CLXXXIV. OBSERVATIONS ON THE POLY-SACCHARIDE COMPLEX PRESENT IN SERUM PROTEINS AND ON THE EFFECT OF PEPSIN ON SERUM PROTEIN FRACTIONS

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It was shown in a previous paper [Hewitt, 1938] that fractional precipitation with acid acetone following peptic digestion of various proteins afforded a convenient method of separating carbohydrate-rich peptones. The use of proteolytic enzymes to separate the carbohydrate component in proteins has been described by a number of workers [Rimington, 1929; Iseki, 1934; Neuberger, 1938], but the subsequent fractionation process is in general lengthy or involves somewhat drastic chemical treatment so that repetitions of the fractionation process become cumbersome and it is difficult to ensure that the final product has suffered no chemical modification during the manipulations. The relatively mild procedure involved in the acid acetone precipitation and the extreme rapidity of the process rendered it worthy of investigation as a method of isolating the polysaccharide residues from serum proteins, amongst which seroglycoid and globoglycoid have been shown to be particularly rich in "bound carbohydrate" [Hewitt, 1934; 1936; 1937; 1938]. Among other points requiring investigation was the question of the initial products of the attack of enzymes on the protein fractions.

Experimental

The protein fractions used were obtained from horse serum. Globoglycoid was prepared from either the globulin fraction precipitated between the limits 45 and 55 % saturation with ammonium sulphate or from the albumin fraction as previously described [Hewitt, 1938]. Seroglycoid was precipitated from the mother liquor after successive removal of globulin fractions and crystalalbumin by $(\text{NH}_4)_2\text{SO}_4$ [Hewitt, 1936; 1937].

Carbohydrate determinations were carried out using the orcinol method [Tillmans & Philippi, 1929; Sørensen & Haugaard, 1933; Hewitt, 1934], colorimetric comparisons being made photoelectrically in most cases. Results are quoted in terms of galactose-mannose-hexosamine.

Fractionation experiments

Peptic digests of seroglycoid, globoglycoid and ovalbumin at pH 1.5 were treated with acetone (5.5 vol.) and after removing the precipitate by centrifuging a second precipitate was produced by adjusting the pH to 4.8. After centrifuging the supernatant fluid was evaporated to dryness. The properties of the fractions are summarized in Table I.

It will be seen that the bulk of the carbohydrate is to be found in the acid acetone precipitate. A more detailed fractionation on a larger scale was now carried out as follows. A solution of globoglycoid was brought to pH 1 by

Table I. *Acetone fractionation of peptic digests*

Fraction	Seroglycoid		Globoglycoid		Ovalbumin	
	Weight mg.	Carbo- hydrate %	Weight mg.	Carbo- hydrate %	Weight mg.	Carbo- hydrate %
Acid acetone	91	20.0	33	24.6	50	18.6
Neutralized acetone	248	1.8	58	3.0	133	0.3
Evaporated residue	96	0	49	0	79	1.3

addition of HCl, 0.3 % of pepsin was added and the mixture was incubated at 50° for 22 hr. Acetone (4 vol.) was added, the precipitate was removed by centrifuging, and more acetone (4 vol.) was added. Again the precipitate was centrifuged down and a further fraction was obtained by neutralizing the supernatant fluid with NaOH. The precipitates were washed with acetone and ether and dried *in vacuo*. The composition of the fractions is summarized in Table II.

Table II

Fraction	Weight g.	Carbohydrate content %
80 % acid acetone	0.44	39
90 % acid acetone	0.88	9.7
Neutralized acetone	1.61	1.0

It is clear that the bulk of the polysaccharide-containing material is precipitated by acid acetone. An experiment, using tryptic digestion of heated globoglycoid in place of peptic digestion, was conducted as follows. A globoglycoid solution was heated in a boiling water bath and cooled, the pH was adjusted to 8, trypsin was added and the mixture was incubated at 45° for 24 hr. For the first 8 hr. small additions of trypsin, together with NaHCO₃ to maintain the pH at 8, were made at 2 hr. intervals. After incubation the digest was acidified to pH 1 by addition of HCl, acetone (4 vol.) was added and the precipitate was collected and dried *in vacuo* after dehydration with acetone and ether. The carbohydrate content (25 %) was rather lower than in comparable experiments with pepsin, when figures between 27 and 39 % were observed. On the basis of these experiments a method of preparing the polysaccharides from serum proteins was worked out.

Preparation of polysaccharides

Proteins (globoglycoid and seroglycoid) were first subjected to peptic digestion at pH 1 for 24 hr. at 50°. The carbohydrate-rich fraction of peptone was then precipitated by addition of acetone (4 or 5 vol.) to the acid solution, centrifuged down, washed with acetone and ether and dried *in vacuo*. The dried powder was then dissolved in water and subjected to tryptic digestion. The solution was made alkaline by addition of NaHCO₃, trypsin was added and the mixture was incubated at 45° for 24 hr., further small quantities of trypsin and NaHCO₃ being added at 2 hr. intervals for the first 8 hr. of incubation. In all about 0.2 % of trypsin was added. After incubation the trypsin digest was acidified to pH 1 with HCl, acetone (5 vol.) was added and the precipitate was collected and dried as before. Following the peptic digestion two or three successive tryptic digestions, conducted under similar conditions, were used in the preparation of each specimen of polysaccharide. Typical experiments are summarized in Table III.

Table III. *Properties of typical digest products*

Digest	Globoglycoid			Seroglycoid		
	Weight g.	Carbo- hydrate %	N content %	Weight g.	Carbo- hydrate %	N content %
Pepsin	2.7	30	8.2	5.3	20	10.5
1st Trypsin	1.45	44	5.9	2.2	37.5	7.3
2nd Trypsin	1.0	53	4.8	1.4	43.5	5.9
3rd Trypsin	—	—	—	1.15	51	5.3

It appeared that seroglycoid was more resistant to digestion than globoglycoid although in other experiments the differences were less than those shown in Table III.

Properties of the polysaccharides

When first precipitated with acid acetone from aqueous solution the polysaccharides were in the form of sticky gums, but when treated with acetone and ether they were obtained as fine white powders. These were readily soluble in water, giving clear, colourless solutions. Like the polysaccharide from *B. dysenteriae* [Morgan, 1937] the serum protein polysaccharides were soluble in ethylene glycol but were precipitated by addition of acetone. The polysaccharides were not dissolved by glacial acetic acid. The carbohydrate contents, as determined by the colorimetric orcinol method, ranged from 51 to 55%, and the N content from 4.8 to 5.3%. The Molisch reaction was strongly positive at a dilution of 1:1,000,000. The reactions with the biuret reagent, the phenol reagent and with phosphotungstic acid were all negative or very faintly positive. The colorimetric reaction for cystine with the uric acid reagent was very faintly positive after acid hydrolysis. After acid hydrolysis the polysaccharides possessed strong reducing properties as will be seen later, but before hydrolysis the trace of reducing power that could be detected was due to the presence of traces of amino-acids as described in a previous communication [Hewitt, 1938].

Samples of the polysaccharides were hydrolysed by heating for 4 hr. in a boiling water bath with 2.5 *N* HCl, or with 1.5 *N* *p*-toluenesulphonic acid for 2 hr. The reducing power was determined by a modified Hagedorn-Jensen method [Hanes, 1929; Hulme & Narain, 1931; Hewitt, 1938] and hexosamine was determined by a colorimetric method [Hewitt, 1938] based on that of Zuckerkandl & Messiner-Klebermass [1931], Elson & Morgan [1933], Nilsson [1936], and Meyer *et al.* [1936]. Acetyl determinations were carried out after hydrolysis with *p*-toluene sulphonic acid using the technique of Elek & Harte [1936]. Considerable humin formation occurred during the hydrolyses. The results are summarized in Table IV.

Table IV. *Analysis of hydrolysed polysaccharides*

Source	Method of hydrolysis	Carbo- hydrate %	Hexos- amine %	Acetyl %
Globoglycoid	2.5 <i>N</i> HCl	55	22	—
"	1.5 <i>N</i> MePhSO ₃ H	55	17	4.4
"	2.5 <i>N</i> HCl	61	23	—
Seroglycoid	2.5 <i>N</i> HCl	51	18	—
"	1.5 <i>N</i> MePhSO ₃ H	52	16	5.1
"	2.5 <i>N</i> HCl	—	20	—
"	2.5 <i>N</i> HCl	56	23	—

The hydrolysates when heated with orcinol and sulphuric acid developed a colour similar to that given by an equimolecular mixture of galactose and mannose. Assuming, therefore, that the polysaccharide has the composition galactose-mannose-acetylhexosamine the analytical figures for a total carbohydrate content of 55% should be hexosamine 18.3% and acetyl 4.4%. It will be seen that the above figures are in reasonable agreement with the theoretical expectation.

The amount of "diamino-nitrogen" precipitable by phosphotungstic acid was determined after hydrolysis with concentrated HCl for 4 hr. on an electric sand bath in a flask fitted with an air condenser. Considerable discoloration due to humin formation was again observed. The HCl was removed by evaporation *in vacuo*, the residue was dissolved in water and the humin filtered off. The solution was made alkaline and NH_3 was removed by gently warming the solution *in vacuo*. The solution was acidified with H_2SO_4 and boiled and the diamino-N was precipitated by addition of 5% of phosphotungstic acid in the presence of 5% H_2SO_4 to the boiling solution. After standing overnight in the ice-chest, the precipitate was centrifuged down, washed with ice-cold phosphotungstic acid solution and dissolved in dilute alkali for N determinations. The diamino-N accounted for about 21% of the total N in the polysaccharide. Although some colour was developed when the diazo-reagent was added to the dissolved phosphotungstic acid precipitate, the Knoop bromine reaction was extremely weak so that it would appear that the content of histidine was not great. It is interesting to note that in the case of egg albumin polysaccharide Neuberger [1938] was unable to account for that portion of the phosphotungstic acid precipitate which was not ammonia.

The optical rotatory powers of the polysaccharides were examined and they were found to be laevorotatory, but after acid hydrolysis they became dextro-rotatory as shown in Table V.

Table V. *Specific rotations of polysaccharides*

Polysaccharide from	[α] ₅₄₆₁	
	Before hydrolysis	After hydrolysis
Globoglycoid	-20°	+32°
"	—	+37°
Seroglycoid	-20°	+34°
"	-17°	+31°

Ultra-violet absorption spectra

In view of the value of ultra-violet absorption spectra in differentiating proteins according to their content of certain aromatic amino-acids [Dhéré, 1909; Stenström & Reinhard, 1925; Smith, 1929; Hewitt, 1929; Coulter *et al.* 1936; Holiday, 1936] the absorption spectra of the polysaccharides and of other digest products were examined using a Hilger quartz spectrograph and photometer. In Figs. 1 and 2 are seen the absorption spectra in alkaline solution during successive stages in the digestion of globoglycoid and seroglycoid. The gradual diminution of the absorption in the neighbourhood of wave-lengths 2800 and 2900 Å is seen, showing that tyrosine and tryptophan are split off at a relatively early stage in the digestion, leaving the polysaccharide almost entirely free from these amino-acids. The absorption spectra of the polysaccharides themselves under different conditions are shown in Fig. 3, plotted on a different scale, the solutions examined being four times more concentrated than

Fig. 1.

Fig. 2.

Fig. 1. Ultra-violet absorption spectra during successive stages of seroglycoid digestion (1 cm. layer; 0.1%).

Fig. 2. Ultra-violet absorption spectra of globoglycoid and its digestion products (0.1%; 1 cm. layers).

Fig. 3. Ultra-violet absorption spectra of globoglycoid polysaccharide in 0.1 *N* NaOH and 0.1 *N* HCl (0.4% solution; 1 cm. layers).

those in Figs. 1 and 2. It will be seen that an absorption band in the neighbourhood of 2650 Å. is clearly seen in acid solution but this is apparently obscured by an increase in the general absorption in alkaline solution. The position of the peak of the band is fairly close to that of phenylalanine but is well removed from that of tryptophan or tyrosine. Polysaccharides like agar show no absorption bands in this region of the spectrum.

Trypsin and pepsin polysaccharides

The method outlined above for isolating polysaccharides was applied to trypsin and pepsin themselves. No appreciable amount of polysaccharide was obtained from commercial trypsin, but from commercial pepsin a small amount of polysaccharide was isolated. Pepsin (10 g.) in aqueous solution was allowed to

Fig. 4.

Fig. 5.

Fig. 4. Pepsin autolysis products; ultra-violet absorption spectra in 0.1 *N* HCl (0.4 % solutions; 1 cm. layers).

Fig. 5. Ultra-violet absorption spectra of pepsin; autolysis products in 0.1 *N* NaOH (0.4 % solutions; 1 cm. layers).

autolyse at pH 1.1 for 18 hr. at 45°. Addition of acetone (2.5 vol.) produced a precipitate which, when dehydrated, was a white powder weighing 0.2 g., containing 54 % of carbohydrate and having a rotatory power $[\alpha]_{5461}$ of -35° . Addition of more acetone (2 vol.) to the mother liquors yielded a peptone (1.3 g.) containing 19 % of carbohydrate and 11 % of N with $[\alpha]_{5461} -105^\circ$. The ultra-violet absorption spectra of these products are shown in Figs. 4 and 5 and it will be seen that the general characteristics of the serum protein polysaccharides are encountered again, with the absorption band visible in acid solution, but obscured under alkaline conditions, although the extinction coefficients are quite

different. Landsteiner & Chase [1936] have prepared from pepsin, by a process involving heating, a polysaccharide reacting with blood group A immune sera and this polysaccharide had rather different properties. The amount of polysaccharide contributed by the enzyme in the protein digests was negligibly small.

Initial effects of enzymes on proteins

A preparation of seroglycoid containing 6.3% of carbohydrate was subjected to the action of pepsin at pH 1.6 for only 5 min. at 45°. The mixture was then neutralized and fractionated with $(\text{NH}_4)_2\text{SO}_4$. The properties of the fraction are summarized in Table VI.

Table VI. *Properties of fractions of seroglycoid digested for 5 min.*

Saturation with $(\text{NH}_4)_2\text{SO}_4$ %	Amount of fraction g.	Carbohydrate content %	Optical rotation [α] ₅₄₆₁	Heat coagulability
33	0.28	3.0	-90°	+
50	0.88	6.6	-90°	+
66	0.26	15.0	-94°	-
100	0.18	24.0	-66°	-

It will be seen that this very brief treatment with pepsin had split seroglycoid into fractions of entirely different properties from the original protein. The last fraction in the table is not coagulated on heating and has a very high carbohydrate content, properties which render it of considerable interest which will be discussed later.

The behaviour of globoglycoid after brief pepsin digestion was different. After 5 min. treatment with pepsin at 43° about half the protein was precipitated by one-third saturation with $(\text{NH}_4)_2\text{SO}_4$ although the original material required 45–55% saturation for precipitation, but it was not found possible to isolate a high-carbohydrate fraction as in the case of seroglycoid.

Antitoxic sera have been purified by peptic digestion [Parfentjev, 1936; Pope, 1939]. A pseudoglobulin solution containing haemolytic streptococcus antitoxin was submitted to a process similar to that of Pope [1939]. After brief peptic digestion at room temperature in the presence of triresol some inactive proteins were removed by heat coagulation at 55° and the antitoxin was precipitated by 50% saturation with $(\text{NH}_4)_2\text{SO}_4$. Addition to the mother liquors of more $(\text{NH}_4)_2\text{SO}_4$ precipitated a sticky gum which dissolved readily when dialysed. This material comprised some 5% of the original protein, it gave a strongly positive Molisch reaction and contained over 8% carbohydrate whilst the original protein contained only half this amount. The material was not precipitated by 3% trichloroacetic acid and the main bulk remained uncoagulated after heating, but it was precipitated by phosphotungstic acid.

DISCUSSION

That the polysaccharide present in proteins forms an integral part of the protein molecule and is not merely a loosely bound contaminant is shown by the difficulty of removing this "bound carbohydrate". Prolonged fractionation with salts, isoelectric precipitation, dialysis, heat coagulation and even specific flocculation between antigen and antibody all failed to remove the carbohydrate present in serum proteins [Hewitt, 1934] and Neuberger [1938] has shown that recrystallization and ultra-filtration failed to remove the carbohydrate from egg-albumin. In order to separate the carbohydrate moiety from

the protein drastic chemical hydrolysis or proteolytic enzyme digestion is necessary. It is not proposed to review historically the development of knowledge of the nature of the carbohydrate group present in proteins but it should be mentioned that the presence of glucosamine was detected very early [Seeman, 1898; Langstein, 1902] and later mannose was found [Fränkel & Jellinek, 1927; Levene & Mori, 1929; Rimington, 1929; 1931; Neuberger, 1938]. Modification of the Tillmans & Philippi [1929] orcinol colour reaction led Sørensen & Haugaard [1933] to conclude that the carbohydrate present in serum proteins is galactose-mannose-glucosamine. The high figures of Sørensen [1938] herself for glucosamine are not in agreement with this view, but it is possible that all her glucosamine figures are too high since her ratio of glucosamine to mannose in ovalbumin is 0.82 compared with Neuberger's [1938] ratio of 0.5.

Neuberger [1938] has evolved a method of isolating the polysaccharide from protein digests in extremely good yield and of a high degree of chemical purity. The object of the present method was to prepare a product which should have suffered the very minimum of chemical change so that the final product should approximate to the complex present in the protein molecule even at the expense of chemical purity and, to this end, even the mild acetylation and deacetylation used by Neuberger has been omitted. It is hoped that step-wise enzymic digestion followed by extremely mild and simple methods of isolating the product may eventually lead to further knowledge of the mode of linking of polysaccharide in the protein molecule.

In the present work hexosamine and acetyl radicle determinations are in agreement with the presence in serum proteins of galactose-mannose-acetyl hexosamine. The colour reactions with orcinol and sulphuric acid of both the intact proteins and of the separated polysaccharides also agree with the presence of that carbohydrate unit. Colorimetric evidence can never be considered as entirely satisfactory, but in view of the mixture of sugars present it seems that the complete elucidation of the structure of serum protein polysaccharides is likely to be a matter of some complexity requiring considerable amounts of material.

The presence of acetylhexosamine in the polysaccharide is not surprising since it is a constituent of the polysaccharides obtained from a number of natural sources. Like the polysaccharides obtained from *B. dysenteriae* [Morgan, 1937] and ovalbumin [Neuberger, 1938] by methods avoiding the use of drastic chemical hydrolysis, the carbohydrate from serum proteins still contains some non-carbohydrate nitrogenous material. That it is not due to contamination with undigested protein is shown by the absence of various colour reactions and by the ultra-violet absorption spectra which differs from those of serum proteins in the absence of bands corresponding to tyrosine and tryptophan. Rimington [1931] experienced great difficulty in removing nitrogenous material present in the polysaccharide complex even when he used drastic hydrolytic processes which also decomposed some of the carbohydrate itself.

Seroglycoid and serum mucoid

The behaviour of serum proteins when subjected for a very brief period (e.g. 5 min.) to the action of pepsin is of some interest. In the case of seroglycoid, the protein was split into fractions of entirely different properties. One of these fractions was precipitable between the limits of 66 and 100 % saturation with $(\text{NH}_4)_2\text{SO}_4$, it was not heat-coagulable and it contained some 24 % of carbohydrate. It is remarkable that these properties correspond to those ascribed to serum mucoid [Zanetti, 1897; Bywaters, 1909; Rimington, 1933]. Despite

several attempts the present author has failed to detect the presence in normal blood serum of a protein corresponding to mucoid and it seems probable that the mucoid previously described was due to the decomposition of seroglycoid either by the drastic method of heating with acid or by the action of proteolytic enzymes present in serum. It has been shown above that after brief treatment with proteolytic enzymes material with properties corresponding to mucoid may be obtained from seroglycoid, and the protein-disaggregating effect of the fibrinolysin present in serum has been described by Pope [1939].

Globoglycoid did not yield a product of this kind when subjected to brief proteolysis and this presents additional evidence of the difference between seroglycoid and globoglycoid.

SUMMARY

1. A series of digestions with pepsin and trypsin followed by acid acetone precipitations provide a simple method of isolating the carbohydrate complex present in the protein molecule.

2. A study has been made of some of the properties of the polysaccharides present in the serum proteins seroglycoid and globoglycoid.

3. The evidence so far available is compatible with the view that the carbohydrate present in serum proteins contains galactose-mannose-acetylhexosamine.

4. Like polysaccharides from other natural sources the preparation contains non-carbohydrate nitrogenous material which may be removed by more drastic chemical procedures.

5. Very brief treatment of seroglycoid with pepsin yields a partial break-down product with the same properties as those ascribed to "serum mucoid" and it is suggested that this latter product, which could not be detected in normal serum, is produced by disaggregation of seroglycoid.

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