LXXVIII. THE INFLUENCE OF GLUTATHIONE ON REDUCTION AND ROTATION OF BLOOD-FILTRATES.

BY JUDA GROEN AND JACOB WILLEM MEYER.

From the Laboratory for General Pathology, University of Amsterdam.

(Received April 13th, 1932.)

DURING the last decade numerous reduction methods have been published for the determination of blood-sugar. The literature on the optical rotation of protein-free filtrates of blood is less extensive. The majority of investigators in this field have determined the rotation after concentration of the blood-filtrates *in vacuo*. During this procedure which takes a fairly long time and is accompanied by increase of acidity, the possibility of hydrolysis of complex molecules with production of optically active bodies is obvious. Moreover a loss of reducing substances during concentration has been observed [Opler, 1910, 1911; Stepp, 1919; Barenscheen *et al.*, 1926]. Some workers have used dialysates, but the results obtained in this way can be applied to plasma only. It seemed therefore worth while to adapt a method for micropolarisation that had been worked out by Meyer [1930] to the determination of the rotation of protein-free blood-filtrates. For technical details the reader is referred to the original publication. The principle of the method is the photometric measurement of the intensity of light, penetrating a solution of an optically active substance, placed between crossed Nicols. The method permits of the determination of 0.01 % solutions of glucose to within 5 %; its accuracy is not affected by the presence of the Folin-Wu protein precipitants or by variations in p_H from 3.5 to 7.0.

Reduction and rotation.

Hedon [1898] was the first to point out that the rotation method always yielded lower results for blood-sugar than reduction methods. This fundamental observation has been confirmed by nearly all subsequent investigators who have used the greatest variety of ways of protein precipitation, reduction, concentration of the filtrates, *etc.* The discrepancy between reduction and rotation was only denied by Opler [1910, 1911], Takahashi [1911], Griesbach and Stützer [1913] and Maase and Tachau [1915], who used respectively phosphotungstic acid, colloidal iron hydroxide and mercuric chloride with hydrochloric acid as protein-precipitating agents, a fact to which we shall refer later on.

Most of these older investigators ascribed the discrepancy between the two methods to the presence of non-sugar reducing substances in the blood; this

seemed the more probable since Stepp [1919, 1922] demonstrated that the "sugar values" obtained by rotation agreed much better if they were not compared with apparent reduction figures, but with the amount of true fermentable sugar present in the blood.

Winter and Smith [1923, 1, 2, 3, 1924] tried to explain the discrepancy between reduction and rotation by assuming that the sugar in normal blood was γ -glucose, whilst in diabetic blood, in which they observed no discrepancy, it was α , β -glucose. Their conclusions have been questioned by van Creveld [1923], Denis and Hume [1924], Foster [1923], Hewitt [1923], Laquer [1925], MacLeod [1924], Mozotowski [1924], Thannhauser and Jenke [1924], and Vischer [1926].

A few years later Lundsgaard and Holbøll [1924, 1925] suggested the existence in blood of a "new glucose" on the basis of the observed discrepancy between reduction and rotation in dialysates of blood-plasma. Barbour [1926] and Anderson and Carruthers [1926] who repeated their work have not followed them in their conclusions.

Wright, Herr and Paul [1928] have also compared reduction and rotation of protein-free dialysates of blood-plasma. They found but a small discrepancy between both methods if the blood was fresh and suggested that the difference might be partly due to the presence of lactic acid in the plasma. It appeared that the discrepancy increased while glycolysis proceeded, the initial values showing hardly any difference at all; furthermore they showed that after acidification of the dialysates the low rotation values increased until they corresponded almost exactly to the reduction figure. As the sodium lactate from the blood is laevorotatory and lactic acid itself dextrorotatory but much less active, this phenomenon would fit in very well with their explanation. However, as these workers themselves have pointed out, the quantity of lactic acid present under normal circumstances in the blood (20 to 30 mg. per 100 cc.) is far too small to account for the whole of the difference observed. Furthermore if one uses not plasma but whole blood for these comparisons a difference is found which is not changed at all by addition of acid, so that lactates can account for but a small part of the discrepancy.

In this paper evidence is brought forward to support a quite different explanation of the low polarisation values of protein-free filtrates, based on the presence of non-sugar reducing substances in the blood. Recent work by Somogyi [1927, 1928] and others has considerably extended our knowledge of these reducing substances. Their influence on various blood-sugar methods depends on their behaviour towards every special reducing agent as compared with glucose [Herbert and Groen, 1929; Somogyi and Kramer, 1928]. Their chemical nature has been studied by Benedict and Newton [1929], Herbert, Cotonio-Bourne and Groen [1930] and Herbert and Cotonio-Bourne [1930].

Uric acid, creatine, creatinine and ergothioneine can be dismissed as possible causes of the residual reduction, as normally these substances occur in far too small amounts to have any appreciable effects on blood-sugar methods.

The paramount importance of glutathione in this respect has been specially emphasised by Herbert and Cotonio-Bourne [1930] who regarded glutathione as the only substance responsible for the phenomenon. They pointed out that the magnitude of the non-glucose reducing fraction as determined by various methods corresponded almost exactly with the results of a calculation based on the assumption that the normal glutathione content of the blood is about 52 mg./100 cc. The fact that these saccharoids (the name given by Benedict [1931] to the reducing non-sugars in the blood) are chiefly confined to the corpuscles formed another of their arguments.

In a recent paper Benedict [1931] has subjected Herbert and Cotonio-Bourne's postulate to a vigorous criticism. The actual amount of glutathione in the blood is probably less than 52 mg./100 cc. and the non-sugar reducing fraction of the plasma, small as it may be, cannot consist of glutathione, which substance is confined exclusively to the corpuscles. In the present state of affairs therefore glutathione cannot be regarded as the only reducing non-sugar of the blood, but to our mind there can be little doubt that it is by far the most important saccharoid. This is also in accord with Benedict and Newton's [1929] former conception.

The idea that glutathione is responsible for the long known discrepancy between blood-sugar estimations by rotation and reduction was originally suggested by Holden [1925], and formed the starting-point of the present investigation.

EXPERIMENTAL.

For the first series of experiments we used blood of normal adults, convalescent patients or of patients with local affections who did not show evidence of any metabolic disturbance. Blood was taken fasting, after ordinary meals, or after administration of glucose by mouth; none of these factors appeared to influence the results. Clotting was prevented by addition of oxalate (20 mg. per 10 cc. of blood), or by defibrination; immediately after the collection of the blood the protein was precipitated with sodium tungstate and sulphuric acid after Folin and Wu, centrifuged and the supernatant fluid filtered through Jena sintered glass funnels (No. 17-G-4). This extra filtration is necessary to obtain absolutely clear filtrates for the photometric polarisation method. As reduction method the Somogyi [1926] modification of the Shaffer-Hartmann technique was used throughout. All estimations were carried out in duplicate; these duplicates never showed a difference exceeding 5 mg./100 cc. As standard solution for the polarimetric determination two glucose solutions were used, containing respectively 10 and 20 mg. glucose per 100 cc. corresponding to a glucose content of 100 and 200 mg./100 cc. in the undiluted blood. These standard solutions were made up fresh every time by dilution from a 1% stock solution, to avoid errors due to mutarotation. The results of this series of simple comparisons between rotation and reduction are shown in Table I.

In every instance we found a considerably lower value by rotation than by

Table I. *Glucose in mg./100 cc. of blood.*

Experiment	Reduction	Rotation	Difference
1	89	40	49
2	71	50	21
3	92	70	22
4	82	50	32
5	82	46	36
6	103	74	29
7	86	50	36
8	118	84	34
9	81	50	31
10	106	67	39
11	114	64	50
12	74	42	32
13	118	90	28
14	114	70	44
15	112	64	48
16	107	75	32
17	113	88	25
18	97	63	34

reduction, the difference in Table I showing a maximum of 50, minimum of 21 and an average of 34 mg./100 cc. expressed as glucose.

A second series of experiments was carried out in the blood and plasma simultaneously. Glutathione is present in the corpuscles only and if it were the cause of the discrepancy between physical and chemical methods one might expect both methods to agree, if applied to plasma. Table II shows that this

Table II. *Glucose expressed in mg./100 cc.*

Blood			Plasma		
Reduction	Rotation	Difference	Reduction	Rotation	Difference
116	82	34	110	114	-4
85	59	26	81	79	2
82	59	23	78	77	1
135	111	24	131	139	-8
107	80	27	103	106	-3
			97	100	-3
			97	90	7
			94	100	-6
			83	79	4

is actually the case. For plasma precipitation only half the quantities of sodium tungstate and sulphuric acid of those used for blood were added; in a few cases we used plasma-ultrafiltrate, but the final dilution was always 1 : 10.

The samples of blood in this series showed differences between rotation and reduction varying between a minimum of 23 and a maximum of 34, the average difference being 27 mg./100 cc., which may be called in fairly good agreement with the results of Table I. For the plasma, maximum, minimum and average differences are respectively - 8, + 7, - 1, which shows that the cause of the observed discrepancy is present in the corpuscles only. This result in itself renders the hypothesis of a γ - or "new glucose" extremely improbable, as it is difficult to imagine how a substance like glucose, which can diffuse freely between corpuscles and plasma, could be present in the usual α , β -form

in the plasma and in an isomeric state in the corpuscles. It also makes it very improbable that lactic acid is responsible, as this substance is also equally divided between corpuscles and plasma.

A third series of determinations was carried out in blood-filtrates, which had been obtained by the so-called "sulphate precipitation technique" [Herbert and Cotonio-Bourne, 1930] in which haemolysis of the corpuscles does not take place and glutathione does not pass out into the surrounding fluid. In Table III we have compiled our results of a threefold comparison

Table III. *Glucose in mg./100 cc.*

Blood: "Folin and Wu"			Blood: "Sulphate technique"			Plasma		
Reduction	Rotation	Difference	Reduction	Rotation	Difference	Reduction	Rotation	Difference
82	63	19	73	88	-15	87	84	3
74	50	24	65	77	-12	76	74	2
81	52	29	69	80	-11	84	79	5
87	58	29	65	70	-5	—	—	—
93	61	32	77	81	-4	84	79	5
102	50	52	82	87	-5	99	103	-4
92	58	34	81	88	-7	88	98	-10
82	47	35	67	72	-5	79	84	-5
98	60	38	80	86	-6	96	94	2
90	65	25	72	75	-3	88	96	-8
87	59	28	71	80	-9	84	80	4
119	96	23	97	102	-5	113	108	5

between rotation and reduction as carried out in the blood, treated both with the ordinary Folin and Wu precipitation and with the Herbert and Bourne method, and in the plasma.

The Table shows that if one prevents glutathione from passing into the filtrate a much better agreement between rotation and reduction is obtained than in the case of the ordinary Folin and Wu filtrates. The differences for whole blood are by the Folin and Wu method minimum 19, maximum 52, average 31, and for the plasma minimum -10, maximum +5, average 0, mg./100 cc., which confirms the results of the preceding Tables. For the "sulphate technique" the differences vary between -12 and -3, showing an average of -7 mg./100 cc. The agreement in this case is not so perfect as found in the plasma, but, as the magnitude of the differences is hardly outside the limit of experimental error, we are inclined to attach more value to the gross agreement than to small discrepancies. In this state of the work the good agreement obtained between results of reduction and rotation by Opler, Takahashi, Griesbach and Stützer, and Maase and Tachau became clear. These investigators used for the protein precipitation respectively iron hydroxide, phosphotungstic acid and mercuric chloride with hydrochloric acid, which reagents are all known to precipitate or destroy reduced glutathione; the same holds true for uranyl acetate of which Vischer claimed that it was able to yield a protein-free filtrate in which polarisation and rotation agreed.

Reduction and rotation of glutathione.

Finally we have used our figures for a calculation in support of the view that glutathione can explain the different results of chemical and physical methods. The glutathione content of normal human blood is still under discussion; the majority of investigators give values for reduced glutathione between 30 and 50 mg./100 cc. of blood. The amount of this substance varies with the corpuscle volume of the blood, but as the haemoglobin contents of our subjects were about normal, we are justified in leaving out this factor.

The results obtained by us by various methods have led us to regard 40 mg./100 cc. as the average figure for the glutathione of normal human blood.

The glucose equivalent of a certain quantity of glutathione, as determined by various blood-sugar methods, has been worked out by Herbert, Cotonio-Bourne and Groen [1930]. For the Shaffer-Hartmann method they found that 100 mg./100 cc. of glutathione yield a reduction equivalent to 39 mg./100 cc. of glucose. 40 mg./100 cc. of glutathione present in human blood would therefore raise the true sugar figure by 16 mg./100 cc., a value somewhat lower than that actually found for the reducing non-sugars, the remaining 6 mg./100 cc. being caused by less important constituents of the blood.

What will be the influence of 40 mg./100 cc. of glutathione on the rotation of a protein-free blood-filtrate? In the literature on glutathione, we could find for its rotation only two exact figures. For the mercury green light the specific rotation of reduced glutathione amounts according to Hopkins [1929] to -18.5° , and according to Mason [1930] to -20° . With samples of glutathione, obtained from the Eastman Kodak Co. and from the Hoffmann La Roche Co. we found for the filtered yellow light, used in our experiments, a specific rotation of -17.5° ; this figure was determined with an ordinary Lippich polarimeter. We also determined the specific rotation of glutathione with the photometric micropolarisation method. As glutathione is laevorotatory we used fructose of high purity for comparison; in this way one finds the rotation equivalent of glutathione compared with fructose and, as the specific rotation of the sample of fructose used by us was -90° , we were able to calculate the specific rotation of glutathione. We also measured the rotation of glucose solutions of known strength, to which varying quantities of glutathione had been added; the decrease in rotation of the glucose solutions was used for the calculation of the specific rotation of glutathione.

The results are laid down in Tables IV and V.

Table IV. *Rotation of glutathione compared with fructose.*

Exp. No.	20 mg./100 cc. GSH rotation equivalent to 4.00 mg./100 cc. fructose: $[\alpha]_D$							
1	30	"	"	"	6.00	"	"	: -18.0
2	40	"	"	"	8.40	"	"	: -18.9
3	10	"	"	"	2.34	"	"	: -21.0
4	20	"	"	"	4.55	"	"	: -20.5
5	10	"	"	"	1.99	"	"	: -17.9
6	30	"	"	"	6.72	"	"	: -20.2

Table V. *Rotation of glutathione compared with glucose.*

Exp. No.

1	8 mg./100 cc.	GSH rotation equivalent to 2.7 mg./100 cc. glucose : $[\alpha]_D$ -17.1						
2	8	"	"	"	"	2.6	"	" : -17.7
3	40	"	"	"	"	16.0	"	" : -21.0
4	60	"	"	"	"	24.0	"	" : -21.0

The average specific rotation of glutathione calculated from the figures in these Tables is -19.2° , which is in sufficient agreement with the optically determined rotation of much stronger solutions, to demonstrate the reliability of the method. If we use the optically determined figure -17.5° as the basis of our calculation, we find that 100 mg. of glutathione show a rotation of opposite sense and equivalent magnitude to 33 mg. of glucose, and a glutathione content of 40 mg./100 cc. in the blood would therefore diminish the polarisation figure of the true sugar in the blood by about 13 mg. The reduction figure being 16 mg. too high, the polarisation figure 13 mg. too low, the difference between the two methods would be 29 mg./100 cc. of glucose. Actually the average observed figure, calculated from the results in Tables I, II and III, for the difference between reduction and rotation was 32 mg./100 cc. If the experimental errors of both methods and the physiological variations in the glutathione content of the blood are taken into consideration, the agreement between observed and calculated figures is remarkable and in itself a strong argument in favour of the important rôle played by glutathione in both reduction and rotation estimations.

Diabetes.

The behaviour of diabetic blood has been studied in a series of experiments, in which the ordinary Folin and Wu method, the "sulphate technique" for precipitation of the protein and the plasma values were compared. The experiments were carried out on fasting patients, after various meals, with and without insulin administration. The results are tabulated in Table VI and show that all these factors do not influence the behaviour of diabetic blood, which in this respect yields the same results as that of normal people.

Table VI. *Diabetes.*

Glucose in mg./100 cc.

Name	Blood: "Folin and Wu"			Blood: "Sulphate technique"			Plasma			Remarks
	Reduction	Rotation	Difference	Reduction	Rotation	Difference	Reduction	Rotation	Difference	
M.	218	167	51	—	—	—	—	—	—	Coma
M. H.	290	247	43	—	—	—	—	—	—	No insulin
d L.	137	114	23	—	—	—	133	132	+1	No insulin
S. A.	246	218	28	—	—	—	259	253	+6	8 hrs. after 10 units insulin
S. B.	243	216	27	—	—	—	256	259	-3	No insulin
F.	92	71	21	80	82	-2	—	—	—	Fasting, no insulin
R.	276	246	30	259	259	0	—	—	—	1 hr. after 20 units insulin
B. G.	231	201	30	208	212	-2	—	—	—	No insulin
G.	221	197	24	200	201	-1	—	—	—	No insulin
R.	155	126	29	131	128	+4	160	157	+3	½ hr. after 30 units insulin
V.	282	251	31	254	255	-1	304	300	+4	4 hrs. after 20 units insulin
P.	196	162	34	175	179	-4	204	200	+4	8 hrs. after 15 units insulin
B.	113	80	33	103	103	0	112	106	+6	8 hrs. after 10 units insulin

The bloods in this Table show differences varying from a minimum of 21 to a maximum of 51, on the average 31 mg./100 cc.; the plasma shows a difference between both methods of maximum + 6, minimum - 3, average 3; for the "sulphate technique" these differences are maximum + 4, minimum - 4, average - 1 mg./100 cc.

We have often left our filtrates to stand overnight at room temperature and carried out experiments after 24 hours. We were never able to find a rise in rotation as described by Winter and Smith [1923, 1, 2, 3]. Unfortunately we could not succeed in keeping the filtrates sterile for more than 24 hours; after prolonged standing faint turbidities appeared, which would have been no obstacle for any ordinary optic polarimetric estimation, but rendered the application of the photometric technique impossible. We cannot exclude therefore the possibility of an increase in rotation of the blood filtrates after prolonged standing. But as glutathione is liable to be decomposed if the medium does not show a marked acid reaction, it is quite possible that the disappearance of this laevorotatory substance from the solution may have caused the rise in rotation observed by Winter and Smith. In any case the phenomenon in itself is not sufficient to uphold the theory of the presence of γ -glucose.

In his latest publication Winter [1930] has succeeded in isolating the glucose from human blood; he identified it as the α -form; no evidence of a separate isomeric modification was found.

SUMMARY.

1. A method is described for the determination of very small rotations in non-concentrated protein-free filtrates of blood and plasma.

2. In Folin and Wu filtrates of normal human blood the difference between reduction, estimated with the Shaffer-Hartmann-Somogyi method, and the rotation amounts on the average to 32 mg./100 cc. expressed as glucose.

3. Plasma filtrates show almost complete agreement between the results of reduction and rotation.

4. In filtrates of blood prepared with the "sulphate technique" of Herbert and Bourne, reduction and rotation methods agree within the limits of experimental error.

5. The reduced glutathione present in the blood is the main cause of the differences between the results of reduction and rotation methods. The absence of this substance from plasma and "sulphate filtrates" explains the agreement mentioned under 3 and 4.

6. 100 mg. of glutathione show a reduction equivalent to 39 mg. of glucose and a rotation equivalent to 33 mg. of glucose, but oppositely directed. If the glutathione content of human blood amounts to about 40 mg./100 cc., a discrepancy of 29 mg. glucose per 100 cc. between reduction and rotation may be expected, which is in agreement with the observed value of 32 mg./100 cc. The influence of some less important substances (lactates among them) affords

a satisfactory explanation of the small discrepancy between the observed and calculated figures.

7. The blood of diabetic patients does not differ from normal blood, as far as the differences between reduction and rotation are concerned.

8. The hypothesis of a γ -glucose or "new glucose" for the explanation of the differences studied in this paper is unnecessary and untenable.

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