112. SEROMUCOID AND THE BOUND CARBOHYDRATE OF THE SERUM PROTEINS

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Received 29 April 1940

THE past few years have witnessed a renewal of interest in the carbohydrate groups of proteins, particularly those of serum. Earlier investigators had claimed that serum albumin and globulin afforded a positive Molisch reaction, but the complex polysaccharide was isolated for the first time from each of these proteins by Rimington [1929; 1931]. Glucosamine and mannose were detected as components of this complex [Rimington, 1929] and the presence of galactose also has been rendered probable [Sörensen & Haugaard, 1933; see also Dische, 1928; Bierry, 1929]. For a more detailed historical presentation see Rimington [1933]. Although, in these experiments, evidence was presented that the carbohydrate groups were firmly united to the protein, it was shown later by Sörensen & Haugaard [1933] that, by a suitable fractionation procedure, there could be produced from crystalline serum albumin a series of crystalline sub-fractions differing widely in their carbohydrate contents and other properties, those most readily soluble containing approximately 50 times as much carbohydrate as those least soluble. This finding was regarded as further evidence for Sörensen's theory of the nature of soluble proteins as associations of mutually interacting component systems [Sörensen, 1930].

More recently still, Hewitt [1936; 1937; 1938; 1939] has obtained from serum albumin both carbohydrate-rich and practically carbohydrate-free materials. His interpretation, however, is that the pure albumin ("crystalbumin") is devoid of carbohydrate but that, as usually prepared, it contains quantities of two other proteins, "seroglycoid" and "globoglycoid", both of which contain carbohydrates. His view thus differs sharply from that of Sörensen. The nature of the association of crystalbumin with the other two proteins is not precisely defined, but Hewitt seems to regard it as a simple admixture. He advances reasons for considering "seroglycoid" to be different from the "seromucoid" of earlier investigators.

Seromucoid appears to have been first discovered by Freund [1892] who, however, overlooked the presence of nitrogen in his preparation which he named "Tiergummi". It was later investigated by Zanetti [1897; 1903], who gave it the name "seromucoid", considering it to be similar to ovomucoid. Bywaters [1909] studied the protein more thoroughly, obtaining it in the same way as his predecessors from the filtrate from the heat-coagulated serum albumin and globulin by precipitation with alcohol. The yield of purified material was 0·3–0·9 g./l. of blood, but varied according to the nutritional state of the animals. The protein contained 25% of carbohydrate and pentabenzoylglucosamine was obtained from the acid hydrolysate. Ozaki [1936] has also described the preparation of seromucoid by an essentially similar method and claims that it contains 13·4% of reducing sugars, calculated as glucose after hydrolysis.

I have recently been able to resume a study of seromucoid begun many years ago. The present paper deals with the isolation and properties of this constituent from ox serum, and with methods for determining the total hexose and glucosamine contents of proteins. The question of the relation of the seromucoid of horse serum to other constituents of the albumin fraction, with their specific immunological relations, is considered in the following paper with Dr M. Van den Ende.

Source of materials

In the present investigation, ox blood was employed, and as it was necessary to work with large amounts of material, the preliminary operations were carried out for me by Messrs Boots Pure Drug Co. of Nottingham. To them and to the biochemist in charge of the operations, Mr H. Calam, I am very greatly indebted for their generous assistance. The method employed was as follows.

Ox blood, collected and defibrinated at the slaughter-house, was mixed with an equal volume of 0·9 % NaCl containing "Chinosol" to prevent putrefaction. After removal of the erythrocytes, the serum was acidified with acetic acid to about *p*H 4·7 and then heated to coagulate the albumin and globulin, which were filtered off after very careful adjustment of the *p*H to ensure maximum precipitation.

The clear filtrate was concentrated *in vacuo* at 50° to a small volume and treated with 10 vol. of alcohol. The precipitate, containing the crude seromucoid and much inorganic material, was washed with alcohol and ether and dried *in vacuo*. In all, approximately 1 kg. of this material was prepared (with a mean yield of 3 g./l. of blood). It was found, however, to contain over 92 % of incombustible ash. It was a light brown powder, soluble in water and giving strong biuret and Molisch reactions; 2 % of trichloroacetic acid produced only a faint opalescence.

Purification and fractionation of the crude seromucoid

Concentrated solutions of the crude seromucoid, when boiled at *p*H 4·7, developed only a faint opalescence, so that coagulable protein was virtually absent. The ratio of total carbohydrate[1] to total N before dialysis was of the order of 1·4 : 1 and was reduced only slightly by prolonged dialysis in collodion sacs. 500 g. crude seromucoid were stirred mechanically with 2 l. of water and some insoluble material removed by filtration. The solution was then dialysed against running tap water for 8 days, thymol being added as a preservative. At the end of this time, some dark brown material had separated in the solution and as an encrustation upon the dialysing membrane; it was removed by centrifuging. The solution now had a carbohydrate/N ratio of 1·14 : 1.

Addition of 2·75 vol. of alcohol produced a bulky precipitate which was collected at the centrifuge. When stirred with water again this material did not completely redissolve; the residue gave a red colour with H_2SO_4, and with acetic anhydride and chloroform a typical Liebermann-Burchard reaction, indicating the presence of sterols; the biuret reaction was also positive. Material of this description was invariably present in crude seromucoid preparations and was very difficult to remove. Each time a clear mucoid solution was precipitated with alcohol, there remained, on attempting to redissolve it, a further quantity

[1] Expressed as glucosamine-galactose-mannose (see later). Ratios of carbohydrate to total N are preferable to calculations as percentages, since in the latter case the N content of the protein has to be assumed.

of this lipin-containing material, but by subjecting the crude protein to prolonged and repeated treatments, with absolute alcohol and ether, the lipin could be almost completely removed. It is difficult to decide whether or not seromucoid is actually combined with these sterol and lipin bodies in serum; the presence of carbohydrate in the insoluble residues from crude material may be due merely to mechanical co-precipitation, and in this connexion one may recall the separation of similar material with the albumin crystals from natural serum not freed from lipins. Purified seromucoid, as finally obtained, is not rendered insoluble by contact with alcohol; in this respect, as in its non-coagulability on heating, it resembles other mucoids such as ovomucoid.

Fractional precipitation with alcohol of seromucoid freed from lipins was carried out by adding alcohol in successive stages to the solution and removing the precipitates formed. These were redissolved and analysed with the results shown in Table 1.

Table 1

Fraction	Vol. of alcohol added	Total carbohydrate/total N	Remarks
Original	—	1·14	—
Precipitate A	1	0·95	Bulk of material
Precipitate B	1·5	1·72	—
Precipitate C	4	4·40	Very small quantity
Do.	— *	4·43	—

* After dialysis.

The bulk of the material was represented by fraction A. This was redissolved in 400 ml. of water and an equal volume of alcohol added, thereby producing a fairly abundant precipitate P and a filtrate F; the treatment of each will be separately described.

The precipitate P was redissolved in 250 ml. water with the addition of a few drops of alkali (to *p*H 6·5), 75 ml. of alcohol were added (to 23 %), the reaction adjusted to *p*H 4·0 and the small precipitate *a* removed; a further 25 ml. of alcohol (to 29 %) brought down precipitate *b*, and so on, as shown in Table 2, until the filtrate finally gave no further test for protein.

Table 2

Fraction	Alcohol added % conc.	Total carbohydrate/total N	Remarks
Original	—	0·82	—
Precipitate *a*	23	0·68	Small precipitate; requires alkali for resolution
Precipitate *b*	29	0·79	Combined and reprecipitated affording main precipitation 0·79, called M
Precipitate *c*	37·5	0·79	
Precipitate *d*	50	0·80	
Precipitate *e*	66	1·25	Small precipitate

It will be seen from Table 2 that, with the exception of the two end fractions *a* and *e*, the material had a constant carbohydrate/N ratio, corresponding to about 10·7 % of glucosamino-dihexose in the protein. The three fractions, *b*, *c* and *d*, comprising the bulk of the material, were therefore combined and once more reprecipitated and named fraction M.

Returning now to the filtrate F from 50 % alcohol mentioned above, when the alcohol concentration was further raised to 66 %, there separated a small quantity of material with carbohydrate/N ratio of 0·91, and at still higher

alcohol concentration (80%) the remaining material, very small in amount, was recovered and had a ratio of 3·0. This material, designated "G", was used later when determining hexose/glucosamine ratios (see that section). It is thus clear that, accompanying the main protein, there was a small quantity of material which required a relatively high concentration of alcohol for precipitation, and which was probably a mixture of peptone-like bodies. The substances present in this fraction were comparatively rich in carbohydrate, and may well have been derived from the seromucoid itself by decomposition of a portion of the latter during the process of isolation, though the possibility that they actually exist in native serum cannot be excluded.

Analysis of the different fractions

Whilst the greatest interest naturally attached to the homogeneous, main fraction M, it was considered instructive to analyse in detail also the fractions *e* and C, having carbohydrate/N ratios of 1·25 and 4·43 respectively, and also a small preparation A 2 from another batch, prepared in a slightly different way, with a ratio 1·26. The materials were dehydrated by alcohol and ether, and then dried to constant weight *in vacuo*. The ash contents were determined and the other percentages calculated upon an ash-free basis. The results are assembled in Table 3.

Table 3. *Analytical data for certain fractions*

Fraction	Ash %	C %	H %	N %	S %	Acetyl	Carbohydrate as glucosamino-dihexose	Total carbohydrate/total N
Main seromucoid M	0·37	50·32	6·89	13·60	1·59	Present	10·7	0·79
Slightly more soluble fraction *e*	0·46	50·76	7·02	12·52	0·94	,,	15·6	1·25
Differently prepared A 2	0·69	51·73	6·99	13·31	0·64	,,	16·8	1·26
Very soluble end-fraction C	4·35	48·81	6·71	9·67	0·44	2·48	42·8	4·43

On fraction C it was possible to make a reasonably accurate acetyl determination and the figure of 2·48% obtained is suggestively near to that of 2·65%, which would be required by the assumption that glucosamine is present in the carbohydrate complex as the *N*-acetyl derivative.

Determinations of optical rotations and of contents of certain amino-acids are reproduced in Table 4. The figures given are the means of several concordant determinations. Tyrosine and tryptophan were determined by the Folin & Marenzi [1929] method.

Table 4

Fraction	$[\alpha]_{5461}$	Cystine	Tyrosine	Tryptophan	Carbohydrate content
M	−53·99°	2·28	2·98	1·03	10·7
e	−55·13°	2·06	2·63	0·98	15·6
C	—	—	1·08	1·04	42·8

The determination of total hexose in glycoproteins

For the determination of non-amino sugars in proteins, the colorimetric method employing orcinol, originally put forward by Tillmans & Philippi [1929], has been widely adopted. It was used by the writer [Rimington, 1931] in his

early work upon the carbohydrate complex of serum albumin and globulin and in a slightly modified form by Lustig & Haas [1931] in their sub-fractionation experiments. Sörensen & Haugaard [1933] made a careful study of the method and, on the basis of the time curves of colour development given by various sugars, used it to identify the hexose constituents present in a number of proteins. In connexion with later experiments on lactalbumin, Sörensen [1936] showed that the data given by the time curves are not entirely reliable owing to interference, particularly by tryptophan. She now concludes that both mannose and galactose are present in this protein, and states that casein may also contain another hexose in addition to galactose. Hewitt [1934] made the important observation when using the orcinol method that the solutions must be kept shielded from the light. The Japanese workers have employed both this and the indole colorimetric method for the differentiation of hexoses.

For the present investigation, a closer study of the conditions affecting colour development has been made. The points requiring emphasis are as follows: In order to obtain reproducible results the temperature of the water bath should be controlled at $80 \pm 0{\cdot}5°$, and the time of immersion of the solutions accurately timed (20 min. is a convenient time). The concentration of the H_2SO_4 should be 60 % by volume or 68·5 % by weight (sp. gr. 1·592 at 20°), and the acid orcinol solution should be stored in the dark and rejected if it acquires a pinkish brown tint. The colour developed by 1 ml. of sugar solution + 2·5 ml. of orcinol (1·6 % in 30 % by volume H_2SO_4) + 15 ml. of 60 % by volume H_2SO_4, heated at 80° for 20 min., then cooled by immersion in tap water, was examined in a Hilger Spekker absorptiometer. Using the green Ilford Spectrum filter No. 604 (max. transmission 5200 Å.) mannose, galactose and glucose give identical readings under these conditions; with the blue filter No. 602 (4700 Å.) there is some divergence, mannose affording somewhat higher readings than galactose [compare Lustig & Langer, 1931].

Throughout these investigations each unknown solution was read against both the green and blue filters and standard reference curves, one for each filter, derived from a mixture of equal quantities of mannose and galactose, were employed, but no significant deviation from this ratio was found in any of the fractions analysed. Thus it may be concluded that in seromucoid, as in the serum albumin fractions investigated later, mannose and galactose are present in the polysaccharide complex in equimolecular proportions. As is reported below, the ratio of non-amino sugar to glucosamine was similarly constant throughout the series at 2 : 1. Thus the polysaccharide unit in seromucoid consists of glucosamine-galactose-mannose, and all figures for total carbohydrate have been

Table 5. *Distribution of bound carbohydrates in 500 ml. normal horse serum*

Fraction	Hexose by orcinol method (g.)	Ratio: Hexose/total N
Total serum proteins	1·116	0·339
Globulin	0·531	0·333
Crystalline albumin	0·170	0·100
Insoluble lipoid material accompanying crystalline albumin	0·019	—
66 % $(NH_4)_2SO_4$ fraction	0·220	0·315
Insoluble material accompanying above	0·023	—
75 % $(NH_4)_2SO_4$ fraction	0·014	0·538
Insoluble material accompanying above	0·001	—
100 % $(NH_4)_2SO_4$ fraction	0·014	1·370
Total	0·992	

calculated and expressed accordingly. The total quantity of bound carbohydrate in normal horse serum is approximately 0·2%, and a typical determination of its distribution between the individual fractions is shown in Table 5. No subfractionation of the globulins was attempted; Lustig and his associates [1931] have provided abundant data concerning these groups and the present figures for the albumin fraction confirm, in general, his findings for his albumins I, II and III.

The determination of glucosamine

For the colorimetric determination of glucosamine in simple solutions, the procedure of Elson & Morgan [1933] is satisfactory. Difficulties have been encountered, however, in attempting to apply this method to the analysis of glycoproteins. When my work was begun, the only methods available were those of Masamune & Nagazumi [1937] and Nilsson [1936]. The conditions for hydrolysis, etc. advocated in these two papers differ widely; Nilsson used 3 hr. hydrolysis in *N* HCl, and the Japanese workers 5·5*N* acid for 5 hr., followed by a rather elaborate procedure for the removal of excess Cl ions. Again, the compositions of the alkaline acetylacetone solution are different in the two cases. A trial of both methods showed that neither was completely satisfactory, but the following procedure has proved both convenient and reliable.

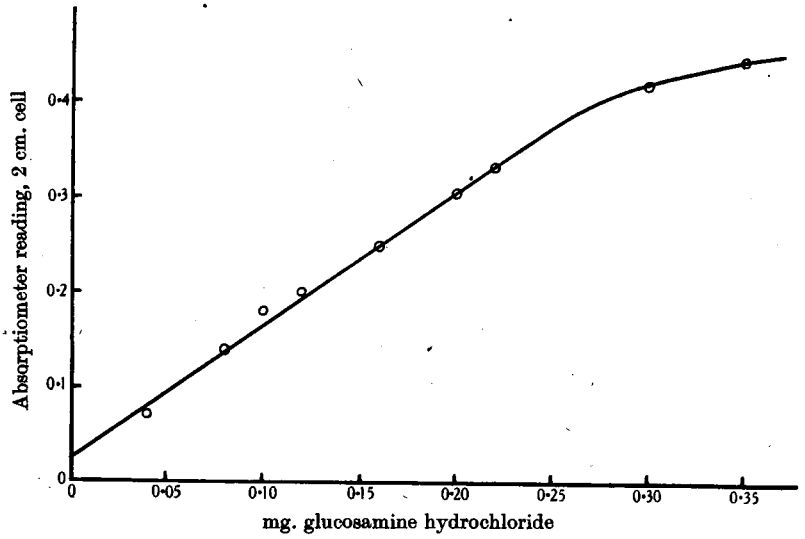

Fig. 1. Proportionality between colour developed and amount of glucosamine present.

10–20 mg. of protein, depending upon the glucosamine content, are placed in a small flask and refluxed for 4 hr. with 5–10 ml. of *N* HCl and 2 drops of capryl alcohol. This procedure is sufficient to liberate all the glucosamine, as is illustrated by Fig. 2. The equivalent quantity of *N* NaOH is then added to neutralize the solution and the deposit of humin removed at the centrifuge. 4 ml. of the hydrolysate, containing about 0·2 mg. of glucosamine, are used for the determination. The quantity of NaCl present does not interfere with colour development. 2 ml. of Elson & Morgan's acetylacetone reagent (1 ml. freshly dissolved in 50 ml. of half *N* Na_2CO_3) are added and the tube, provided with a glass bulb to prevent evaporation, immersed for 20 min. in a boiling water bath.

After cooling, 5 ml. of alcohol and then 2 ml. of Ehrlich's reagent (0·8 g. repeatedly recrystallized *p*-dimethylaminobenzaldehyde dissolved in 30 ml. alcohol + 30 ml. conc. HCl) are added, and 45 min. are allowed for colour development. 2 ml. of alcohol are then added to adjust the volume to 15 ml. and the colour intensity is measured in the Hilger Spekker absorptiometer, using the Ilford green filter No. 604. A blank tube containing no protein solution is run at the same time. By reference to a standardization curve, the amount of glucosamine is read off. This curve (Fig. 1) was obtained with pure glucosamine hydrochloride, and it will be seen that up to about 0·25 mg. of glucosamine the relation between extinction and concentration is linear.

Since this technique was evolved, Sörensen [1938] has published a study of the problem, but recommends a stronger acid for hydrolysing the protein. In other respects her method does not differ greatly from that here described.

The rate of liberation of glucosamine during hydrolysis and determination of the hexose/glucosamine ratio

The progress of the liberation of free glucosamine from a glycoprotein was followed, using a preparation of ox pseudoglobulin.

2 g. of protein were refluxed with 100 ml. of *N* HCl in a tared flask and 5 ml. samples withdrawn at intervals. A few drops of capryl alcohol were present to prevent foaming. After neutralization of the sample, 1 ml. was used for non-amino sugar determination by the orcinol method and 4 ml. for glucosamine as described above. Total N was determined upon 5 ml.

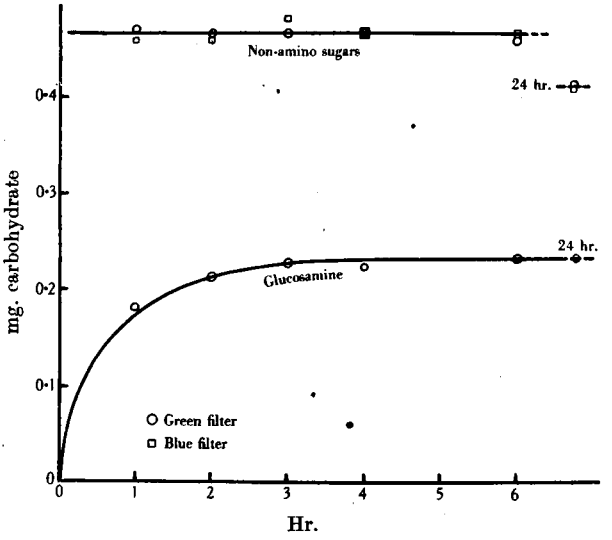

Fig. 2. Hydrolysis of ox pseudoglobulin by *N* HCl.

The results are shown on a graph in Fig. 2. Liberation of glucosamine is complete within 4 hr., the value then remaining unchanged up to 24 hr. The hexoses are slowly destroyed by the boiling acid, so that the 24-hr. value for these is appreciably lower than the earlier determinations. The mean of these gave 16·8 mg./100 g., and the correspondence between the values using the blue and green filters indicated equimolecular proportions of galactose and mannose.

The glucosamine was 8·43 mg./100 g., hence the ratio galactose-mannose : glucosamine = 1·99 : 1.

Several of the individual seromucoid fractions were analysed in this way, making the determinations after 4 hr. hydrolysis; 10–20 mg. of protein sufficed in each case, and all colorimetric determinations were made in duplicate. The results are shown in Table 6.

Table 6

Fraction	Total carbohydrate/ total N	Hexose	Glucosamine	Hexose/ glucosamine
Main seromucoid M	0·79	0·116	0·0561	2·07
Small fraction G separated at 66 % alcohol concentration	3·0	0·057	0·025	2·28
Very soluble peptone-like fraction C	4·43	0·123	0·0606	2·03

It is thus clear that in all fractions 2 mol. of hexose are associated with 1 of glucosamine.

Discussion

Since the original claims of Zanetti [1903] and Bywaters [1909] that there exists in serum a non-coagulable protein which is rich in carbohydrate, considerable doubt has been felt about the status of the so-called "seromucoid". The method of preparation employed by these workers involved drastic treatment, and no satisfactory evidence other than elementary analyses was offered to indicate the homogeneity of the materials obtained.

I have confirmed the fact that a protein, rich in carbohydrate and not coagulated by heating, is left behind in serum after the separation of the coagulable proteins. By employing more gentle methods for its preparation and purification, the greater part of this material has been obtained in an apparently homogeneous form, since fractional precipitation with increasing quantities of alcohol yields products with a constant carbohydrate/N ratio of about 0·79. If the term seromucoid is to be given a definite significance, it seems rational to limit its use to this homogeneous fraction. There are also present, however, in the filtrate from the heat-coagulated proteins, other substances less readily precipitated by alcohol and probably of peptone-like nature, in which carbohydrate may account for nearly 50 % of the whole molecule. These materials have not been purified, but like the homogeneous seromucoid, they are incoagulable by heat, and their carbohydrate component appears to be the same. It seems probable that they may be split-products derived from the native seromucoid during the course of its isolation.

The purification of seromucoid is further rendered difficult by the presence in the crude filtrates from serum of lipin-protein complexes, which give the reactions for sterols and also appear in many instances to contain carbohydrate. Only by thorough treatment of the crude seromucoid fraction with absolute alcohol and ether was it possible to remove the water-insoluble constituents. The work of Kleczkowski [1938] is of interest in this connexion.

Seromucoid is similar to other proteins of the mucoid class such as ovomucoid. It is not heat-coagulable nor is it precipitated by 2 % trichloroacetic acid, but it is precipitated by alcohol and by saturated $(NH_4)_2SO_4$ and it gives a rose-red biuret and an intense Molisch reaction. The polysaccharide, accounting for 10·7 % of the molecule, contains *N*-acetylglucosamine, galactose and mannose in equimolecular proportions, and in addition there are approximately 3 % tyrosine, 1 % tryptophan and 2·3 % cystine in the preparation from ox serum. The optical rotation of $[\alpha]_{5461} - 54°$ is considerably lower than that of albumin.

The question arises, as to the relation between the "seroglycoid" of Hewitt and the apparently homogeneous fraction of the carbohydrate-containing protein incoagulable by heat, which I have here termed "seromucoid", on the ground that this name was already in use for a less purified preparation having these properties. In many respects seroglycoid and seromucoid are indeed similar. Hewitt dismissed the possibility of their identity, because his best seroglycoid preparations contained only about 10–11 % of carbohydrate, whereas Bywaters had given the carbohydrate content of seromucoid as in the neighbourhood of 20 %, and my own earlier preparations similarly contained 22 % carbohydrate [Rimington, 1933]. It will be seen, however, that when a substance with the properties of seromucoid is thoroughly purified from accompanying peptone-like substances which may contain as much as 43 % carbohydrate, the carbohydrate content falls to 10·7 %, a figure very close to that for Hewitt's best seroglycoid preparations. The relation between the two substances is considered in the following paper.

The physiological significance of the protein-bound carbohydrate in serum is not known. The French school of workers and Lustig and his associates [1931] have paid some attention to this aspect of the subject, and fluctuations in protein-sugar under certain conditions are reported. Bywaters also stated that there was less seromucoid in the serum of a starving animal than in one well-fed, but he did not carry out exact determinations.

From the immunological point of view, it is naturally of interest to know whether the polysaccharide present in the serum proteins behaves like a haptene or influences in any way the immunological specificity. Recently Coghill & Creighton [1938], using Rimington's polysaccharide prepared under such conditions that any acetyl groups would remain intact, have been unable to demonstrate such activity. Neuberger & Yuill [1940], dealing with ovalbumin and ovomucoid, have also failed to find any evidence that the carbohydrate complexes of these proteins enter into or influence in any way their immunological specificities.

Summary

Seromucoid, the carbohydrate-rich, non-coagulable protein of serum has been reinvestigated and an improved method for its isolation worked out, leading to a main fraction of uniform properties.

This contains 10·7 % of carbohydrate, consisting of *N*-acetylglucosamine, galactose and mannose in equimolecular proportions, and also approximately 3 % tyrosine, 1 % tryptophan and 2·3 % cystine. The specific rotation ($[\alpha]_{5461} - 54°$) is lower than that of serum albumin.

Methods are described for the determination of non-amino sugars and glucosamine in proteins. The ratio of hexose/glucosamine was found to be 2/1 for all fractions examined.

My sincere thanks are due to Messrs Boots Pure Drug Co. and to Mr H. Calam of their staff, who kindly undertook the preparation for me, according to instructions, of crude seromucoid from 13·5 gallons of ox blood.

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