XLVII. THE NATURE OF THE REDUCING SUBSTANCE IN HUMAN BLOOD.

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A rapid and convenient method of estimating sugar in blood is required for physiological investigations. With this purpose in view a research was undertaken conjointly between the physiological and biochemical departments, and a large number of estimations have been carried out by the recent method of MacLean [1919].

By this method the estimation of sugar in small quantities of blood, *e.g.* 1 cc. and even 0·2 cc. is possible. The process depends on the removal of proteins by precipitation. A measured volume of blood is first added to a 15 % solution of sodium sulphate. The mixture is then boiled, and into it whilst still hot freshly prepared "dialysed iron" is poured, which renders the protein insoluble. After filtration MacLean's solution (copper carbonate with potassium iodide and iodate) is added to a known volume and boiled. The sugar in the blood reduces the copper carbonate to cuprous oxide in presence of the iodide and iodate. The cuprous oxide is then converted into cuprous chloride by acidification, whereby a proportional amount of the liberated iodine is fixed and precipitated as cuprous iodide, and the remaining iodine is titrated with *N*/100 thiosulphate.

We decided that further work on the following lines was desirable.

(1) To ascertain whether the reducing substance estimated by the above method is entirely sugar or whether an interfering substance is present, and

(2) To discover the chemical nature of the sugar present.

The main results are summarised below. In the course of the experiments some matters bearing on technique have also been considered.

1. Technique.

(*a*) In MacLean's method the total volume is calculated by adding together the volumes of the blood, salt solution and iron, and it is assumed that there is no change in volume during precipitation. The bulky precipitate is then removed by filtration through starch-free paper and the sugar estimated in an aliquot portion of the filtrate, usually 20 cc. We found it more convenient to make up the mixture to a definite volume before filtration, *i.e.* 1·5 cc. blood to 50 cc. with distilled water. (The volume of the precipitate is negligible.) We at first cleared this by centrifuging. Subsequently we gave

up the centrifugal method for filtration through glass wool. A clear solution can easily be obtained in this way.

In order to show that no sugar was carried down in the adsorbed state by the protein precipitate, the supernatant liquor was poured off and the precipitate shaken with distilled water. On pouring off and analysing the water, no trace of sugar could be detected.

Also on adding a known weight of pure glucose to blood the reducing action of the filtrate was increased to the extent of the amount added, thus showing that none of the added sugar had been adsorbed by the protein.

(*b*) In the original method the conditions of heating the mixture of blood solution and copper salt are strictly standardised. It is recommended by MacLean that the solution should be brought to the boil in exactly 100 seconds, and that boiling should be continued for 6 minutes. In our experience the rate of initial heating has made no perceptible difference to the results. Very early in our researches we found that occasionally the results obtained were somewhat erratic which shows the importance of not relying on a single determination in pathological work. Impure distilled water, such as is supplied on the market in carboys, is one factor causing such irregularity and care should therefore be taken to ensure a pure supply of distilled water.

We have also found that the blood extract retains its reducing power without perceptible change for at least 48 hours.

2. THE NATURE OF THE REDUCING SUBSTANCES IN HUMAN BLOOD.

(*a*) Is the reducing substance in human blood entirely sugar, or are interfering substances present?

(*b*) Is the sugar entirely or largely a monosaccharide, or are di-saccharides and more complex sugars present in appreciable amount?

The results of our experiments in regard to these points are as follows:

(1) Uric acid is known to have a reducing action on copper solutions and occurs normally in the blood to the extent of 0·006 %. We therefore prepared solutions varying in strength from 0·01–0·4 % and proceeded to estimate these solutions by the method used for blood. We found that there was no reducing action under the conditions of MacLean's method.

(2) Creatinine similarly reduces copper solutions and occurs normally in the blood to the extent of 0·004 %. We prepared solutions of this strength and also 0·2 %, and proceeded in the same way as with uric acid. No reduction occurred. On adding small amounts of creatinine corresponding to the above percentages to the blood-sugar solutions, there was also no increase in reducing power.

We therefore conclude that the reducing substance is neither uric acid nor creatinine.

(3) As in the case of glucose, the reducing substance was entirely destroyed by boiling with ammonia, and was not extracted from the blood solution by means of ether.

(4) *The method of osmotic compensation.* Michaelis and Rona [1908] dialysed blood against sugar solutions of different strengths and found that when the concentration of sugar in the dialysate was the same as that found in the blood by determination, there was a state of balance. The polarimetric method was used in these experiments for estimating the sugar.

Hess and Mcguigan [1914] obtained similar results by means of *in vivo* dialysing experiments. The above authors conclude from these experiments that the sugar exists in the free state in blood and is not in the form of a colloidal complex.

We have carried out further experiments on these lines, employing the process of MacLean for estimating the sugar instead of the polarimetric method.

Small dialysers made of "viscose" were used in the experiments and the protein-free blood solution was dialysed against a solution of pure glucose of concentration slightly less than that found in the blood solution by analysis. It was found that the readjustment in concentration was such as would be expected if the reducing substance were entirely or almost entirely glucose.

We have since considered however that such experiments are by no means conclusive, because the concentrations of sugar employed are so small, that even if other substances, possessing a reducing power, were present in the blood they might not be detected by this method.

(5) *Formation of the osazone.* Several investigators, *e.g.* von Jaksch [1886], Kutz, Pickhardt [1893], and Miura [1895], have demonstrated the presence of glucose in mammalian, but not apparently human blood, by the isolation of the osazone with the characteristic microscopic appearance and melting-point.

As we intended to employ human blood throughout our experiments an attempt was made to isolate the osazone from that blood by the following procedure.

The proteins from 15 cc. of blood were precipitated by MacLean's method, and the protein-free blood solution was evaporated to dryness on the water-bath. The residue was extracted in a Soxhlet with absolute alcohol and the extract, freed from alcohol, was dissolved in a little water. The solution was then filtered and boiled for 30 minutes with 0·25 cc. of pure phenylhydrazine and glacial acetic acid. On cooling, the osazone crystallised out, and was identified by microscopical examination as glucosazone.

Pavy [1894] and many other investigators have isolated another osazone, apparently that of isomaltose, from horse blood and also from human urine.

Pavy and Siau [1901] also subsequently showed that after hydrolysing the sugar-containing solution obtained from the blood of various animals, there was a marked increase in reducing power. They calculated a factor, denoted by K, which was the percentage ratio of the reducing power determined before and after hydrolysis. This ratio varied from 66–75, according to whether the blood was obtained from the dog, cat, rabbit, or horse. Similar results were obtained in the case of human urine. They concluded that there exists in

blood and urine in addition to glucose a sugar of the maltose type, possibly isomaltose.

Cammidge [1920] has also shown that there is a hydrolysable substance in human blood, which he believes to be a dextrin. This substance increases in amount in pancreatic disease.

It seemed desirable to make a further investigation of this question, as it is likely to be of importance in clinical work, especially in following the course of certain metabolic diseases. At first we employed Pavy's original method [1894] which consists in extracting the blood with alcohol and removing the colloidal matter present in the extract with sodium sulphate. In some experiments we added a small amount of "dialysed iron" to the alcoholic extract, and dispensed with the salt altogether. The alcohol was removed by evaporation, and the extract made up to a known volume with distilled water and filtered through glass wool. The sugar was estimated in a portion of this extract by MacLean's method, and the remaining portion was hydrolysed by boiling with $N/100$ HCl for 4 hours. After neutralisation the sugar content was again determined, the results being expressed as a percentage ratio in the following way.

If a = % in sugar expressed as glucose before hydrolysis,
b = „ „ „ „ after „
then $K = \frac{a}{b} \times 100.$

We found this ratio to be very variable for different normal individuals, the range being from $K = 21$ to $K = 100$.

$K =$ 21·25	48·0	100
21·66	72·0	100
		100

When $K = 100$ there is, of course, no increase in reducing power after hydrolysis.

This method is slow and needs much manipulation; furthermore, the reducing power of a given specimen of blood was occasionally found to be much lower when this method was employed than when the usual salt-process was used.

Subsequently we simplified the process, so as to render it more convenient as a clinical method. The proteins were removed by sodium sulphate and dialysed iron as in MacLean's process, and the salt-extract was hydrolysed with HCl ($N/1$–$N/100$). In a few cases an increase in reducing power was noticed after 4 hours' hydrolysis, the ratio K varying from 36 to 58.

In many of these experiments however there was no increase in reducing power after hydrolysis for 4 hours; in fact, in some experiments there was a diminution, and even a total disappearance of the sugar originally present in the blood, pointing to destruction through the action of the acid. The amount of destruction was usually proportional to the concentration of the acid. Even when the reducing power of the blood solution was unchanged at the end of the period of 4 hours, a temporary diminution or even absence of reducing

power was sometimes noticed at the end of 1 or 2 hours, followed by a reappearance of this property after 4 hours' hydrolysis.

In the case of one person on one occasion sugar was shown to be entirely absent from the blood, as there was no reducing power before or after 4 hours' hydrolysis.

In the case of another person glucose was also absent, but the reducing power appeared after acid hydrolysis. This may mean that although there was no glucose, a hydrolysable complex sugar was present in the blood. In both these persons on every other occasion on which the blood was examined, the reducing power (before hydrolysis) was found to be normal.

There was no evidence that the presence of the sodium sulphate inhibited the hydrolytic action of the acid, as cane-sugar was easily inverted under the same conditions.

It is evident from these results, that at the present time the exact determination of the hydrolysis factor K is not available as a simple clinical method.

It was possible that the glycogen present in blood might occasionally be incompletely precipitated by the salt and "dialysed iron" and traces thus be left in the solution. This would account for the occasional increase in reduction observed after hydrolytic experiments. We therefore added a small amount of glycogen to blood and proceeded in the usual way, but found by hydrolysis that it was completely precipitated by the salting-out method.

We next attempted to ascertain the reason for the destruction of the reducing-substance present in blood during hydrolysis. In the case of $N/10$ and $N/100$ HCl the destruction is undoubtedly due to the direct action of the acid, as the amount of salt formed on neutralisation was found to have no influence at all on the reducing power of the solution. In the case of the experiments with $N/1$ acid however we were surprised to find that the presence of sodium chloride to the extent of 5 % (an approximately $N/1$ solution) had a very marked inhibitory effect on the reducing power of the blood-extract, and also on that of pure glucose and maltose.

This is illustrated by the following tabulated results.

Table I. % *of sugar.*

		In absence of NaCl	In presence of NaCl (5 %)
Blood-sugar:	(1)	0·10	0·05
	(2)	0·085	0·024
		0·243 (+added glucose)	0·199
Glucose:	(1) In water	0·102	0·087
	In 15 % Na_2SO_4	0·102	0·082
	(2) In water	0·100	0·075
	In 15 % Na_2SO_4	0·105	0·057
	(3) In 15 % Na_2SO_4	0·091	0·035
		0·231	0·139
Maltose:	(1) In water	0·071	0·032
	In 15 % Na_2SO_4	0·090	0·055
	(2) In water	0·080	0·078
	In 15 % Na_2SO_4	0·070	0·000

The above results are percentages of sugar in the original blood or sugar solution, but the actual titrations were of course carried out in much more dilute solutions (about $\frac{1}{30}$th strength). These results show that the reducing power of the blood-extract and also that of glucose and maltose is considerably diminished by the presence of sodium chloride in 5 % concentration.

But sodium sulphate has no perceptible effect, and there is thus no objection to its use as a protein-precipitant in the method of MacLean for the estimation of blood-sugar.

The difference in behaviour of sodium chloride and sulphate suggests that it is the halogen ion which exerts the specific effect.

The experiments tabulated below show that the inhibitory effect is due to a diminution in the velocity of reaction between the sugar and copper reagent, since we found by extending the period of boiling from 6 to 10 minutes that somewhat higher results were obtained.

Table II. *Glucose.*

		%	%	%
Without NaCl	6 mins. boiling	0·0070	0·0040	0·0034
Without NaCl	10 mins. boiling	—	0·0047	0·0047
With NaCl	6 mins. boiling	0·0042	0·0028	0·0028
With NaCl	10 mins. boiling	0·0066	0·0039	0·0037

It is well known that glucose forms additive compounds with halogen salts. These compounds can be obtained in a crystalline form, but are very unstable in dilute solution and are readily dissociated, and the reducing and rotatory powers of the compounds have been shown to correspond exactly to their content of glucose. This is not in agreement with the above experiments, but we have used extremely dilute solutions of sugar with a large excess of salt, so that it is still possible that the formation of the additive compound is a cause of the observed retardation. It is remarkable that this retardation is still observed even when the salt is added after the sugar solution has been raised to the boiling point, as it is unlikely that such an unstable compound would be formed under these conditions.

It is evident from the above results that in order to determine the ratio K an acid will have to be employed for hydrolysis which has no perceptible destructive effect on sugars and which forms a salt which has no retarding effect on the reducing process.

Summary.

We have carried out a number of experiments by the method of MacLean for estimating blood-sugar with the object of discovering the nature of the reducing substance or substances present.

1. Instead of filtering the bulky precipitate of protein through filter paper, it is suggested that the blood to which the sodium sulphate and "dialysed iron" have been added be made up to a known volume (1·5 cc. to 50 cc.)

with distilled water and be then filtered through glass wool. In this way a clear solution can readily be obtained.

2. Uric acid and creatinine in amounts much greater than those normally present in human blood exert no reducing action under the conditions of the above method.

3. The reducing substance is destroyed by boiling with ammonia and is insoluble in ether, as is the case with reducing sugars.

4. Phenylglucosazone with the characteristic microscopic appearance has been isolated from human blood.

5. We have confirmed the observations of Pavy and Cammidge that the reducing power of blood increases after hydrolysis with hydrochloric acid, indicating the presence of a hydrolysable substance.

6. The exact determination of the increase in reducing power after hydrolysis is complicated by at least two factors:

(*a*) The destructive effect of HCl even in low concentration upon the reducing substance of blood.

(*b*) A retarding effect on the reducing process due to the presence of the sodium chloride formed after neutralisation.

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