ON THE NATURE OF THE SUGAR IN BLOOD.

By L. B. WINTER, B.A. and W. SMITH, B.A.

(*From the Biochemical Laboratory, Cambridge.*)

The experiments of Minkowsky(1) first established the importance played by the pancreas in the metabolism of carbohydrate. Clark(2) showed that the consumption of sugar by the heart was increased when the perfusing fluid was also passed through the pancreas. In a later paper(3) he established the remarkable fact that perfusion of the pancreas with Locke's solution, *i.e.* one containing dextrose caused the optical rotation to be diminished whereas the copper reducing value remained unaltered. He suggested that the pancreas contained an enzyme which was responsible for converting dextrose into another form of sugar, which then could be utilised by the body.

The recent work of Embden and his co-workers(4) has emphasised the importance of hexose-phosphoric acid in the carbohydrate metabolism of muscle. Since on hydrolysis hexose-phosphoric acid yields fructose, it seemed possible that the sugar in normal blood might be other than glucose. The primary aim of our experiments—begun in 1921—was, then, to investigate the nature of the sugar in the blood by a comparison of the polarisation, and copper reducing values. For this it was necessary to prepare a protein-free filtrate.

Method.

After testing the various protein precipitation methods usually employed for blood sugar estimations, we found that they all gave on concentration a fluid which was quite unsuitable for polarimetric determination. The tungstic acid method of Folin and Wu(5) was least tedious, and gave a filtrate which was almost free from the precipitant; that all protein was not removed was made clear by nitrogen determinations, and by the turbidity which appeared when the filtrate was concentrated to a small bulk. It was necessary, therefore, to employ some further process in order to remove the remainder of the proteins and to extract the sugar. The use of alcohol in a suitable strength to effect both processes simultaneously suggested itself. In a series of trials 85 p.c.

alcohol was found to be the best extraction medium; this strength extracts all the sugar likely to be present and is sufficiently strong to coagulate all the remaining proteins. Until a neutral sample of sodium tungstate was obtained care was taken to adjust the reaction in the way recommended by Folin(6). Neutral samples were finally obtained from Messrs Harrington.

The method finally adopted is as follows: The blood is precipitated according to the method of Folin and Wu. Filtration is effected by filtering in succession through two funnels. In the upper one is placed a coarsely grained paper, in the lower a Whatman paper No. 40. The double filtration removes all of the precipitate which it is possible to remove by filtration at this stage. The filtrate is concentrated almost to dryness *in vacuo*, not allowing the temperature to rise above 40° C. Caprylic alcohol is used to prevent foaming. A volume of 85 p.c. alcohol equal to the original volume of blood is then added to the distillation flask, and an immediate precipitation of the remaining proteins and tungstate takes place. The alcohol is allowed to stand in contact with the residue thus formed at 40° C. for 20 minutes with occasional shaking, so as to ensure complete extraction of the sugar from the solid mass. The alcohol is filtered into a small distillation flask and a further quantity of alcohol equal to half the original amount is added to the first flask in order to complete the extraction. This is allowed to stand for ten minutes with shaking as before. The combined filtrates are concentrated *in vacuo* at 40° C. until almost dry. The residue is taken up in a small quantity of water, the amount depending upon the original volume of blood used, and then filtered (usually 20 to 30 c.c.). The filtrate is perfectly clear and suitable for polarimetric determinations. A one decimetre tube is usually employed owing to the amount of fluid available being small, and a considerable amount being required for the copper reducing estimation.

The final filtrate was tested by the protein and biuret colour reactions; negative results were obtained. A determination of the nitrogen showed that it was no greater than could be accounted for by the extraction by the alcohol of urea and similar substances. In order that an accurate comparison may be made between the polarimetric and copper reducing values of the blood sugar, it is necessary that every trace of protein be removed; if this condition be not obtained, not only are the optical properties of the solution liable to be affected to an unknown and variable extent in each experiment, but in determining the copper reducing value of the sugar, there is a danger of traces of protein giving rise to substances

which will reduce the alkaline copper solution. Owing to the careful control of temperature, and the very small concentration of acid, we do not consider that the method would tend to split off copper reducing substances from possible gluco-genetic bodies.

To determine how completely the sugar was extracted by the alcohol employed, the residue left behind after the filtration of the alcohol was shaken up and extracted with water. The filtrate now was perfectly clear showing that the remaining protein was not readily redissolved. Copper reducing determinations and polarimetric readings showed no measurable amount of sugar present. To test whether the relation between the polarimetric and copper reducing powers of glucose would be altered by this treatment, a solution of pure glucose was concentrated in the presence of tungstic acid employed for precipitation, extracted with alcohol, and made up with water. The copper reducing power was then, if anything, slightly lower than the polarimetric value. Possibly the larger amount of free acid caused slight disaccharide formation(7); normally all but a small amount of tungstic acid combines with the protein and is so removed.

The filtrate is invariably slightly acid and therefore most likely to stabilise the sugar originally present. It soon however became evident that the instability of the original sugar made it essential that concentration of the aqueous filtrate should be effected as quickly as possible. This we have always done, and as soon as the final filtrate was obtained we have made a quantitative comparison of the sugar by polarimeter and copper reducing power. We have made no attempt to obtain in our final filtrate all the sugar originally present in the blood. To have attempted quantitative extraction would have lengthened the process very considerably. Our sole concern was to obtain the sugar in a form as nearly as possible approximating to that normally in the blood. Our experiments from the beginning of protein precipitation to the polarimetric reading usually occupied five hours for 100 c.c. blood; when less blood was used the time taken was much less. Of this time only a comparatively short period is occupied in what we find to be the dangerous stage, viz. the concentration of the aqueous filtrate.

The copper reduction method which has been adopted throughout is that of Bertrand as modified by Wood and Berry(8); it had been previously determined by us that this gives reliable and consistent results; the large amount of fluid used and the convenience of a permanganate titration being points in its favour. A comparison of this method with values obtained from the polarimeter was undertaken with

a sample of pure glucose (for this we are indebted to Dr C. G. L. Wolf). The degree of accuracy obtained may be seen from the following figures.

Glucose weight taken	·2435 gm. made up to 100 c.c. with water.
Copper reduction value	·23 %
Polarimeter	·242 „

The polarimeter used is a three field instrument made by Hilger of London, the illumination being the mercury green line. This has the advantage of giving a higher specific rotation than that obtained from the sodium light. The light also is steady and can easily be varied in intensity by the resistances with which it is connected. By means of the instrument used readings to ·01 degree can be obtained. The mean of a series of readings by each of us was taken whenever possible. As a rule complete agreement was obtained; it was noticeable though that when one of us had been bled this agreement no longer held; in these cases the reading from the other was accepted. It had been previously noticed that a comfortable position and normal mental state are necessary for reading colorimeters and similar instruments.

The amount of sugar extracted is approximately $\frac{1}{3}$ to $\frac{1}{2}$ of that indicated as being present by the micro estimation. There is no possibility of more than one sugar being present and of one being preferentially extracted by the solution of alcohol employed, as no trace of sugar was found to be extracted by water after the alcohol treatment had been completed. From the final filtrate in some of our experiments an osazone was prepared. This was recrystallised. Microscopic examination showed only glucosazone to be present; melting point determination gave 205° C., which was sharp and well defined. This figure corresponds to the accepted value of the melting point of glucosazone.

The figures obtained by Bang's old method (this was used by us for the quantitative estimation of the blood sugar content) give only an approximation to the actual content in the whole quantity of blood drawn owing to the sample being obtained at one period only of the operation. This should be noted in connection with the nervous factor which is apparent in one of the cases discussed below.

Experiments with animal blood.

A series of experiments was carried out using the blood of different animals, ox, sheep, cat and rabbit. That from the ox and sheep was obtained from the slaughter house; that of the other animals, from those killed for the purpose in the laboratory without anæsthetics. 25 to 100 c.c. were usually employed, depending upon the animal. This blood

was treated as has been described above. The final filtrate when read in a one decimetre polarimeter tube usually gave a value considerably below that obtained by the copper reduction method. The copper reduction value was calculated as glucose. Calculating also the polarimeter reading as glucose the difference between the two results was very apparent.

The work of Hewitt and Pryde(9) suggested that an unstable form of glucose might be present; the tubes were therefore read for at least three days after the completion of the experiment. The readings in each case gradually approached, and usually reached at the end of this time, a rotation corresponding to the α, β equilibrium form of glucose. The copper reducing value was unaltered at the end of this time. As mentioned above, the speed at which the concentration of the aqueous filtrate is carried out is of the greatest importance. If faulty water-pressure delays the concentration, or the temperature is allowed to rise unduly, the polarimetric is very close to, or even above, the copper value. It was found that rapid concentration was most readily effected by employing large distilling flasks of two litre capacity. The distillation was begun with only about one half of the filtrate, the remainder being added at intervals by means of a funnel connecting with the capillary. The effect of this is to enable the distillation to proceed smoothly and continuously while the fluid is being added, and at the same time avoids the necessity of allowing air to enter the flasks in order to stir up the fluid.

To test whether appreciable alteration in the state of the sugar took place at room temperature when in contact with the precipitated proteins, two samples of the same blood were taken: one was precipitated, filtered immediately, and proceeded with as usual; the other was precipitated, allowed to stand for six hours, and then filtered and treated as usual. The readings of the final products were both identical as regards their copper reducing and polarimetric values.

Some experiments were carried out to determine whether a ferment or ferments are present in blood which will convert added glucose to another form of sugar. The sugar was added before and after precipitation of the proteins and allowed to stand at room temperature, 20° C., for one hour. The amount of glycolysis being inappreciable under these conditions, almost identical results were obtained from the two samples, indicating that no such ferment can be present, or at any rate, that it is unable to work under the conditions of our experiments. Hewitt and Pryde's experiments(9) suggested that if any change were to take place

it would occur with great rapidity. Our negative results are in agreement with the conclusion of Cooper and Walker(10), who state that no such enzyme is present in the blood.

The only experiments which we can find in which the sugar of the blood has been investigated as regards its copper reducing power and its polarimetric value are those of Oppler(11). We attribute the little or no difference he found in the two values to the long time required to obtain a concentrated protein free solution by the method he employed, and to the sugar being in aqueous solution throughout—conditions which would allow the sugar to be converted into the α, β equilibrium form.

Experiments with human blood.

In view of the results obtained from the blood of animals, it became of interest to determine the condition of the sugar circulating in the blood of man. The blood was drawn from an arm vein by means of a hypodermic needle and syringe into a known amount of ·1 p.c. ammonium oxalate solution in a measuring cylinder, with constant shaking to prevent clotting of the blood. The precipitation was then carried out at once. These operations were carried out at Addenbrooke's Hospital, except in two diabetic cases. At the same time a sample of blood was taken on which a quantitative estimation was effected by Bang's old method.

In normal persons with one exception the first polarimeter reading on the final filtrate showed that sugar in the blood had a specific rotation which was considerably below that of α, β glucose as deduced from the copper value. This is entirely comparable with our experiments with the blood of animals. The curves approach the copper value in a similar fashion; in some cases this value was not reached, and some subsidiary action was evidently occurring. In Fig. 1 are shown the curves of the polarimetric readings of three normal individuals. It will be seen that in each case they start below their respective copper values. These are calculated for glucose. The curve of H.M. did not quite reach its end point in four days. Both of the others were in equilibrium on the third day.

Hewitt and Pryde's experiments(9) suggest that solutions of glucose and fructose in contact with the mucous membrane of the intestine are converted into γ glucose; presumably they enter the blood in this form; to test this it seemed desirable to determine whether any alteration in the nature of the blood sugar could be brought about by ingestion of considerable quantities of these sugars. While the experiments with glucose might be open to doubt on the grounds of a small difference in

the curve being possibly produced by delay in the concentration of the filtrate, any considerable amount of fructose present in the blood would

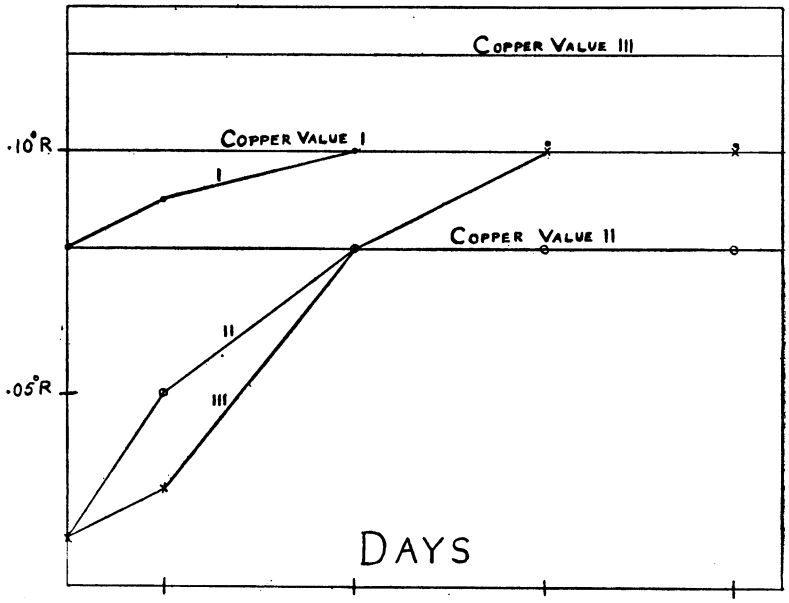

Fig. 1. Increase of polarimeter reading on successive days.
Normal subjects: I. J. B. S. H. II. G. M. D. III. H. M.

be more likely to be detected, since the specific rotation of fructose is so much greater than that of glucose, and still greater than that of the normal sugar of blood. It might reasonably be expected that with fructose lævo rotations would be obtained.

The normal blood sugar content which we obtain by Bang's old method is ·08 p.c. to ·1 p.c. After meals of 100 to 150 grams of glucose or fructose this rises to ·13 or ·14 p.c., *i.e.* the content of the blood sugar has increased by 50 p.c. The blood was drawn half-an-hour after the meal. If this sugar had been in the condition as ingested, a marked alteration of the ratio copper reduction to polarimeter should have been apparent. This however did not occur. The ratio of copper reducing power to polarimeter reading was similar to that of normal blood. We conclude therefore that glucose and fructose enter as, or at any rate are very rapidly converted into, the form normally present in the blood. In Fig. 2 the results of three experiments on L. B. W. are shown. Quantities of blood varying from 55 to 100 c.c. were taken, but for purposes of comparison one copper line is taken in order to emphasise the fact that the ratio copper value to polarimeter was approximately the same

at the beginning of each curve. This shows that the sugar which recently entered from the intestine had been converted into normal blood sugar.

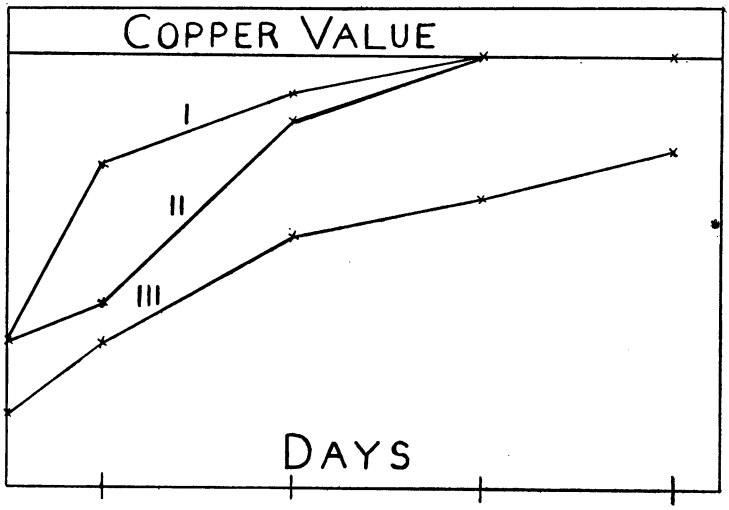

Fig. 2. Increase of polarimeter reading on successive days. L. B. W. I. Normal. II. After 100 grams of glucose. III. After 150 grams of fructose. (The curves are scaled to a common copper value.)

In view of the facts mentioned above, it became of interest to see whether the blood of patients suffering from diabetes mellitus differed from that of normal persons. The cases investigated of subjects suffering from diabetes have all been of the severe type. As a consequence it has only been possible to obtain a comparatively small quantity of blood from each. It is important to note that owing to the high sugar content a much smaller quantity of blood is required with a consequent acceleration of the manipulation. If the blood sugar was even approximately normal in quality it should be very evident, since there would be less time elapsing between the precipitation and the final product. In three cases the polarimeter reading was slightly above the copper reducing value. Any considerable amount of β-oxybutyric acid present in the final filtrate would tend to throw the rotation in a lævo direction, as the l-form is the only one present in these cases. The curves reached the copper value on the second or third days. In one case the polarimeter reading was considerably above that of the copper reducing value; the possible meaning of this is discussed later.

It is probable then, that in severe cases at any rate the normal blood sugar is present only in very small amounts and it is possible that the failure to utilise sugar is due to the lack of some ferment, which has

the property of converting glucose and fructose to the form normally present in the blood, and that the sugar in cases of diabetes is mainly the α, β equilibrium form of glucose.

In Fig. 3 curves are shown of two diabetic cases with one normal for

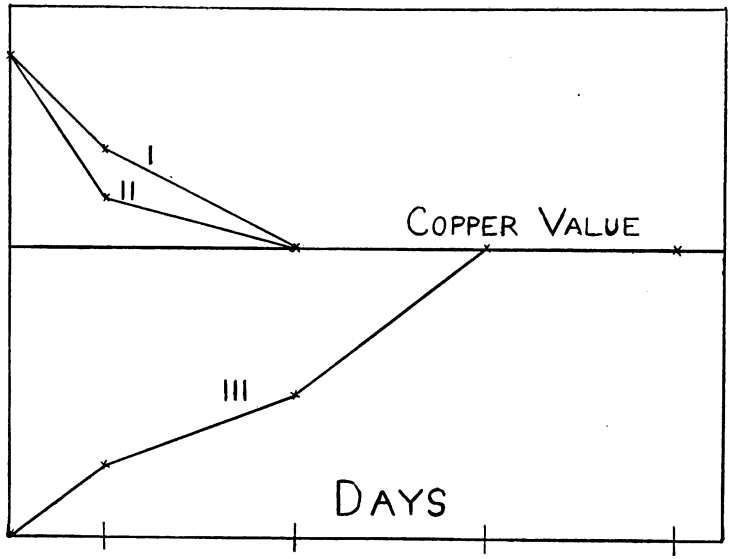

Fig. 3. Curves of polarimeter reading on successive days. I. Diabetic. II. Diabetic. III. H. L. W. (normal). (The curves are scaled to a common copper value.)

comparison. As in Fig. 2 one copper line only has been taken, the curves showing the polarimeter readings being scaled so that the ratio polarimeter reading to copper reducing value is unaltered. It will be seen that the diabetic curves approach the copper line from the opposite side to that in the normal case.

One of the "normal" cases (J. C. C. P.) presented features differing from those of the others. The subject had no diabetic history, and was perfectly healthy. Determination of his blood sugar by Bang's old method gave ·17 p.c., which is abnormally high as is shown in the protocols. Even in the cases of feeding with sugar, this amount was not reached. The maximum amounts so obtained were ·13 p.c. with G. F. T. after 150 grams of glucose, and ·14 p.c. with W. S. after taking 100 grams of glucose. Further, the ratio of polarimeter to copper reduction value was higher than in other experiments on normal persons. The results we think were due to the psychological condition of the subject. He was somewhat nervous about the operation, which is not entirely painless, and nervous influences can affect the sugar content of the blood

in a variety of ways. We determined his blood sugar next day by Bang's method, and obtained the figure ·12 p.c., which would tend to support this view.

One experiment was made on a case of cerebral hæmorrhage in which there was sugar in the urine. The subject was on a milk diet, was comatose and apparently unconscious that blood (100 c.c.) was being drawn. A large amount of sugar was present in the blood. The ratio polarimeter to copper reducing value was however much below that of the diabetic cases. The polarimeter value attained the copper value in 12 hours, the short time being no doubt due to the difference between them being originally slight. Thus the change was like that of normal, and not like that of diabetic subjects in which the polarimeter value approaches the copper line from the upper side.

Discussion.

The nature of the normal blood sugar we leave for the present undecided. Since the sugar yields an osazone having the same melting point as glucosazone, and the same microscopic appearance, it is probably a hexose. The failure to obtain lævo-rotation may possibly have been due to a rapid change in the sugar notwithstanding the care taken to prevent it. Professor Hopkins suggested that if alcohol were used throughout as a precipitant, alteration of the sugar might be prevented. This was tried but the clear filtrate was dextro-rotatory.

The method takes much more time than that with tungstic acid. Alcohol allows fatty substances to pass into the filtrate, whilst by the tungstic acid method almost the whole of the fatty bodies remain entangled in the precipitate; and it was only after repeated filtering in the various stages that a clear fluid suitable for polarimetric observations was obtained.

On applying Seliwanoff's test for fructose, negative results were obtained, indicating the absence of lævulose and pseudo-lævulose (iso-glycuronic acid).

The sugar we consider is some form of glucose. We have seen that the polarimetric readings finally reach a value corresponding to that given by α, β glucose in equilibrium. Further experiments on the final filtrate as soon as obtained showed that $KMnO_4$ (12) was decolourised with great rapidity when compared with a solution of glucose of equal concentration; but after being allowed to stand for some days until the polarimeter reading has risen to a value corresponding to that of α, β glucose, the decolorisation is slow and approximates to that of α, β glucose. It is suggested therefore that the decolorisation by the fresh filtrate is due to the sugar and not to some impurity. It is in harmony with this view that, according to Hewitt and Pryde (9), sugar solutions

introduced into the intestine undergo a rapid downward muta-rotation. The enzyme responsible for the conversion being apparently absent from the blood, and the reaction of the blood being alkaline, suggests that it is impossible for so reactive a sugar to exist free in the blood. The combination, if combination there is, must be of the nature of an unstable glucoside for it is well established that the sugar of the blood is readily diffusible.

Experiments after taking glucose and fructose indicate that these sugars are rapidly changed into the normal form present in blood. Nef(13) suggested that the reaction *d*-fructose → glucose must take place with the intermediary formation of an enolic form. It is probable then that the normal blood sugar is γ glucose, and that it arises from the enolic form.

While the work of Hewitt and Pryde(9) may account for the formation of γ glucose owing to passage through the intestinal wall, some further explanation is necessary as regards the sugar which is formed from glycogen. The experiment with J. C. C. P. suggests that γ glucose is formed not directly by glycogen breakdown, but as the result of the interaction of some other mechanism. We may therefore picture the processes:

$$\text{glycogen} \rightarrow \alpha, \beta \text{ glucose} \rightarrow \gamma \text{ glucose occurring.}$$

For the first stage of this process the liver diastase is the active agent, for the second stage the work of Clark(2, 3) suggests that the pancreas is responsible.

We have confirmed Hewitt and Pryde's observations(9) that extracts of the mucous membrane of the intestine do not alter the specific rotation of glucose solutions *in vitro*, and we can only suggest that as the result of some stimulus the pancreas secretes an enzyme, or enzymes, which cause the change α, β glucose → γ glucose. Some experiments on which we are at present engaged tend to support this view.

The marked difference shown between the curves of polarimetric readings obtained from normal subjects and severe diabetic patients throws a new light on the etiology of the disease. It is probable that glucose can only be stored and utilised after passing through the γ form, and that the severity of diabetes depends on the degree to which the power to form this sugar is lost. In the severe cases which we have examined there was no γ glucose which we were able to detect, any small amount present being masked by the large amount of other sugar in the solution. This would be especially the case in the diabetic where the very high ratio polarimeter to copper value suggests that a certain

amount of sugar may be present of which the ratio is higher than that of α, β glucose. We were unable to test this by acid hydrolysis owing to the small amount of filtrate in the final product. The further fact that the polarimeter curve does not reach the original copper value tends to support this hypothesis. It seems feasible to conclude that the direct cause of diabetes is the lack or inactivation of the enzyme which causes the conversion α, β glucose $\rightarrow$ γ glucose.

SUMMARY.

1. A method is described for obtaining the sugar of blood in a concentrated solution free from proteins.

2. By means of this it is shown that the sugar in normal blood of man, and of the ox, sheep, cat and rabbit, is an unstable form of glucose, with an initial low rotatory power. It is suggested that the sugar is γ glucose.

3. Glucose and fructose taken in large quantities *per os* cannot be detected as such in the blood, their conversion into normal blood sugar being very rapid.

4. Nervous influences alter the nature and quantity of the blood sugar.

5. The blood sugar of persons suffering from severe diabetes mellitus is of an abnormal nature. It appears to be the α, β form of glucose.

6. An enzyme is postulated whereby the α, β equilibrium form of glucose is converted into γ glucose. This enzyme is absent from the blood.

7. The absence or inactivation of this enzyme is suggested as the cause of diabetes.

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PROTOCOLS.

ANIMALS. One experiment only is given of those made on each kind of animal.

	Initial polarimeter reading	Final reading	Copper reducing value
	R.	R.	R.
Rabbit, 50 c.c. blood	·02	·07	·08
Cat, 80 c.c. blood ...	·03	·08	·08
Sheep, 100 c.c. blood	·04	·08	·08
Ox, 100 c.c. blood ...	·03	·12	·12

EXPERIMENTS ON MAN.

	Polarimetric readings on successive days	Copper reducing value	Bang
	R.	R.	%
H. L. W. Normal. 95 c.c. blood	·07, ·08, ·09, ·11, ·11	·11	—
H. L. W. 150 gm. fructose. 100 c.c. blood	·04, ·06, ·09, ·10, ·10	·10	·13
G. F. T. Normal. 85 c.c. blood	·03, ·06, ·05, ·10, ·09	·10	—
G. F. T. 150 gm. glucose. 55 c.c. blood ...	·04, ·06, ·08, ·08, ·08	·08	·13
H. M. Normal. 72 c.c. blood	·02, ·03, ·08, ·10, ·10	·12	·10
J. C. C. P. Normal. 100 c.c. blood ...	·20, ·19, ·20, ·18	·21	·17
J. B. S. H. Normal. 70 c.c. blood... ...	·08, ·09, ·10, ·10, ·10	·10	·17
G. M. D. Normal. 70 c.c. blood	·02, ·05, ·08, ·08, ·08	·08	·08
W. S. 100 gm. glucose. 65 c.c. blood ...	·05, ·07, ·09, ·08, ·08	·08	·14
L. B. W. Normal. 100 c.c. blood	·07, ·11, ·13, ·14, ·14	·14	·09
L. B. W. 100 gm. glucose. 55 c.c. blood ...	·04, ·05, ·08, ·09, ·09	·09	·13
L. B. W. 150 gm. fructose. 100 c.c. blood	·06, ·08, ·10, ·13, ·14	·16	·12
Diabetic (Dr J. Aldren Wright's patient). 18 c.c. blood	·11, ·10, ·09, ·09	·09	—
Diabetic (Dr J. Aldren Wright's patient). 20 c.c. blood	·11, ·10, ·10, ·10	·10	—
Diabetic (Dr G. Graham's patient). 21 c.c. blood	·11, ·10, ·08, ·08	·08	—
Diabetic (Dr G. Graham's patient). 13 c.c. blood	·07, ·06, ·05, ·05	·03	—
G. P. (cerebral hæmorrhage. Dr Lloyd-Jones' patient). 100 c.c. blood ...	·21, ·26, ·24, ·25	·25	—