administration of glucuronidogenic compounds to animals increased the glucuronidase content of certain tissues (Fishman, 1940; Fishman & Fishman, 1944). As an explanation of his experimental data, it would seem at least equally plausible to suppose that ' β -glucuronidase' acts purely as a hydrolytic enzyme in the body, and that increase in the amount of enzyme in an organ occurs in response to the presence of excess glucuronide formed from the aglycone administered. The work of Mills (1946), who found that splenectomized rabbits fed with menthol conjugated larger fractions with glucuronic acid than normal, could be more readily explained by the latter theory.

Since the reducing material formed from menthylglucuronide can be accounted for as glucuronic acid, it appears that estimates of β -glucuronidase activity based on the former are sound. Considerable practical difficulties may, however, be encountered in assaying the enzyme in this way if the preparation is at all impure. The preparation may initially contain large amounts of reducing substances, and these may increase when the enzyme is incubated in absence of substrate (Graham, 1946). Heat inactivation of the enzyme prevents further production of reducing material. Graham therefore recommended that controls in determining glucuronidase activity should consist of enzyme incubated for the same length of time as the reaction mixture, instead of heat-inactivated enzyme as used by Fishman (1939). Graham's procedure would not, however, be

correct if the reducing material in the controls were derived from a glucuronide, since in the experimental tubes the latter would merely add to the excess substrate. Enzyme preparations with very high reducing powers were found to give completely negative reactions with naphthoresorcinol, indicating the absence of glucuronic acid, free or in combination as a glucuronide. It appears safe to assume that production of the unknown reducing substances will proceed at the same rate in experimental and control tubes, independently of the enzyme's action, and that Graham's procedure is therefore justifiable.

SUMMARY

1. The reducing material formed when menthylglucuronide was incubated with freshly purified beef spleen β -glucuronidase was accounted for by free glucuronic acid, determined by the colour reaction with naphthoresorcinol.

2. The glucuronic acid in enzyme hydrolysates of menthylglucuronide was isolated and characterized as the dibenziminazole derivative of D-glucosaccharic acid.

3. Crude preparations of β -glucuronidase, containing large amounts of reducing material, did not give the colour reaction for glucuronic acid and conjugated glucuronides.

4. The theoretical implications of these findings are discussed.

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Preliminary Studies on the Carbohydrate-rich Fractions of Ox Serum

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The glycoproteins of blood have formed the subject of numerous researches. Their existence having been once established (Bywaters, 1909; Biery & Ranc, 1914; Rimington, 1929), investigators have attempted either their isolation or the elucidation of the nature of the carbohydrate-containing complex. Attempts at isolation (Meyer, 1938; Sørensen, 1938;

Hewitt, 1936, 1937; Rimington, 1931, 1940) have not led to a product which has been characterized as pure by the modern physico-chemical criteria available (e.g. electrophoresis, ultracentrifugation). McMeekin (1940) has recently announced the isolation of a crystalline carbohydrate-containing albumin fraction which was also electrophoretically

homogeneous, but these two criteria alone are insufficient evidence of purity, and we decided therefore to apply to this substance further tests including the rigorous one of constant solubility under a given set of conditions. Unfortunately the procedure of isolation recorded is extremely laborious, and although we have seen crystals answering to the description given by McMeekin we have not been able to obtain workable quantities of the substance.

The constitution of the polysaccharide moiety of the blood glycoproteins has not as yet been unequivocally settled. Are we dealing with a galacto-mannose-glucosamine, a gluco-galactose-glucosamine or even with several different polyosides? Penet (1940), Dumazert & Memmi (1943) and Dumazert, Lévy & Marszak (1944) favour the latter view. Dumazert & Lévy (1943) isolated and attempted to identify a carbohydrate fraction from the plasma which is not present in the free state but in an easily detachable form. This fraction, which they call 'a loosely combined carbohydrate', would be the same in their view as the 'sucré virtuel' of Lépine (1921). It contains no glucosamine but only glucose and might, they suggest, be composed of three molecules of glucose united to one of phosphoric acid. Such a substance would clearly be of considerable physiological interest.

It is with two main problems in mind, purification of starting material and elucidation of chemical constitution, that we have undertaken the more extended study of a glycoprotein, a seromucoid fraction previously obtained by one of us (Rimington, 1940) from serum. In particular we have attempted (1) to explore the solubility curve of our mucoid in the presence of a precipitant, and (2) to cleave the carbohydrate-protein linkage by methods more gentle than acid or alkaline hydrolysis and to investigate the polyosides so obtained.

MATERIAL AND METHODS

Starting material

This was a seromucoid fraction obtained after removal of heat-coagulable protein from ox blood and precipitation by 50% (v/v) ethanol at pH 4. It was thus equivalent to seromucoid fraction *e* described by Rimington (1940, p. 933).

Analytical methods

Total hexose. Both the orcinol method as described by one of us (Rimington, 1940) and the carbazole method of Gurin & Hood (1939) have been employed, the latter affording somewhat more consistent figures. A mixture of equal parts of mannose and glucose served as reference standard except when examining one fraction by the carbazole method when a glucose standard was used (see discussion). Comparison was made in a Pulfrich step photometer, the orcinol colours always being read through both filters S 47 and

S 50, whilst for carbazole colours, filter S 50 was employed. Results obtained by the carbazole method were higher by a factor of about 1.4 than those obtained with orcinol but, allowing for this factor, agreement was very good. (Gurin & Hood (1939) have already observed that carbazole reacts to some extent with amino-acids.) In any one series of experiments the same method was used throughout.

Glucosamine. The acetylacetone and Ehrlich reagent technique has been employed under the conditions already described (Rimington, 1940) using filter S 53.

Nitrogen was determined by the micro-Kjeldahl method using selenium oxide catalyst.

Precipitation curves were based upon the specific property phase rule relationship enunciated by Falconer & Taylor (1946). Since the hexose/nitrogen (*H/N*) ratio is characteristic for a glycoprotein, we have constructed curves expressing (a) soluble nitrogen and (b) soluble hexose in relation to concentration of precipitant, and (c) soluble hexose as a function of soluble nitrogen.

All equilibrations were carried out at 37° when using Na_2SO_4 and at laboratory temperature when using other salts or ethanol. The different weights (or volumes) of precipitants were introduced into Emich micro-filtration vessels and then a constant volume of the seromucoid solution. The vessels were stoppered, and after the required conditions of time and temperature had been established, filtration was effected through the sintered glass disk by applying a positive pressure of nitrogen.

RESULTS

Precipitation curves and fractionation by Na_2SO_4

Preparation of the seromucoid solution

For all experiments, a solution of the starting material was made as follows. To a weighed quantity of mucoid, distilled water was added to yield a 0.2–0.3% (w/v) solution and the mixture dialyzed against distilled water for 2–3 days. It was then filtered through no. 1 Whatman paper, the latter washed with washings of the dialyzing bag and the volume adjusted to 1 g./l., assuming complete solubility of the protein. This technique afforded more complete solution than simple addition of distilled water (Table 1, compare solution 1 with 2, 3 and 4). The solutions which had a pH between 4 and 5 were used without neutralization and afforded very constant values for the ratio hexose/nitrogen (see Table 1).

Table 1. *Analytical data from different seromucoid solutions prepared as described in text from the same raw material*

Solution	Nitrogen ($\mu\text{g./ml.}$)	Hexoses by orcinol method using glucose-mannose standard	Hexose Nitrogen
		($\mu\text{g./ml.}$)	
1	155	148	1.04
2	200	196	1.02
3	200	190	1.05
4	200	194	1.03

Choice of precipitant

The characteristics of several precipitants were investigated with the following results.

Ethanol. Although in presence of sodium acetate this caused complete precipitation of seromucoid, the concentration zone of precipitation was too restricted for useful application to the purpose in hand; moreover the volatility of ethanol and the unavoidable dilution of the starting material introduced troublesome sources of error.

Magnesium sulphate and alkaline phosphates were found not to precipitate seromucoid.

Ammonium sulphate. Although capable of precipitating seromucoid, the use of this salt was abandoned for analytical reasons, since no agent could be found which would remove the protein completely from solution and thus enable the micro-Kjeldahl

method to be employed without preliminary dialysis. Seromucoid is neither heat-coagulable nor is it completely precipitable by phosphotungstic acid, hot tungstic acid or trichloroacetic acid.

Sodium sulphate. Both the anhydrous salt and $\text{Na}_2\text{SO}_4 \cdot 12\text{H}_2\text{O}$ precipitated at 37° the major part of the seromucoid and below are given the results obtained with the anhydrous salt, all operations having been conducted in a hot room.

Solubility curves in presence of sodium sulphate

Quantities of anhydrous sodium sulphate varying from 1 to 2.75 g. were weighed and then to each were added exactly 5 ml. of a seromucoid solution prepared as described above. Solution was effected as rapidly as possible by warming the vessel and contents in a water bath at 37° , but with mixtures containing a high concentration of salt the solution

Table 2. *Salting out of seromucoid by sodium sulphate; data for solubility curves*

(Two different starting solutions were used (A and B) and the data were obtained in four separate series of experiments performed at different dates indicated by the numbers 1, 2, 3 and 4 respectively.)

Na_2SO_4 added (mg./ml.)	Correction factor $k = (1 + x)/b$	Sero- mucoid solution	Experi- mental series	Nitrogen in filtrate		Hexoses in filtrate by orcinol method	
				Found ($\mu\text{g.}$)	Corrected ($\mu\text{g.}$)	Found ($\mu\text{g.}$)	Corrected ($\mu\text{g.}$)
200	1.042	A	1	153	159	160	167
225	1.049	A	1	155	162	168	176
		A	2	144	151	155	163
		A	4	140	147	155	163
250*	1.054	A	1	146	154	155	163
		A	2	152	160	155	163
		B	3	168	177	175	184
260	1.059	A	1	138	146	155	164
		A	2	140	148	150	159
		A	4	132	140	155	164
275	1.062	A	1	132	140	150	159
		A	2	146	155	140	149
		A	4	140	149	150	159
300	1.068	A	1	98	105	120	128
		A	2	96	102	125	133
		A	4	96	102	135	144
		B	3	90	96	120	128
325	1.075	A	1	48	52	85	91
		A	2	50	54	95	102
		A	4	54	58	100	107
		B	3	60	64	102	109
350	1.080	A	1	32	34	82	88
		B	3	30	32	87	94
375	1.087	A	1	16	17	65	70
		B	3	28	30	65	70
400	1.095	A	1	14	15	57	62
		B	3	26	28	55	60
425	1.101	B	3	16	18	50	55
450†	1.104	A	1	9	10	30	33
475	1.104	B	3	16	18	50	55
500	1.104	B	3	14	15	50	55
525	1.104	B	3	17	19	50	55
550	1.104	B	3	12	13	50	55

* Ppt. first visible.

† Saturation.

was slow and the vessels required periodic shaking for the first few hours. After 24 hr. a precipitate of

pressure of N_2 and samples of 1 ml. of filtrate taken for duplicate nitrogen and hexose determinations. Hexose was frequently determined in triplicate.

In order that all results should refer to 1 ml. of the original solution of glycoprotein, the experimentally determined values were corrected by a factor k

A. Relation between soluble nitrogen and salt added.

B. Relation between soluble hexoses and salt added.

C. Relation between hexoses and nitrogen.

Fig. 1. Salting out of seromucoid by sodium sulphate at 37° (for explanation of lettering see text).

protein was present in the series which contained a concentration of Na_2SO_4 greater than 250 mg./ml. Filtration was carried out at 37° under a positive

Fig. 2. Filtering device used for fractionation of large quantities of seromucoid by sodium sulphate at 37°. *a*, wooden block; *b*, wax-lined groove; *c*, rubber sleeve.

derived from the formula $k = (a + x)/b$, where a = sp.gr. of the original solution of seromucoid, x = weight of Na_2SO_4 added to 1 ml. of this solution and b = sp.gr. of the final solution. In practice, since the solution of seromucoid is very dilute, we have calculated values of k for solutions of Na_2SO_4 of various concentrations in distilled water from the expression $k = (1 + x)/b$.

The results of solubility curve determinations carried out in four series of experiments on different solutions of seromucoid exhibit a satisfactory concordance (Table 2 and Fig. 1). They will be discussed in detail below.

Fractionation of seromucoid by Na₂SO₄

On the basis of information obtained from the solubility curves, a fractionation of seromucoid was carried out as follows: to 450 ml. of the seromucoid solution were added 123.75 g. of anhydrous Na₂SO₄ (0.275 g./ml.) and the mixture kept at 37° for 24 hr., agitation being frequent during the first few hours. Filtration through a large sintered glass filter (porosity G 4) without loss from evaporation was accomplished by the use of a device (Fig. 2) sealing hermetically the solution-container to the body of the filtering funnel. The precipitate was dissolved from the funnel with successive quantities of water and dialyzed, yielding fraction Na I. After further addition of 22.5 g. Na₂SO₄ (0.05 g./ml.) to the filtrate, a further precipitate was similarly removed affording fraction Na II. The final fraction Na III was obtained after a further 78.75 g. Na₂SO₄ (0.175 g./ml.) had been added to the filtrate to achieve full saturation with the salt.

Nitrogen, hexose and hexosamine determinations were made on fractions Na I and Na III but fraction Na II was unfortunately lost before analysis. The analytical results are presented in Table 3 and are further discussed below.

Cleavage of seromucoid by phenol

Seromucoid in the form of a powder was added to 92% (w/v) phenol according to a procedure used by

Morgan & Partridge (1941) and dialyzed. The hexose/total N ratio was determined in the water-soluble and water-insoluble fractions afforded by the dialysis.

Fractionation of phenol solutions by ethanol. The solutions of the seromucoid in phenol were either dialyzed directly against distilled water, or were first precipitated by addition of ethanol, the insoluble material being subsequently suspended in water and dialyzed. The latter procedure was more satisfactory, the *H/N* ratio of the water-soluble fraction after dialysis being higher (Table 4). Moreover this carbohydrate-rich fraction is precipitated completely from the phenolic solution by the addition of 1 vol. of ethanol, further additions of ethanol only bringing down products with a *H/N* ratio of 0.24–0.70 as compared with 3–4.

Study of the fraction insoluble in water after dialysis of the ethanol-phenol precipitate. A portion of the precipitate was dissolved in a little 0.05N-NaOH, the solution neutralized and dialyzed. Analysis of the solution indicated a fall in the ratio of hexose to nitrogen (*H/N* = 0.77) relative to that of the original (or 'standard') preparation of seromucoid (*H/N* = 1.04). The remainder of the precipitate was washed with water, dried *in vacuo*, powdered and re-extracted by 92% (w/v) phenol. Only a small quantity dissolved and this was worked up as on the former occasion by addition of ethanol, dialysis, etc. The residual water-insoluble material was dissolved in NaOH, neutralized, dialyzed and analyzed as above, but no further decrease of the *H/N* ratio was apparent. It does not seem possible, therefore, to separate by this means the residual polyside from

Table 3. *Composition of fractions obtained from seromucoid by fractionation with either sodium sulphate or phenol and chloroform*

Fraction	Nitrogen (%)	Hexoses by carbazole method: (a) glucose- mannose standard, (b) glucose standard	Glucosamine (%)	Hexose	Glucosamine
		(%)		Nitrogen	Hexose
Seromucoid	13.0	17.8 (a)	8.0	1.44*	0.45
Na I	11.8	11.6 (a)	6.7	0.94	0.58
Na III	12.5	15.2 (a)	8.7	1.15	0.57
C I	3.9	66.0 (b)	10.0	17.0	0.15
C II	11.4	12.4 (a)	7.4	1.08	0.60

* The difference from the figure given in Table 1 is due to the use of the carbazole in place of the orcinol method (see text).

Table 4: *Fractions obtained from a solution of seromucoid in phenol by dialyzing directly or by dialyzing after a preliminary ethanol precipitation*

Origin of fraction	Dry wt. (mg./ml.)	Nitrogen (%)	Hexoses by orcinol method: glucose- mannose standard	Hexose
			(%)	Nitrogen
Dialysis of phenol solution: fraction soluble in water (B)	0.82	8.5	25.0	3
Precipitation of phenol solution by 1 vol. ethanol, ppt. dialyzed: fraction soluble in water (A I)	0.72	7.2	28.5	4

the protein with which it remains in combination in this fraction of the seromucoid.

Purification of the water-soluble fraction after dialysis of the ethanol-phenol precipitate (A I). Two methods were employed, (1) repeated precipitation by ethanol, and (2) agitation with chloroform.

(1) *Ethanol precipitation.* Since a trial fractionation by addition of 1, 2 or 3 vol. of ethanol did not lead to a fraction with appreciably augmented H/N ratio, the material was precipitated twice by 5 vol. of ethanol, the final precipitate being completely soluble in water. This fraction A II is characterized by an increase of the H/N ratio to 5.

(2) *Agitation with chloroform.* A solution (approx. 0.5% (w/v)) of the above material A II was shaken repeatedly with chloroform and a few drops of amyl alcohol as described by Sevag (1934). In the first series of experiments the reaction was neutral. When a gel of protein was no longer formed, the material was precipitated by ethanol and redissolved in water yielding fraction A III in which the H/N ratio had been raised to 10. The chloroform treatment was continued with fraction A III at an acid reaction (pH 4-5, by addition of HCl) until a thin layer of protein no longer formed at the interface. Fraction A IV was thus obtained which, although containing hexose, contained so little nitrogen that, with quantities available, determinations fell within the range of error of the micro-Kjeldahl method. The ratio H/N was subsequently determined upon larger quantities of material (fraction C I, see below), and found to be 17. The above results are collected in Table 5.

Preparation and analysis of two fractions resulting from seromucoid by phenol cleavage

The application of the results of the small scale experiments to a larger quantity of seromucoid, yielded two fractions C I and C II as follows (see scheme, Fig. 3):

Table 5. *Further fractionation of the carbohydrate-rich fraction of seromucoid. Hexoses determined by orcinol method*

Manipulations	Fraction Seromucoid	Hexose Nitrogen 1:04
Solution in phenol, precipitation by 1 vol. ethanol, dialysis	A I	4
Two successive reprecipitations of A I by 5 vol. ethanol	A II	5
Repeated shaking of A II with chloroform and amyl alcohol in neutral solution	A III	10
Repeated shaking of A III with chloroform and amyl alcohol in solution at pH 5	A IV	17

Seromucoid (1 g.) was ground in a mortar with 10 ml. of 92% (w/v) phenol and the mixture shaken and left for some hours at room temperature, when all dissolved. An equal volume of absolute ethanol was then added and the insoluble fraction separated, suspended in water and dialyzed until free from phenol. The precipitate was collected on the centrifuge, washed with water, dissolved in the minimal quantity of 0.05N-NaOH, then neutralized and again dialyzed, affording fraction C II.

The aqueous mother liquor, to which were added the water washings from the preparation of C II, was treated with 5 vol. of absolute ethanol, the precipitation repeated, the material dissolved in 30 ml. of water, and shaken repeatedly with 6 ml. portions of chloroform until a protein gel no longer formed. The collected chloroform phases were washed with water to remove any non-coagulated protein, and these washings themselves treated with fresh chloroform as above until gel no longer formed, and the aqueous residue united with the main fraction. To the aqueous solution was then added 5 vol. of ethanol, the resulting precipitate was

Fig. 3. Scheme of fractionation of seromucoid by phenol and chloroform.

redissolved in 20 ml. of water, acidified to c. pH 5 and again treated with chloroform as already described. A final precipitation with ethanol, re-solution and dialysis afforded fraction C I.

Table 3 records the analytical findings upon fractions C I and C II with, for comparison, those derived from the fractions obtained by Na_2SO_4 precipitation.

DISCUSSION

A study of the curves A (nitrogen : concentration of Na_2SO_4), B (hexoses : concentration of Na_2SO_4) and C (nitrogen : hexoses) of Fig. 1 shows that the greater part of the seromucoid is precipitated between the limits of 225 and 425 mg./ml. of Na_2SO_4 , the latter figure being close to the figure for full saturation at 37° (440 mg./ml.). It also shows that although the seromucoid studied was not a pure substance (the relationship in curve C would in that case have been represented by a straight line) the amount of impurity was relatively small (compare *KN* with the total curve, Fig. 1 A). To facilitate discussion, we will consider consecutively the four portions of the curves, corresponding to Na_2SO_4 concentrations of 200–275, 275–350 and 350–550 mg./ml., viz. *QN*, *NK* and *KP*, and finally the fraction of the glycoprotein not precipitated by Na_2SO_4 .

QN. Our results in this region are indefinite owing to the very fine nature of the precipitates which form in small quantity and are difficult to retain by the G 3 filters employed. On curve C the portion *NQ* may be co-linear with the mean experimental values grouped about *N*, but curves A and B, on the other hand, might suggest that the protein precipitating in this region differs from that precipitating subsequently. Nevertheless the differences between the determinations are often only slightly greater than the limit of experimental error of the methods, and it is not possible to form an unequivocal conclusion concerning this small portion of the seromucoid studied.

NK. All three curves show that over this region it is possible to speak of a characteristic glycoprotein constituting the main fraction of the seromucoid (containing 70% of the nitrogen and 40% of the hexose of the whole material) and having a ratio H/N of 0.72 (orcinol method) or 1.00 (carbazole method) calculated from curve C.

Electrophoretic analysis at pH 8 of this fraction afforded some confirmation of its homogeneity (Fig. 4), but one must not overlook the fact that the electrophoresis was carried out at one pH only, and furthermore that it is possible by this method to overlook impurities which comprise less than 10% of the main material.

KP. The striking difference in the character of the curves A and B over this region and the much steeper slope of C clearly indicate the presence of

a glycoprotein richer in carbohydrate than that previously precipitating and having a ratio H/N of 1.0 or 1.4 according to whether orcinol or carbazole is used for determination.

Fig. 4. Electrophoretic pattern of the main fraction obtained from seromucoid by salting out with sodium sulphate. Phosphate buffer, pH 8.0, ionic strength 0.2.

Residual fraction. A fraction representing approx. one-third of the total hexoses and a lesser fraction (approx. one-twelfth) of the total nitrogen remains unprecipitated by Na_2SO_4 , the ratio H/N of this residual material being greater than 3.

An analytical study of fraction Na I (precipitation up to *N*) and Na III (precipitation from *M* to full saturation) agreed with expectations based upon deductions from the three curves (see Table 3). Unfortunately a laboratory mishap deprived us of the supposedly pure fraction Na II (precipitation between *N* and *M*) and we were unable in this instance to confirm by analysis the ratio $H/N = 1.0$ deduced from the straight line *MN*. The value found for Na I (0.94) does not differ greatly from this figure, but we are unable to say whether the slight deviation is due to experimental error or to the presence here of a glycoprotein different from Na II and slightly poorer than it is in hexoses. On the other hand the difference between the value of 1.0

for Na II and 1.15 found for Na III is much beyond the possible limits of experimental error and agrees well with the values deducible from curve C.

It is possible indeed to calculate a theoretical value for the ratio H/N for fraction Na III knowing the values of H/N for the portions *MK* (1.00) and *KP* (1.4) and the relative proportions of these two materials in the fraction Na III, viz. 2.5/2 deduced from inspection of curve C.

One finds

$$H/N \times 4.5 = 2.5 \times 1.0 + 2 \times 1.4, \text{ i.e. } H/N = 1.17.$$

The actual value of the ratio H/N , found by experiment was 1.15, an excellent agreement.

In addition to the above results, the study of Table 3 allows interesting conclusions to be drawn concerning the ratio of glucosamine to hexoses in the different fractions. One finds, indeed, an enrichment in glucosamine in Na I and Na III relative to the original seromucoid (0.57 and 0.58 respectively as against 0.45). Moreover these values are the same within the limits of experimental error as that afforded by the fraction C II obtained by phenol cleavage. This enrichment in glucosamine necessitates an impoverishment in the residual fraction not precipitated by Na_2SO_4 , if one supposes, as seems reasonable, that fraction Na II would possess the same ratio as Na I and Na III (both of which contain more or less Na II). It would have been interesting to know whether the glucosamine : hexose ratio of the residual fraction, which unfortunately was not kept, was the same as that (0.15) of fraction C I obtained during fractionation with phenol.

It would appear, then, that the seromucoid which we studied contained different proteins united to two different polysides, one of which was easily detachable by phenol (and perhaps by Na_2SO_4) and poor in glucosamine, whilst the other was more firmly bound to protein and relatively rich in glucosamine. Substantiation is thus afforded of the results obtained by Penet (1940) and Dumazert *et al.* (1943, 1944) and mentioned in the introduction to this paper. It seems indeed possible either that the presence of glucosamine in the easily hydrolyzable polyside studied by them was not revealed on account of the very small quantities with which they worked, the glycoproteins being examined in the whole plasma, or that the small quantity was destroyed during hydrolysis.

Our determinations of hexose made with carbazole afforded in the case of fraction C I the red colour characteristic of glucose (which finding led us to use a reference standard of glucose while investigating this fraction), whilst all other fractions, viz. Na II, Na III, C II and seromucoid itself invariably gave the brown colour observable when the carbazole method is applied to galactose or to mixtures of glucose and mannose. The few μg . of tryptophan

which would be present during these determinations made on glycoproteins could not be responsible for the difference in tint observed. This also accords with the results of Penet (1940) and Dumazert *et al.* (1943, 1944) who found that those carbohydrates which could be liberated by a mild hydrolysis were composed of glucose only.

A chromatographic study of hydrolysates of the two fractions C I and C II would doubtless permit an elucidation of the nature of the hexoses (Partridge, 1946) they contain; we were not able to do more than carry out orientating experiments in this direction owing to the departure of one of us having interrupted the work before definite conclusions could be reached.

CONCLUSION

We have separated from a seromucoid, previously prepared by one of us, a glycoprotein the solubility curve of which entitles it to be regarded as a pure substance, and also a fraction rich in carbohydrate but with a nitrogen content too high to allow it to be classed as a polyside. This material resembles more closely the group of polyosido-polypeptides familiar to immunologists.

The carbohydrate moieties of these two fractions are distinctly different both as regards their glucosamine contents and the nature of their hexoses. In connexion with the latter one may recall the 'sucre virtuel' of Lépine (1921) which however has never up to the present time been isolated in the form of a polysaccharide from a glycoprotein. Penet (1940) stresses that it is not possible to affirm 'that it involves a linkage to protein'.

SUMMARY

1. Further investigation has been made of a seromucoid fraction previously prepared from ox blood.

2. Salting out studies with sodium sulphate at 37° showed that a fraction could be separated representing about 70 % of the nitrogen and about 40 % of the total bound hexose in the starting material, and satisfying the criterion of constant solubility and composition (hexose/nitrogen) demanded by a pure substance. The homogeneity of this fraction was supported by electrophoresis.

3. Cleavage of a fraction, rich in carbohydrate and very poor in nitrogen, from the seromucoid could be accomplished by fractionation with phenol-ethanol and repeated shaking with chloroform and amyl alcohol at suitable reactions. The residual material was correspondingly poorer in carbohydrate than the parent seromucoid.

4. The ratio of glucosamine to hexoses in these two fractions has been compared with that in the materials obtained by sodium sulphate fractionation. The particularly low ratio of glucosamine to

hexoses in the carbohydrate-rich fraction is emphasized.

5. The possibility is considered that the treatment of our seromucoid with sodium sulphate at 37° also results in the detachment of a carbohydrate-rich fraction.

6. The bearing of these preliminary findings upon

current views concerning the nature of the serum glycoproteins is briefly discussed.

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The Reduction of Methaemoglobin in Red Blood Cells and Studies on the Cause of Idiopathic Methaemoglobinaemia

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It is well established that methaemoglobin produced in the blood of normal animals by the injection of drugs disappears quite rapidly on withdrawal of the drug (Dennig, 1900; Stadie, 1921), and that its disappearance is due to reactions taking place in the red cell itself (Cox & Wendel, 1942). The last named authors showed that reduction of methaemoglobin taking place *in vivo* continued at the same speed in the shed blood, and did not depend on reducing substances in the plasma. Drug methaemoglobinaemias in man appear to be closely similar to those in animals, the foreign pigment usually disappearing within 24 hr. after the last dose of the drug.

In idiopathic methaemoglobinaemia, a rare disease which may be familial, it has been found that there is little or no tendency for the methaemoglobin to disappear from the shed blood (Lian, Frumusan & Sassier, 1939; Barcroft, Gibson, Harrison & McMurray, 1945). This observation has recently been confirmed by Sievers & Ryon (1946) who showed that the stability of the methaemoglobin probably does not depend on an abnormality of the plasma, since plasma from their patient did not

affect the rate of reduction of methaemoglobin in normal human erythrocytes, nor did normal plasma alter the behaviour of methaemoglobin in erythrocytes from the case.

The work described here has been carried out on blood from six persons belonging to two families in which idiopathic methaemoglobinaemia occurred. It has been possible to obtain evidence of a specific deficiency in the enzyme system of erythrocytes from the cases, and also to draw conclusions concerning the reactions involved in the reduction of methaemoglobin in the blood of normal persons. Apart from their application to the study of idiopathic methaemoglobinaemia, the results are of interest since they throw light on the mechanism by which the haemoglobin of normal persons is maintained in a functional state. The mechanism of regeneration of haemoglobin from methaemoglobin is of importance as it has been suggested that methaemoglobin is constantly being formed in the blood (Peters & Van Slyke, 1931), and Paul & Kemp (1944) have shown that methaemoglobin is present in small amounts in normal human blood.