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Carbohydrates in Protein

2. THE HEXOSE, HEXOSAMINE, ACETYL AND AMIDE-NITROGEN CONTENT OF HEN'S-EGG ALBUMIN*

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(*Received* 15 *March* 1960)

One of us described more than 20 years ago (Neuberger, 1938) the isolation, from an enzymic hydrolysate of crystalline egg albumin, of a polysaccharide containing mannose, glucosamine and some unidentified nitrogenous material. Comparison of the hexose and hexosamine content of the protein and of the isolated polysaccharide together with the estimation of the molecular weight of the latter indicated that all the carbohydrate was present as one unit in the egg-albumin molecule. The analytical data obtained also suggested that, on the basis of the then accepted value of 40 000 for the molecular weight of the protein, egg albumin contains four residues of mannose and two of glucosamine. As the methods of isolation used involved treatment with acetic anhydride, no information could be obtained as to whether the glucosamine residue was present in the protein as its *N*-acetyl derivative. By the methods then available it was not possible to obtain any indication of the linkage between the carbohydrate and the peptide chain. Some revision of the data is also necessary in view of the fact that the molecular weight of egg albumin is now generally believed to be 45 000 (see Warner, 1954).

This work was taken up again some years ago and a short report of some new findings has appeared (Johansen, Marshall & Neuberger, 1958). Preliminary communications on the same subject from other Laboratories have also been recently published (Cunningham, Nuenke & Nuenke, 1957; Jevons, 1958). In the present paper the analytical methods used are critically examined and some of the earlier figures revised. Chromatographic evidence obtained with a hydrolysate of the polysaccharide, the preparation of which from egg albumin has been briefly described (Johansen *et al.* 1958), indicated that the only sugars present are mannose and glucosamine.

Mannose

Carbohydrate in proteins has generally been determined by reactions involving heating the material with conc. H_2SO_4 in the presence of an appropriate phenol, nitrogen base or related compound, giving rise to products, the intensities of the colours of which are measured. These methods have been applied to egg albumin. The orcinol–H_2SO_4 method as described by Winzler (1955) was used; but the extinction was read at a wavelength of 505 mμ, since it was found that, with a mannose standard, the spectrum of the coloured product had a steep curvature at a wavelength of 540 mμ but not at 505 mμ, and secondly that rectilinearity was achieved on plotting absorption against amount of mannose at 505 mμ but not at 540 mμ. A five-times recrystallized [$(NH_4)_2SO_4$] preparation

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of egg albumin (preparation M) was found to contain 1·93, 1·83 and 1·98, 1·88 % of mannose, and a five-times recrystallized (Na_2SO_4) sample (preparation P) 1·95, 1·96, 2·02 and 2·02 %. The spectra obtained with mannose, whole-egg albumin and the isolated polysaccharide (Johansen *et al.* 1958) were identical.

The orcinol–H_2SO_4 procedure as described by Hewitt (1937), in which the reagent concentrations and the time of heating are slightly different from those of Winzler (1955), was also applied. This method also gave about 2 % of mannose and the spectra obtained with mannose and egg albumin were identical. Neither of the methods appears to be influenced by the presence of amino acids or amino sugars when present in concentrations as high as are normally found in egg albumin. Previous results obtained have tended to be slightly lower than these values: 1·7 % by Sørensen (1934) and 1·8 % by Neuberger (1938) and Hewitt (1938).

With a solution of tryptophan-free purified glycopeptide, the estimation of mannose by the anthrone method, as described by Dische (1955), and by the Winzler orcinol method gave approximately the same value.

A problem which arises in connexion with the measurement of carbohydrate by such methods lies in ascertaining whether a solution containing a given quantity of a sugar will give a colour yield identical with one containing the same amount of sugar but combined in polysaccharide linkage. The work of Vasseur (1948) indicates that mannan after treatment for 20 min. with orcinol–H_2SO_4 reagent gives a spectrum 'which almost completely corresponds with that of mannose, and the dextran curve nearly parallels that of the glucose analogue'. The extinction of the polysaccharides, however, amounted to only about 90 % of those of the monosaccharides. It seems that values might tend to be a little low when obtained by such techniques.

The neutral-sugar content of hen's-egg albumin is thus probably five residues (2 %)/45 000 g. of protein, and that this sugar is largely mannose is indicated by (i) the identity of the spectra obtained with mannose and egg albumin in the orcinol–H_2SO_4 methods, and (ii) the preparation of the phenylhydrazone and the *p*-bromophenylhydrazone of mannose in about 70 % yield of that calculated from the orcinol value from a purified polysaccharide (Neuberger, 1938). Further support for such conclusions in the case of the polysaccharide is given when one considers the agreement obtained between the carbohydrate as estimated by the orcinol–H_2SO_4 methods (47–50 %) and the amount of fermentable sugar (56 %) in a 1·5N-HCl hydrolysate (Neuberger, 1938). However, confirmation of this conclusion by methods not based on colorimetry is desirable. Difficulties may still be encountered if the neutral sugars are combined with C-1 of hexosamine (Gottschalk & Ada, 1956).

Hexosamine

The estimation of the amino sugar content of proteins presents some special difficulties. The discussion below is centred largely on glucosamine, since egg albumin appears from the evidence given below to contain only this amino sugar. The principal methods available for the determination of amino sugars are the colorimetric methods of Elson & Morgan (1933) and its many modifications, that of Dische & Borenfreund (1950) and the deamination method due to Tracey (1952), as well as procedures involving isolation. All of these methods were applied to the amino sugars which have been liberated from the mucoprotein by acid hydrolysis and two problems arise in connexion with this: the hexosamine must be quantitatively liberated, and the possibility of its destruction must be considered.

Moggridge & Neuberger (1938) pointed out that substances such as α-methyl *N*-acetylglucosaminide can be cleaved under conditions of acid hydrolysis in two ways, as shown in Fig. 1. Hydrolysis may proceed either by cleavage of the glycosidic bond followed by that of the acetamido linkage (pathway I), or the reactions may occur in inverse order (pathway II), the former being the favourable pathway since it was shown that α- and β-methyl glucosaminide are extremely resistant to acid hydrolysis. Thus, for example, the rate of

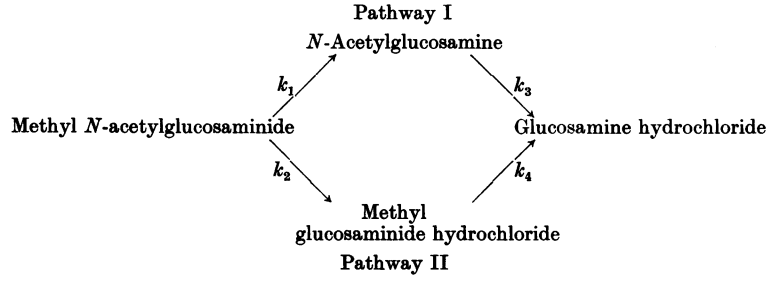

Fig. 1. Alternate pathways for hydrolysis of methyl *N*-acetylglucosaminide.

hydrolysis of the glycosidic linkage in α-methyl *N*-acetylglucosaminide in N-HCl at 100°, calculated from the results of Moggridge & Neuberger, is $1{\cdot}05 \times 10^{-3}$ hr.$^{-1}$, and the rate of hydrolysis of α-methyl glucosaminide under identical conditions is about $4{\cdot}05 \times 10^{-6}$ hr.$^{-1}$, the ratio of rates being of the order of 250 (Moggridge & Neuberger, 1938; Neuberger & Pitt-Rivers, 1939). The bearing of these results on the quantitative estimation of the amino sugar and neutral sugar content of mucoids and glycoproteins was appreciated at the time, but the problem was not clearly pointed out (see, however, Kent & Whitehouse, 1955) until the paper by Gottschalk & Ada (1956) appeared. Foster, Horton & Stacey (1957) made a study of the acid hydrolysis of a number of glucosamine derivatives and showed that glucosamine was not quantitatively liberated in general on hydrolysis in 1·16N-HCl at 100°. Any glucosamine combined in glycosidic linkage which was to be cleaved by the unfavourable pathway II would then be liberated only very slowly indeed, and possibly at such a rate that irreversible destruction balanced its rate of liberation. In order to ensure that the reaction should proceed by the more favourable pathway I, conditions of hydrolysis must be chosen so that the rate of hydrolysis of the glycosidic bond exceeds that of the acetamido bond as much as possible.

From the information available it would appear that the heat of activation of the glycosidic bond in α-methyl *N*-acetylglucosaminide is of the order of 36 000 cal./mole, while that of the acetamido bond is about 19 000 cal./mole (Moggridge & Neuberger, 1938). Although these values may vary from substance to substance it seems reasonable to assume that in general an increase in temperature will favour rupture of the glycosidic bond relative to that of the acetamido bond. Thus in N-HCl the ratio of the rate constants for glycoside to acetamido hydrolysis is 1·6 at 61·25° and 3·4 at 80°, so that a higher temperature would seem to favour the more desirable pathway I. The effect of acid concentration on the relative rates of hydrolysis (k_1/k_2) must also be considered. With methyl glucosaminide an increase in acid concentration from N- to 2·5N-HCl at 100° causes a nearly fivefold increase of rate constant (Moggridge & Neuberger, 1938). Such large increases in the rate of hydrolysis with increase in acid concentration have been observed in general for glycosides (Bunton, Lewis, Llewellyn & Vernon, 1955; Hantzsch & Weissberger, 1927). The amide-type bond shows much smaller increases in its rate of hydrolysis with acid concentration, however (Krieble & Holst, 1938). Thus conditions for obtaining the maximum yield of hexosamine from a glycoside would seem to involve the use of high acid concentrations and high temperatures, but the conditions must not be too vigorous since destruction of the amino sugar may occur.

We do not know the intrinsic stability of the glycosidic bonds in egg albumin and have therefore obtained values with various acid concentrations for different periods of time, hoping that the maximum value obtained approximates to the true glucosamine content of the protein. However, complete liberation of the glucosamine may not have occurred, some of the carbohydrate having undergone hydrolysis by the unfavourable pathway II. In such a case, disaccharides may have been formed which are almost completely resistant to acid hydrolysis, as in the hydrolysis of heparin. This might be particularly important if two *N*-acetylglucosamine residues were linked by 1:3 or, less likely, 1:1 (trehalose) linkages.

The glucosamine having been liberated, the most widely used method for its measurement is that due to Elson & Morgan (1933) or one of the numerous modifications thereof. This depends on the condensation of acetylacetone in mildly alkaline solution to give a pyrrole which Elson & Morgan thought to be 3-acetyl-2-methyl-5-(tetrahydroxybutyl)pyrrole. On reaction of the pyrrole with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) a red colour is produced and its intensity is measured. It was not until 1951 that Schloss (1951) showed that there were indeed at least two chromogens formed in the condensation of the amino sugar with acetylacetone, one of which was steam-volatile. This material condensed with Ehrlich's reagent to give a product absorbing maximally at a wavelength of 550 mμ. It was identified as 2-methylpyrrole by Cornforth & Firth (1958) and shown to be the major chromogen in the Elson–Morgan reaction. In the formation of such a pyrrole both the 3-acetyl and 5-tetrahydroxybutyl groups are eliminated, the latter by what is essentially a retroaldol fission involving C-3 of the original glucosamine molecule.

We were very fortunate in receiving in 1957 from Dr C. Cessi, then working at the Rowett Institute, Bucksburn, Aberdeen, details of a method for the quantitative estimation of hexosamine in which the steam-volatile chromogen noted by Schloss is distilled into the Ehrlich's reagent. The method is a modification of an earlier procedure (Cessi, 1952). The new procedure appears to decrease the interference, in the usual Elson–Morgan method, which has been observed when neutral sugars and amino acids are heated together (e.g. Horowitz, Ikawa & Fling, 1950; Vasseur & Immers, 1950; Sideris, Young & Kraus, 1938; Aminoff, Morgan & Watkins, 1952; Immers & Vasseur, 1952). The Cessi modification is also more specific than the original Elson–Morgan method in that it depends on the possibility of a

retroaldol fission occurring. Such a reaction is unlikely to take place with a 3-substituted amino sugar such as muramic acid or 3-methylglucosamine (Neuberger, 1941), and it has in fact been observed by us that these compounds do not give any colour in the Cessi modification of the Elson–Morgan procedure. Differences in the spectra obtained in the direct Elson–Morgan reaction with 3-substituted compounds and 2-amino sugars with a free hydroxyl group in the 3-position have been recorded. Cifonelli & Dorfman (1958) have shown that 3-methylglucosamine and hyalobiuronic acid exhibit maxima at a wavelength of 510 mμ, whereas glucosamine gives a maximum at about 535 mμ. Crumpton (1959) studied the reaction of muramic acid and demonstrated that there is not only a shift in the spectrum compared with that of glucosamine but also that the time of development of the full colour is delayed. It seems fairly clear that 2-methylpyrrole is not the chromogen produced in the case of 3-substituted hexosamines and it seems reasonable to suggest that it is 2-methyl-5-(2:3:4-trihydroxy-1-methoxybutyl)pyrrole where 3-methylglucosamine is involved. The Cessi procedure coupled with the original Elson–Morgan conditions could be used as a further diagnostic method in determining the possible presence of 3-substituted hexosamines.

The determination of the glucosamine content of two preparations of egg albumin, one a five-times crystallized [$(NH_4)_2SO_4$] sample and the other a five-times crystallized (Na_2SO_4) sample, was made. Hydrolysis of each preparation was carried out for 3 and 6 hr. in both 4N- and 5·7N-HCl. Glucosamine was estimated by the direct Elson–Morgan reaction as described by Rondle & Morgan (1955) and also by the Cessi modification. It is noteworthy that both the direct Elson–Morgan and the Cessi procedures give approximately the same results (Table 1). All the values obtained indicate that about three equivalents of glucosamine were liberated from both preparations of egg albumin and this is the minimum amount of the amino sugar that the protein contains. It is not impossible, as discussed above, that there are more than three residues of glucosamine in egg albumin.

The possibility existed that as well as the three moles of glucosamine, a 3-substituted hexosamine was also contained in egg albumin. A synthetic mixture was therefore prepared containing glucosamine and muramic acid in a molar ratio of 3:1, and a hexosamine assay carried out by the method of Rondle & Morgan (1955). The spectrum was observed after 5 min. and again after 18 hr. (cf. Crumpton, 1959). A hydrolysate of egg albumin (preparation M) was similarly examined. These results are shown in Fig. 2, where it is seen that by reading only on the 5 min. curve confusion might arise, yet after 18 hr. there is such a noticeable change in the spectrum of the material containing muramic acid that we may safely conclude that egg albumin does not contain this amino sugar and probably no other 3-substituted hexosamine stable to the hydrolysis under the conditions used.

Quantitative examination of the hexosamine content of a 4N-HCl hydrolysate (8 hr. at 100°) of the purified glycopeptide from egg albumin (Johansen *et al.* 1958), by the procedure of Moore & Stein (1951) as developed by Eastoe (1954), has been carried out. A sample of the glycopeptide containing 720 μg. of mannose was found to give 2·28 μmoles of an amino sugar in a position on the column corresponding to that of glucosamine, which is equivalent to 2·85 residues of glucosamine on the basis of 5 moles of mannose; there were also 1·55 moles of ammonia.

Table 1. *Glucosamine content of egg albumin after hydrolysis* (100°) *under various conditions*

Egg-albumin preparation	Concn. of HCl (N)	Time of hydrolysis (hr.)	Method of analysis	Glucosamine in protein (%)	Moles of glucosamine/ 45 000 g. of protein
$(NH_4)_2SO_4$-crystallized (preparation M)	4	3	Rondle & Morgan	1·13, 1·18	2·83, 2·95
	4	6	Rondle & Morgan	1·29, 1·23	3·21, 3·07
	5·7	3	Rondle & Morgan	1·35, 1·23	3·37, 3·07
	5·7	6	Rondle & Morgan	1·17, 1·18	2·93, 2·95
	4	3	Cessi	1·15, 1·12	2·89, 2·82
	4	6	Cessi	1·17, 1·17	2·94, 2·94
	5·7	3	Cessi	1·10, 1·15	2·77, 2·90
	5·7	6	Cessi	1·11, 1·10	2·89, 2·87
Na_2SO_4-crystallized (preparation P)	4	3	Rondle & Morgan	1·29, 1·28	3·21, 3·26
	4	6	Rondle & Morgan	1·15, 1·13	2·88, 2·83
	5·7	3	Rondle & Morgan	1·13, 1·26	2·83, 3·15
	5·7	6	Rondle & Morgan	1·07, 1·20	2·70, 3·00
	4	3	Cessi	1·10, 1·10	2·87, 2·87
	4	6	Cessi	1·22, 1·25	3·05, 3·14
	5·7	3	Cessi	1·21, 1·20	3·04, 3·02
	5·7	6	Cessi	1·17, 1·16	2·94, 2·92

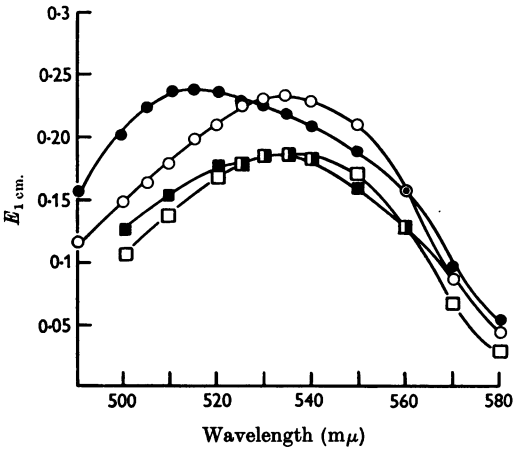

Fig. 2. Spectra given in the Rondle & Morgan (1955) determination of 2-amino sugars. Glucosamine (32 μg.) and muramic acid (3·28:1 molar ratio): ○, extinctions read after 5 min.; ●, extinctions read after 18 hr. Egg-albumin hydrolysate (4N-HCl): □, after 5 min.; ■, after 18 hr.

Table 2. *Hexosamine content (Cessi procedure) of a purified glycopeptide from egg albumin after various conditions of acid hydrolysis* (100°)

Concn. of HCl (N)	Time of hydrolysis (hr.)	Residues of glucosamine/5 moles of mannose
2	3	2·49, 2·51, 2·52, 2·52
4	3	2·96, 2·98
4	6	3·02, 3·02
5·7	3	3·07, 3·07
5·7	6	3·01, 3·07

Hydrolysis of the purified egg-albumin glycopeptide was also carried out in 2N- (3 hr.) and 4N- and 5·7N-HCl (3 and 6 hr.) and the amount of hexosamine liberated was measured by the Cessi procedure. The results of these experiments are given in Table 2, where the number of moles of glucosamine/5 moles of mannose are shown. The hydrolysis in 2N-HCl appears to be less complete than those carried out in 4N- and 5·7N-HCl, results of a similar nature having been noted in connexion with the acid hydrolysis of ovomucoid (Marshall & Neuberger, 1960). The results again indicate that 3 moles of glucosamine/5 moles of mannose are liberated on acid hydrolysis.

Hewitt (1938) has reported a value of 0·8% of hexosamine in egg albumin after hydrolysis of the protein for 3 hr. in 2N-HCl, whereas the results we have obtained under these conditions (Table 2) approximate to 1·0%. Sørensen (1938) has reported a value of 1·4% of hexosamine in the whole protein after hydrolysis in 5N-HCl for periods of 3 and 6 hr., 0·54, 0·91, 1·13 and 1·22% after hydrolysis for 1, 3, 6 and 12 hr. respectively, in N-HCl and 1·25 and 1·19% after 2 and 3 hr. respectively in conc. HCl. Our results, we believe, demonstrate that there are certainly not less than three residues of glucosamine in egg albumin (1·2%), with the possibility of a fourth molecule.

During the analysis of the purified glycopeptide for its amino acid content by the method of Moore & Stein (1951) as described by Eastoe (1955), further data for glucosamine were also obtained. Hydrolytic conditions were rather more vigorous than those described above, namely 5·7N-HCl at 115°; after 18 hr. hydrolysis values of 1·68 mole of glucosamine and, after 36 hr. hydrolysis, 0·79 mole of glucosamine/5 moles of mannose were obtained. Considerable destruction of glucosamine had occurred under these conditions, the degree being of a similar order of magnitude to that observed by Folkes, Grant & Jones (1950) when glucosamine was autoclaved for 5 hr. in 3N-HCl in a sealed tube at 15 lb./in.^{2} pressure (approx. 120°).

Amide nitrogen

Analytical data for the amide nitrogen of egg albumin have been reported on a number of occasions (Hausmann, 1899; Osborne & Harris, 1903; Pittom, 1914; Shore, Wilson & Stueck, 1935–36; Harington & Mead, 1936; Rees, 1946; Steven & Tristram, 1958). These determinations have all been carried out with acid medium for hydrolysis and are reported in Table 3. Alkaline conditions of hydrolysis have been used in one study (Warner & Cannan, 1942) and, although a result was obtained agreeing fairly well with that of Shore *et al.* (1935–36), such conditions are not recommended in view of the alkali lability of various amino acids.

Because of the instability of some of the amino acids under hot, strongly acid conditions, it would seem that the milder procedures should give the more accurate results. In particular, Rees (1946) showed that, under the hydrolytic conditions he employed, serine and threonine are deaminated only to a negligible extent.

All these earlier results are subject to error, however, for since that work was done it has become apparent that special precautions are necessary for determining the amide nitrogen content of proteins containing amino sugars. These are deaminated to a greater or lesser extent under the alkaline conditions generally used for the liberation of amide ammonia from a protein hydrolysate. Ammonia is quantitatively liberated from glucosamine and galactosamine provided that the conditions are rigorously standardized (Tracey, 1952).

In order, therefore, to determine the amide nitrogen in a hexosamine-containing protein, the following conditions must be observed: the hydrolysis of the protein must occur in such a way that

Table 3. *Amide-nitrogen content of egg albumin as reported by various workers*

Workers	Hydrolytic conditions	Amide N as % of protein
Hausmann (1899)	Conc. HCl. 5 hr. Boiling	1·28
Osborne & Harris (1903)	5·7 N-HCl. 7–10 hr. Boiling	1·35
Pittom (1914)	5·7 N-HCl. Time curve. Boiling	1·26
Shore, Wilson & Stueck (1935–36)	Time curves in 0·2 N-, N- and 5 N-HCl. 85° and 100°	0·97
Harington & Mead (1936)	5·7 N-HCl. 3 hr. 100°	1·06
	0·2 N-HCl. 20 hr. 100°	0·99
Rees (1946)	10 N-HCl. Time curve. 37°	1·04
Steven & Tristram (1958)	2 N-HCl. 3 hr. Boiling	1·04

the constituent amino acids are not deaminated; the protein hydrolysate must be steam-distilled under conditions whereby the hexosamine gives up its ammonia quantitatively without causing deamination of the amino acids; thirdly, care must be taken that the protein is not contaminated by free ammonia (Chibnall, Mangan & Rees, 1958). Since the result obtained will be a measure of the amide nitrogen plus the hexosamine nitrogen liberated, the latter must be subtracted from the value obtained to give the amide-nitrogen content. The amide nitrogen should also be directly measurable by precipitation of the ammonia in acid solution as its phosphotungstate, a method used by Berlin, Neuberger & Scott (1956), and this has been used in the present work.

Several preparations of egg albumin, details of which are given in the Experimental section, were used in this work. Hydrolyses of the various preparations were carried out with 10 N-HCl at 37° (Rees, 1946), and the amide and liberated glucosamine ammonia distilled by using the saturated sodium borate in saturated trisodium phosphate medium of Tracey (1952). In one experiment with protein preparation P, after periods of 18, 45, 96, 164, 216 and 264 hr., the apparent 'amide'-nitrogen values were 0·84, 0·96, 1·04, 1·07, 1·07 and 1·06% respectively. Similar time curves were obtained in the other preparations and gave asymptotic values for the 'amide'-nitrogen content of 1·04% for preparation M, 1·04–1·05% for preparation N, and 1·03–1·06% for preparation O. These final values indicate that the total amide nitrogen plus liberated hexosamine-nitrogen content of egg albumin lies between 33·1 and 34·4 residues per 45 000 g. of protein.

Glucosamine is either partially destroyed, or, more likely, incompletely liberated with the low temperature and high acidity used, as was indicated by following its rate of liberation from the protein by the Cessi procedure. After 24, 48, 96, 167, 217 and 266 hr. the apparent glucosamine content of the protein (preparation P) was 0·23, 0·34, 0·49, 0·64, 0·74 and 0·72% respectively. Results obtained after hyrolysis for 250 hr. with the other preparations are 0·70 (M), 0·73 (N), 0·72 (O) and 0·69% (P). The highest values correspond to slightly less than 2 equiv. of glucosamine/mole of protein.

The ammonia obtained on acid hydrolysis for 250 hr. under the same conditions, by precipitation as the phosphotungstate, was also measured. Values of 0·96 (M, 30·6 residues), 0·93 (N, 29·9), 0·99 (O, 31·9) and 0·97% (P, 31·2) were obtained. A similar method has been used previously for determining the content of the amide nitrogen in ovomucoid by Marshall & Neuberger (1960).

Our results indicate that in whole-egg albumin there are about 31 ± 1 residues of amide nitrogen, which is equivalent to about $0{\cdot}96 \pm 0{\cdot}03$% of amide nitrogen/100 g. of protein.

Acetyl content

Since in hexosamine-containing substances the amino groups of the amino sugars are almost always acetylated, an estimate of the amount of acetic acid produced on acid hydrolysis of both whole-egg albumin and the purified glycopeptide was carried out. The steam-volatile acid from a 2 N-HCl hydrolysate of the protein was distilled and subjected to column chromatography by the procedure of Bueding & Yale (1951). Yields of acetic acid under the conditions we used were of the order of 90%. Determinations of the acetic acid produced from egg-albumin hydrolysates gave values of 3·9 and 3·8 moles/45 000 g. of protein, allowing for a 90% recovery. After hydrolysis for 6 hr. the equivalent of 3·8 moles was produced.

Direct determinations of the amount of steam-volatile acid which were distilled from the (pH 3) hydrolysate were also carried out. The results obtained indicated values of 4·18, 4·15 (preparation M), 4·29 (N), 4·19 (O) and 4·00 (P) moles/45 000 g. of egg albumin. In all cases no acid other than acetic acid could be recognized by the gas–liquid partition chromatography method of James & Martin (1952).

Hydrolysis of the purified glycopeptide (3 hr., 2 N-HCl) gave rise to steam-volatile acid which was shown to be acetic acid (James & Martin, 1952), in amounts equivalent to 2·7 and 3·1 moles/5 moles of mannose. Thus the isolation of the glycopeptide

from whole-egg albumin results in a loss of one acetyl residue. It is possible that such a group is the substituent at the *N*-terminus of the protein chain, as egg albumin has no *N*-terminal amino acid when the usual dinitrophenyl techniques are used (Porter, 1950; Steven & Tristram, 1958). An *N*-terminal acetyl residue has been recognized for the tobacco mosaic virus protein (Narita, 1958*a*, *b*; Tsugita, 1960) and also in the α-melanocyte-stimulating hormone (Harris, 1959).

At least some of the three acetyl residues found in the glycopeptide are combined with the amino groups of the glucosamine residues, since acetyl glucosamine could be detected by paper chromatography after hydrolysis of the material in 0·5 N-HCl at 100° for 5 hr.

EXPERIMENTAL

Materials

Egg albumin. Preparations M, N and O were crystallized by the procedure of Sørensen & Høyrup (1915–17) as modified by Warner (1954). Recrystallization was carried out five times in all cases, followed by thorough dialysis at 4° against distilled water. One preparation was dried from the frozen state (M) and its moisture, ash and nitrogen contents determined (Chibnall, Rees & Williams, 1943). On a moisture-free, ash-free basis the nitrogen content of the protein was found to be 15·73%. A second preparation (N) was heat-denatured by the procedure of Chibnall *et al.* (1943), and a third (O) prepared by the same method and denatured by pouring an aqueous solution into cold methanol. Their nitrogen contents were 15·77 and 15·72% respectively. Preparation P was obtained by the method of Kekwick & Cannan (1936) whereby recrystallization was carried out five times with Na_2SO_4. The protein was dialysed and freeze-dried. Its nitrogen content was 15·66%. All these preparations were examined for the presence of free ammonia by the procedure of Chibnall *et al.* (1958) and found to be completely free from it.

A brief description of the isolation of the glycopeptide from egg albumin has been given by Johansen *et al.* (1958).

Methods

Carbohydrate estimations. These were carried out on the whole protein by the orcinol–H_2SO_4 methods of Winzler (1955) and Hewitt (1937), with mannose as a standard. Extinctions were read on a Unicam SP. 600 spectrophotometer. For the Winzler method the wavelength at which the extinction was read was 505 mμ. Orcinol from the British Drug Houses Ltd. was purified by sublimation. A protein solution (1 ml.) (1–1½%, w/v) was generally used. Amounts of glycopeptide were used containing similar levels of carbohydrate.

Glucosamine determinations. A sample of protein or purified glycopeptide solution (0·2 ml.) containing 30–40 μg. of glucosamine was placed in a tube fitted with a ground-glass stopper. The sample was dried *in vacuo* at room temperature over KOH and P_2O_5. A suitable dilution of constant-boiling HCl (1 ml.) was added, the stopper sealed in, and the solution heated in a boiling-water bath for the required time. The tube was cooled, the stopper removed and the contents were washed to the bottom of the tube. The solution was dried over KOH and P_2O_5 *in vacuo* at room temperature. The glucosamine content of the residue was determined either by the procedure of Rondle & Morgan (1955) or by the Cessi technique. The following description includes a slight modification which we have made to the method that Dr Cessi developed and kindly made available to us.

Cessi method for glucosamine analysis. The reagents used were: (i) acetylacetone reagent; 1 ml. of freshly distilled acetylacetone was dissolved in 25 ml. of M-Na_2CO_3 solution plus 20 ml. of water. The pH, where it differed from 9·8, was adjusted to that value, and the volume made up to 50 ml. This solution was never used more than 30 min. after it was prepared. (ii) Ehrlich's reagent; 2 g. of *p*-dimethylaminobenzaldehyde was dissolved in absolute ethanol, containing 3·5% of conc. HCl to a final volume of 250 ml. This solution was stored in the refrigerator.

In ground-glass stoppered tubes was placed 2 ml. of standard glucosamine hydrochloride solutions containing up to about 40 μg. of glucosamine (as the free base)./ml. Water (2 ml.) was used for the protein hydrolysates and for blank experiments. To each tube was added 5·5 ml. of acetylacetone reagent, and the tubes, with stoppers inserted, were heated together in a vigorously boiling water bath for 20 min. After being cooled in ice–water, the contents of each tube, in turn, were transferred to a 25 ml. flask together with 3 × 2 ml. of water washings. The flask was heated over a Bunsen flame and the steam-volatile chromogen distilled into a 10 ml. volumetric flask containing 8 ml. of Ehrlich reagent. Most of the chromogen came over in the first few drops of distillate. The extinctions were read after at least 30 min. at a wavelength of 548 mμ with a Unicam SP. 600 spectrophotometer in 1 cm. cells. Under these conditions 25 μg. of glucosamine (free base)/ml. gives an *E* 0·42, and the results are highly reproducible. Rectilinearity has been observed when a graph of *E* against μg. of glucosamine is plotted for values of between 4 and 40 μg. of amino sugar/ml. The colour values obtained are

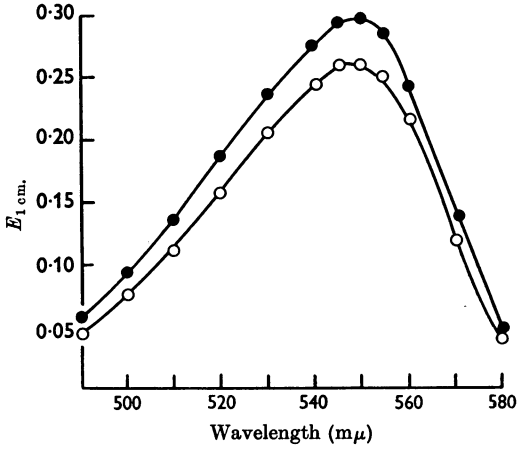

Fig. 3. Spectra given in the Cessi method for 2-amino sugars. ○, Glucosamine (46·4 μg.); ●, 4 N-HCl egg-albumin hydrolysate.

stable for at least 18 hr. The identity of the spectra obtained from glucosamine and from an egg-albumin hydrolysate is shown in Fig. 3.

Muramic acid and 3-methylglucosamine were subjected to a similar procedure at levels in each case of about 500 μg. of amino sugar/ml.

Precipitation of ammonia by phosphotungstic acid. A sample of hydrolysed protein (dried over KOH and P_2O_5 *in vacuo* at room temperature) in a centrifuge tube was dissolved in 5 ml. of water and 10 ml. of 30% (w/v) phosphotungstic acid which had been purified by the procedure of Van Slyke & Rieben (1944) was added. The pH was about 1. The tube was left at 4° overnight and centrifuged to separate the precipitate completely. The latter was washed twice by resuspending in 5 ml. of cold (4°) 30% phosphotungstic acid and centrifuging. The residue was steam-distilled with saturated Na_3PO_4 solution and the ammonia determined. In control experiments, with about 6–7 mg. of ammonia under these conditions, recoveries are of the order of 97–98%.

Acetyl estimations. The protein or the glycopeptide was hydrolysed in 2N-HCl, usually for 3 hr. The solution was cooled and neutralized to pH 3, and the steam-volatile acid distilled. The distillate was neutralized to phenolphthalein with NaOH and subjected to column chromatography by the method of Bueding & Yale (1951). In control experiments, sodium acetate gave rise to a yield of 86% of acetic acid, and acetic acid, when added to bovine-serum albumin and then hydrolysed, was recovered to the extent of 89%. The acetic acid level in such experiments was about 100 μmoles.

In the determinations in which direct measurement of steam-volatile acid was made, care was taken to ensure that CO_2 was excluded. The distillate was collected to a fixed volume (usually 25 ml.) and titrated against NaOH solution with phenolphthalein or thymolphthalein as indicator. Further fractions were collected and titrated until the titre value was equal to that of a similar volume of distillate from distilled water.

SUMMARY

1. Mannose is the only neutral sugar that has been recognized in egg albumin by paper chromatography. Colorimetric techniques have been applied and they indicate the presence in the protein of about 2% of this sugar, or five residues/45 000 g.

2. The difficulties which are liable to be encountered in liberating glucosamine from polysaccharide linkage have been discussed. The glucosamine liberated by hydrolysing egg albumin and the isolated polysaccharide from it under various conditions has been measured by both the direct Elson–Morgan reaction and also the Cessi modification of it. Both methods indicate a value of about 1·2% as the glucosamine content of the protein (three residues). The possibility of a fourth molecule of this or some other amino sugar cannot be completely excluded, for reasons which have been discussed. The Cessi procedure, which depends on the formation and steam-distillation of a steam-volatile chromogen (2-methylpyrrole), has been shown to be more specific than the original Elson–Morgan method. Thus 3-substituted glucosamines are not determined at all by this method.

3. Most of the recorded amide-nitrogen values of glycoproteins probably need correction, because 2-amino sugars are deaminated under the alkaline conditions used in the liberation of ammonia. This complication has been overcome by precipitating the ammonia with phosphotungstic acid. The amide nitrogen content of egg albumin is believed to be 31 ± 1 residues/mole.

4. The acetyl content of egg albumin has been shown to be four residues, and at least three of these have been shown to be associated with the carbohydrate moiety in the protein. The glucosamine is partly, if not wholly, *N*-acetylated. The possibility that the substituent at the *N*-terminus of the protein chain is an acetyl group has been discussed.

We wish to thank Dr C. Cessi, of the University of Bologna, Italy, who gave us details of his method for hexosamine analysis. Dr J. E. Eastoe of the Royal College of Surgeons kindly allowed us the use of his laboratory for the Moore & Stein analyses, as did Dr G. Popják of the Postgraduate Medical School, Hammersmith, in some of the early work on the fatty acid analysis by James–Martin chromatography. Dr M. J. Crumpton of the Microbiological Research Establishment, Porton, gave us a sample of muramic acid which he had prepared. Acknowledgement is made of the financial assistance given by the Medical Research Council to P.G.J. and by the British Empire Cancer Campaign to R.D.M.

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Some Factors Affecting the Permeability of Large-Granule Membranes to Mouse-Liver Catalase *in vitro*

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(*Received* 26 *February* 1960)

Approximately 70% of the catalase in mouse liver is present in the large granules, the remainder being in the extraparticulate cytoplasm (Adams & Burgess, 1957). Adams & Burgess (1959*a*) showed that when slices from mouse liver were incubated at 38° in a phosphate medium, catalase migrated from the granules to the extraparticulate cytoplasm. Also, when granules were isolated and incubated at 38° in 0·25 M-sucrose, catalase migrated into the surrounding medium. They produced evidence suggesting that no appreciable rupture of granules occurred under these conditions, and concluded that the migration was due to permeability of the intact granule membranes to catalase. In the same paper it was also shown that catalase migration was greatly reduced when slices in phosphate, or preparations of granules in sucrose, were incubated at 20°. No catalase migrated from granules prepared and incubated in phosphate medium at 38°. It appeared therefore that the granule membrane permeability depended greatly on the applied conditions, and the purpose of the present paper is to study these conditions further.

EXPERIMENTAL

Animals. Young adult mice of the CBA strain (Committee on Standardized Genetic Nomenclature for Mice, 1960) were used. The diet of the animals consisted of commercial rat cubes and water (both without restriction). The catalase migration from granules prepared from the livers