CXIX. THE CARBOHYDRATE COMPLEX OF THE SERUM PROTEINS.

II. IMPROVED METHOD FOR ISOLATION AND RE- DETERMINATION OF STRUCTURE. ISOLATION OF GLUCOSAMINODIMANNOSE FROM PROTEINS OF OX BLOOD.

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In a previous communication [Rimington, 1929] the isolation was reported of a nitrogen-containing polysaccharide from both the albumin and globulin of horse's blood-serum. The yield from each protein was about 2 %. It was also shown that this non-reducing complex gave rise, upon acid hydrolysis, to glucosamine and a hexose which was identified as mannose. On the basis of analytical figures, more particularly the nitrogen content of the isolated complex, it was concluded that the simple structure consisted of 1 molecule of glucosamine associated with 1 molecule of mannose and a structural formula was proposed which would account both for the lack of reducing property and for the liberation of nitrogen from the amino-group when acted upon by nitrous acid.

Shortly after this paper was published, a communication appeared from Levene and Mori [1929] who claimed to have isolated a carbohydrate complex from ovalbumin and ovomucoid in which 1 molecule of glucosamine was associated with 2 molecules of mannose. From molecular weight determinations by the diffusion method, Levene and Rothen [1929] came to the conclusion that the complex was a polymeride consisting of 4 units of the trisaccharide, *i.e.* 12 hexose residues in all. These workers found that relatively prolonged hydrolysis by baryta was necessary to free the carbohydrate completely from accompanying protein material.

Since the carbohydrate isolated from egg-protein by Levene and Mori was in many respects similar to that obtained from serum, it was thought to be desirable to reinvestigate the conditions under which the latter could be obtained in pure form and to establish with certainty its ultimate di- or trisaccharide nature.

It was found that by further prolonged hydrolysis of the preparations previously reported upon, some nitrogenous material was eliminated, leaving

a substance with a nitrogen content which became constant at 2.7 % agreeing with that required by a trisaccharide structure. The impurity present in the original preparations was only removed with difficulty and by the application of relatively vigorous methods. It proved to consist largely of histidine. By comparison of the analytical figures for histidine with those required by the di- and tri-saccharide formulae it will readily be appreciated that its presence in small quantity might be overlooked. The pure carbohydrate has now been

	C	H	N
Histidine	46.42	5.85	27.10
Disaccharide (glucosamine + 1 mannose)	42.21	6.79	4.10
Trisaccharide (glucosamine + 2 mannose)	42.94	6.56	2.78

obtained from the serum proteins of the horse and ox. In each case the composition indicates a trisaccharide nature and as far as it is possible to tell, the preparations appear to be identical.

Levene and Mori found that from ovomucoid as much as 5 % of carbohydrate could be isolated and concluded, therefore, that this protein was the source of the material isolated from crystallised ovalbumin preparations. Although the carbohydrate content of egg-white appeared to decrease on crystallisation of the protein it is difficult to believe that a three times recrystallised preparation could have contained as much as 5 % of associated ovomucoid, the figure demanded by the quantities of carbohydrate isolated from each individual protein (5.1 % from ovomucoid, 0.26 % from thrice recrystallised ovalbumin).

Sørensen [1930] has shown that egg-albumin dissociates to some extent in solution, so that by successive recrystallisation fractions are continually being left behind in the mother-liquors which differ from the crystalline phase by possessing a relatively higher solubility; these fractions also show differences in chemical composition. It might well be that, by this process, carbohydrate-rich fractions are eliminated from the bulk of the protein, a hypothesis which would explain the decreasing carbohydrate content observed by Levene and Mori, of repeatedly recrystallised egg-albumin more readily than the supposition that only ovomucoid contains carbohydrate, the albumin being free from carbohydrate.

In the case of blood it is known that there is present in the serum a protein to which the name seromucoid has been given [Zanetti, 1897, 1903; Bywaters, 1909]. Although present to the extent of only 0.1 to 0.5 g. per litre of blood, seromucoid has been credited with a high carbohydrate content, 20 % or more, which if true, would mean that the sugar so found, accounted for as much as 10 % of the total protein-sugar of the blood.

An investigation of this protein is in progress and will be published shortly. It appears to contain a carbohydrate complex similar to that present in the albumin and globulin and in quantity approaching 25 % (determined colorimetrically by comparison with glucose and recalculated in terms of glucosaminodimannose).

For reasons similar to those outlined above in the case of egg-proteins, it is considered unlikely that the carbohydrate isolated from serum-albumin and globulin preparations was in reality derived from admixed seromucoid. The quantity obtained, about 2 % of the weight of the protein, was too great to render this likely, moreover evidence is presented below which proves all but conclusively that, in the case of horse serum-globulin, the carbohydrate enters into the protein molecule as a true constituent to the extent of approximately 3.7 %. Final solution of the question may be deferred until the identity of the seromucoid carbohydrate has been established. It is noteworthy, however, that Lustig and Haas [1931] have recently investigated the carbohydrate content of blood-albumin and globulin preparations obtained by an elaborate series of fractionations and have recorded values which strongly support the view that carbohydrate enters into each of these proteins as an integral constituent.

That the carbohydrate complexes isolated from the proteins of horse and ox bloods appear to be identical does not, at first sight, appear to support the view put forward in the first paper of this series [Rimington, 1929] that the polysaccharide may play an active part in immunity reactions. This point is, however, under consideration and may be the subject of further communications. That what is apparently the same carbohydrate appears in so many different situations and species is undoubtedly a remarkable fact.

With regard to the structure of the trisaccharide complex there seems to be no reason why the argument originally put forward in support of a betaine-like linkage involving the nitrogen atom of the glucosamine molecule should not be equally applicable to the case of three hexose residues united together. Only exhaustive methylation and hydrolysis would show the point of attachment of the constituents. The slow liberation of nitrogen from the amino-group indicates however, as formerly, a union similar to that existing in glucosamine methyl glucoside. Karrer [1930] has put forward a similar view to account, on structural lines, for the known properties of chitosan. More recently Bertho *et al.* [1931] have discussed this question in relation to their work upon synthetic N-acyl derivatives of glucosamine and suggest that proximity of the —NH_2 to the —CHO group may alone be sufficient to cause abnormality in the reactions of the latter. The lack of activity of the aldehydic group towards the usual reagents would be interpreted by these authors as due to steric hindrance rather than to the existence of a true betaine grouping.

As to how the carbohydrate complex is united to the protein, no suggestion is at present ventured. It is however certain that, whatever the linkage, it is of a type that is markedly resistant to prolonged alkaline hydrolysis. Different proteins appear to vary in this respect, since in the present case 30 to 36 hours' boiling with 10 volumes of 10 % baryta was found necessary to eliminate all accompanying material from the blood-serum carbohydrate, whilst Levene

and Mori found that pure material could be obtained from the various egg-proteins after only 7 or 8 hours' boiling with the same reagent.

Unfortunately, appreciable destruction of the carbohydrate occurs during the more extended periods of hydrolysis with consequently lower yields, and no way has been found up to the present of obviating this loss of material.

EXPERIMENTAL.

As a result of previous work the conclusion had been drawn that the carbohydrate complex obtainable from serum-albumin and globulin was a disaccharide structure (possibly polymerised) containing one molecule of glucosamine and one of mannose and having therefore a nitrogen content of 4.1 %. Preparations isolated after 3 hours' hydrolysis of the protein by 10 % baryta appeared to approximate fairly closely in composition to this figure, provided glacial acetic acid had been used in the final stages as a precipitating reagent. If this treatment, which involved considerable loss of material, was omitted, nitrogen contents prevailed of 4.5 % and upwards. As a result of the investigations detailed below it appears that the carbohydrate is in reality a polymeride of a trisaccharide containing 2 molecules of mannose associated with every one of glucosamine, and thus possessing a nitrogen content when pure of 2.78 %. Certain modifications have been made in the method of isolation, details of which will be found below.

Preparation of carbohydrate from mixed proteins of horse-serum.

100 g. mixed albumin and globulin were boiled under reflux with 1 litre of 10 % barium hydroxide for 8 hours. (This proved to be too short a time; in later experiments 30–36 hours' hydrolysis was allowed.) To the filtered solution, sufficient 50 % sulphuric acid was added to render the reaction faintly acid to litmus and the barium sulphate removed by filtration and washed. 150 cc. of saturated basic lead acetate were added and then saturated baryta until maximal precipitation was attained. After removal of this precipitate on the Büchner funnel the filtrate was found still to give a faintly positive Molisch test. A further 75 cc. of basic acetate was therefore added and baryta as before. After this precipitation the solution was carbohydrate-free. The lead-barium precipitate was ground into a thin cream with water and sufficient 33 % acetic acid added to make the mixture faintly acid to litmus. The bulk of the precipitate dissolved leaving an orange-yellow material. If this gives a positive Molisch reaction, positive addition of acetic acid is cautiously continued until all the carbohydrate is in solution. The undissolved residue is filtered off and to the yellow-brown solution (allowing the acid solution to stand for 12–24 hours is recommended, since during this time a deposit of dark orange material settles out which can be filtered off and discarded) is added 3.0 cc. of basic lead acetate followed by baryta as before. The carbohydrate-lead compound is soluble in excess of baryta, so care should

be taken during the addition. After repetition at least three times of the solution in acetic acid and reprecipitation by baryta, the final lead-baryta precipitate is suspended in water and decomposed by a stream of carbon dioxide at 50–55°. Re-suspension of the lead carbonate and treatment with CO_2 is repeated until all the carbohydrate has passed into solution. To the combined liquids, a moderate excess of Hopkins's reagent (10 % HgSO_4 in 5 % H_2SO_4) is added and the mixture allowed to stand for several days. It is important that the total concentration of acid should not exceed 5 % whilst this precipitation is going on. As will be described later the material removed by mercuric sulphate has been shown to consist largely of histidine, and Calvery [1929] has shown that the histidine-mercury compound is soluble in sulphuric acid exceeding 5 % in strength.

After filtration lead and mercury are removed by hydrogen sulphide and the sulphuric acid by baryta. The solution is then concentrated *in vacuo* to a small bulk and the final elimination of SO_4 or Ba ions effected at this stage. Further concentration is performed in a vacuum desiccator over sulphuric acid, and to the syrup remaining is added about 20 cc. of dry methyl alcohol followed by an equal volume of ether. The precipitated carbohydrate is centrifuged, reprecipitated once or twice from concentrated watery solution and finally dehydrated by absolute alcohol and ether. Should the nitrogen content prove to be high, a further hydrolysis for 6–10 hours by 10 % baryta is necessary, followed by isolation as above, but in this case the basic lead acetate-baryta precipitation may be omitted.

Levene and Mori observed that pure material, from which all that was precipitable by mercuric sulphate had been removed, yielded nevertheless an additional precipitate with this reagent after further hydrolysis had been performed.

Precisely similar findings were encountered in the present case, which might be taken to indicate that the carboxyl group of the histidine, chief constituent of the mercury precipitate, is involved in some way in the union with the carbohydrate. Too great weight is not to be attached to this interpretation; it is put forward rather as a tentative suggestion as to how the complex may be united to the protein.

A yield of 3.5 g. of carbohydrate having a nitrogen content of 5.23 % was obtained. Since it was obviously still impure a further 8 hours' hydrolysis by baryta was performed and the isolation repeated. Yield 0.9 g. having 3.48 % of N; after 10 hours' more hydrolysis, 0.38 g. of pure material was obtained with 2.79 % of N.

In order to demonstrate that the nitrogen content remained constant at this figure and that the material was therefore pure, a further hydrolysis for 10 hours in 10 % baryta was carried out and 0.18 g. of material recovered with 2.66 % of N.

The steps in this purification, the yields and analytical data are summarised on p. 1067.

100 g. mixed proteins of horse-serum.

Hydrolysis (hr.)	Yield g.	Ash-free			Reaction with diazobenzenesulphonic acid HgSO ₄ ppt.	
		C	H	N*		
8	3.5	44.30	6.56	5.23	+	+
8 more	0.9	41.50	7.05	3.48	+	+
10 „	0.3	41.58	6.39	2.79	-	-
10 „	0.1	41.91	6.31	2.66	-	-
Calculated for C ₁₈ H ₃₃ O ₁₅ N		42.94	6.56	2.78		

* Micro-Kjeldahl by Mr A. R. Colwell, Cambridge. It was found that determinations on the same material by micro-Dumas gave constantly high results. C and H micro-determination by Dr Schoeller, Berlin.

Since histidine is the most tenaciously adhering impurity, applying the diazo-reaction with diazotised sulphanilic acid affords an indication of the progress of purification. The pure carbohydrate gives no reaction, neither does any precipitate form when mercuric sulphate reagent is added to its solutions after a further hydrolysis. Either test may be used to check the purity of material during the course of isolation.

Properties of the pure carbohydrate.

As precipitated from watery solution by alcohol and ether, the carbohydrate complex is obtained as a hygroscopic, creamy-white amorphous material. Its solutions are pale straw-coloured, viscous when concentrated, and show no measurable optical activity. They do not reduce Fehling's solution until after acid hydrolysis. The absence of optical activity may be due to racemisation during the prolonged hydrolysis. In the Van Slyke apparatus nitrogen is slowly evolved indicating the presence of a free amino-group as discussed previously. After acid hydrolysis no sugar except mannose and glucosamine could be detected.

Isolation from mixed proteins of ox-serum.

100 g. of the heat-coagulated mixed proteins from ox-serum¹ was boiled under reflux with 10 times the quantity of 10 % baryta. The hydrolysis was allowed to proceed uninterruptedly for 30 hours, after which the process of isolation was gone through precisely as described for the horse-serum carbohydrate. A yield of 1.1 g. was obtained. The N content was 3.72 %, thus indicating that nitrogenous impurities were still present. The whole of the material was therefore again hydrolysed with 10 % baryta for 12 hours. In carrying out the isolation the entire process was repeated without omission of any of the stages. Yield 0.48 g. having N 2.70 %. The full analyses were as follows:

	Ash-free			Yield g.
	C	H	N	
Initial 30 hours' hydrolysis	42.94	6.31	3.72	1.1
After further 12 hours' hydrolysis	44.65	6.53	2.70	0.45
Calculated for C ₁₈ H ₃₃ O ₁₅ N	42.94	6.56	2.78	—

In general properties, the material was similar to that isolated from horse-serum proteins.

¹ For the preparations of ox-globulin and albumin I am indebted to Messrs Boots Pure Drug Co., Ltd., Nottingham, who kindly placed this quantity at my disposal.

Detection of histidine among the impurities present in the crude preparation.

As mentioned previously, it was found that crude preparations of the carbohydrate still contained nitrogenous impurities which were not precipitable by Hopkins's reagent, but which could be liberated by further boiling of the complex with baryta and were then removable as their mercury salts.

A small quantity of the mercury precipitate was suspended in water and decomposed by prolonged saturation with hydrogen sulphide gas. After removal of the sulphide, which showed a marked tendency towards the colloidal form, a portion of the solution was subjected to the following tests.

Biuret reaction	Very faintly positive; pink
Ninhydrin reaction	Intense
Millon reaction	Negative
Glyoxylic reaction	Positive, but atypical
Molisch reaction	Faintly positive, but accompanied by a dark brown colour.
Diazo reaction (sulphanilic acid)	Intense, deep red
Diazo reaction (sulphanilic acid) as modified by Totani	Clear golden yellow after addition of hydrogen peroxide
Phosphotungstic acid	Immediate precipitate, soluble on warming but re-appearing as the solution cooled

The remainder was purified through the phosphotungstate and then gave all the typical reactions of histidine; the quantity present, however, was not great. The filtrate from the histidine phosphotungstate no longer gave any reaction with ninhydrin. It would appear from the above that histidine constitutes the major part of the impurity in crude carbohydrate preparations. It must be in some way combined with the polysaccharide since acid mercuric sulphate fails to precipitate it from solution.

Determination of the carbohydrate content of the serum-proteins by colorimetric methods.

Since isolation of a substance from a complex mixture is invariably attended by some degree of unavoidable loss, an attempt was made to determine the true carbohydrate content of the serum-proteins by an indirect means.

Methods available for the determination of carbohydrate materials fall into two classes, those depending upon the reducing property of the free aldehydic group and colorimetric methods. The former are inapplicable in this case since the conditions under which the protein gives rise to reducing sugars (acid hydrolysis) are also those favourable to secondary reactions whereby carbohydrate and amino compounds combine to form "humins" [see Gortner, 1916].

A colorimetric method has been proposed by Dische and Popper [1926] based upon the interaction between carbohydrates and indole in the presence of 70 % (by volume) sulphuric acid as condensing agent. The colour value is not strictly proportional to the quantity of sugar but this deviation is easily

corrected for¹; moreover Dische and Popper showed that the same value was given by sugars whether in the free or combined state, thus starch and glycogen could be matched directly against glucose. Mannose gives only about 65–70 % of the colour of glucose whilst glucosamine does not react at all. The method is applicable to the determination of the combined carbohydrate in proteins and in the present writer's hands has proved most serviceable. A very similar method is that proposed by Tillmans and Phillipi [1929] who employ orcinol together with 60 % (by volume) sulphuric acid. This was the method employed by Lustig and Haas [1931] in their examination of serum-globulin subfractions. Mannose reacts quantitatively like glucose, but the amino-sugars fail to give any colour, as in the indole method.

From a comparison of the two methods one would expect that the pure glucosaminodimannose complex would have a higher glucose equivalent by the latter than by the former, which is indeed the case. Further support is thus offered to the structure already assigned to the complex. In the investigations with proteins, Dische and Popper's method was more frequently employed although both this and Tillman and Phillipi's orcinol method yielded the same result (see below).

Pure glucosaminodimannose (N, 2.70 %).

1.675 mg. was dissolved in 5 cc. of water and 1 cc. aliquots compared with a 0.02 % glucose solution.

By Dische and Popper's method 1.675 mg. polysaccharide $\equiv$ 0.6695 mg. glucose. Hence 39.96 % of the colour of glucose.

By Tillman and Phillipi's method 1.675 mg. polysaccharide $\equiv$ 1.099 mg. glucose. Hence 65.60 % of the colour of glucose.

Assuming mannose to react quantitatively, theory would require 66.6 %.

Ox-serum globulin*:

Dissolved 0.1024 g. in 5 cc. of 0.05 N sodium hydroxide.

By micro-Kjeldahl N = 11.825 mg. in all.

Total carbohydrate (on 1 cc. aliquot) = 1.468 % as glucose by Dische and Popper's method, *i.e.* 3.67 % of the protein when calculated as glucosaminodimannose.

Total carbohydrate (on 1 cc. aliquot) = 2.279 % as glucose by Tillman and Phillipi's method, *i.e.* 3.47 % of the protein when calculated as glucosaminodimannose.

* For this preparation I am indebted to Messrs Boots Pure Drug Co., Ltd., of Nottingham. The total nitrogen was taken as 15.85 % in calculating the carbohydrate present.

The agreement between the two methods is satisfactory. The preparation of horse-serum globulin utilised by the present writer in the experiments culminating in the isolation, for the first time, of the carbohydrate complex was now examined. This protein sample had been very thoroughly purified [see Rimington, 1929], however, its carbohydrate content was found to be very similar to that of the ox-globulin above, and moreover this figure was found to suffer no decrease after thorough washing of the suspended protein particles, a treatment which would, undoubtedly, have removed any seromucoid still adhering.

¹ An empirically determined correction curve was employed, not the formula of Dische and Popper.

Horse-serum globulin, highly purified preparation.

Dissolved 0.1005 g. in 5 cc. of 0.05 *N* sodium hydroxide.

By micro-Kjeldahl *N* = 13.873 mg. in all.

Total carbohydrate (on 1 cc. aliquot) = 1.3 % as glucose, *i.e.* 3.74 % of the protein when calculated as glucosaminodimannose.

A further sample of about 0.1 g. of protein was placed in a stoppered centrifuge tube with 30 cc. of distilled water and subjected to vigorous mechanical shaking for 6 hours. After centrifuging and washing it was dissolved in dilute alkali as above and the carbohydrate content redetermined.

By micro-Kjeldahl *N* = 14.755 mg. in all.

Total carbohydrate (on 1 cc. aliquot) = 1.73 % as glucose, *i.e.* 4.32 % of the protein when calculated as glucosaminodimannose.

It seemed of interest to compare the carbohydrate content of the protein as determined colorimetrically with the actual yield obtainable by isolation. From the above figures it is clear that this sample of horse-serum globulin contained about 3.8 % of glucosaminodimannose. After an initial 3½ hours' hydrolysis there had been isolated 3.91 % of material having a nitrogen content of 4.92 %. As it is now known that the substance when pure has 2.78 % *N*, and that the major portion of the impurity is in all probability histidine (*N*, 27.10 %) it is permissible to deduct 9 % by weight corresponding to this amino-acid and to assume that the remaining material would be pure glucosaminodimannose. The corrected yield works out at 3.6 % in very good agreement with the content of 3.8 % determined colorimetrically. Of course, much loss is incurred if the crude material is boiled for any considerable length of time with baryta in order to effect further purification, so that yields of pure material as high as this are not directly attainable. In the previous investigation the yield had dropped to a little over 2 % by the time the nitrogen content was reduced to 4 %.

It seems quite certain that the carbohydrate content of the serum-proteins is considerably higher than has previously been supposed.

SUMMARY.

1. A reinvestigation of the conditions under which the carbohydrate complex of the serum-proteins can be isolated in the pure state has led to modifications in the scheme of separation originally proposed.

2. It also appears that the carbohydrate is composed of an association of one molecule of glucosamine with two of mannose, being thus similar to that obtainable from egg-proteins.

3. The impurity accompanying the original preparations has been shown to consist largely of histidine. This amino-acid appeared to be united chemically to the complex.

4. Glucosaminodimannose has been isolated from the mixed serum-proteins of both horse- and ox-blood.

5. Colorimetric methods have been used to determine the carbohydrate

content of proteins. By reference to the colour value of the pure complex, such figures are expressible directly as glucosaminodimannose. The values obtained by two different methods are in agreement.

6. Serum-globulin appears to contain approximately 3.7 % of carbohydrate.

7. This figure is not decreased by thorough washing of the coagulated protein and is taken therefore to represent carbohydrate in actual structural combination. Decreasing values obtained after repeated crystallisation of native proteins should be interpreted with caution, since, it is not improbable that dissociation of the component systems comprising the protein takes place in solution, the carbohydrate-rich fractions being left in the mother-liquors.

My thanks are due to Mr A. T. King for his continued interest in these studies and to Mr A. R. Colwell of the Biochemical Laboratory, Cambridge, for his kindness in performing numerous micro-Kjeldahl determinations.

I also wish to acknowledge the benefit I have derived from correspondence with Prof. P. A. Levene upon the subject of the presence of carbohydrate groups in the serum- and egg-proteins.

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