LI. THE ISOLATION OF A CARBOHYDRATE DERIVATIVE FROM SERUM-PROTEINS.

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MANY investigators in the past have, at different times, called attention to the fact that preparations of proteins made by them persistently gave positive Molisch or orcinol tests. In no case, however, was any satisfactory evidence brought forward in support of the view that carbohydrate substances could be separated from proteins until Seemann [1898] obtained glucosamine from egg-albumin as the hydrochloride, after acid hydrolysis of the protein. Numerous other investigators have reported the isolation of osazones as a result of treating the acid hydrolysates of various proteins with phenylhydrazine hydrochloride in the customary manner. Thus Mörner [1893] obtained from serum-globulin an osazone melting at 170° – 172° . Pavy [1895], Krawkoff [1896] and Jacewicsch [1897] also reported positive findings, whilst Langstein [1902] succeeded in isolating benzoylglucosamine after having benzoylated the mixture obtained by hydrolysing crystalline serum-albumin.

A very large amount of work has also been done upon the mucins, clearly proving that they are glucoproteins and establishing their conjugate character.

In the case of the simpler proteins—especially the albumins and globulins—agreement is by no means so general. Whilst researches such as those quoted above seem to indicate the probable presence of carbohydrate groups as integral constituents, the criticism is always possible that the starting material was not put through a sufficiently rigorous process of purification. Moreover, evidence of a more indirect nature has been sought by examining the power of hydrolysed proteins to reduce alkaline copper solutions and whilst many investigators have claimed positive findings in this direction the results of others have been directly opposed [Hofmann and Pregl, 1907]. Abderhalden, Bergell and Dörpinghaus [1904] deny the presence in serum-albumin of any carbohydrate capable of giving a positive Molisch reaction. Among such contrary findings it is difficult to draw any conclusion. The present author would suggest that the lack of unanimity is very probably due in large measure to the differing experimental conditions. Most previous workers have employed relatively concentrated acid to effect hydrolysis of the proteins. Under these conditions secondary reactions are liable to occur, involving amino-acids

and any carbohydrate that may be formed, and resulting in humin production. It is significant that in nearly all cases the carbohydrate recognised, or claimed to have been present, is glucosamine or an amino-sugar. Now glucosamine gives neither the Molisch nor the orcinol tests and therefore if these tests were originally positive the protein must also have contained some other carbohydrate substance.

EXPERIMENTAL.

In the present investigation very carefully purified proteins have been used and a method of hydrolysis employed which, whilst effective in breaking up the protein, had little or no action upon the carbohydrate substances formed.

Preparation of the serum-proteins.

Freshly drawn horse's blood was allowed to stand at 0° for 24 hours. The expressed serum (2 vols.) was separated off and centrifuged. It was then diluted with 5 vols. of water and 3 vols. of saturated ammonium sulphate were added. The small precipitate was filtered off and discarded. Ammonium sulphate was added to the remainder until half saturation. The precipitated globulin was separated by suction and the albumin precipitated by fully saturating with solid ammonium sulphate.

These two precipitates were purified by eight successive reprecipitations from large volumes of solution, the end fractions being discarded.

Each was then denaturated by addition of 5 vols. of alcohol and 30 cc./litre of a salt buffer mixture¹ (p_H 4.7) to its aqueous solution. After removal of the alcohol, the denaturated protein was washed by repeated extraction with large volumes of distilled water (5 litres to 150 g. protein) filtering by suction between each washing. Dehydration was finally accomplished by alcohol and absolute ether. The preparations so obtained were pure white in colour. Coloured filter papers were employed throughout so that any fine particles adhering to the precipitates could be easily seen and removed. Preparations of both albumin and globulin used in reasonable quantity were found to give a faint, but distinctly positive, Molisch reaction. The colour appeared to form somewhat slowly and was most intense after about half an hour's standing. Possibly hydrolysis in the presence of the acid was occurring, liberating the reactive agent.

Isolation of a carbohydrate derivative.

Hydrolysis was accomplished by boiling under a reflux condenser 150 g. of albumin or globulin with an equal weight of crystallised baryta and ten times its weight of water.

¹ Made up as follows.

Sol. A. 40 g. potassium sulphate, 202.5 g. sodium sulphate (cryst.), 360 cc. water heated to effect solution, then cooled. Sol. B. Normal acetic acid. Sol. C. Normal sodium acetate. Equal volumes of A, B and C mixed: p_H = 4.7-4.8.

After $3\frac{1}{2}$ hours the golden-coloured solution, which gave a strongly positive Molisch reaction, was cooled, filtered from a little insoluble material and the bulk of the barium removed by passing a stream of carbon dioxide through the solution at 60° .

After filtration, saturated lead acetate was added, and the slight precipitate filtered off and discarded, as it was invariably found to give no Molisch reaction. The carbohydrate was precipitated, however, by addition of ammonia which was continued as long as any precipitate formed. The filtrate gave no Molisch reaction, showing that separation was complete.

The lead-ammonia precipitate was filtered by suction, washed repeatedly on the filter with weak ammonia, finally with distilled water, and then ground into a cream with water. Upon acidifying with acetic acid most of the precipitate dissolved; the insoluble matter, which was usually pigmented, could be filtered off and discarded as it gave no Molisch reaction. Addition of excess of ammonia to this filtrate once more precipitated the carbohydrate. It was found by experience that much difficulty could be saved at later stages by repeating this washing and reprecipitation of the lead compound several times.

Finally it was suspended in water and decomposed by a stream of carbon dioxide at 60° . The point at which decomposition is complete can be easily ascertained with practice, for at this stage the suspended lead carbonate rapidly sinks to the bottom of the containing vessel.

The liquid was filtered. It was light yellow in colour, gave an intense Molisch reaction and pink biuret reaction, showing that it still contained protein substances. Most of the latter were removed by addition of mercuric chloride solution (saturated). It was necessary to add the reagent until further addition caused no turbidity and then to allow the mixture to stand in the laboratory for several days—10 or 12 preferably—as precipitation continued slowly for some time. The filtered liquid was now treated with hydrogen sulphide to remove excess of mercury and lead and after filtration a stream of air was drawn through to remove excess of gas. At this stage the solution was colourless or very pale yellow. It still gave a faint biuret reaction (pink) and an intense Molisch reaction but did not give any trace of turbidity with the following protein precipitants: uranium acetate, sodium tungstate, copper acetate. Phosphotungstic acid in 5 % sulphuric acid gave a white precipitate. This contained the carbohydrate but precipitation was not complete until a large excess of the reagent was present. The final stages of isolation took place as follows. The clear filtrate from the mercuric sulphide was concentrated under reduced pressure to a small volume. Four volumes of methyl alcohol were then added, followed by 5 vols. of absolute ether.

The carbohydrate material was precipitated as a sticky mass which adhered to the sides of the vessel and stirring rod. By leaving it in contact with absolute ether, its character changed completely, for on dehydration it broke up into a fine white powder, having an almost crystalline appearance.

After 5 or 6 reprecipitations by means of methyl alcohol and ether, it was

found that the nitrogen content remained constant at 4.4 to 4.9 % (in place of 5.5 % after the first precipitation). The material at this stage still gave a pink biuret reaction suggesting admixture with protein substances.

Much biuret-giving material was eliminated by addition of glacial acetic acid to the concentrated aqueous solutions and, after 2 or 3 precipitations by ether, the anhydrous material was found to have a constant nitrogen content of 4.1 %. The products isolated from albumin and globulin respectively were apparently identical. The yield from each protein was just under 2 % of pure material.

Microanalysis¹ yielded figures which, after correction for a slight amount of ash (about 0.2 %), agreed with the formula $C_{12}H_{23}O_{10}N$. A small but variable amount of chlorine was also present (about 3 %) and this has been allowed for in reckoning the percentages.

Carbohydrate from albumin.

Prep. I. Total N 3.99, 4.28, 3.94 %.

Prep. II. C 41.57, 42.89; H 7.26, 7.61; N 3.99, 4.14 %.

Calcd. for $C_{12}H_{23}O_{10}N$: C 42.21, H 6.79, N 4.10 %.

Carbohydrate from globulin.

Prep. I. Total N 3.98, 4.17 %.

Prep. II. C 41.73, 42.71; H 7.01, 6.75; N 4.19 %.

Calcd. for $C_{12}H_{23}O_{10}N$: C 42.21, H 6.79, N 4.10 %.

Properties of the carbohydrate derivative.

The substance isolated appears to be a nitrogen-containing polysaccharide. It is extremely hygroscopic, dissolves readily in water, giving a pale yellow solution, but is without measurable optical activity. The Molisch reaction is intense, the biuret reaction negative and the orcinol test gives merely a greenish-red colour quite different from the reaction of glycuronic acid or the pentoses. Solutions of the substance do not reduce Fehling's solution but do so after hydrolysis with acid.

On account of these properties it was thought desirable to obtain an accurate determination of the molecular weight. The cryoscopic method was used, the solvent being water, and the following results obtained:

Carbohydrate from globulin.

$c = 6.406$; $\Delta = 0.348^\circ, 0.347^\circ, 0.350^\circ$; Mean 0.348 . $M = 340.6$.

A disaccharide $C_{12}H_{23}O_{10}N$ would have $M = 341$. The substance is correctly represented, therefore, by its empirical formula.

Identification of the component sugars. An attempt was made to isolate the constituent sugars in the following way. 0.595 g. substance (from serum-globulin) was heated with 18.3 cc. of 20 % hydrochloric acid for 6 hours in

¹ All microanalyses were performed either by Dr Ing. A. Schoeller of Berlin or by Mr A. R. Colwell of the Biochemical Laboratory, Cambridge.

a sealed tube at 100°. The solution contained some dark tarry matter which was filtered off and the filtrate concentrated *in vacuo* over sulphuric acid and sodium hydroxide until nearly dry. Some crystalline material which separated was removed, washed with a little alcohol and dried for analysis. It proved to be a hydrochloride containing nitrogen.

Analysis (micro):					C	H	N	Cl
Found	...	...	...	...	33.02	6.41	6.25	16.31
Calcd. for $C_6H_{14}O_5NCl$	...	...	...	...	33.40	6.54	6.50	16.45
(glucosamine hydrochloride)								

A solution of this substance when heated on the water-bath for 2½ hours with phenylhydrazine hydrochloride and sodium acetate yielded an osazone which was identified as glucosazone microscopically and by its melting point 202°–206°. It was concluded therefore that the substance was *glucosamine hydrochloride*. The yield was 0.122 g. or 41 % of the theoretical.

The residual solution from which the glucosamine had separated was evaporated to dryness and the residue repeatedly extracted with alcohol, the alcoholic extracts being combined, and the alcohol evaporated on the water-bath. The residue was taken up in a little water and to this solution phenylhydrazine hydrochloride and sodium acetate were added and the mixture left in a boiling water-bath. An osazone formed resembling glucosazone in microscopical appearance. The crystals were washed with 20 % alcohol then dissolved in hot absolute alcohol; water was added until turbidity resulted and the solution allowed to cool. Typical glucosazone crystals formed, having melting point 204°–205°. It was thus shown that the sugar associated with glucosamine in this carbohydrate derivative obtained from serum-globulin was a hexose capable of yielding glucosazone and therefore either glucose, fructose or mannose.

Reducing power and optical activity of hydrolysate.

In order to gain further evidence concerning the nature of the hexose some periments were ex performed in which the carbohydrate material was hydrolysed by boiling with acid and the optical rotation and reducing power of the solution determined when hydrolysis was complete.

The method employed for determining reducing power was that of Willstätter and Schudel [1918] where the active agent is sodium hypoiodite which distinguishes between aldoses and ketoses, only the former being oxidised. 0.4689 g. of the carbohydrate from globulin was dissolved in 50 cc. of *N* HCl and the mixture boiled. At intervals, samples were taken and reducing power determined by the iodine method.

Time (hours)	Reducing power expressed as glucose (mg.)
0	—
½	78.7
1	229.0
2	425.4
3½	459.7
5	481.2
Theoretical possible	493.5

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Within 5 hours hydrolysis was practically complete, the reducing power being 97·5 % of theory (all expressed as glucose). The remaining solution, amounting to 14 cc. in all, was neutralised by sodium hydroxide, filtered and its rotation observed.

Rotation in 2 dm. tube	= + 0·40°
Concentration (from reducing power) = 567·3 mg./100 cc.	
$[\alpha]_{5461}^{18^\circ}$	= + 35·28°.

Assuming glucosamine and mannose to be present in equal proportions, calculation from the known constants gives, for the mixture, an estimated¹ $[\alpha]_{5461}$ of + 38·64°, whilst for glucosamine + glucose $[\alpha]_{5461} = + 61·27^\circ$. Considering the difficulties of measurement, using such small quantities, the agreement is sufficiently close. It may be pointed out that since both components of the disaccharide are oxidised in the iodine method the possibility of fructose being present is excluded. The substance must therefore consist of glucosamine together with either glucose or mannose and the above polarimetric evidence strongly suggests that the hexose is mannose.

Isolation of mannosephenylhydrazone.

For the isolation of mannosephenylhydrazone from the hydrolysis mixture 1 g. carbohydrate material was boiled with 10 cc. *N* HCl for 5 hours. When cold, the brownish-yellow liquid was neutralised to litmus paper by the very cautious addition of sodium hydroxide solution. It was then filtered and warmed to about 37°, and solid phenylhydrazine hydrochloride (0·4 g.) and crystalline sodium acetate (0·38 g.) were added. Solution took place very rapidly, whereupon the liquid was again filtered, allowed to cool and gently stirred as soon as crystals began to separate. The tube was stoppered and left in ice overnight. The crystalline material (which consisted of extremely fine microscopic prisms or needles) was separated by centrifugation and washed repeatedly, in the same manner, first with distilled water, then with cold 60 % alcohol and finally desiccated over sulphuric acid *in vacuo*.

By elementary analysis and melting point it was identified as mannosephenylhydrazone. Preparations contained a little ash (about 0·9 %), which has been allowed for in calculating the percentages.

Analysis (micro):		C	H	N
Carbohydrate from globulin. Found	...	52·95	6·78	10·85
Carbohydrate from albumin. Found	...	53·11	6·54	10·82
Calcd. for mannosephenylhydrazone	...	53·33	6·67	10·37
		(C ₁₂ H ₁₈ O ₅ N ₂)		

The substances had M.P. 198–199° and 199–200° (uncorr.) respectively. The values given in the literature for the melting point of mannosephenylhydrazone vary from 195° to 200°. A specimen prepared from pure mannose had M.P. 200°; mixed M.P. with substance isolated 199°.

¹ The factor 1·18 has been used to convert values of $[\alpha]_D$ into those for the line 5461 Å.

*Isolation of glucosamine hydrochloride from the carbohydrate
obtained from albumin.*

The carbohydrate materials isolated from the albumin and globulin respectively agree in having the same elementary composition and molecular weight and also in their physical and general properties. They appear in fact to be identical. Glucosamine was identified in the substance from albumin in the following manner.

0.2 g. of the carbohydrate was boiled for 5 hours with 50 cc. of 10 % hydrochloric acid. The resulting solution was concentrated over sulphuric acid and sodium hydroxide *in vacuo* until crystals began to separate. After standing overnight the crystals were separated from the dark syrupy mother liquor, washed with 97 % alcohol and then dried. They were yellowish white in colour, but after recrystallisation from dilute hydrochloric acid were obtained colourless.

Analysis (micro):				C	H	N	Cl
Found	...	...	...	33.43	6.92	6.49	16.96
Calcd. for glucosamine hydrochloride				33.40	6.54	6.50	16.45
				(C ₆ H ₁₄ O ₆ NCl)			

Preparation of the carbohydrate material by tryptic hydrolysis of the protein.

The carbohydrate groups present in the mucins and other glucoproteins have the structure of polysaccharides in which glucosamine and glycuronic acid are united. It has been shown by Levene and La Forge [1913], however, that these substances also contain sulphur, in the form of sulphuric acid in ester linkage with the sugars, whilst the amino-group of the glucosamine is not free, but is acetylated. Since the conditions of hydrolysis obtaining in the work here recorded were such as would probably remove any ester-linked sulphuric acid, were this originally present in the carbohydrate complex obtained from the serum-proteins, and also such as to eliminate any acetyl groups from their union with amino-nitrogen, it was felt that possible criticism should be met by endeavouring to obtain the carbohydrate by other methods.

As identical substances were obtained from the albumin and globulin respectively of horse-serum, in this experiment their separation was omitted. In all other details the preparation of the proteins resembled that previously described, but addition of ammonium sulphate was carried to full saturation and the albumin and globulin filtered off together.

200 g. of the carefully purified and dried mixed serum-proteins were suspended in 2 litres of water together with a little toluene. After standing overnight the protein was soft and evenly dispersed throughout the liquid. Sufficient sodium hydroxide to bring to p_H 8.3 was then added, followed by 10 cc. of Allen and Hanbury's "Trypsin." This enzyme preparation was selected as being the most suitable since it gave an almost entirely negative Molisch reaction; other commercial preparations contained considerable quantities of carbohydrate material. After 2 days' incubation at 38° a further 10 cc. of enzyme solution was added and the p_H readjusted to 8.3.

Hydrolysis was allowed to proceed at 38° for 4 weeks, the mixture being shaken occasionally. It was then filtered through pleated filter papers. The filtrate was clear yellow in colour and gave an intense Molisch reaction. A scheme of isolation was followed identical with that previously described. Much trouble was encountered in the final stages, however, in ridding the carbohydrate of admixed peptone material, and a repetition of the precipitation with lead acetate and ammonia, followed by all the subsequent stages, was necessary before satisfactory analytical figures were obtained. These showed the substance to be identical with that isolated by the baryta hydrolysis method. The yield was slightly smaller (about 1.5 %).

Sulphur was tested for but was found to be absent from the preparation.

Analysis (micro):				C	H	N
Found	...	...	...	42.68	7.39	4.65
Calcd. for $C_{12}H_{23}O_{10}N$	...	...	...	42.21	6.79	4.10

It is clear as a result of these experiments that the carbohydrate substance present in the serum-proteins is of a type quite different from that occurring in the mucins and has nothing in common with the latter save the presence in each of glucosamine as one of the constituent units.

Structure of the carbohydrate derivative.

The molecular weight of the substance isolated is in accordance with that required by the simple formula $C_{12}H_{23}O_{10}N$. Hence it is clear that the substance is of the disaccharide type and not a complex polymer. The absence of reducing properties would in this circumstance suggest a linkage similar to that present in trehalose, involving the two reducing groups of the component sugars.

On the other hand, a union of the α - or β -glucoside type would result in a molecule exhibiting the same behaviour towards copper solutions if, at the same time, the reducing group of one or other of its components was involved in some way—for example, with the amino-group of the glucosamine—so that its potentialities were in abeyance.

In order to gain further insight into the constitution of the compound attention was directed towards the behaviour of the amino-group. Glucosamine and its methyl ester give off nitrogen quantitatively when acted upon by nitrous acid. When the disaccharide was investigated, using the Van Slyke microapparatus, the following results were obtained, from which it is clear that the amino-group, though slowly attacked, is capable of reacting quantitatively to form nitrogen.

Solution (a). 11.768 mg. in 2.5 cc.

Reaction time 3 mins. ... 1.77 cc. gave 0.22 cc. N_2 at 763 mm. and 20° = 1.66 % N_2 .

Solution (b). 50 mg. in 10 cc.

Reaction time	$\frac{1}{2}$ hr.	...	2 cc.	gave 0.41 cc. N_2 at 769 mm. and 19° = 2.37 % N_2
"	1 "	...	2	0.56 765 " 20 = 3.23 "
"	2 "	...	2	0.65 762 " 21 = 3.67 "
"	6 "	...	2	0.70 760 " 20 = 3.99 "
Theoretical = 4.10 "				

Hynd and Macfarlane [1926] have recently examined the behaviour towards nitrous acid of a number of amino-compounds. Glucosamine they found to react only slowly in presence of 20 % acetic acid, taking 18 hours for complete decomposition, although its salts like the hydrochloride, or the free base in presence of mineral acid, yielded all their nitrogen as gas within half an hour at 20°.

They conclude from these observations that glucosamine must contain the modified betaine ring suggested by Irvine and Hynd [1913], whilst in the case of its salts, or of the base itself in presence of mineral acid, this linkage is unstable and gives way to form the free amino-group.

In many ways the properties of the carbohydrate compound are in keeping with the suggestion that the nitrogen atom of the glucosamine is involved in a ring of the betaine type, the other hexose being attached in a manner similar to the methyl group in Irvine and Hynd's α -aminomethylglucoside. Like this substance it exhibits a stability towards acid hydrolysis that is quite irreconcilable with a simple glucosidic linkage, and the slowness with which the reaction takes place between the amino-group and nitrous acid in the presence of a weak organic acid like acetic acid suggests that the substance belongs to the "free-glucosamine" type, containing ring-nitrogen rather than that of the glucosamine salts as typified by the hydrochloride.

It is impossible, however, to overlook a point of some difficulty arising at this juncture, should the suggestion be accepted that the nitrogen atom is part of a betaine ring.

The work of Kenner and Earl [1927] has shown that a grouping, in which a nitrogen atom occurs in the quinquivalent state, bears a positive charge. In most cases this will give rise to a negatively charged ion but in the case of a disaccharide such as that described here it is difficult to see how this condition could be fulfilled. It is, however, noteworthy that in all preparations a very great difficulty was encountered in attempting to eliminate traces of chlorine from the compound during its purification, an attempt which, in fact, proved unsuccessful. There was in each case a small quantity of chlorine, amounting to some 2 to 4 %, which could not be removed by the precipitation procedure adopted. This quantity is smaller than would be required by one atom of combined chlorine and was moreover variable, but its presence accords with the view that there was resident somewhere in the molecule a sphere of electropositive influence.

Experiments were next undertaken to determine whether any of the usual enzymes acting on carbohydrates were capable of hydrolysing the compound. Reducing power was determined by the method of Hagedorn and Jensen, at intervals of time up to 4 days. The enzyme preparations were all freshly prepared and the activity of each was tested upon a suitable substrate. In all cases buffer solutions of the appropriate p_H were added to the test and control solutions.

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The following results were obtained:

Enzyme	Effect
Emulsin	Nil
Ptylain	Nil
Pancreatic amylase	Nil
Maltase	Slight hydrolysis?

With maltase the observed increase in reducing power was but slightly greater than the experimental error; trehalase, unfortunately, has not yet been available for trial.

The evidence furnished by these enzyme experiments militates against the view that an α - or β -glucosidic linkage is present. It is in keeping with the suggestion that the two sugar molecules are united as in trehalose, or as in the modification of this linkage possible when one constituent is an amino-sugar, in which the betaine ring occurs.

In the absence of further evidence it is perhaps unwise to consider the structure of this carbohydrate as proven. The following formula is, however, put forward with a certain degree of confidence as indicating its probable constitution.

There are two possible ways in which such a structure could enter into combination with the amino-acid complexes of the protein molecule. In the first place attachment might occur through the nitrogen atom, or, secondly, it is possible that condensation might occur between one or other of the hydroxyl groups and some constituent of the protein molecule. In the absence of further knowledge there is no way of deciding between these two alternatives.

Deductions concerning the molecular weights of the serum-proteins.

Both the albumin and globulin of horse-serum yielded about the same quantity of pure carbohydrate material, namely 2 %. Since the carbohydrate possesses a molecular weight of 341, the minimal molecular weight of each protein is given by the expression $M = \frac{341 \times 100}{2} = 17050$. Of course such a calculation can give only approximate figures since it is not possible to determine the percentage content of carbohydrate more exactly than by direct isolation; however, this figure is in quite good agreement with the values recorded in the literature and found by methods very different from that here employed [see Svedberg and Sjögren, 1928; Cohn, Hendry and Prentiss, 1925].

DISCUSSION.

The demonstration that a carbohydrate complex is one of the structural units present in both the albumin and globulin of horse-serum is important and raises several problems of biological, as well as of chemical, interest. It is somewhat surprising to find that this new constituent is present in relatively large amounts—approximately 2 % of the dry weight of each protein—and one naturally wonders whether any particular biological function is subserved by its presence. Reference will be made later to the work of Glassmann [1925, 1926].

The part which the plasma-proteins play in immunity reactions has been the subject of many researches. In view of the very interesting results which have been obtained recently in a related branch of immunology by Heidelberger and Avery and other workers it is desired to call attention to the fact that the soluble specific substances obtained by these authors from pathogenic bacteria have been shown to be carbohydrate derivatives of a relatively simple structure. Many of these specific substances contain nitrogen, presumably as the amino-group of an amino-sugar.

It is so suggestive to find carbohydrate structures having a complexity at least comparable with that of the carbohydrate present in the serum-proteins and possessed of specific immunological properties, that, whilst avoiding undue emphasis, the present writer would suggest that future investigations concerning the immunological rôle of the plasma-proteins should not overlook the fact that both albumin and globulin contain carbohydrate material as an integral constituent of their molecules.

Returning to purely chemical issues, the question arises of the relationship existing between the serum-proteins and the class of conjugated proteins known as glucoproteins. The work of Levene and La Forge [1913] has shown that the mucins contain a carbohydrate complex which is quite different from the disaccharide complex of the serum-proteins. Moreover, the relations existing between the carbohydrate and the serum-proteins are not those usual between prosthetic group and protein moiety; the carbohydrate appears to enter into their structure just as an amino-acid would or as the phosphorus-containing complex enters into the structure of caseinogen [see Rimington, 1927].

Throughout the course of the present research attention has been confined strictly to the two proteins derived from horse-serum. It is hoped, at a later date, to extend and develop the work and, as a preliminary result, it has been ascertained that a carbohydrate substance is present also in wool-keratin. Whether or not it will prove to be the case that carbohydrate complexes are of general occurrence in the protein molecule it is impossible to say, but recently Fränkel and Jellinek [1927] claimed that a carbohydrate composed of glucosamine and mannose occurred in egg-albumin. Unfortunately these authors did not determine the molecular weight of this product, neither did they investigate its structure. Whilst there are points of resemblance between

the disaccharide isolated by me from serum-proteins and the substance obtained by Fränkel and Jellinek, in other characteristics it is notably different. Thus Fränkel and Jellinek state that their preparation was hydrolysed by ptyalin, and furthermore that it yielded no nitrogen gas when treated with nitrous acid in the Van Slyke apparatus. The substance obtained by me from serum-proteins was not attacked by any of the carbohydrase preparations—including ptyalin—which were allowed to act upon it. Its nitrogen, on the other hand, is evolved completely though slowly as nitrogen gas in the Van Slyke apparatus.

Soon after this work was completed Dische [1929] reported some investigations upon the "protein-bound" sugar of the blood. His interest appears to centre round the clinical significance of protein-bound sugar, but by adding phenylhydrazine hydrochloride and sodium acetate to an acid hydrolysate of whole plasma which had been treated with phosphotungstic acid, he succeeded in isolating mannosephenylhydrazone and also in detecting the presence of another, non-fermentable, reducing substance which, he considers, may be glucosamine.

The conditions employed by Dische were such as would hydrolyse not only the plasma-proteins but also the glucosamine-mannose disaccharide which would be obtained, during the course of the hydrolysis, from the albumin and globulin present.

Since, largely owing to the work of Glassmann, the whole subject of protein-bound sugar has recently taken on a new clinical significance, it is of interest to notice how the present research illuminates several long disputed points. Lèpine [1918], to whom the conception of "bound sugar" is due, considered that this was sugar held in glucosidic linkage. Bierry and Fandard [1911], on the other hand, rejected this view since they found that neither emulsin nor invertase increased the reducing power of plasma, boiling acid alone being capable of so doing. The present findings clearly substantiate Bierry and Fandard's views. Secondly, the values obtained by different authors for the proportion of bound sugar have not always agreed. Thus Glassmann [1925, 1926] arrived at figures six or seven times as large as those of Bierry. Dische has shown that alkaline mercuric nitrate—employed as a protein precipitant by Bierry—removes some reducing material from solution. In the light of the present investigations it is clear that the material so lost was glucosamine derived from the glucosamino-mannose.

There remains for discussion one point more; namely, the chemistry of humin formation. Gortner [1916 and elsewhere] has shown that the humin produced from proteins undergoing hydrolysis by boiling acids is derived from an interaction between tryptophan and some substance, most probably of an aldehydic nature, formed together with the amino-acid during the hydrolysis of the protein. Glucose, starch and other carbohydrates were shown to cause an increase in the yield of humin when added along with the protein for hydrolysis. Nothing further is known concerning humin formation,

but the discovery of a carbohydrate complex in two proteins at least, and in relatively large amount, opens up a new field for experimental attack. This carbohydrate may be the precursor of the aldehyde reacting with tryptophan, and the failure of investigators in the past to appreciate its presence is no doubt due in large part to the fact that acid hydrolytic agents were customarily employed in any research upon the nature of the constituent units of the protein. The bulk of the carbohydrate would thereby be lost as a result of secondary reactions.

A systematic examination of these and allied problems is contemplated in the near future. So also is a thorough examination of the different classes of proteins with a view to ascertaining whether the nitrogenous disaccharide described in the above pages is peculiar to the proteins of horse-serum or whether it, or some related compound, occurs universally as a structural unit of protein material.

SUMMARY.

1. From the carefully purified proteins of horse-serum, a carbohydrate derivative has been obtained which is regarded as a definite structural unit of these proteins.

2. The substance isolated has the composition $C_{12}H_{23}O_{10}N$, is devoid of reducing properties and exhibits in solution no measurable optical activity.

3. The constituent sugars have been identified as glucosamine and mannose and it has been shown that the compound has a molecular weight corresponding to that required by the simple disaccharide formula. Identical products are obtained from serum-albumin and globulin respectively and in a yield of approximately 2 % in each case.

4. Calculation from the yield gives figures for the minimal molecular weights of the proteins which are in good agreement with values previously recorded in the literature.

5. Exactly the same product has been obtained from the serum-proteins when trypsin was employed as the hydrolytic agent, thereby establishing the distinction between this carbohydrate and the sulphur-containing tetrasaccharide of the glucoproteins.

6. A structure has been suggested for the carbohydrate substance.

7. Some chemical considerations, arising out of the occurrence of carbohydrate material in these two proteins, have been discussed in connection with physiological and immunological problems.

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