CCII. THE POLYSACCHARIDE CONTENT AND REDUCING POWER OF PROTEINS AND OF THEIR DIGEST PRODUCTS

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THE carbohydrate contents of proteins are of value in differentiating individual serum proteins and in following the course of fractionation processes [Hewitt, 1934–1938]. Apart from these practical considerations, however, the presence of carbohydrate groups or polysaccharides in proteins is of theoretical interest also, from the point of view of the structure of the protein molecule.

In order to separate the polysaccharide completely from protein material very drastic chemical treatment is necessary [Levene & Mori, 1929; Rimington, 1931; Bierry, 1934; Onoe, 1936]. Whilst this may be taken as evidence that the polysaccharide forms an integral part of the protein molecule, it also raises doubts whether the constitution of the polysaccharide present in the protein molecule is necessarily the same as that of the isolated material, particularly when the yield of purified polysaccharide is very low. Any data obtainable without previous isolation of the polysaccharide are therefore of interest, so it was decided to attempt to correlate the reducing powers of various proteins with their polysaccharide contents. Below are presented data on the carbohydrate contents, glucosamine contents and reducing powers of various proteins, and of the effect of various hydrolytic and enzymic digestion processes.

EXPERIMENTAL

Carbohydrate determinations. A modification of the orcinol method [Tillmans & Philippi, 1929; Sørensen & Haugaard, 1933] was used as previously described, colorimetric comparison being made either in a Stufenphotometer, or photoelectrically using a green filter. For serum proteins results are quoted in terms of galactose-mannose and for egg proteins in terms of mannose.

Reducing powers. The reducing powers of solutions were determined by the Hagedorn & Jensen [1923] method as modified by Hanes [1929] and Hulme & Narain [1931] except that deproteinization with $ZnSO_4$ and NaOH was omitted, the protein solution being heated with the ferricyanide reagent.

Glucosamine determinations. The colorimetric methods described by Zuckerkandl & Messiner-Klebermass [1931], Elson & Morgan [1933], Nilsson [1936], and Palmer *et al.* [1937] were modified as follows for the determination of the glucosamine content of proteins. Hydrolysis was effected by heating the protein solution in a boiling water bath for 4 hr. with 2*N* HCl, and the solution was cooled and neutralized with NaOH. The temporary existence, during the neutralization, of a slightly alkaline reaction was not found deleterious, although the importance of avoiding alkalinity has been stressed by some workers. After filtration through filter paper to remove humin 1 ml. of the solution, diluted to contain 0·05–0·3 mg. glucosamine was heated with 1 ml. acetylacetone solution (2 % in 0·5*N* Na_2CO_3) in a boiling water bath for 15 min. Irregular results were obtained until a simple but effective form of reflux condensation was resorted to. The solutions were contained in $6 \times 0{\cdot}75$ in. test tubes drawn out into a

constriction at the upper end and resting in this narrow neck was placed a 3 × 0·4 in. test tube filled with cold water. After heating, the tubes were cooled by immersion in cold water and 5 ml. of alcohol were added. The ethyl alcohol had been purified by heating with Ag_2O and NaOH and redistilled. 1 ml. of Ehrlich's reagent was added and the colour was allowed to develop for 30 min. Ehrlich's reagent was prepared by dissolving 0·8 g. purified dimethylaminobenzaldehyde in 30 ml. purified ethyl alcohol and adding 30 ml. concentrated HCl. The *p*-dimethylaminobenzaldehyde was purified by dissolving in concentrated HCl, diluting with water and adding sodium acetate solution. The first yellowish precipitates obtained were rejected, and further addition of sodium acetate produced a white precipitate, which yielded a satisfactory reagent. Colorimetric comparisons were carried out in a Stufenphotometer using 1 cm. cells and an S 53 filter, glucosamine solutions being examined at the same time.

Protein preparations. The blood serum proteins used were prepared as described previously. The casein, edestin, gelatin and amino-acids investigated were commercial specimens. The ovalbumin specimens used were repeatedly crystallized and the ovomucoid was obtained from the mother liquors after the successive removal of globulins, albumin and conalbumin. The ovalbumin was quite colourless but the ovomucoid was lemon yellow in colour and when pure contained no heat-coagulable protein.

Results. In Table I are summarized mean values for the glucosamine and carbohydrate contents of various proteins. The seroglycoid and globoglycoid preparations contained some crystalbumin and had correspondingly decreased carbohydrate contents.

Table I. *Galactose-mannose and glucosamine contents of proteins of blood serum and egg white*

(g. per 100 g. protein)

Protein	Galactose-mannose (G.-M.)	Glucosamine (Ga.)	Ratio $\left(\frac{\text{G.-M.}}{\text{Ga.}}\right)$
Seroglycoid	5·6	2·7	2·1
Crystalbumin	<0·05	<0·05	—
Globoglycoid	6·2	2·9	2·1
Pseudoglobulin	2·2	1·1	2·0
Euglobulin-II	2·7	0·8	3·5
Ovalbumin	1·8*	0·8	2·3
Ovomucoid	10·5*	9·5	1·1

* In the case of egg white proteins results are quoted in terms of mannose.

It has been shown in previous communications that purified crystalbumin contains no carbohydrate and in the present experiments it was found, as anticipated, that no glucosamine could be detected in crystalbumin hydrolysates. In the case of the other serum proteins in which the polysaccharide present is generally considered to be galactose-mannose-glucosamine the ratio of galactose-mannose content to glucosamine content should be 2 : 1. With seroglycoid, globoglycoid and pseudoglobulin the deviation from this ratio does not exceed the probable experimental error; in the case of euglobulin-II the ratio appears to be high but the reason for this is not at present apparent.

In the case of ovalbumin the mannose : glucosamine ratio is in agreement with the presence of dimannose-glucosamine but with ovomucoid there are equimolecular amounts of mannose and glucosamine suggesting that the polysaccharide present is built up of mannose-glucosamine units. This will be commented upon later.

In Table II are summarized the reducing powers of various protein substances, using the Hagedorn-Jensen method; the figures quoted are in terms of glucose. The polysaccharide contents are calculated in terms of galactose-mannose-glucosamine for the serum proteins, dimannose-glucosamine for ovalbumin and mannose-glucosamine for ovomucoid.

Table II. *Polysaccharide contents and reducing powers of various proteins, etc.*

(g. carbohydrate per 100 g. protein)

Protein	Polysaccharide content	Reducing power (glucose)
Euglobulin-II	4·1	11·9
Pseudoglobulin	3·3	12·5
Globoglycoid	9·3	13·5
Crystalbumin	>0·05	17·0
Seroglycoid	8·4	14·4
Ovalbumin	2·6	9·5
Ovomucoid	20·0	23·0
Casein	0·5	7·5
Edestin	>0·1	7·5
Gelatin	0·5	0·9
Cystine	0	15·0
Glutamic acid	0	0·1
Glycine	0	0
Tryptophan	0	80·0
Tyrosine	0	100·0

It will be seen at once from Table II that the reducing power of the proteins is not dependent upon the polysaccharide content. Of the serum proteins crystalbumin has the highest reducing power although it contains no carbohydrate.

Carbohydrate, when present, probably contributes to the reducing power but it is necessary to seek some other constituent of the protein molecule, to account for a considerable portion, or in some cases all, of the reducing power. Glycine and glutamic acid were found to have no reducing power, cystine had about one-seventh of the reducing power of glucose, tyrosine had approximately the same reducing activity as dextrose and tryptophan four-fifths of the reducing power.

It is significant that gelatin has an extremely low reducing activity since it is the only protein investigated containing negligibly small amounts of cystine, tryptophan and tyrosine in addition to having a low carbohydrate content.

The Hagedorn-Jensen method for the determination of reducing power includes the liberation of free iodine at one stage and it was thought possible

Table III. *Comparison of the apparent reducing powers of proteins, etc., at room temperature and when heated with ferricyanide*

Protein	Apparent reducing power: When heated	Apparent reducing power: At room temperature
Crystalbumin	17·0	0·5
Pseudoglobulin	12·5	0·4
Globoglycoid	13·5	1·1
Seroglycoid	14·4	0·9
Casein	7·5	1·5
Edestin	7·5	1·5
Cystine	15·0	1·0
Tryptophan	80·0	60·0
Tyrosine	100·0	45·0

that iodination of the protein might account for a substantial portion of the reducing power. In the next series of experiments, therefore, heating of the protein with the ferricyanide was omitted and the protein was mixed with the reagents in the cold and titration was conducted immediately. The figures obtained are summarized in Table III.

In the case of tryptophan, and to a less extent with tyrosine "cold" reducing power, probably associated with iodination, is very marked, but in the case of the serum proteins from 92 to 97 % of the Hagedorn-Jensen reducing power is observed only when the protein is heated with ferricyanide.

Effects of acid hydrolysis

Heating with 2*N* HCl for 4 hr. was sufficient to liberate the glucosamine present in the protein, but this heating had no appreciable effect on the galactose-mannose content of the protein as determined by the usual orcinol method. In most cases the effect of the heating with acid on the reducing power was also negligible. The exceptions were ovomucoid, in which the reducing power increased from 23 to 27 % (calculated as dextrose), and crystalbumin, in which the reducing power fell from 17 to 8 % during the heating with acid.

Enzymic hydrolysis

Solutions of pseudoglobulin, seroglycoid, globoglycoid and ovalbumin containing 0·125*N* HCl and 0·4 % pepsin were incubated at 52° for 18 hr. Acetone (from 4 to 9 vol.) was added to the cooled digestion mixture and the precipitate was centrifuged off, washed with acetone and ether and dried *in vacuo*. The polysaccharide contents of the dried products were 19, 19, 24 and 18 % respectively—values from three to six times higher than those of the original proteins. Products containing even higher carbohydrate contents may readily be obtained by modification of the conditions, as the following example illustrates. A globoglycoid solution of *p*H 1·03 containing 0·4 % pepsin was incubated at 52° for 18 hr. The digest product precipitated by 80 % acetone contained 35 % of polysaccharide (calculated as galactose-mannose-glucosamine).

The effect of experimental conditions on the digestion of pseudoglobulin containing diphtheria antitoxin is seen in Table IV. In each case 5 ml. of 3·5 % pseudoglobulin were treated with varying amounts of HCl and pepsin and the mixture was incubated for 2 hr. at 52°. After neutralizing the digestion mixture the flocculation titre was determined under standardized conditions.

Table IV. *Pepsin digestion of diphtheria antitoxin under different experimental conditions*

Digest no.	0·1*N* HCl ml.	0·1 % pepsin ml.	Flocculation Titre A.U./1 ml.	Flocculation Time min.
1	2·0	0·5	0	—
2	1·1	0·5	250	14
3	0·9	0·5	350	14
4	0·9	0·1	350	80
5	0·7	0·5	450	22
6	0·5	0·5	500	45
7	0·5	2·0	550	14
8	0	0	600	38

It will be seen that the destruction of antitoxin appears to be due to acid and that the presence of pepsin has some protective effect as claimed by Parfentjev [1936]. The digest products in some cases actually flocculate more rapidly than the original product although chemical manipulation usually lengthens the flocculation time. The accelerated flocculation of digested material was noted by Pope & Healey [1938]. An attempt was made to purify digest product No. 3 by first removing the material precipitated by addition of 0·5 vol. acetone and then collecting and drying the product precipitated on addition of a further 1·5 vol. of acetone. When dissolved in water the flocculation time was still only 17 min. This indicates a much greater degree of stability than that of undigested antitoxin. Purification of digest No. 7 by this method was less successful, the flocculation time increasing to 43 min. The carbohydrate content of an experimental batch of digest antitoxin kindly supplied by Mr Pope was 2·5%, a figure falling within the limits observed for normal horse serum pseudoglobulin.

Discussion

It has been suggested that ovalbumin contains no polysaccharide and that the carbohydrate detected in specimens of this protein is due to the presence of ovomucoid [Levene & Mori, 1929]. The figures given by Levene & Mori for the polysaccharide content of these proteins were obtained by actual isolation, a process which would be expected to give a low yield. The results reported, 0·26% for ovalbumin and 5·1% for ovomucoid are, in fact, much lower than the figures, 2·7 and 21% respectively, obtained in the present investigation. Figures of the same order as the present values have been reported by Sørensen [1934] and Onoe [1936]. Repeated crystallization has failed to reduce the carbohydrate content of ovalbumin.

In ovalbumin the ratio of mannose to glucosamine approximates to 2 : 1 and in ovomucoid it is close to 1 : 1. It would seem, therefore, that the polysaccharides present in the two proteins are different, in one case being composed of dimannose-glucosamine and in the other of mannose-glucosamine units. In the present work no attempt has been made to distinguish between glucosamine and its acetyl derivative. The latter is said to be present in ovomucoid by Onoe [1936]. There is thus considerable weight of evidence that ovalbumin contains polysaccharide in the molecule, unlike serum crystalbumin: the carbohydrate content of serum albumin has been traced to the presence of seroglycoid, globoglycoid, etc. [Hewitt, 1934; 1937; 1938].

It was thought possible that determination of the reducing power of proteins might be of value in gauging the amount and nature of the polysaccharides present in the protein molecule and in following the liberation of polysaccharides during the hydrolysis of proteins. The Hagedorn-Jensen method has been used for this purpose. It has been found, however, that the reducing power of proteins bears little relation to the carbohydrate content. Crystalbumin, for example, which contains no polysaccharide has the highest reducing power of all the serum proteins investigated. With each protein the reducing power was greater than could be accounted for by the carbohydrate content even assuming that the whole of the carbohydrate was free to exert its whole reducing effect.

Various amino-acids were examined and it was found that although glycine and glutamic acid did not reduce the reagents used, other amino-acids had very pronounced reducing effects. Cystine had 15% of the reducing power of dextrose and tryptophan 80%, whilst tyrosine had about the same reducing activity as

an equal weight of dextrose. That the reducing power of proteins is due in part to amino-acids such as cystine, tyrosine and tryptophan, receives support from the behaviour of gelatin. Gelatin was the only protein material investigated which is deficient in these particular amino-acids as well as in carbohydrate and it was the only protein with a negligibly small reducing activity. Four hours' heating with 2*N* HCl is sufficient to liberate glucosamine from serum and egg white proteins, but the reducing power is not increased by this treatment except in the case of ovomucoid where there was an increase from 23 % (calculated as glucose) before heating to 27 % after hydrolysis. Meyer *et al.* [1936] have attempted to show by measurements of reducing power that the bacteriolytic activity of lysozyme is due to splitting off polysaccharides from glycoproteins, but it would seem from the present experiments that the technical difficulties in following the liberation of carbohydrates from proteins by reducing power determinations are very great. In the case of crystalbumin the effect of acid hydrolysis was an unexplained fall in the reducing power to about half its original value.

By digesting serum and egg proteins with pepsin and precipitation with acetone, peptones may readily be prepared with high carbohydrate contents, the highest carbohydrate content so far observed being 35 %. Such carbohydrate-rich peptones will receive further study. In a recent short note Ogston [1938], using a commercial enzyme preparation, split off rather less than half the carbohydrate from a specimen of pseudoglobulin, leaving one-third of the protein with an unchanged ultracentrifugal sedimentation constant. Full experimental details are lacking but it is possible that one of the constituents of the pseudoglobulin fraction [Tiselius, 1937; Hewitt, 1934; 1938] may be preferentially hydrolysed.

Summary

1. The glucosamine contents of pseudoglobulin, globoglycoid and seroglycoid are in rough agreement with the generally accepted view that the polysaccharide present in the proteins is composed of units having the composition galactose-mannose-glucosamine. Crystalbumin contains no glucosamine.

2. The glucosamine contents of the egg white proteins are in agreement with the view that ovomucoid contains mannose-glucosamine and that ovalbumin contains dimannose-glucosamine.

3. Evidence is presented disproving the view that the polysaccharide present in ovalbumin is due to the presence of ovomucoid.

4. The reducing power of proteins has been traced to the presence of various amino-acids such as tyrosine, tryptophan and cystine as well as to carbohydrates.

5. The reducing power of many proteins is not increased after acid hydrolysis and the course of hydrolysis cannot therefore be followed by reducing power determinations.

6. Carbohydrate-rich peptones may readily be prepared from proteins by peptic digestion followed by acetone precipitation.

7. A few observations have been made on the effect of peptic digestion on diphtheria antitoxin globulins.

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