LXXIII. STUDIES IN CARBOHYDRATE METABOLISM.

- I. THE RELATION BETWEEN OPTICAL ACTIVITY AND REDUCING POWER OF NORMAL BLOOD FILTRATES.
- II. A NOTE ON THE INTERACTION OF MUSCLE TISSUE, INSULIN AND GLUCOSE.

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THERE is considerable confusion as to the nature of the sugar in normal blood, arising largely out of the fact that workers in this field differ as to the relations between optical and reducing power of protein-free blood filtrates. There is disagreement, not only in the interpretation of results, but also in the actual figures obtained. The experimental procedure adopted may be responsible for these discrepancies, Visscher [1926] having shown that different protein precipitants give different results. In much of the previous work dilute solutions were used and the criticism is made that the error involved is probably sufficient to account for the small changes in rotation. Consequently, a year ago, we endeavoured to secure concentrated ultrafiltrates of blood and plasma. The observations which were made showed in all cases a much lower value for glucose calculated from the polarimeter readings than for those obtained by reduction methods.

Shortly after this work was done Lundsgaard and Holbøll [1925] published a series of papers reporting similar discrepancies in dialysates from normal blood, but adding that mutarotation occurred so that after standing for 48 hours at room temperature the values were in agreement.

The dialysates examined by them were not concentrated, and in consequence the actual readings on the polarimeter and, still more important, the changes noted were very small. We therefore thought it advisable to repeat their experiments but to modify them slightly by using concentrated solutions, as in our previous experiments.

At the very outset it must be mentioned that the procedure we have employed could not be applied readily to the study of human blood since large quantities (about 1 litre) are necessary.

EXPERIMENTAL.

Ultrafiltration and dialysis.

Collodion sacs, made from 6 % pyroxylin in equal parts of alcohol and ether and treated with 70 % alcohol, were used in both ultrafiltration and dialysis. These sacs were impermeable to protein but freely permeable to glucose. Polarimetric observations were made in 2 dm. tubes using a Hilger polarimeter adapted for mercury green. All solutions and glass-ware were sterilised before use. Polarimeter tubes were made as sterile as possible by washing with alcohol-ether and drying in a current of sterile air. Using these precautions, solutions could be kept for four or five days in a polarimeter tube without showing any signs of bacterial growth. Reducing values were determined by the method of Hagedorn and Jensen [1923], these determinations being made in quadruplicate, except in the case of a few preliminary observations, when the method of Wood-Ost was used.

Blood from sheep, bullock or pig was collected in large glass bottles containing sufficient oxalate to make a final concentration of 0.2 %, chilled and used immediately. All ultrafiltrates and dialysates were concentrated by distillation *in vacuo* in sterile flasks at a temperature of 30–35°.

For ultrafiltration glass tube adapters were fitted into the collodion sacs and the joints sealed with collodion solution. The sacs were filled with blood or plasma and then connected to an apparatus containing compressed air, and filtration carried out at a pressure of 80–100 mm. Hg. The ultrafiltrate was collected in large boiling tubes, the joints between the boiling tubes and adapters being plugged with sterile cotton wool, and the tubes then surrounded by ice. As many as 12 sacs were used at one time, containing in all about 500 cc. After 10–12 hours' ultrafiltration, 80–100 cc. of filtrate were obtained, concentrated *in vacuo* to a volume of 20–25 cc. and then examined. The results of a few experiments on ultrafiltration are given in Table I.

Table I. *Ultrafiltration.*

Exp. No.	Blood	Rotation	Percentage glucose			Remarks
			Calculated from rotation	Hagedorn and Jensen	Wood-Ost	
1	Pig plasma	+0.42°	0.34	0.500	0.512	—
2	Ox „	+0.54	0.43	—	0.634	—
3	„ „	+0.19	0.15	0.225	0.237	Kept for 4 days in polarimeter tube; no change in rotation
4	Pig corpuscles (laked)	-0.15	—	—	Negligible	—
5	Ox corpuscles (laked)	-0.412	—	—	So small as to be negligible	—
6	Ox corpuscles (laked)	-0.56	—	—	Very small	Kept for 4 days in polarimeter tube; no change in rotation

It will be observed that Exps. 1, 2 and 3 (Table I) were performed with plasma. The figures given show very marked differences between the glucose values calculated from the two methods of determination.

After the plasma had been removed the corpuscles were laked and used as well. Exps. 4, 5 and 6 were all performed on laked corpuscles and in every case the concentrated solutions were very distinctly laevo-rotatory and the reducing power was so very small that it could not be estimated by the method of Wood-Ost with any accuracy.

Owing to the small volume of filtrate obtained, and the length of time taken for ultrafiltration, it was decided to adopt the procedure outlined by Lundsgaard and Holbøll. Briefly the procedure was as follows: whole blood or plasma was introduced into collodion sacs, the sacs placed in tubes containing 30 cc. sterile 0.9 % NaCl solution for whole blood dialysis, and sterile water for plasma dialysis. It was not possible to sterilise the collodion sacs, but after filling with blood or plasma they were washed rapidly with sterile water. Only freshly prepared sacs were used. The tubes were plugged with sterile cotton wool, and the dialysis allowed to proceed for 2 hours at room temperature. At the end of this time the dialysates were examined for the presence of small amounts of protein, and wherever protein was suspected the solution was discarded. The combined dialysates, about 250 cc., were concentrated *in vacuo* below 37° to about 30 cc. Polarimetric readings and reductions were then performed on these concentrated solutions.

Table II. *Dialysis.*

Exp. no.	Blood	Rotation	% Glucose		Rotation after 4 days	Amount of concentrated HCl in cc. added to 10 cc. solution	Acidified solution		
			Calculated from rotation	Reduction			Rotation	% Glucose calculated from rotation	% Glucose by reduction
1	Pig whole blood	+0.27°	0.22	0.322	+0.28	—	—	—	—
2	Pig plasma	+0.31	0.25	0.38	+0.32	1.5 cc. mixture at 100° for $\frac{1}{2}$ hr.	+0.42°	0.34	0.327
3	Ox plasma	+0.63	0.50	0.617	+0.63	2.0 cc.	+0.62	0.50	0.505
4	Pig plasma	+0.77	0.62	0.700	—	Considerable dilution with water + 2 cc. acid	+0.32	0.26	0.21
5	Sheep plasma	+0.24	0.19	0.27		2.0 cc.	+0.30	0.24	0.22

The results, some of which are given in Table II, show an appreciable difference between the glucose values as calculated by the two methods of determination. The solutions were allowed to stand for 4 days in a polarimeter tube; during this time no alteration in rotation was observed (see Exps. 1, 2 and 3).

In some cases a slight alteration in procedure was adopted. After dialysis for 2 hours the sacs were removed from the tubes, and the tubes then plugged

with sterile cotton wool and allowed to stand at room temperature for 2 days. At the end of this time the dialysates were combined, concentrated and examined. Again, however, as Exp. 5 shows, a difference was found between polarimetric and reducing values. It seemed possible that concentration immediately after dialysis might in some way or other interfere with the mutarotation as observed by Lundsgaard and Holbøll. On further reference to Table II it will be noticed that a second series of results is recorded, namely those obtained after treatment with HCl.

At first it was thought that possibly the differences between the figures for glucose were due to the presence of some glucose complex which could be broken down on hydrolysis. In Exp. 2 is recorded the result of heating, for half an hour at 100° under reflux, 10 cc. of the solution with 1.5 cc. conc. HCl. It will be observed that the polarimetric value increased so that there was now good agreement between the results of polarimetric and reduction determinations. It was then thought advisable to try the action of making acid, without heating. The original concentrates were quite alkaline, having a p_H 8.0–9.0, and it was noticed that owing to the large amount of bicarbonates present a considerable quantity of acid was necessary to make the solution acid. It was found that 2 cc. of conc. acid added to 10 cc. of concentrate was adequate to effect a large change in p_H . Examination of the acid solutions then showed that the polarimetric values had increased so that there was now fairly good agreement between the concentration of glucose as calculated from the two methods. In some cases it will be observed that the polarimetric value was slightly greater than the reducing value. It is significant that the change in reducing value was merely due to the dilution.

At this stage it appeared worth while to attempt work on a larger scale with the idea of isolating substances responsible for the phenomenon. In order to avoid large changes in p_H alcohol was chosen as the protein precipitant. Because alcohol extracts large amounts of pigments, difficulty was experienced in obtaining solutions suitable for polarimetric observations. It was found that pig's plasma, which is only slightly pigmented, gave the most satisfactory solutions. Oxalated blood was centrifuged as soon as possible after collection, and, to the plasma obtained, sufficient 95 % alcohol added to make the final mixture approximately 80 % alcohol.

The mixture was shaken, allowed to stand over night at 0° and the precipitated proteins filtered off. The clear alcoholic filtrate was concentrated *in vacuo* at 30–35° until practically all the alcohol was removed. The watery solution was extracted with ether twice or thrice, thus removing fat and a considerable amount of pigment. Ether was then removed by distilling again *in vacuo* and the solution further extracted with butyl alcohol. After removal of butyl alcohol the solutions were a greenish colour due to bile pigment. Addition of 5 % $BaCl_2$ solution, drop by drop, until no further precipitate was obtained yielded, after filtration, a solution clear enough for good optical work. A few typical results are shown in Table III.

Table III. *Solutions obtained after precipitation of proteins of pig's plasma by alcohol and removal of fat and pigments.*

Exp. No.	Rotation	% Glucose		Solution acidified with HCl conc. $p_H < 4.0$		
		Calculated from rotation	By reduction	% Glucose		
				Rotation	From rotation	By reduction
1	+0.33°	0.27	0.41	+0.50°	0.40	0.39
2	+1.00	0.80	1.36	+1.68	1.35	1.24
3	+1.26	1.00	—	+2.13	1.70	—
4	+0.90	0.72	1.16	(0.90 p_H 6.0) +1.04	0.84	0.83

Again the solutions showed marked differences between the glucose values. The effect of the addition of acid to the solutions was just as in previous experiments. One rather important observation, reported in Exp. 4, was made at this point. Here the p_H of the solution was adjusted to 6.0. No change in the rotation was observed even on allowing the solution to stand for 4 days. On making the $p_H < 4.0$, however, the rotation increased in the usual manner.

Some attempts were now made to see if it were possible to obtain any fractional separation of the optically active substances by means of alcohol. The watery solution, after removal of fat and most of the pigment, was taken down to dryness, first by distillation *in vacuo*, and finally in a vacuum desiccator over sulphuric acid. The dry residue was extracted with 99 % alcohol, the extract filtered and taken down to dryness *in vacuo* and the residue dissolved in water.

The residue remaining after extraction with 99 % alcohol was then dissolved in a small volume of water and sufficient absolute alcohol added to make the final concentration of alcohol 90 %. The mixture was filtered, the filtrate taken to dryness *in vacuo* and the residue dissolved in water. The types of results obtained are seen in Table IV.

Table IV. *Optical rotations of solutions after addition of acid and alkali.*

Exp. No.	Solution used	Original rotation	Acid solution		Alkaline solution		
			Rotation	% Glucose from rotation	Rotation	% Glucose from rotation	% Glucose by reduction
1	Solution after removal of pigments	+0.47°	+0.65°	0.52	+0.33°	0.27	—
2	Dilute 99 % alcohol extract	-0.06	+0.15	0.12	+0.03	0.02	—
3	90 % alcohol filtrate of residue from No. 2	+0.15	+0.52	0.42	+0.19	0.15	—
4	99 % alcohol extract	+0.19	+0.43	0.35	+0.17	0.14	0.305
5	90 % alcohol filtrate of residue from No. 4	+0.33	+0.51	0.41	+0.25	0.20	0.335

It will be observed that the changes in rotation were common to both the 99 % and 90 % alcohol extracts. In each case, too, the solution showed a low rotation (in Exp. 2 a laevo-rotation is reported) and a large increase on

making the p_H less than 4.0 by the addition of HCl. The effect of the addition of alkali sufficient to restore the original p_H 8.0–9.0 was now tried. The polarimetric values decreased, in some cases almost to the original values, if allowance is made for dilution. The results make it clear that no separation of optically active substances had been effected.

Two experiments in which carbohydrates were removed from the solutions by means of copper sulphate and calcium hydroxide are given in Table V.

Table V.

No.	Rotation	Reduction calculated as % glucose	Acidified with conc. HCl $p_H < 4.0$	
			Rotation	Reduction calculated as % glucose
1	-0.08°	0.037	+0.10°	0.020
2	-0.03	—	+0.10	0.017

When the solutions were alkaline the rotations observed were laevo-rotatory, but on acidifying to $p_H < 4.0$ the solutions became dextro-rotatory. The solutions showed only a very small reducing power.

NOTE ON INTERACTION OF MUSCLE TISSUE, INSULIN AND GLUCOSE *IN VITRO*.

In an early communication Lundsgaard and Holbøll [1924] described experiments in which they incubated muscle tissue, insulin and glucose at 37°. At intervals of time over 2 hours samples of these mixtures were withdrawn and dialysed through collodion sacs into 0.9 % NaCl solution. They then compared the polarimetric and reducing values of the dialysates and found that these were not in agreement. The polarimetric values became increasingly lower than the reducing values, and this they attributed to the fact that the glucose had been partially converted into a form called "new-glucose." It seemed desirable that this important communication should be confirmed and, if possible, extended.

Table VI. *Examination of dialysates from glucose, insulin and muscle mixtures.*

Exp.	Incubation mixture	Time after beginning exp. at which sample was taken for analysis Minutes	% Glucose	
			Reduction by Hagedorn and Jensen	Calculated from rotation
1	17.5 g. guinea-pig muscle + 40 units insulin + 200 cc. 2 % glucose solution	15	0.575	0.55
		30	0.430	0.42
		60	0.555	0.56
		135	0.492	0.51
2	20 g. guinea-pig muscle + 40 units insulin + 200 cc. 2 % glucose solution	Immediately	0.700	0.65
		15	0.765	0.73
		60	0.655	0.64
		120	0.780	0.75
		180	0.720	0.71

The methods employed in the experiments were exactly the same as those described by Lundsgaard and Holbøll, and following the procedure of these workers muscle from guinea-pigs was used. The insulin used was that of

Burroughs, Wellcome Co. In no case could we confirm the findings of Lunds-gaard and Holbøll. Only a few results are given here (see Table VI) as, since we performed this work, Barbour [1926] has published a paper on the question. Barbour states that he was unable to detect any difference between the polarimetric and reducing values, or any changes in these values in dialysates from muscle, insulin and glucose mixtures incubated at 37° for 2 hours.

DISCUSSION.

We have obtained concentrated ultrafiltrates from laked corpuscles of pig and ox blood showing strong laevo-rotation and little or no reducing power. Holden [1925] called attention to the presence of a laevo-rotatory substance in the corpuscles of sheep's blood which he identified as glutathione. Hunter and Eagles [1925] report the presence of a substance confined to corpuscles and having a laevo-rotation $[\alpha]_D = -155^\circ$. Benedict, Newton and Behre [1926] also report the isolation of a substance "thiasine," which however has a dextro-rotation $[\alpha]_D = +116^\circ$. This introduces some confusion, but it would appear that there are at least two laevo-rotatory substances in corpuscles. Thus conclusions on the nature of blood sugar, which are based on the rotatory behaviour of blood filtrates prepared from whole blood and involving disintegration of corpuscles, are open to criticism.

Our observations on solutions prepared from plasma have also demonstrated peculiar rotatory phenomena. In alkaline solution, $p_H > 8.0$, the rotation is low, on making acid to $p_H < 4.0$ the rotation immediately alters so that now there is an approximation for glucose values calculated from rotatory and reducing powers. That this rise in rotation is not a hydrolysis effect would seem to be certain because (1) the reducing value remains constant, and (2) the rotation falls again on the addition of alkali. In none of our preparations from plasma have we been able to detect the presence of glutathione.

Visscher [1926] has called attention to the importance of p_H when studying the optical activity of blood filtrates. However, he prepared his filtrates by the technique outlined by Winter and Smith [1923], which involved the use of whole blood and a disintegration of the corpuscles. In any discussion on the results we have obtained mention must be made of papers by Denis and Hume [1924], and Hynd [1926]. Denis and Hume prepared "synthetic" blood extracts which gave a rotatory power lower than the reducing power. Hynd reports that solutions containing urea and glucose, when heated for some time, show a diminution in rotation, but that this effect is obtained at 37° only after incubation for 3 months. In our experiments on concentrated dialysates a period of only 6–8 hours elapsed between the time of receiving the blood and making the first polarimetric reading. The occurrence of a similar reaction *in vitro* in our experiments is, therefore, very unlikely. Hynd suggests that this reaction may occur *in vivo* and so account for the different values found for blood filtrates. Unfortunately he does not

mention if there is any change in rotation with alteration in the p_H of his solutions. If the change caused by urea is similar to a Lobry de Bruyn transformation, as Hynd suggests, then it is difficult to reconcile this with our results which show alteration in rotation with changes in p_H .

At present it seems impossible to say definitely what substance or substances are responsible for the changes in optical activity of blood filtrates at varying p_H , but there are indications that they are not of the nature of carbohydrates.

SUMMARY.

1. Methods of preparation of protein-free concentrated ultrafiltrates and dialysates of normal blood, laked corpuscles and plasma, and of alcohol extracts of plasma of ox, sheep and pig are described.

2. Laked corpuscles are shown to yield strongly laevo-rotatory ultrafiltrates which have very little reducing power.

3. The concentrated ultrafiltrates, dialysates and alcohol extracts of plasma are observed to have a low rotatory power as compared with the reducing power (calculating values as for $\alpha\beta$ -glucose).

4. The optical rotation changes with large alterations in p_H and this change is reversible.

5. It is suggested that these results are probably explained by the presence in plasma of one or more optically active substances other than glucose.

These substances are slightly soluble in 99 % and soluble in 90 % alcohol, and attempts to separate them from glucose by means of alcohol were not successful. After treatment with copper sulphate and calcium hydroxide, laevo-rotatory solutions having practically no reducing power are obtained. The rotation becomes dextro at a $p_H < 4.0$.

6. No mutarotation was observed in any of the solutions examined.

7. Experiments on glucose-muscle-insulin mixtures are in agreement with those of Barbour and do not confirm the findings of Lundsgaard and Holbøll.

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