XCIII. THE NATURE OF THE BLOOD-SUGAR.

By LEWIS BLAND WINTER (*Grocers' Company Research Scholar*).

From the Sherrington School of Physiology, St Thomas's Hospital, London.

(Received April 8th, 1930.)

ISOLATION of the blood-sugar has been seldom attempted. Hédon [1898] worked with the blood of depancreatized dogs. The proteins were separated by dialysis for several days at low temperature, and the solution containing the sugar was evaporated to a syrup in open vessels at 60–70°. Partial purification was effected by precipitation with neutral lead acetate; the filtrate after removal of lead was evaporated. The solid precipitated from the syrup by addition of ether gave glucosazone on heating with phenylhydrazine. The reducing power determined by titration with Fehling's solution showed a considerably greater glucose content than that deduced from the optical activity. Similar results were obtained with the blood-sugar of the horse; the glucose content estimated by the polarimeter was rather more than half that found by titration. Hédon found substantial agreement between the two methods in the case of sugar prepared from urine in diabetes, and concluded that the urinary sugar differed from that of the blood, undergoing some modification at the moment of its secretion. Hanriot [1898] used horse blood, which was deproteinised by boiling with sodium sulphate; the filtrate was concentrated and the syrup purified by repeatedly dissolving in methyl alcohol and precipitating with ether. A dry powder was obtained, and when the sugar was estimated by Fehling's solution or polarimetrically there was good agreement. The sugar could be converted into glucosazone or parachloralose. Neither Hédon nor Hanriot records analyses or melting-points of the substances isolated by them, nor was the sugar pure enough to show mutarotation. The sugar content given by the polarimeter was deduced from the equilibrium value of α - and β -glucose. This value was also used in the calculation of the sugar content of blood-filtrates by Winter and Smith [1923] and compared with that found by estimation of the copper-reducing power. It was found that blood-filtrates from normal men and animals showed a marked discrepancy between the figures obtained by the two methods, but that this difference disappeared in the course of some days. On the other hand, in diabetes the values agreed from the outset, and it was suggested that the sugar was of a different type in the two cases. Subsequently, Lundsgaard and Holbøll [1925] came to a similar conclusion. Denis and Hume [1924] repeated the experiments of Winter and Smith [1923] and concluded that the changes in optical activity on keeping the sugar solutions were due to contamination

with micro-organisms; the changes recorded by them were, however, in the opposite direction. In all the above experiments observations were made on sugar solutions contaminated with varying amounts of other substances. The object of the present work was to separate the sugar from normal blood in such a way as to enable it to be identified with certainty.

EXPERIMENTAL.

The blood of the rabbit has been used throughout. The animal having been killed by a blow on the head, the throat is cut and the blood received into a shallow basin. The blood is defibrinated by whipping and the volume measured. The proteins are precipitated with the Folin-Wu reagents, seven volumes of water being used for dilution. After vigorous shaking the mixture is allowed to stand for an hour and then filtered through papers which have been very thoroughly washed with water. When filtration is complete the precipitate is washed with a little cold water. The total volume of filtrate measures about 800 cc. for 100 cc. blood. Concentration, which is carried out *in vacuo* at 35–40° until the volume is rather more than half the original volume of blood, should be completed in 2–3 hours. The concentrated solution is transferred to a flask, and the distilling flask washed out with saturated lead acetate, 0.5 cc. per cc. blood. The lead acetate is added to the sugar solution. After shaking, the mixture is allowed to stand for an hour. The bulky precipitate is centrifuged, and washed with small amounts of water. The clear solution is cooled in ice, and made alkaline with strong ammonia. The precipitate is allowed to settle for a few hours, being kept cold, and then separated by the centrifuge. A fifth of the previous quantity of lead acetate is added to the fluid, and a further small amount of ammonia. The second precipitate is centrifuged in the same cups as contain the first. The precipitate is stirred up with 80 % alcohol until all lumps have disappeared and then spun down. The washing is repeated. The cups and precipitate are dried in a vacuum over sulphuric acid. The lead compound may be kept for some time in the dry state if convenient. The dry precipitate is weighed and replaced in a centrifuge cup. For each gram 2 cc. 2*N* sulphuric acid are added, and the precipitate is disintegrated with a glass rod; almost continuous stirring for 10 minutes is advisable for complete decomposition. If not acid to litmus further acid is cautiously added, with stirring, until a test sample is distinctly acid. The fluid is separated in the centrifuge and transferred to a measuring cylinder. The precipitate is stirred with a small amount of water, separated, and the washing added to the first solution. The volume is noted. The solution is transferred to a small flask, and 4 volumes of strong alcohol are added gradually, shaking between each addition. The lead precipitate is washed twice with 5 cc. of 80 % alcohol, and the washings are added to the flask. It is advisable to allow a day in the cold chamber for complete precipitation; haste at this stage is liable to cause difficulty later. The solution of the sugar in alcohol is centrifuged, and the fluid, which

should be quite clear, is separated. It is better not to wash the precipitate as there is a tendency to form a colloidal suspension. The solution is cooled in ice, and a slight excess of 10 % baryta in methyl alcohol is added. After standing for an hour in ice, the barium compound is separated in the centrifuge and washed twice with cold 80 % alcohol. The precipitate is suspended in 80–100 cc. of 80 % alcohol, warmed to 40°, and decomposed with a rapid stream of CO₂ for 2 hours. After 24 hours in the cold room the carbonate is filtered off, and the solution completely evaporated under reduced pressure. To the distilling flask 15 cc. of 80 % alcohol are added, a trace of insoluble matter is filtered off, and the solution allowed to evaporate slowly in a small basin over sulphuric acid. There is left a colourless syrup which begins to crystallise on stirring. Very soon there is a hard crystalline mass which is removed, rapidly washed with alcohol, and dried. The yield from 100 cc. blood averages 36 mg. To obtain pure α -glucose the crude product is twice re-crystallised from dry methyl alcohol, best by allowing the solvent to evaporate very slowly at room temperature; the sugar crystallises in fine needles which are washed with alcohol and dried in a vacuum over sulphuric acid.

Melting point. 145–146° (uncorr.).

Analysis. (Micro-analyses by Dr Ing. A. Schoeller, Berlin.)

C 39.87, 39.73 %; H 6.71, 6.64 %.

C₆H₁₂O₆ requires C 40.00 %; H 6.66 %.

Optical activity. All readings were taken in a 1 dm. tube of 4 mm. bore. Only 1.5 cc. of fluid are required, and it is possible with this tube to obtain as great a degree of accuracy as when a tube of standard bore is used. The polarimeter was a three field instrument; a half shadow angle of 1° was used. All readings agreed within 0.02°. The substance shows mutarotation in water and dry methyl alcohol; the rate of change in the latter solvent is slow. 17.4 mg. were dissolved in 1.6010 g. water. $c = 1.08$.

Time (hrs.)	0.08	1	1.5	3.1	9.0	18
α	+1.36	1.00	0.90	0.80	0.66	0.66°
$[\alpha]_{5461}^{14^\circ}$	+125	92	83	74	61	61°

8.4 mg. were dissolved in 1.6155 g. methyl alcohol ($d. 0.799$). $c = 0.41$.

Time (hrs.)	0.08	7	22	44
α	+0.56	0.52	0.50	0.46°
$[\alpha]_{5461}^{14^\circ}$	+136	126	121	112°

Molecular weight. Determinations were carried out by Barger's method. Methyl alcohol containing 25 % of water was used as solvent, and urea as standard. 10.5 mg. sugar were dissolved in 0.522 g. methyl alcohol of $d. 0.878$, giving a concentration of 17.6 g. per litre. This solution was sealed in capillary tubes with the urea solutions. Tube 1 contained 0.09 M , tube 2, 0.1 M , tube 3, 0.11 M urea. After reading, the tubes were left for 15 hours at room temperature. In tube 1 the urea was hypotonic, in tube 3 hypertonic. In tube 2 no increase or decrease took place in the size of the drops, giving a molecular weight of 176. In another experiment a value of 188 was obtained.

Preparation of the phenylosazone. 85 mg. of the sugar were dissolved in 4.5 cc. of water and 0.25 g. phenylhydrazine hydrochloride and 0.55 g. sodium acetate were added. The mixture was heated on a boiling water-bath for half an hour and allowed to cool slowly. The crystals were separated, washed with water, and recrystallised from dilute alcohol. Yield 32 mg. Rapidly heated the melting-point was 208–210°, unchanged when the substance was intimately mixed with a known sample of glucosazone. The optical activity of the product was observed when dissolved in a mixture of 6 parts of pyridine and 4 parts of absolute alcohol. 8.7 mg. in 1.5 g. solvent (*d.* 0.873). *c* = 0.50.

Time (hrs.)	0.25	22
α	–0.40	0.22°
$[\alpha]_{5461}^{13^\circ}$	–80	40°

The following figures were obtained on analysis:

C 59.76, 59.92 %; H 6.28, 6.18 %; N 15.57, 15.54 %.

$C_6H_{10}O_4(N : HN.C_6H_5)_2$ requires C 60.33, H 6.14, N 15.64 %.

Preparation of the β -penta-acetate. A mixture of 0.14 g. of the sugar and 0.07 g. of freshly fused sodium acetate was heated with 0.7 cc. of acetic anhydride in a small tube fitted with a reflux condenser. After 2 hours at 100° 4 cc. of cold water were added. The oil which precipitated rapidly crystallised on stirring. The product was washed with several changes of cold water, and three times recrystallised from 90 % ethyl alcohol, in order to remove as much of the α -compound as possible. Yield 34 mg. m.p. 124–125°. $[\alpha]_{5461}^{13^\circ} = +5.5^\circ$ (in chloroform).

C 48.84, 49.54 %; H 5.77, 5.90 %.

$C_6H_7O_6(OC.CH_3)_5$ requires C 49.23; H 5.64 %.

From a consideration of the experiments described it seems clear that the blood-sugar consists largely of glucopyranose. It has not been possible during the present work to obtain definite evidence as to the presence of any other sugar. The accepted figure for the concentration of reducing substances in normal blood varies from 0.08 to 0.1 %, and the yield of crystalline glucose obtained, taking into account the losses attendant on the various precipitations, does not suggest that more than traces of other sugars can be present; this question is being considered in connection with the metabolism of lactose and will be dealt with in a later communication. In numerous experiments the mother-liquor has been worked up further after crystallisation of the glucose; the syrup obtained has a strongly positive rotation, and gives glucosazone on treatment with phenylhydrazine. The source of the fasting blood-sugar is liver-glycogen. Recent views as to the constitution of this polysaccharide agree that glycogen consists of a conjugated chain of α -glucose units [Haworth, Hirst and Webb, 1929]. When α -glucose is liberated by the action of the liver diastase *in vivo* it seems likely that under the influence of body temperature and blood alkalinity a mixture of the α - and β -stereoisomers will be rapidly established, unless there are some factors involved of which

we have no knowledge. Allott [1928] working with rabbits, found no definite difference between the blood-sugar curves after injection of either isomer, or of the equilibrium mixture. The explanation of the low values obtained when the sugar in deproteinised blood-filtrates is estimated by the polarimeter is not yet clear. It has been suggested [Holden, 1926] that glutathione, or ergothioneine [Sjollema, 1927] may be responsible, but the experiments with rabbits after insulin [Winter and Smith, 1924] are difficult to reconcile with this view. When the blood-sugar was removed under the action of insulin and the blood-filtrates examined polarimetrically, a small positive value was always obtained. If any strongly laevo-rotatory substances pass into the Folin-Wu filtrates they should become apparent when the glucose is removed, unless insulin has the effect of diminishing their concentration *pari passu* with the blood-sugar.

SUMMARY.

The blood-sugar of the rabbit has been isolated as α -glucopyranose.

I wish to thank Professor W. N. Haworth for his interest and advice. Part of the expense of this work was defrayed by a grant from the Government Grant Committee of the Royal Society.

REFERENCES.

- Allott (1928). *Biochem. J.* **22**, 773.
Denis and Hume (1924). *J. Biol. Chem.* **60**, 603.
Hanriot (1898). *Compt. Rend. Soc. Biol.* **50**, 543.
Haworth, Hirst and Webb (1929). *J. Chem. Soc.* 2479.
Hédon (1898). *Compt. Rend. Soc. Biol.* **50**, 511.
Holden (1926). *Biochem. J.* **20**, 363.
Lundsgaard and Holbøll (1925). *J. Biol. Chem.* **65**, 343.
Sjollema (1927). *Biochem. Z.* **188**, 465.
Winter and Smith (1923). *J. Physiol.* **57**, 100.
—— — (1924). *Proc. Roy. Soc. Lond. B* **97**, 20.