CLXXXIX. CARBOHYDRATES IN PROTEINS

I. THE CARBOHYDRATE COMPONENT OF CRYSTALLINE EGG ALBUMIN

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THE question of the occurrence of carbohydrate groups as essential parts of the structure of proteins (apart from mucins and mucoids) has been the subject of many investigations, none of which however has yielded an unequivocal result.

Before the peptide theory of proteins was generally accepted, numerous papers were published concerning the carbohydrate content of proteins. Pavy [1893] hydrolysed coagulated egg-white first with alkali and then with dilute acid and obtained glucosazone from the hydrolysate. He assumed that all proteins contain fairly large amounts of carbohydrate and that the preformed sugar in the protein molecule is responsible for the conversion of protein into carbohydrate which occurs in the animal body. These contentions were the subject of a heated controversy and, although Pavy's physiological hypotheses were somewhat bizarre, his chemical observations were correct. Eichholz [1898] confirmed Pavy's findings by showing that egg albumin, from which mucin and mucoid had been carefully removed, gave on hydrolysis a substance which reduced Fehling's solution and from which an osazone of m.p. 208° could be obtained. Later, when crystalline egg albumin had been prepared, Hofmeister [1898] and Langstein [1902] obtained an osazone from the hydrolysate of the crystalline protein and Seemann [1900] isolated glucosamine hydrochloride from a thrice crystallized sample. The amounts isolated, however, were very small and it was generally assumed that ordinary proteins are composed of amino-acids only and that the presence of sugars in hydrolysates is due to impurities such as mucoids (see Levene [1923] and Plimmer [1917]).

Much later, the question was taken up again by Fränkel & Jellinek [1927] who hydrolysed apparently non-crystalline egg albumin with baryta, removed amino-acids with neutral lead acetate and precipitated the polysaccharide with ammoniacal lead acetate. They repeated this separation several times and finally obtained, by precipitation with alcohol, an optically inactive polysaccharide. This substance on acid hydrolysis gave mannose and glucosamine. Levene & Mori [1929], following the work of Fränkel & Jellinek, introduced as an additional step in the purification of the polysaccharide the use of mercuric sulphate for the removal of nitrogenous impurities. These authors, however, obtained only small amounts of polysaccharide from repeatedly recrystallized egg albumin and concluded therefore that the carbohydrate is not derived from the egg albumin itself but from ovomucoid. Rimington [1929; 1931] applied the same method of hydrolysis and isolation to mixed serum proteins and also obtained mannose and glucosamine hydrochloride as ultimate hydrolysis products.

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Sørensen [1934], using a colorimetric technique, detected carbohydrates in nearly all proteins examined by her, and estimated that crystalline egg albumin contains 1.8% of mannose.

There remains therefore considerable doubt as to whether crystalline proteins do in fact contain carbohydrates as part of their molecular structure. The positive colour reactions for carbohydrates which are given by highly purified albumin preparations are not sufficient to establish the point with certainty, whilst the failure of workers such as Levene & Mori to obtain more than traces of carbohydrate from purified proteins may well be due to defects in the methods employed.

Thus, prolonged hydrolysis with strong alkali as used by Levene & Mori is well known to be destructive of many carbohydrates and it will in fact be shown later in this paper that such treatment is harmful to the polysaccharide obtained from egg albumin. Moreover, it can hardly be claimed that the method used by these workers for the isolation of the carbohydrate, depending as it does on quantitative removal of amino-acids by neutral lead acetate and mercuric sulphate and selective precipitation of the polysaccharide by ammoniacal lead acetate, is well adapted to the separation of a small amount of carbohydrate in a pure state from a protein hydrolysate.

The object of the work to be described in the present paper has been first to develop a new method for the isolation of carbohydrates from proteins which should be free from the objections mentioned above and secondly to apply the new method to the study of what is perhaps the best-defined protein at present available, namely crystalline egg albumin.

Attempts to separate carbohydrate from egg albumin by physical means

The possibility of the separation of carbohydrate from crystalline proteins by physical means was first investigated, and for this purpose the carbohydrate content of crystalline egg albumin was estimated by the orcinol method after successive recrystallizations, after denaturation and after ultrafiltration. The justification for the use of a colorimetric method for the estimation of carbohydrate will appear later from the fact that the amount of sugar finally estimated by actual isolation agrees satisfactorily with the colorimetric value.

Table I. *Change of carbohydrate content with recrystallization*

Crystallization no.	% Carbohydrate
1	2.8
2	2.0
3	1.8
4	1.75
5	1.8
6	1.8
7	1.8

Table I shows that the carbohydrate content of crystalline egg albumin falls during the first two recrystallizations but is not affected by further repetitions of the process. The final value of 1.8% carbohydrate calculated as mannose obtained agrees well with that recorded by Sørensen [1934]. Crystallization in itself therefore, so far from effecting a separation of carbohydrate from the protein, leads to a product of constant carbohydrate content.

An attempt was then made to separate the carbohydrate from the protein by ultrafiltration through filters of known porosity. Table II shows that a membrane of average pore diameter 5.5 μ , which retains egg albumin completely

[Elford & Ferry, 1936], is also impermeable to the carbohydrate associated with it; moreover, for membranes of intermediate porosity the ratios of carbohydrate and nitrogen in the ultrafiltrates are constant within the limits of experimental error. It is clear therefore that the carbohydrate is associated with particles of molecular size similar to that of egg albumin.

Table II. *Ultrafiltration of egg albumin*

Protein	Average pore diameter of membrane in $m\mu$	Carbohydrate content		Protein content		Carbohydrate content of protein	
		Supernat. mg. per ml.	Ultrafiltr. mg. per ml.	Supernat. mg. per ml.	Ultrafiltr. mg. per ml.	Supernat. %	Ultrafiltr. %
Native	35	0.324	0.321	1.786	1.714	1.80	1.86
"	11	0.424	0.095	2.370	0.452	1.77	2.10
"	9	0.464	0.043	2.740	0.224	1.70	2.00
"	5.5	0.545	0.010	3.260	0.003	1.70	—
Acid denat.	15	0.325	0.010	1.820	—	1.77	—
Heat denat.	15	0.039	0.060	0.207	0.010	1.88	—

Finally Table II also shows that no carbohydrate is split off on denaturation with acid. The fact that the carbohydrate, which passes readily through the $15m\mu$ membrane when combined with the native protein, is completely retained with the protein after the latter has been denatured is convincing evidence that the carbohydrate must be associated with a denaturable protein and not, for instance, with contaminating mucin. This is confirmed by the fact that no carbohydrate is liberated during denaturation by heat.

The impossibility of separating carbohydrate from recrystallized egg albumin by any of the physical processes described affords reasonable ground for the assumption that the carbohydrate forms an integral part of the protein molecule. With respect to denaturation the possibility remains that the carbohydrate group may play some part in the intramolecular mechanism of this process while remaining attached to the protein molecule.

New method for the isolation of carbohydrate from protein hydrolysates

From the experiments of Rimington [1931], it can be deduced that trypsin does not hydrolyse the polysaccharide derived from serum proteins. It is reasonable to expect that the carbohydrate in egg albumin will be similarly resistant and hydrolysis by trypsin was therefore chosen for the present work. Introduction of extraneous carbohydrate with the trypsin was minimized by keeping the amount of enzyme as low as possible; since the (colorimetric) carbohydrate content of the trypsin preparation used was very small, the amount of carbohydrate introduced with the enzyme was actually less than 0.2% of the total.

The new method of isolation of the carbohydrate from the hydrolysis mixture was based on the following considerations. Ketene reacts quantitatively with α -amino-acids (and α -imino-acids) [Neuberger, 1938] in aqueous solutions of neutral and alkaline reaction to form *N*-acetyl compounds [Bergmann & Stern, 1930]. In the basic amino-acids the amino groups only are acetylated; thus the action of ketene on histidine and arginine yields α -*N*-acetylhistidine and α -*N*-acetylarginine respectively; these acetylated acids retain a zwitterion structure, and are therefore insoluble in organic solvents. Aliphatic hydroxyl groups on the other hand are not acetylated by ketene in aqueous solution, as shown by the fact that ketene reacts with glucosamine to form *N*-acetylglucosamine only

[Bergmann & Stern, 1928]. It is to be expected therefore that if a mixture of amino-acids and carbohydrate is treated with ketene, all amino-acids except histidine and arginine will be converted into compounds which are soluble in organic solvents, whereas carbohydrates will remain unchanged, apart from acetylation of the amino group of glucosamine or other amino-sugars.¹

By treatment of a tryptic hydrolysate of protein with ketene and subsequent exhaustive extraction of the acidified solution with chloroform, after removal of basic amino-acids with phosphotungstic acid, it should therefore be possible to remove a large proportion of nitrogenous material without affecting the carbohydrate.

Now carbohydrates in general are readily and completely acetylated by acetic anhydride and pyridine; treatment of the material remaining in the chloroform-extracted solution with these reagents should therefore convert the carbohydrate into a chloroform-soluble condition in which it can easily be separated from the remaining nitrogenous contaminants. It should then be possible to deacetylate the chloroform-soluble acetylated carbohydrate under mild conditions, and to isolate the free carbohydrate by precipitation with alcohol.

A difficulty might arise in the application of the procedure just outlined owing to the formation of chloroform-soluble acetaminoketones by the reaction studied by Dakin & West [1928]; actually it has been shown [Neuberger, 1938] that if the temperature of the acetic anhydride-pyridine mixture is kept low such formation of acetaminoketones from amino-acids is almost entirely suppressed in favour of the formation of acetamino-acids; in any case, even if small amounts of acetaminoketones and/or acetamino-acids should pass into the final chloroform extract, these will be unaffected by the deacetylation process and, being readily soluble in alcohol, will remain in the mother liquor when the free carbohydrate is precipitated.

Table III. *Distribution of nitrogen (N) and carbohydrate (CH) during process of fractionation*

¹ The differential extraction of acetamino-acids by organic solvents as a means of separating amino-acids is being examined by Mr R. L. M. Synge in the Biochemical Laboratory, Cambridge.

The procedure finally adopted for the isolation of the polysaccharide has been as follows. The hydrolysate is first treated with phosphotungstic acid to remove basic amino-acids and incompletely digested protein. The material not precipitated by phosphotungstic acid is then treated with ketene, and acetylated amino-acids are removed so far as possible by chloroform extraction. The material obtained on evaporating the aqueous solution is treated with pyridine and acetic anhydride at 0°. The acetylated polysaccharide thus obtained is then extracted from the aqueous solution of the reaction product by chloroform and carefully deacetylated by dilute alkali in the cold, and the free carbohydrate is precipitated by addition of alcohol.

Table III shows the distribution of nitrogen and carbohydrate in the different fractions in a typical experiment; it will be seen that very little carbohydrate is lost by precipitation with phosphotungstic acid and no carbohydrate at all passes into the first chloroform extract. It is clear from Table III that the partition coefficients of some acetamino-acids between chloroform and water are not very favourable for their complete removal from the aqueous phase.

It also appears from Table III that only 60 % of the polysaccharide is obtained in the second chloroform extract after one treatment with pyridine and acetic anhydride. In later experiments it was found that 95 % of the total carbohydrate could be obtained in the final alcohol precipitate by repeating the acetylation process on the residue.

Properties of the carbohydrate

General. The substance obtained, having the appearance and general properties of a polysaccharide, was homogeneous, since samples obtained by fractional precipitation with alcohol had identical rotations, molecular weights and nitrogen and carbohydrate contents (Table IV). Colour reactions indicated

Table IV. *Physical and chemical properties of different preparations and fractions obtained by fractional precipitation with alcohol*

	Nitrogen content %	$[\alpha]_D$	Mol. wt. uncorr. for ash	Carbo- hydrate content %
Preparation 1	4.85	+21.5	1150	47.0
Preparation 2	4.95	+22.0	—	48.0
Preparation 3	5.05	+22.0	—	50.0
Preparation 4	4.95	+22.5	1130	48.0
Fraction I precipitated by 50 % alcohol	4.85	+22.0	—	46.5
Fraction II precipitated by 65 % alcohol	4.90	+20.5	1160	50.1
Fraction III precipitated by 75 % alcohol	5.10	+22.5	1110	49.0
Fraction IV precipitated by 90 % alcohol	5.15	+22.0	—	—

the presence of hexose and acetamino-sugar, whilst the presence of significant amounts of ketoses, pentoses and uronic acids could be excluded. The substance gives the ninhydrin reaction after acid hydrolysis; this does not necessarily imply the presence of amino-acids however since glucosamine hydrochloride gives a typical ninhydrin test. The negative biuret test showed that the polysaccharide was not contaminated by higher peptides. Negative colour reactions further indicated the absence of tyrosine, tryptophan, histidine, arginine and cystine from the preparation.

Component sugars. The polysaccharide itself had no free reducing group, but reducing sugars were liberated on acid hydrolysis. Fig. 1 shows that the maximum reducing value on hydrolysis with 1.5*N* HCl at 100° is obtained after about 5 hr.

If correction is made for the "salt error" and the slightly higher reducing equivalents of mannose and glucosamine as compared with glucose, the amount of reducing sugar in the polysaccharide can be calculated to be about 75%. On the other hand the amount of sugar other than amino-sugar, as determined colorimetrically, was about 48%, a figure nearly identical with that obtained for the fermentable sugar. Thus, about 50% of the polysaccharide or 68% of the reducing sugars is present as fermentable hexose. On the other hand direct colorimetric estimation of amino-sugar indicated a content of 25% calculated as glucosamine [Elson & Morgan, 1933]. Thus, it can be concluded that the reducing sugar obtained on hydrolysis consists of two-thirds of a fermentable hexose and one-third of an amino-sugar.

Fig. 1. Liberation of reducing sugar on hydrolysis of polysaccharide with 1.5 *N* acid at 100°.

Mannose was isolated from a hydrolysate of the polysaccharide in the form of the phenylhydrazone and the *p*-bromophenylhydrazone. The amounts corresponded to 36 and 38% of the polysaccharide, whilst in model experiments under the conditions used for the isolation of the hydrazones, mannose could be recovered to the extent of 75–80%. If such a correction for the incomplete recovery of mannose is applied to the values obtained, the content of mannose in the polysaccharide falls between 45 and 48% which is in satisfactory agreement with the values obtained from the fermentable reducing sugar content and from colorimetric determination.

Glucosamine was isolated by a newly devised method as 2:4-dihydroxy-benzylideneglucosamine. The amounts isolated in different experiments corresponded only to about 60% of the value found colorimetrically. The presence of an amino-sugar other than glucosamine can therefore not be excluded with complete certainty.

Nitrogenous components other than amino-sugars. The maximum content of amino-sugar (calculated as glucosamine) accounts for no more than 40% of the total nitrogen of the polysaccharide; on the other hand, after acid hydrolysis under conditions which would not destroy amino-sugars, 60–70% of the total nitrogen appears as amino-nitrogen (Van Slyke) and about 20% as ammonia.

In addition to amino-sugar(s) there must therefore be present in the polysaccharide a nitrogenous compound which yields its nitrogen partly as amino-nitrogen and partly as ammonia on acid or alkaline hydrolysis, the proportion of ammonia being probably larger in the latter case (see next paragraph). This compound has not so far been identified; colour reactions and consideration of its properties seem to exclude all known amino-acids and its instability both towards acid and alkali make its closer investigation difficult. It is hoped that

its further study may become possible if success is attained in the search for an enzyme which will hydrolyse the polysaccharide.

Action of alkali. Fig. 2 shows that 50 % of the total nitrogen is liberated from the polysaccharide as ammonia by *N* NaOH at 100° after 16 hr. The fact that the yield of mannose as phenylhydrazone after a subsequent acid hydrolysis was greatly diminished indicated that the sugar had been partially destroyed by the alkaline treatment. Similarly, the fact that only 60 % of the remaining nitrogen or 30 % of the original nitrogen was present as amino-nitrogen showed that some

Fig. 2. Rate of liberation of ammonia on hydrolysis of polysaccharide with *N* NaOH at 100°.

glucosamine also had been destroyed. This apparent instability of glucosamine bound in a polysaccharide towards strong alkali is in contrast to the behaviour of methyl glucosaminide which is very stable under the conditions used [Moggridge & Neuberger, 1938].

After alkaline hydrolysis the polysaccharide gives a positive test with Ehrlich's reagent which is not easy to explain, since *N*-acetyl methyl glucosaminide and *N*-acetyl phenyl glucosaminide, when heated with alkali under the same conditions, do not give this reaction. A possible explanation is that a part at least of the glucosamine is bound to the unknown nitrogenous substance by a linkage more unstable to alkali than the *O*-glucoside linkage. On alkaline treatment, this linkage might be split more quickly than the *N*-acyl linkage, and the compound thus partly deaminated and partly condensed to a ring system giving a positive test with Ehrlich's reagent.

Acetyl content. Acetyl estimations showed that the polysaccharide contained 10.65 % acetyl groups, presumably in the form of *N*-acetyl. The amount of acetyl corresponds nearly exactly to the amount of amino-nitrogen obtained after acid hydrolysis, and it may be concluded that the acetyl groups are linked partly to glucosamine and partly to the unknown nitrogenous component. Since the isolation of the polysaccharide involves acetylation it is impossible to say whether the glucosamine in its original combination in the protein is *N*-acylated or not.

Content of the polysaccharide in egg albumin. As will be shown later, 3.5 g. of the polysaccharide complex are obtained from 100 g. of egg albumin. If the yield of the polysaccharide is assumed to be 95 %, this corresponds to a value of 3.65 g. per 100 g. of protein. Since the mannose content of the polysaccharide complex is 50 %, a value of 1.8 g. of mannose per 100 g. of protein is obtained which is in perfect agreement with the colorimetric value. From the analytical figures therefore egg albumin with mol. wt. 40,000 would contain 4 mol. of mannose and 2 mol. of glucosamine.

Estimations of the mol. wt. of the polysaccharide complex indicated a value of 1250, which, on the basis of mol. wt. 40,000 for egg albumin, corresponds to a polysaccharide content of the protein of 3.15%. The agreement with the value obtained from isolation and colorimetric methods is sufficiently good to justify the assumption that four mannose and two glucosamine residues are linked up as one polysaccharide chain with the protein to form one large prosthetic group.

EXPERIMENTAL

Preparation of egg albumin

Egg albumin was crystallized according to Kekwick & Cannan [1936]. Samples from each crystallization were dialysed against frequent changes of distilled water for 10 days and nitrogen and carbohydrate were estimated. For the estimation of carbohydrate the method of Sørensen & Haugaard [1933] was used employing mannose as standard. The nitrogen content of egg albumin was assumed to be 15.65%.

Effect of heat-denaturation on carbohydrate content

Five times recrystallized egg albumin (20 g.) was denatured by heating in aqueous solution at 80° for 20 min. and the suspension was dialysed against frequent changes of distilled water at 0°. The combined dialysates were evaporated to dryness *in vacuo*. A minute amount of solid matter giving a very weak Molisch reaction was obtained. It was impossible to obtain from it an osazone, even after acid hydrolysis, nor could a phenylhydrazone or phenylhydrazide be prepared; the presence of hexoses and hexonic acids was thus excluded.

Ultrafiltration experiments

The membranes were made and standardized by Dr Elford and the technique used was that described by Elford & Ferry [1934]. Nitrogen and carbohydrate were estimated in samples of the supernatant fluid and of the ultrafiltrate. Heat-denatured protein was prepared by keeping an electrolyte-free solution of the protein at 85° for 20 min. The suspension was centrifuged at 2000 r.p.m. for 5 min. and the supernatant fluid was filtered through a 1.5 μ membrane which allowed about 12% of the protein originally present to pass through. Acid-denatured protein was prepared by keeping a protein solution for 10 hr. at pH 1 at a temperature of 30° and then adjusting the pH to the isoelectric point. Protein thus denatured passes readily through a 1.5 μ membrane, but is completely retained by a 15 μ membrane.

Isolation of the polysaccharide from egg albumin

Egg albumin, five times recrystallized and nearly completely freed from sodium sulphate by dialysis, was used in all experiments. A 5% solution of the protein was denatured by heating and the reaction of the suspension was adjusted to pH 7.8. Trypsin (Merck) was then added in an amount corresponding to 0.3% of the dry weight of the protein. Alkali was added frequently to adjust the pH of the digest to 7.8 and further quantities of trypsin were added about twice a week. The enzymic hydrolysis was followed by alkalimetric titration in 90% alcohol according to Willstätter & Waldschmidt-Leitz [1921], correction being made for the alkali added; the digestion was continued for 4-6 weeks. The total amount of trypsin added varied between 0.8 and 1.1 g. per 100 g. of protein.

The working up of this digestion mixture may be described in detail for a typical experiment.

The enzymic hydrolysate of 120 g. of egg albumin was acidified to pH 1 with HCl. Phosphotungstic acid (150 g.) in 0.1 *N* HCl was added and the mixture was left for 3 days in the cold room. The precipitate was filtered off and washed with a 10 % solution of phosphotungstic acid in 0.1 *N* HCl. The precipitate was suspended in warm 50 % acetone in which most of it dissolved and the suspension was extracted five times with an amyl alcohol-ether mixture. The aqueous suspension was filtered and the solution was analysed for carbohydrate and nitrogen (fraction I). The precipitate was again suspended in water and dissolved by addition of NaOH (fraction II). The phosphotungstic acid was removed by Ba(OH)₂ and the excess Ba(OH)₂ precipitated by H₂SO₄.

The filtrate from the phosphotungstic acid precipitation was freed from excess phosphotungstic acid by amyl alcohol-ether extraction until a sample gave no precipitation with Ba(OH)₂. The solution was then neutralized with 2 *N* NaOH and concentrated to low bulk.

To the neutralized solution sufficient NaOH was then added to adjust the reaction to pH 9 and ketene was passed in until the ninhydrin reaction became negative; this took about 18 hr. Small additions of alkali were made at intervals to maintain the pH at about 6. The solution was then concentrated at low pressure and acidified to pH 1.6 with HCl. Acetylated amino-acids were now removed by continuous extraction with chloroform in the apparatus shown in Fig. 3. The extraction was generally stopped after 12 hr. since it was found that continuance for a further 50 hr. did not appreciably increase the amount of nitrogen in the chloroform layer. The chloroform solution was evaporated to dryness, the residue was taken up in water and nitrogen and carbohydrate were estimated.

The aqueous solution was concentrated *in vacuo* and dried in a desiccator first over conc. H₂SO₄ and finally over P₂O₅ to a thick syrup. To this syrup were added pyridine (250 ml.) and acetic anhydride (350 ml.), and the mixture was shaken mechanically for 2 days at 2°. It was then poured into ice-water and left for 5 hr. The solution was concentrated under very low pressure, the residue diluted with water and the latter again evaporated. The aqueous solution of the residue was then made acid to Congo red and extracted repeatedly with chloroform, the combined chloroform extracts being washed with water containing NaCl and dried. After removal of the chloroform an oil was obtained which was dissolved in alcohol. Sufficient lithium hydroxide was added to keep the solution alkaline to phenolphthalein for at least half an hour. After 2 hr. more in the ice-chest the alkali was exactly neutralized with the required amount of dilute HCl and the solution concentrated under reduced pressure nearly to dryness; the residue was dissolved in a small amount of water and alcohol was added to a final concentration of 95–97 %. The precipitate was centrifuged off

Fig. 3.¹ Apparatus for continuous extraction of an aqueous solution with chloroform.

¹ This apparatus can be purchased from Messrs Quickfit and Quartz, Ltd.

after standing for several hours in the ice-chest, washed several times with ice-cold absolute alcohol and dried over P_2O_5 .

The mother liquor from the alcohol precipitation was evaporated and a further small amount of precipitate was obtained on addition of alcohol. Altogether 2.9 g. of material were obtained.

The aqueous solution obtained from the last chloroform extraction and containing unacetylated polysaccharide was evaporated to dryness, and subjected to reacetylation followed by further treatment as above; 1.2 g. more of material were obtained, identical in all respects with that derived from the first acetylation. A third acetylation gave no further product.

General properties of the polysaccharide

The substance obtained by the method described is very slightly pigmented and fairly hygroscopic; it is very soluble in water, somewhat soluble in aqueous alcohol and insoluble in absolute alcohol and acetone. Its aqueous solution has pH 5.5–6.0. The nitrogen contents of different samples as estimated by the Kjeldahl method varied between 4.85 and 5.1%. (It was found that reliable nitrogen values for glucosamine or glucosamine-containing compounds could only be obtained by the ordinary Kjeldahl method if ashing with H_2SO_4 were continued for about 6 hr.; with SeO_2 as catalyst however half an hour sufficed.)

The polysaccharide gives no biuret reaction even in a 20% solution; it gives a ninhydrin reaction only after acid hydrolysis. The Millon and Sakaguchi reactions are negative both with the untreated polysaccharide and with samples hydrolysed with 2*N* HCl and with conc. HCl. In view of the claim of Rimington [1931] that histidine is combined with the polysaccharide obtained from serum proteins the Pauly reaction was carried out on samples hydrolysed under many different conditions: 5 mg. samples were hydrolysed with 2*N* HCl at 100°, conc. HCl at 125° and with 10% $Ba(OH)_2$ at 100° all for 5 hr. In all cases only a slight yellow-orange colour was obtained with diazobenzenesulphonic acid in sodium carbonate solution, which was very different from the colours given by histidine and tyrosine. The Hopkins-Cole and Rosenheim tests for tryptophan were negative before and after alkaline hydrolysis. Tests for sulphur and phosphorus were likewise negative.

The Molisch reaction was positive up to a dilution of 1:1,000,000. The polysaccharide itself did not reduce Fehling's solution, but became strongly reducing on mild acid hydrolysis. A strongly positive test for *N*-acetylglucosamine [Morgan & Elson, 1934] was obtained after hydrolysis with 0.01*N* HCl at 100° for 1 hr. The naphthoresorcinol, phloroglucinol and Seliwanoff tests were all negative on samples hydrolysed under difficult conditions. Carbohydrate estimations by the method of Sørensen & Haugaard gave values of 47–50% using mannose as a standard (see Table IV).

Estimation of the molecular weight

The molecular weight was estimated by an isopiestic method worked out by Dr G. S. Hartley similar to that described by Robinson & Sinclair [1934]. A rectangular silver plate containing three circular grooves was placed in a bulb-shaped glass apparatus which could be exhausted. Polysaccharide solutions of different concentrations were placed in the outside grooves while a solution of KCl of known strength was put into the middle groove. The apparatus was carefully exhausted on an oil pump and left for 2 days at 25°; it was then opened and the amounts of solutions in the three grooves were determined by weighing.

Osmotic equilibrium was reached, and the concentrations of polysaccharide in the two outside grooves were identical. \

The method may be described in detail for one experiment. 0.0896 g. of a solution containing 0.00706 g. of polysaccharide was placed in groove 1, 0.05840 g. of a solution containing 0.00132 of polysaccharide in groove 3, whilst in groove 2 0.09168 g. of 0.05 *N* KCl was introduced. At the end of the experiment grooves 1 and 3 contained 0.0928 and 0.0178 g. of solution respectively indicating concentrations of polysaccharide of 7.45 and 7.61 %. The values show that osmotic equilibrium between the two polysaccharide solutions had in fact been established, and it can be assumed that equilibrium had also been reached between the polysaccharide and the KCl solutions. In groove 3 containing KCl, the amount of solution had increased to 0.117 g. indicating that the two polysaccharide solutions are in equilibrium with a 0.0409 *N* solution of KCl. Using an osmotic coefficient for KCl of 0.943 and recalculating the concentrations of polysaccharide in g. per 1000 g. of solvent, values for the mol. wt. of 1120 and 1150 respectively were obtained.

Estimation of ash content

10.62 mg. of the polysaccharide were ashed by heating at 500° for 6 hr. The residue amounted to 0.0192 mg. corresponding to an ash content of 0.1735 %. If it is assumed that the ash consists only of lithium chloride a correction for the value of the mol. wt. can be made. The values of the mol. wt. corrected on such a basis are 1245 and 1275.

The homogeneity of the polysaccharide

450 mg. of the polysaccharide were dissolved in 1 ml. of water and 1 ml. of alcohol was added. The precipitate was centrifuged off and dried. To the supernatant solution pure alcohol was added and the precipitate formed again centrifuged off. By thus increasing the alcohol concentration several fractions were obtained. Optical rotations, mol. wt. and nitrogen and carbohydrate contents of the different fractions were estimated; the results are recorded in Table IV.

Estimation of reducing sugars

66.6 mg. of the polysaccharide were dissolved in 11 ml. of 1.5 *N* HCl and heated at 100° using a reflux condenser. 1 ml. samples were taken out at intervals, neutralized to 99 % with NaOH and made up to 2.5 ml. The reducing values were estimated according to Hanes's [1929] modification of the Hagedorn-Jensen method. The results in terms of glucose are recorded in Fig. 1. The reducing value rises rapidly on hydrolysis until it reaches a maximum at about 6 hr.; thereafter it decreases slowly, presumably owing to destruction of the sugar. The maximum reducing value expressed as glucose corresponds to a content of 85 % of reducing sugar in the polysaccharide. This value is too high for two reasons. Mannose and glucosamine which occur in the polysaccharide have higher reducing equivalents than glucose; expressed in terms of glucose the molecular reducing equivalents of mannose and glucosamine were found by the Hagedorn-Jensen method to be 101 and 103.5 % respectively. Secondly, it was noted that if an amount of sodium chloride is added to a glucosamine solution similar to that formed on neutralizing the acid hydrolysate, the apparent reducing value of glucosamine is increased by about 10–12 % (see Moggridge & Neuberger, [1938]). If correction was made for these two factors a value of about 75 % of reducing sugar in the polysaccharide was obtained.

Estimation of fermentable sugar

30.2 mg. of polysaccharide were dissolved in 6 ml. of 1.5*N* HCl and heated in a sealed tube at 100° for 6 hr. The solution was then neutralized and made up to 20 ml. One 2 ml. sample of this solution was incubated with washed baker's yeast at 37° for 20 min. according to the method of Somogyi [1929]. The yeast was centrifuged off and the reducing value of the supernatant solution estimated. In a second sample the reducing value was estimated without previous treatment with yeast. Controls with yeast alone were carried out at the same time. The amount of sugar expressed as glucose in the sample not treated with yeast was 2.5 mg., whilst in the sample treated with yeast it amounted only to 0.81 mg. after correction for the reducing value of yeast itself had been made.

Estimation of amino-nitrogen

The polysaccharide itself does not give off any nitrogen in the Van Slyke apparatus. On hydrolysis the following amounts of amino-nitrogen are found:

	%
After 5 hr. hydrolysis with 1.5 <i>N</i> HCl	3.2
After 8 hr. hydrolysis with 2.5 <i>N</i> HCl	3.6
After 8 hr. hydrolysis with 5 <i>N</i> HCl	3.2

These values were obtained on shaking for 5 min. In one experiment—after hydrolysis with 5*N* HCl—shaking was continued for 30 min. and the value was increased to 4.2%. The apparent increase on prolonged shaking is probably due to the ammonia formed on acid hydrolysis. It may be noted that glucosamine hydrochloride gives off all its nitrogen on shaking for 5 min.

Acetyl estimation

Acetyl groups were determined according to Pregl & Soltys [1929]. In two experiments values of 10.61 and 10.67% of acetyl were obtained.

Estimation of amino-sugar

Amino-sugar was estimated by Sørensen's [1938] modification of the method of Elson & Morgan [1933]. A standardization curve was obtained with solutions of glucosamine hydrochloride; the colorimetric readings were done on a Zeiss step-photometer. The mean value from several experiments corresponded to a content of 29.1% of amino-sugar expressed as glucosamine hydrochloride or 24.3% expressed as glucosamine.

Isolation of mannose

(a) *Isolation as phenylhydrazone*. 99 mg. of polysaccharide were dissolved in 1 ml. 1.5*N* HCl and heated in a sealed tube at 100° for 6 hr. The neutralized solution was filtered and made up to 3 ml. To this solution 0.2 ml. of glacial acetic acid and 0.3 ml. of freshly distilled phenylhydrazine were added. After a short time a crystalline precipitate formed which was filtered off after standing for 4 hr., washed with two lots of 0.5 ml. of 15% acetic acid and dried. It amounted to 51 mg. and had m.p. after two recrystallizations 189° (uncorr.); a sample mixed with authentic mannose phenylhydrazone showed no depression of m.p. (Found: N (Dumas), 10.1%. $C_{12}H_{18}O_5N_2$ requires N, 10.33%.)

(b) *Isolation as p-bromophenylhydrazone*. 100 mg. of polysaccharide were dissolved in 10 ml. 1.5*N* HCl and heated at 100° for 5½ hr. The solution was evaporated to dryness at reduced pressure, the temperature of the bath not

exceeding 36°. The residue was taken up in water, neutralized and treated with 150 mg. *p*-bromophenyldiazine in 50 % acetic acid. Crystallization started at once; after 4 hr. standing the precipitate was filtered off, washed with 25 % alcohol and dried. It amounted to 71 mg.

The twice recrystallized hydrazone had m.p. 202° (uncorr.); a sample mixed with authentic mannose *p*-bromophenyldiazine showed no depression of m.p. No osazone could be obtained from the residual solution.

Isolation of glucosamine

Difficulties were encountered in attempts to separate glucosamine from hydrolysates of the polysaccharide until a new derivative of glucosamine was found which is very suitable for purposes of isolation. Glucosamine condenses at slightly alkaline reaction with 2:4-dihydroxybenzaldehyde to form a Schiff's base which crystallizes readily and which, in contrast with other Schiff's bases of glucosamine, is stable towards excess alkali. The reaction is almost quantitative as shown by the following experiments.

Preparation of 2:4-dihydroxybenzylidene-1-glucosamine. 215 mg. glucosamine hydrochloride (1 mol.) and 150 mg. KHCO_3 (1.5 mol.) were dissolved in 2.5 ml. water. 175 mg. 2:4-dihydroxybenzaldehyde (1.25 mol.) dissolved in 2 ml. 40 % ethyl alcohol were added to the solution. Crystallization started after 15 min.; the mixture was left overnight in the ice-chest and the yellow crystals were then filtered off and washed with 2 ml. 40 % alcohol. Yield: 265 mg. or 90 % of the theoretical. The yield is not decreased by the presence of other sugars.

The substance was recrystallized from aqueous alcohol. It is soluble in alcohol and hot acetone, insoluble in water and ether. The substance has m.p. 116° (uncorr.) and decomposes at 118°. (Found: N (Kjeldahl), 4.54 %. $\text{C}_{13}\text{H}_{17}\text{O}_7\text{N}$ requires N, 4.67 %.) The substance mutarotates; the initial $[\alpha]_D = 312^\circ$ (in alcohol); after 42 hr. $[\alpha]_D = 228^\circ$.

If the substance is recrystallized by adding a large amount of water to its concentrated alcoholic solution and leaving the mixture overnight a new compound is formed which is pale yellow in colour and has m.p. 142°. Analysis indicates that the elements of water have been added to the Schiff's base. The same change occurs if the non-hydrated form is kept for some time exposed to the air. (Found: C, 49.15; H, 6.05 %. $\text{C}_{13}\text{H}_{17}\text{O}_7\text{N}$ requires C, 49.27; H, 6.04 %.)

The rotation of the new compound is slightly different from that of the Schiff's base; $[\alpha]_D$ (initial) = 299° (in alcohol), after 42 hr. $[\alpha]_D = 198^\circ$.

Formation of a hydrochloride. 2:4-Dihydroxybenzylideneglucosamine forms with hydrochloric acid a hydrochloride of the hydrated compound containing 1 equiv. HCl. 280 mg. of the Schiff's base were dissolved in hot acetone and 0.25 ml. 5N HCl was added. A white crystalline precipitate formed at once which was filtered off and dried. It amounted to 320 mg. The substance is easily soluble in water and slightly soluble in alcohol, insoluble in ether and acetone. (Found: N, 3.8; Cl, 9.8 %. $\text{C}_{13}\text{H}_{20}\text{O}_8\text{NCl}$ requires N, 3.96; Cl, 10.04 %.)

An alcoholic solution treated with an equivalent amount of aqueous acid gives an identical product.

As found by Dimroth [1902] 2:4-dihydroxybenzylideneaniline is able to add on the elements of water and to form a hydrochloride. By analogy with his interpretation, the reactions here described may be represented as follows:

Recovery of glucosamine. Although the 2:4-dihydroxybenzylidene compound is quite stable to acid in the cold, it is split quantitatively on heating in acid solution.

150 mg. of dihydroxybenzylidene glucosamine were dissolved in 5 ml. 65 % alcohol containing 1.5 *N* HCl and heated under reflux on a water bath for 30 min. The crystalline precipitate which formed on cooling was increased by the addition of ether. The precipitate was filtered off and dried. The product was identified by nitrogen content and rotation as glucosamine hydrochloride, and the yield was 90 % of the theoretical.

Isolation of glucosamine from the polysaccharide. 500 mg. of polysaccharide were hydrolysed with 5 ml. 5 *N* HCl for 3 hr. at 100°. The solution was filtered and evaporated nearly to dryness under reduced pressure; the residue was taken up in water and the solution was again filtered. 500 mg. of phosphotungstic acid dissolved in 2.5 ml. 0.1 *N* HCl were now added and the mixture was left overnight. The precipitate was centrifuged off and washed twice with, altogether, 2.5 ml. of a 5 % phosphotungstic acid solution in 0.1 *N* HCl. The combined solution and washings were extracted with amyl alcohol-ether and the aqueous solution was concentrated to low bulk, again filtered and allowed to evaporate to dryness in a desiccator. The residue which consisted of large plates admixed with amorphous material, was taken up in a small quantity of water, heated with alcohol and again evaporated to remove the last traces of water. Absolute alcohol was now added and the crystalline material was filtered off. It amounted to 120 mg.

The crystalline material was dissolved in 3 ml. 30 % alcohol to which KHCO_3 (80 mg.) and 2:4-dihydroxybenzaldehyde (100 mg.) dissolved in 0.5 ml. 30 % alcohol were added. After 6 hr. in the ice-chest the crystals were filtered off and amounted to 80 mg. The material was dissolved in alcohol and filtered; the solution was evaporated nearly to dryness and the residue recrystallized by addition of water. The substance had m.p. 142°; a sample mixed with the hydrated form of 2:4-dihydroxybenzylideneglucosamine showed m.p. 141°. It agreed with the latter also in optical rotation, having $[\alpha]_D$ (initially) = 304° (in alcohol), after 39 hr. $[\alpha]_D = 204^\circ$. (Found: C, 49.20; H, 6.01 %. $\text{C}_{13}\text{H}_{19}\text{O}_8\text{N}$ requires C, 49.27; H, 6.04 %.)

Isolation of glucosamine hydrochloride. 35 mg. of the Schiff's base were dissolved in 2 ml. 1.5 *N* HCl in 65 % alcohol and the solution heated for 30 min.; the crystalline material was filtered off, washed with anhydrous ether and dried. Yield: 22.8 mg. (Found: C, 32.75; H, 6.51; N, 6.38 %. $\text{C}_6\text{H}_{14}\text{O}_5\text{NCl}$ requires C, 33.4; H, 6.54; N, 6.50 %.) In experiments involving different conditions of hydrolysis no better yields of the dihydroxybenzylidene compound could be obtained.

Nitrogen precipitated by phosphotungstic acid after acid hydrolysis

The phosphotungstic acid precipitate was decomposed with hydrochloric acid and amyl alcohol-ether and the aqueous solution was made up to known volume. A nitrogen estimation showed that 28 % of the total nitrogen of the polysaccharide was precipitable by phosphotungstic acid after acid hydrolysis. The polysaccharide itself is not precipitated by phosphotungstic acid. The Pauly reaction was negative and no reaction for pyridine (chlorodinitrobenzene) could be obtained in the solution recovered from the phosphotungstate. Ammonia estimations by the method of Conway & Byrne [1933] indicated that about 55 % of the nitrogen contained in the precipitate consisted of ammonia. (The ammonia was identified by analysis in the form of ammonium chloride.) Colorimetric

tests with Nessler's reagent indicated that the ammonia is not formed by the alkali used in its estimation. The residual nitrogen contained in the precipitate could not be accounted for; all attempts to prepare derivatives (chloroplatinate, chloroaurate, picrate, picrolonate, reineckate) led only to amorphous gums.

If milder conditions were used for acid hydrolysis the amount of ammonia present in the phosphotungstic acid precipitation was smaller, but it was still impossible to obtain crystalline products from this fraction.

Alkaline hydrolysis

Only small amounts of volatile base are evolved on boiling the polysaccharide with dilute alkali; e.g. 1.4 or 2.8 % of the total nitrogen were obtained as volatile base on boiling the polysaccharide for 1 hr. with 0.01*N* and 0.1*N* NaOH respectively, but much larger amounts are obtained on boiling with *N* NaOH.

51.1 mg. of polysaccharide were dissolved in 8 ml. *N* NaOH and the solution was boiled under reflux, the vapours being trapped in a receiver containing a known amount of 0.01*N* HCl. The acid solution was back-titrated against 0.01*N* NaOH at suitable intervals, and the results are recorded in Fig. 2. The experiment was stopped after 16 hr. In another experiment with 500 mg. polysaccharide the combined solutions containing the volatile base were made alkaline and distilled in the micro-Kjeldahl apparatus into a receiver containing HCl. The distillate was evaporated to dryness and the crystalline residue dried. The total yield was 48 mg. 10.3 mg. were used for nitrogen estimation. (Found: N, 26.05 %. NH_4Cl requires N, 26.16 %.)

The alkaline solution of the polysaccharide was acidified and hydrolysed for 5 hr. with 1.5*N* HCl. Only about 100 mg. of mannose phenylhydrazone could be obtained, a yield of 14 % as against 37 % obtained from non-alkali-treated polysaccharide.

In another experiment in which 500 mg. of the polysaccharide were hydrolysed with 10 % $\text{Ba}(\text{OH})_2$ at 100° for 7 hr. 37 % of the total nitrogen was given off as ammonia. Ba^{++} was removed exactly from the alkaline solution by H_2SO_4 and BaSO_4 was filtered off. The solution of the polysaccharide after alkaline hydrolysis gave a strong reaction with Ehrlich's reagent. To the solution were now added 5 ml. of Hopkins's reagent (HgSO_4 in 5 % H_2SO_4) and the mixture was left for 2 days. The mercuric sulphate precipitate was decomposed by H_2S and $\text{Ba}(\text{OH})_2$ and the solution was evaporated nearly to dryness under reduced pressure. No colour reaction for tryptophan could be obtained nor was it possible to prepare a crystalline derivative from this fraction.

DISCUSSION

It is felt that the work described in this paper places beyond all doubt the question of the occurrence of carbohydrate in crystalline egg albumin. The constancy of carbohydrate content on repeated crystallization and the consistent failure of all attempts at physical separation of protein and carbohydrate fractions, even by processes such as denaturation which must involve intramolecular change, leave no escape from the conclusion that a constant amount of carbohydrate is firmly attached to the protein molecule.

The second conclusion which emerges from the present work concerns the nature of the carbohydrate group, since for the first time a method has been devised for the isolation of this group in a condition which may be assumed to be essentially unaltered. Viewing the properties of the complex isolated from egg albumin in the light of this assumption we find that it consists of

a polysaccharide of molecular weight about 1200, made up for the most part of mannose and glucosamine in the proportion of two molecules of the former to one of the latter, but containing also an unidentified nitrogenous constituent. In all probability the glucosamine occurs in the actual protein-carbohydrate complex in the acetylated condition as it does in other natural products containing glucosamine, such as chitin, but this point cannot be decided by the method at present available; nor can the possibility of the presence of an amino-sugar other than glucosamine in the egg albumin polysaccharide be completely excluded, although it seems extremely remote.

Unfortunately the methods employed in the present work are incapable of giving us any definite information concerning the mode of combination of the polysaccharide with the rest of the protein molecule, except in so far as the liberation of the polysaccharide by tryptic hydrolysis suggests the participation of a peptide linkage; it is possible that the unidentified nitrogenous constituent of the polysaccharide may play some part in the combination and that a knowledge of the nature of this constituent might enable us to assign a definite role to the polysaccharide in the structure of the natural protein. A more exact analysis of the situation must await the development of new methods of attack and further speculation at the present time serves no useful purpose; it is even now however possible to state with some certainty, on the basis of the findings recorded above, that the polysaccharide forms a single prosthetic group with one point of attachment to the molecule of egg albumin, the word prosthetic implying no more than that the group in question is made up of compounds other than amino-acids.

One more point arises which may be briefly considered here. The discovery in recent years of the predominant part which is played by carbohydrate groups in the determination of the specificity (as distinct from antigenic capacity) of many bacterial antigens naturally gives rise to the speculation that a similar state of affairs may exist among the proteins in so far as the latter contain carbohydrate groups. Hitherto it has been impossible to put this question to the test since no method has been available whereby the carbohydrate group could be separated from a protein without subjection to treatment calculated to alter its biological properties entirely. With the aid of the gentle method of isolation described in the present paper it seems that the problem can be tackled with more hope of success, and experiments are in fact already in hand to examine how far, if at all, the immunological specificity of crystalline egg albumin is determined by the carbohydrate group which it contains.

SUMMARY

1. Previous work on the occurrence of carbohydrate in proteins other than mucins and mucoids is briefly reviewed.
2. It is shown that the carbohydrate content of crystalline egg albumin becomes constant on recrystallization and that it is impossible to separate the carbohydrate from the protein by denaturation or ultrafiltration.
3. A method for the isolation of the carbohydrate group in almost quantitative yield from egg albumin is described which is based on tryptic hydrolysis and involves no chemical treatment more drastic than acetylation and deacetylation under the mildest conditions.
4. The properties of the polysaccharide thus isolated are described in detail. It is shown to have mol. wt. about 1200 and to be composed almost certainly of four molecules of mannose and two of glucosamine, together with an unidentified nitrogenous constituent. The complex is regarded as forming a single

prosthetic group with one point of attachment to the molecule of natural egg albumin.

5. The preparation and properties are described of 2:4-dihydroxybenzylideneglucosamine, a compound useful for the isolation and characterization of glucosamine.

6. The possible significance of the carbohydrate group in relation to the structure and immunological properties of egg albumin is briefly discussed.

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