1·65 | 6·6 |
| 15 | 1·65 | 6·6 |
| 20 | 1·65 | 6·6 |
| 30 | 1·65 | 6·6 |

The results in Table II were checked using the following technique. Instead of centrifuging off the cuprous oxide, 5 cc. of the following solution were added:

| | | |
|---|---|---|
| Sodium carbonate ... ... ... ... ... | 1 g. | per litre, |
| Potassium iodide ... ... ... ... ... | 20 g. | |
| Potassium iodate ... ... ... ... ... | 1·4 g. | |
| Potassium oxalate ... ... ... ... | 36·8 g. | |

followed by 5 cc. $2N$ $H_2SO_4$. The whole was then well shaken and titrated with 0·0125$N$ thiosulphate.

The physical nature of the cuprous oxide seems to be related to the concentrations of the alkaline copper solutions. In the experiments reported in Table II the cuprous oxide was flocculent, light brown in colour and difficult to centrifuge, whereas in those of Table III with concentrations of tartaric acid above 1·2 %, the cuprous oxide was red, heavy and easily centrifuged.

Upon examining the results in Table II it will be observed that when the concentration of tartaric acid was increased above 0·75 % there was an increase of reduction up to a concentration of 0·9 %. Further increases in concentration to 1·5 % produced no further increase in reduction except in the case of the highest concentration of glucose examined. In the case of Table III the amount of reduction is also increased with increase in concentration of tartaric acid but when the concentration becomes very high, *e.g.* 2·5 % and 3·0 %, the reduction is decreased. This inhibiting effect becomes apparent in lower concentrations than these, with the highest concentration of glucose used, *i.e.* 40 mg. per 100 cc. With the reagent containing 3·0 % tartaric acid the effect of increasing the concentration of sodium carbonate was studied (Table VI).

From these results it will be seen that an increase in concentration of sodium carbonate increases the reduction to the maximum. It appears then that in high concentration tartaric acid can exert an inhibiting action which can be counterbalanced by a high concentration of sodium carbonate. It is

Table VI.

Alkaline copper solutions containing:

| | | |
|---|---|---|
| Copper sulphate $CuSO_4, 5H_2O$ ... ... | 10 g. | per litre. |
| Tartaric acid ... ... ... ... ... | 30 g. | |
| Sodium carbonate (anhydrous) ... ... | 60–100 g. | |

| Concentration of sodium carbonate (anhydrous) g. per litre | Amount of cuprous oxide by reduction with 10 cc. glucose solution in terms of cc. 0·0125 *N* thiosulphate | |
|---|---|---|
| | 10 mg. glucose per 100 cc. | 20 mg. glucose per 100 cc. |
| 60 | 2·7 | 5·05 |
| 80 | 3·3 | 6·6 |
| 100 | 3·3 | 6·6 |

interesting to note, however, that, when the concentration of sodium carbonate was increased in the reagent containing 0·75 % tartaric acid in Table II, *i.e.* where the concentration of tartaric acid was insufficient to give the maximum reduction, the reduction was unchanged, even when the sodium carbonate concentration was raised to 8·0 %. The maximum reductions obtained in Tables II and III are the same even although the concentrations of copper sulphate were different, *i.e.* 0·5 and 1·0 %. In all the reagents which give a maximum reduction, the amount of reduction is unaffected by increasing either the time of heating or the concentration of sodium carbonate. The amount of reduction with these reagents is directly proportional to the concentration of glucose, with concentrations as widely different as 2·5 to 40 mg. per 100 cc. The blank determination using distilled water increases with increasing concentration of tartaric acid, but this is not a disadvantage in a non-colorimetric method. It was found that carrying out the reduction in an atmosphere of nitrogen had no effect on the results.

*Determination of glucose by the estimation of the unreduced copper.*

Shaffer and Hartmann [1920–21] have determined the amount of glucose by estimating the amount of unreduced copper after the reduction of an alkaline copper solution. An excess of potassium iodide and acid is added when the following reaction takes place:

$$2CuSO_4 + 4KI = Cu_2I_2 + 2K_2SO_4 + I_2.$$

The liberated iodine is titrated with standard sodium thiosulphate solution. This method was investigated by the present writer and the results were compared with those obtained by determining the amount of reduced copper directly. The following alkaline copper solution (cf. Table II) was used for this work:

| | | |
|---|---|---|
| Copper sulphate $CuSO_4, 5H_2O$ ... ... ... | 5 g. | per litre. |
| Tartaric acid ... ... ... ... ... | 15 g. | |
| Sodium carbonate (anhydrous) ... ... ... | 40 g. | |

In an 8 × 1 inch test-tube 10 cc. of glucose solution and 10 cc. of alkaline copper solution were mixed and the whole was placed in a boiling water-bath for 15 minutes. It was then cooled and 10 cc. of saturated potassium iodide

solution were added, followed by 5 cc. of 25 % sulphuric acid. The whole was then well shaken and titrated with 0·0125 *N* thiosulphate. A blank determination using 10 cc. of distilled water was carried out at the same time. The difference between these two results was taken as being equivalent to the amount of copper reduced. This will be termed the indirect method, the other the direct method.

Table VII.

| Concentration of glucose (mg. per 100 cc.) | Amount of copper reduced by 10 cc. glucose solution in terms of cc. 0·0125 *N* thiosulphate | |
|---|---|---|
| | Direct method | Indirect method |
| 30 | 9·9 | 9·9 |
| 20 | 6·6 | 6·65 |
| 10 | 3·3 | 3·3 |

The above agrees with the findings of Shaffer and Hartmann, who found perfect agreement between the two methods.

### The effect of bicarbonates.

Somogyi [1926] states that a decrease of alkalinity of the Shaffer and Hartmann alkaline copper reagent leads to an increased reduction. To obtain a decrease of alkalinity Somogyi substituted sodium bicarbonate for part of the sodium carbonate. He found that as the carbonate was decreased and the bicarbonate increased the reduction increased until the concentration of sodium carbonate was 2·0 % and that of the sodium bicarbonate 2·5 %, but with a further increase of bicarbonate and decrease of carbonate the reduction began to diminish. Somogyi asserts that with the above concentrations the optimum alkalinity for reduction is obtained. Somogyi evolved a modification of the Shaffer and Hartmann reagent from these experiments.

It was decided to investigate this problem along slightly different lines. The oxidising portion of the reagent had the following composition:

| | | |
|---|---|---|
| Copper sulphate $CuSO_4, 5H_2O$ ... ... ... | 6·5 g. | per litre. |
| Rochelle salt ... ... ... ... ... | 12 g. | |
| Sodium carbonate (anhydrous) ... ... ... | 20 g. | |
| Sodium bicarbonate ... ... ... ... | 25 g. | |

In the first set of experiments the concentration of sodium carbonate was varied, keeping the concentration of sodium bicarbonate constant, and in the second set the reverse was carried out. The potassium iodide, iodate and oxalate were not incorporated in the reagent but were added as a separate solution after reduction, otherwise the original technique of the author was carried out. The amount of acid added was varied in accordance with the variation in the concentration of either the carbonate or bicarbonate. The concentration of glucose used in all experiments was 20 mg. per 100 cc.

In the first set of experiments it will be noted that an increase of concentration of carbonate has no effect on reduction. In the second set of experiments the reduction is diminished when the concentration of bicarbonate is

Table VIII.

Concentration of sodium bicarbonate 2·5 %.

| Concentration of sodium carbonate (anhydrous) g. % | Reduction by 10 cc. glucose solution in terms of cc. 0·0125 *N* thiosulphate |
|---|---|
| 1·5 | 7·25 |
| 2·0 | 7·25 |
| 2·5 | 7·25 |
| 3·0 | 7·25 |
| 4·0 | 7·25 |

Table IX.

Concentration of sodium carbonate 2·0 %.

| Concentration of sodium bicarbonate g. % | Reduction by 10 cc. glucose solution in terms of cc. 0·0125 *N* thiosulphate |
|---|---|
| 0 | 4·3 |
| 0·5 | 5·35 |
| 1·0 | 5·95 |
| 1·5 | 6·5 |
| 2·0 | 6·9 |
| 2·5 | 7·25 |
| 3·0 | 7·2 |
| 4·0 | 6·85 |

either increased or decreased from that used by Somogyi in his reagent. The solution in Table IX containing 4 % of sodium bicarbonate was taken and the effect of increasing the concentration of sodium carbonate examined (Table X).

Table X.

| Concentration of sodium carbonate (anhydrous) g. % | Reduction by 10 cc. glucose solution in terms of cc. 0·0125 *N* thiosulphate |
|---|---|
| 2·0 | 6·85 |
| 3·0 | 7·1 |
| 4·0 | 7·25 |

It will be observed that by increasing the concentration of sodium carbonate, the reduction is increased to the maximum value obtained with 2·5 % sodium bicarbonate.

Bicarbonates can act in an alkaline copper reagent in the same manner as a tartrate or citrate (cf. MacLean's and the Wood-Ost reagent). In Somogyi's reagent the concentration of tartrate is insufficient to give the maximum reduction of tartrate reagents, so that by the addition of a substance of similar properties such as sodium bicarbonate, the reduction will be increased. It appears that a high concentration of sodium bicarbonate has an inhibiting action as well, this inhibiting action being neutralised by an increase in concentration of sodium carbonate. It will be noticed that similar results were obtained with solutions containing high concentrations of tartrates (cf. Tables III and VI). These experiments hardly agree with Somogyi's views. It is interesting to note that the maximum reduction obtained with tartrate-bicarbonate solutions is a little higher than with tartrate solutions.

### The effect of amino-acids upon the reduction of alkaline copper tartrate reagents by glucose.

Before a method can be accepted as suitable for the determination of blood-sugar it must be shown that other substances found in a protein-free blood filtrate do not interfere with the reduction of the reagent by glucose. This was first emphasised by Holden [1926]. He showed that under certain circumstances amino-acids are capable of influencing the results considerably, not so much in reducing the reagent but by increasing the amount of reduction by glucose. Normally the blood contains 40–70 mg. of amino-acids per 100 cc. and in certain pathological conditions these figures may be exceeded. Holden investigated the method of Hagedorn and Jensen [1923] using an alkaline ferricyanide reagent and the Wood-Ost method as modified by Cole [1920]. In the latter the reagent contains copper sulphate, potassium carbonate and bicarbonate and the cuprous oxide is filtered off and washed prior to its determination. Holden examined the effects of glycine, aspartic acid, glutamic acid and cystinė. With the alkaline ferricyanide reagent, the amino-acids were found to exert no influence upon its reduction by glucose. Cystine was the only one which reduced the reagent itself and the reduction obtained in the presence of glucose was found to be the sum of the reducing values when determined separately. In the case of the Wood-Ost reagent, glucose in the presence of glycine, glutamic acid and aspartic acid, caused a greater reduction than when alone. None of these amino-acids reduces the reagent itself. Glucose in the presence of cystine, which itself reduces the reagent, reduces more than when alone even when the reduction by cystine is allowed for. Holden investigated the problem for two specific reagents only, both of entirely different composition. It was decided to limit this study to the problem of the effect of these substances on the reduction of alkaline copper reagents generally.

The effect of amino-acids upon the reduction by glucose of the alkaline copper reagents in Tables II and III was examined. The centrifugal technique was employed. The concentrations of the amino-acids were a little higher than are ever present in blood. The following solutions were used:

| | | | |
|---|---|---|---|
| (1) | Glycine ... ... | 10 mg. per 100 cc. | |
| (2) | Cystine ... ... | 10 mg. ,, | |
| (3) | Aspartic acid ... ... | 4 mg. ,, | Total 12 mg. per 100 cc. |
| | Glutamic acid ... | 4 mg. ,, | |
| | Tryptophan ... ... | 4 mg. ,, | |

It was found that when the reagents which gave the maximum reduction were employed, the above amino-acid solutions had no effect upon the reduction by glucose. On the other hand, when those reagents which gave a lower reduction value were used, the amount of reduction by glucose in the presence of the above amino-acids was increased. Results obtained with the latter reagents of Table II are shown.

Table XI.

| Concentration of tartaric acid (g. %) | Concentration of glucose (mg. per 100 cc.) | Reduction by 10 cc. glucose solution in terms of cc. 0·0125 *N* thiosulphate | Reduction by 10 cc. glucose solution in terms of cc. 0·0125 *N* thiosulphate in presence of amino-acids: glycine 10 mg. per 100 cc. | cystine 10 mg. per 100 cc. | aspartic acid glutamic acid tryptophan total 12 mg. per 100 cc. |
|---|---|---|---|---|---|
| 0·75 | 10 | 3·0 | 3·3 | 3·3 | 3·3 |
| | 20 | 6·05 | 6·6 | 6·6 | 6·6 |
| 0·8 | 10 | 3·1 | 3·3 | 3·3 | 3·3 |
| | 20 | 6·05 | 6·6 | 6·6 | 6·6 |
| 0·85 | 10 | 3·1 | 3·3 | 3·3 | 3·3 |
| | 20 | 6·1 | 6·6 | 6·6 | 6·6 |

It will be seen that in the presence of these amino-acids the reduction is increased to the same values as are obtained with reagents giving the maximum reduction. The question therefore arises as to whether amino-acids can function in the same manner as do tartrates in alkaline copper reagents. It was decided to study the effect of substituting the tartrate entirely by an amino-acid such as glycine. Reagents of the following composition were prepared:

| | | |
|---|---|---|
| Copper sulphate $CuSO_4,5H_2O$ ... ... ... | 5 g. | per litre. |
| Sodium carbonate (anhydrous) ... ... ... | 40 g. | |
| Glycine ... ... ... ... ... ... | 5–10 g. | |

It was found that glycine in concentrations above 0·4 % was capable of keeping the copper in solution on boiling. The amount of reduction by pure glucose solutions was determined by the centrifugal technique as described earlier. The following results were obtained:

Table XII.

| Concentration of glycine (g. %) | Reduction by 10 cc. glucose solution in terms of cc. 0·0125 *N* thiosulphate: 2·5 mg. per 100 cc. | 5 mg. per 100 cc. | 10 mg. per 100 cc. | 20 mg. per 100 cc. | 30 mg. per 100 cc. | 40 mg. per 100 cc. |
|---|---|---|---|---|---|---|
| 0·6 | — | — | 3·3 | 6·6 | 9·9 | 13·2 |
| 0·7 | 0·85 | 1·65 | 3·3 | 6·6 | 9·9 | 13·2 |
| 0·8 | — | — | 3·3 | 6·6 | 9·9 | 13·2 |
| 1·0 | — | — | 3·3 | 6·6 | 9·9 | 13·2 |

These results show that glycine can be substituted for tartrate in an alkaline copper reagent. It will be seen that the reductions obtained with these copper-glycine solutions are the same as the maximum values obtained with alkaline copper tartrate reagents. This demonstrates why alkaline copper tartrate reagents giving the maximum reduction are unaffected by the presence of amino-acids whereas those giving lower reductions are affected. The Wood-Ost reagent as used by Holden gives lower reduction values with glucose than those in Table XII, consequently glucose would reduce more in the presence

of amino-acids, as Holden found. It is of interest to note that this reagent contains neither citrate nor tartrate but only copper sulphate, potassium carbonate and bicarbonate, so that this phenomenon is observed with alkaline copper reagents generally.

The effect of amino-acids upon the reduction by glucose of the following reagents was examined: (1) Folin and Wu [1919]; (2) Folin [1926]; (3) Shaffer and Hartmann [1920–21]; (4) Somogyi's modification [1926] of Shaffer and Hartmann. It was found that the first three reagents were reduced in greater amount by glucose in the presence of amino-acids than by glucose alone. The effect of amino-acids upon Somogyi's modification was examined in two ways: (1) the potassium salts were incorporated in the alkaline copper portion of the reagent; (2) the potassium salts were added as a separate solution after reduction. By the second method the amount of reduction by glucose is a little greater than that obtained with the alkaline copper-glycine solutions, consequently amino-acids exert no influence. In the first case the presence of potassium salts during the reduction process exerts an inhibiting action so that the reduction by glucose is much less. Amino-acids therefore tend to increase the reduction. The above deductions were confirmed experimentally.

The Folin and Wu [1919] and Folin [1926] methods are affected by the presence of amino-acids since they give a lower reduction than the maximum value for alkaline copper tartrate solutions. Their low reduction values are due to a low concentration of tartrate. The low reduction values given by the Shaffer-Hartmann method, which is also affected by the presence of amino-acids, is due to the low concentration of tartrate and to the inhibiting action of the potassium salts incorporated in the reagent. Somogyi's modification is only affected when the potassium salts are incorporated in the reagent and not added as a separate solution after reduction.

Holden [1926] states that when cystine is heated with the Wood-Ost reagent, the solution turns green with slight formation of cuprous oxide. With the reagents described in Tables II and III the solutions were turned a greenish tinge but with the concentration of cystine used (10 mg. per 100 cc.) there was no increased formation of cuprous oxide above that obtained with a blank determination using distilled water. The supernatant fluid after centrifuging contains substances which are capable of absorbing iodine. In any iodimetric method these substances would be determined as cuprous oxide, unless the cuprous oxide was removed and washed. Glycine, aspartic acid, glutamic acid and tryptophan were also found to form substances which absorb iodine, after heating their solutions with these alkaline copper reagents. These are removed in the supernatant fluid after centrifuging. These iodine-absorbing substances were found to be formed even in the cold by allowing the amino-acid solution to stand in contact with the alkaline copper reagent. This phenomenon is being investigated further. It was found that creatinine and glucose reduced the alkaline copper reagents to cuprous oxide without any formation of soluble iodine-absorbing substances.

Herbert and Groen [1929] state that when the Folin and Wu alkaline copper reagent is added to a tungstic acid filtrate and without heating the phosphomolybdic acid reagent is added, a blue colour develops. This phenomenon was not obtained with zinc or colloidal ferric hydroxide filtrates. Gulland and Peters [1930] found that if to the zinc filtrates of pigeon's blood, prepared according to the Hagedorn and Jensen technique, the alkaline ferricyanide is added and then, without heating, the potassium iodide mixture is added and the whole acidified, then upon titration with thiosulphate solution a marked absorption of iodine takes place when compared with a similar determination using distilled water.

### Influence of other substances of the blood.

Hiller, Linder and Van Slyke [1925] examined the reducing powers of uric acid, creatine and creatinine in concentrations similar to those found in the blood. They found that in performing a blood-sugar determination the reduction due to these concentrations was negligible.

The present writer found that uric acid and creatinine in the following concentrations reduced the reagents.

*Reduction in terms of cc. 0·0125*N *thiosulphate.*

| | | |
|---|---|---|
| | 10 cc. creatinine solution (10 mg. per 100 cc.) ... | 1·1 |
| | 10 cc. creatinine solution (2 mg. ,, ) ... | 0·2 |
| | 10 cc. uric acid solution (5 mg. ,, ) ... | 0·05 |
| cf. | 10 cc. glucose solution (10 mg. ,, ) ... | 3·3 |

The present writer found that uric acid and creatinine in the above concentrations exerted no inhibiting influence upon the reduction of the alkaline copper reagents by glucose, the reduction obtained being the sum of their separate reduction values. Taylor [1924] states, on the other hand, that creatinine delays the precipitation of cuprous oxide in the Wood-Ost method.

It will be seen that even in pathological cases, where the uric acid and creatinine are increased, their influence will be practically negligible. It will be noted that the concentrations of uric acid and creatinine used in the above experiments were many times higher than are found in tungstic acid filtrates of blood where dilution is 1 in 10.

Holden [1926] states that urea inhibits the reduction of the Wood-Ost reagent by glucose. The effects of urea and potassium oxalate upon the reduction of the alkaline copper solutions by glucose have been examined. The following concentrations have been used: (1) urea 20 mg. per 100 cc; (2) potassium oxalate 0·1 mg. per 100 cc. These concentrations were found to exert no influence upon the reduction.

### The determination of blood-sugar.

As the result of the foregoing work it was decided that the following would be the most suitable method for the determination of blood-sugar.

*Solutions.*

1. Alkaline copper solution:

| | | |
|---|---|---|
| Copper sulphate $CuSO_4, 5H_2O$ ... ... | 10 g. | per litre. |
| Tartaric acid ... ... ... ... ... | 15 g. | |
| Sodium carbonate (anhydrous) ... ... | 80 g. | |

2. Iodide-iodate-oxalate solution:

| | | |
|---|---|---|
| Sodium carbonate (anhydrous) ... ... | 40 g. | per litre. |
| Tartaric acid ... ... ... ... ... | 7·5 g. | |
| Potassium iodide ... ... ... ... | 10 g. | |
| Potassium iodate ... ... ... ... | 0·7 g. | |
| Potassium oxalate ... ... ... ... | 18·4 g. | |

3. $N$ $H_2SO_4$.

*Method.* In a 3 × 1 inch centrifuge tube 10 cc. of a protein-free blood filtrate and 10 cc. of the above alkaline copper solution are mixed. This is placed in a boiling water-bath for 15 minutes. It is then cooled and centrifuged. The supernatant fluid is removed and the cuprous oxide washed twice with distilled water. 10 cc. of the iodide-iodate-oxalate solution are added to the cuprous oxide, followed by 10 cc. $N$ $H_2SO_4$. The whole is well stirred to dissolve the cuprous oxide and then titrated with 0·0125$N$ thiosulphate. A blank determination using distilled water is carried out at the same time.

1 mg. glucose = 3·3 cc. 0·0125$N$ thiosulphate.

Smaller volumes of blood-filtrate and smaller centrifuge tubes may be used if desired but for research purposes more accurate results would be obtained using the larger volume of filtrate. It will be noted that no specific method of preparing blood filtrates is mentioned here. This is being made the subject of a further paper.

## Summary.

1. The amount of reduction of the Shaffer-Hartmann alkaline copper reagent by pure glucose is greater when carried out in an atmosphere of nitrogen than when carried out in air.

2. When the potassium salts are not incorporated in the reagent but added as a separate solution after reduction, the reduction is the same whether carried out in an atmosphere of nitrogen or air and the results are the same as obtained when the reduction of the reagent containing the potassium salts is carried out in an atmosphere of nitrogen.

3. The amount of reduction by glucose of alkaline copper solutions containing copper sulphate, tartaric acid and sodium carbonate depends on the relative concentrations of the two latter substances.

4. Bicarbonates can replace tartrates in alkaline copper solutions.

5. Glycine, aspartic acid, glutamic acid, tryptophan and cystine affect the reduction of alkaline copper reagents by glucose only under certain conditions. These have been discussed.

6. Uric acid and creatinine even in concentrations found in pathological bloods do not appreciably affect the determination of blood-sugar.

7. Urea even when present in a concentration of 200 mg. per 100 cc. blood has no influence upon blood-sugar determinations.

8. Potassium oxalate used as an anti-coagulant even in a concentration of 1 % in blood has no influence on the reduction values.

9. The composition of a modified alkaline copper reagent and a modified technique employed for the estimation of glucose in blood filtrates are described.

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