Studies in Immunochemistry

21. THE PRODUCTS OF THE DEGRADATION OF BLOOD-GROUP-SPECIFIC SUBSTANCES BY FICIN AND PAPAIN

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In earlier work (cf. Morgan, 1954–55; Kabat, 1956; Morgan, 1960) it was shown that the blood-group-specific serological properties of human secretions and body fluids are associated with macromolecular entities which contain carbohydrate and amino acids firmly bound together, and that the part of these complex molecules which is primarily responsible for the characteristic serological behaviour is carbohydrate in nature. There is some evidence, however, that not only is the nature, the mode of linkage and the sequence of the sugar units of importance in the qualitative and quantitative behaviour of the specific substances with their antibodies, but that the orientation of these structures and the overall conformation of the macromolecule play an important part and are probably maintained by the amino acid-containing moiety of the specific substance.

In order to study the part played by the amino acids in mucopolysaccharide structure, the action of the proteolytic enzymes, ficin and papain, on the specific substances was examined (Pusztai & Morgan, 1958, 1961c), and it was found that these enzymes brought about a rapid reduction in the specific serological activity to a value equivalent to a few per cent only of the original figure, when the activity was measured by the haemagglutination-inhibition test. Repeated incubation with fresh enzyme, however, failed to destroy completely the serological activity of the specific substances. These changes occurred irrespective of the specificity of the substances, and the different materials examined were all broken down into smaller, non-diffusible fragments which in each instance had a chemical composition closely similar to that of the substance from which it was obtained.

A more extensive series of experiments has now been made and the results obtained with several highly purified specific substances and crystalline ficin and papain are now given.

EXPERIMENTAL

Group-specific substances. These materials were isolated from human ovarian-cyst fluids and purified as described by Morgan & King (1943), Gibbons, Morgan & Gibbons

(1955) and Pusztai & Morgan (1961b). Except where stated otherwise, all materials were insoluble in 90% (w/w) phenol, soluble in water, insoluble in saturated $(\text{NH}_4)_2\text{SO}_4$ at 60° and precipitated from solution (1% in water) within a narrow range of ethanol concentration. The substances were electrophoretically homogeneous at pH 8 in phosphate buffer, *I* 0.2.

Physical measurements. Ultracentrifugal, electrophoretic and diffusion measurements were made as described by Kekwick (1950, 1952a, b) and Caspary (1954). Intrinsic viscosities were determined from relative-viscosity measurements made at 20° and pH 8 in phosphate buffer (*I* 0.2) containing 0.15M-NaCl (Pusztai & Morgan, 1961a, b). Partial specific volumes were determined pyknometrically.

Chemical analysis. The following methods were used. Nitrogen: Kjeldahl determination (Markham, 1942). Fucose; Gibbons (1955). Amino sugars: Rondle & Morgan (1955). Reducing sugars: Nelson (1944). The last two determinations were made after the material had been hydrolysed in sealed ampoules with 0.5N-HCl at 100° for 18 hr. and the results are expressed throughout as glucosamine base or glucose respectively.

Quantitative amino acid composition was determined as described by Moore, Spackman & Stein (1958) with a column (150 cm.) of Aminex, MS Blend Q-150, particle size 31–45 μ , for acidic and neutral amino acids, and Aminex, MS Blend Q-50, particle size 25–31 μ , for the short (15 cm.) column for basic amino acids.

Serological activity. The serological activity was determined by the capacity of the materials to inhibit the agglutination of a 1% suspension of erythrocytes by the appropriate A, B or Le^a human antibody (Gibbons *et al.* 1955). H activity was measured with rabbit immune H antibody (Annison & Morgan, 1952; Watkins & Morgan, 1955).

Enzymes. Crystalline ($\times 2$ or $\times 3$) papain and ficin, as described earlier, were used (Pusztai & Morgan, 1961c). The enzymes were activated with 2,3-mercaptopropanol (BAL) immediately before use.

Enzymic hydrolysis. A known weight of the specific substance was dissolved in 0.1M-citrate-phosphate buffer, pH 4.0, containing BAL (Pusztai & Morgan, 1961c) to give a 1 or 2% (w/v) solution and incubated with enzyme (1, 2 or 5%, w/w, of substrate). Toluene was added as preservative. In some experiments the substrates were incubated two or more times with enzyme. Where this occurred the material was dialysed and the pH adjusted before the addition of fresh enzyme.

Isolation of the hydrolysis products. After incubation with enzyme the material was dialysed, saturated with $(\text{NH}_4)_2\text{SO}_4$ and heated to 60° for 5–10 min. This treatment

denatured and precipitated the enzyme protein and, where the specific substance was originally insoluble under these conditions, removed unchanged substrate. The specific substances which were originally insoluble in saturated $(\text{NH}_4)_2\text{SO}_4$ at 60° became completely soluble in this reagent after thorough incubation with papain or ficin. The $(\text{NH}_4)_2\text{SO}_4$ was removed by dialysis at $1-3^\circ$, the hydrolysis products were recovered by drying from the frozen state, dissolved to give a solution (1 or 2%, w/v) in water and fractionated by the addition of ethanol containing 1% of potassium acetate. The main fractions obtained were frequently refractionated within the same limits of ethanol concentration. The materials finally obtained were dissolved in water, thoroughly dialysed and dried from the frozen state.

RESULTS

A preparation of group A substance obtained from cyst fluid no. 346 was incubated with ficin at pH 4.0, and after thorough dialysis again treated with ficin under the same conditions. The enzyme and any unchanged substrate were removed as described and the products separated into four fractions by the addition of ethanol (Table 1). The original substrate was completely precipitated from solution between 50% and 55% of ethanol, whereas after treatment with ficin slightly more than half of the substance only could be recovered between these concentrations of ethanol. The remaining material precipitated on the addition of higher concentrations of ethanol and about 20% of the starting material was recovered between 60% and 75% of ethanol. Only about 2% of the material was soluble above 75% of ethanol. Nevertheless, in spite of the changed solubility, the chemical composition of the materials, expressed in terms of fucose and total amino sugar and reducing sugar, remained largely unchanged. The nitrogen values of the recovered materials, however, were less than that of the original substance by a significant amount.

The products of degradation all had a lower relative viscosity than did the original substance, and the more soluble the fractions were in ethanol, the lower was their viscosity. Similarly, all the fractions obtained were less active than the original material, and the first and largest fraction and the last and most soluble fraction possessed no more than about 3% or less than 0.1% respectively of the activity of the starting material.

The material (248 mg.) insoluble in saturated ammonium sulphate at 60° after incubation with enzyme contained the denatured enzyme and some unchanged specific substance. It was recovered, incubated again with ficin and the products were added to the main hydrolysis products and included in the ethanol fractionation which finally gave the materials described in Table 1. The overall yield of recovered substance was rather more than 80% of the original substrate.

A part (550 mg.) of the material (Table 1) precipitating between 50% and 55% (v/v) of ethanol was dissolved in citrate-phosphate buffer and again incubated with ficin for 24 hr. The solution was then saturated with ammonium sulphate, heated to 60° and the soluble material recovered after dialysis and drying from the frozen state and fractionated from solution in water by ethanol. The substrate is seen (Table 2) to be degraded in much the same manner as was the original specific substance, although the formation of material (13%) soluble in 60% ethanol was reduced in amount compared with that (20%) resulting after the first treatment with ficin. The fractions are again found to be closely similar in chemical composition to the original substance.

A portion (350 mg.) of the material which was precipitated between 50% and 55% (v/v) of ethanol (Table 2), after incubation three times with ficin, was again dissolved in buffer and incubated for 16 hr. with 17 mg. of papain. The

Table 1. *Analytical values of materials produced from blood-group A substance no. 346 after digestion with ficin*

The A substance (1.2 g.) was incubated with ficin (21 mg.) in 0.1 M-citrate-phosphate buffer, pH 4, for 48 hr. Products were recovered and treated again with ficin (21 mg.). The digest was fractionated with ethanol.

Concn. of ethanol (% v/v) ...	Original substance	Fractions precipitated			
		50-55	55-60	60-75	Above 75
Material (mg.)	1200	664	98	212	23
Nitrogen (%)	5.3	4.6	4.8	4.8	5.2
Fucose (%)	19.4	21.3	21.1	20.1	16.0
Hexosamine (%)	27.7	29.4	28.5	26.2	22.0
Reducing sugar (%)	48.5	54.3	54.9	52.2	49.6
Viscosity (rel. c, 1% in water)	3.12	1.67	1.24	1.12	—
A activity* (μ g.)	0.025	0.8	12	50	50

* End point of inhibition against natural human anti-A serum.

Table 2. *Analytical figures of material precipitated from A substance after further digestion with ficin*

Material (550 mg.; 50–55% ethanol precipitate, Table 1) was incubated with ficin (10 mg.) in 0.1M-citrate buffer, pH 4, for 24 hr. and fractionated with ethanol.

Concn. of ethanol (% v/v)	...	...	Fractions precipitated		
			50–55	55–60	60–75
		Material before treatment			
Material (mg.)	550		390	35	75
Nitrogen (%)	4.6		4.6	4.7	4.8
Fucose (%)	21.3		22.3	20.5	22.2
Hexosamine (%)	29.4		28.9	25.6	28.3
Reducing sugar (%)	54.3		53.3	50.9	53.8
Viscosity (rel. c, 1% in water)	1.67		1.36	1.18	1.16
A activity* (μ g.)	0.8		0.8	12	25

* See footnote to Table 1.

material (300 mg.) precipitated by ethanol (50–55%, w/v) made up nearly 90% of the starting material and was essentially unchanged in chemical composition and activity (Table 3). The material is apparently resistant to further degradation with ficin or papain at pH 4.

The A substance no. 401, insoluble in 90% phenol, soluble in water and insoluble in saturated ammonium sulphate at 60°, was fractionated from ethanol, and the main fraction, collected between 45% and 50% (v/v) of ethanol concentration, was precipitated three times within this ethanol range from solution in water containing 1% of potassium acetate. The material (2 g.) recovered was thoroughly dialysed, dried from the frozen state and incubated with three successive portions (30 mg.) of ficin, dialysed and recovered. The digestion products were then treated with saturated ammonium sulphate at 60° and separated into material insoluble (0.21 g.) and soluble (1.68 g.) under these conditions. The insoluble material was incubated with 5 mg. of ficin and finally yielded an additional 130 mg. of substance soluble in saturated ammonium sulphate. The total yield of mucopolysaccharide soluble in ammonium sulphate from the original material (2 g.) was 1.8 g. This substance (1.72 g.) was fractionated with ethanol and gave no precipitate up to 48% (v/v) of ethanol, 1.50 g. between 48% and 53% (v/v) of ethanol, no precipitate between 53% and 58% (v/v) of ethanol and 0.19 g. soluble in 58% (v/v) of ethanol. Both materials were incubated again with ficin and recovered after fractionation with ethanol. The overall yield was 1.30 g., precipitating between 49% and 53% (v/v) of ethanol, and 0.26 g. between 58% and 75% (v/v) of ethanol. An attempt to refractionate the latter material gave 100 mg. of substance between 58% and 62% of ethanol, 130 mg. between 62% and 66% of ethanol and 13 mg. between 66% and 70% of ethanol. The first two fractions were combined, as they were considered to be parts of one main

Table 3. *Analytical figures of the main product obtained from A substance after digestion with ficin and papain*

Material (350 mg.; 50–55% ethanol precipitate, Table 2) was incubated with papain (17 mg.) in 0.1M-citrate-phosphate buffer, pH 4, at 37° for 16 hr.

	Material before (a) and after (b) incubation with papain and precipitation between 50% and 55% of ethanol	
	(a)	(b)
Material (mg.)	350	300
Nitrogen (%)	4.6	4.6
Fucose (%)	22.3	21.8
Hexosamine (%)	28.9	29.5
Reducing sugar (%)	53.3	54.5
Viscosity (rel. c, 1% in water)	1.36	1.42
A activity* (μ g.)	0.8	0.8

* See footnote to Table 1.

fraction. The analytical values of those materials finally collected between 49% and 53% and between 58% and 66% of ethanol are given in Table 4. The original mucopolysaccharide material is seen to be divided into two fragments, the major material being precipitated at a lower ethanol concentration than that required to precipitate the second and minor component.

The behaviour of two mucopolysaccharide materials isolated from an ovarian-cyst fluid obtained from a person of blood group A₂B (cyst no. 186) was also studied. In this instance, the specific substances investigated included materials which were (a) insoluble and (b) soluble in saturated ammonium sulphate at 60° (cf. Pusztai & Morgan, 1961b). The materials were each separately incubated at pH 4 with papain and the products were separated and recovered, as described above. The composition and properties of the materials (Table 5) show that the changes occurring in both

materials are essentially similar to those found for the group A substances already described (Tables 1-4).

The group A₁B substance from cyst fluid no. 117 was likewise incubated at pH 4.0 with ficin, dialysed and treated with a second similar quantity of enzyme before the products were recovered.

Table 4. *Analytical values of A substance no. 401 and the two main decomposition products produced by ficin*

The A substance (2 g.) was incubated repeatedly with ficin and the products were fractionated with ethanol.

Concn. of ethanol (% v/v) ...	Original substance	Fractions precipitated	
		49-53	58-66
Material (mg.)	2000	1300	230
Nitrogen (%)	4.9	4.4	4.2
Fucose (%)	22.5	22.1	19.1
Hexosamine (%)	28.5	28.4	24.2
Reducing sugar (%)	51	52	45

Details of the materials obtained are given in Table 6, and show again that the changes induced are similar to those described above.

The results obtained when H-specific substance no. 277 was incubated with three successive quantities of papain at pH 4.0 and the products were separated and examined are given in Table 7. It is again evident that the original material was broken down into smaller units and that the composition of each of the products remained closely similar to that of the original material.

The incubation of an Le^a-specific mucopolysaccharide with papain has already been described (Pusztai & Morgan, 1961a), and analytical details of the products are given in Table 9 in that paper. The changes induced by the enzyme are similar to those described above for the other specific substances.

Behaviour of mucopolysaccharide preparations of different size on incubation with papain

The influence of the size of the mucopolysaccharide molecule on the extent of molecular degradation achieved was studied in the following

Table 5. *Analytical values of materials recovered after incubating a blood-group A₂B substance with papain*

Materials (a) insoluble and (b) soluble in saturated ammonium sulphate at 60° were each digested with papain (10 mg.) and subsequently fractionated with ethanol.

Concn. of ethanol (% v/v) ...	Original substance	(a)				Original substance	(b)			
		50-55	55-60	60-75	Above 75		50-55	55-60	60-75	Above 75
Material (mg.)	530	338	41	62	13	530	300	60	130	7
Nitrogen (%)	5.1	4.6	4.6	4.6	4.2	3.9	3.6	3.5	3.5	3.7
Fucose (%)	20.1	21.5	19.5	19.5	—	22.2	24.6	23.6	24.2	—
Hexosamine (%)	26.0	27.4	25.8	27.4	—	25.5	29.1	29.4	29.0	—
Reducing sugar (%)	49.6	50.0	54.0	52.7	—	57.8	58.0	54.0	57.3	—
Viscosity (rel. c, 1% in water)	2.60	1.80	1.30	1.16	—	1.50	1.45	1.24	1.08	—
[α] _D (c, 1% in water)	-22°	-9°	-11°	-17°	—	-43°	-36°	-41°	-48°	—
A activity* (μg.)	1.0	125	250	250	250	16	>1000	>1000	>1000	>1000
H activity† (μg.)	1.6	6.0	12.5	25	25	3	25	100	100	100

* See footnote to Table 1.

† End point of inhibition against rabbit immune anti-H serum.

Table 6. *Analytical values of materials produced from an A₁B-specific substance after digestion with ficin*

Material was incubated twice with ficin (2%) and fractionated with ethanol.

Concn. of ethanol (% v/v) ...	Original material	Fractions precipitated				
		47-50	50-55	55-60	60-75	Above 75
Material (mg.)	640	19	233	100	140	16
Nitrogen (%)	5.4	4.0	3.8	4.5	4.9	5.3
Fucose (%)	18.2	17.7	19.9	18.0	15.0	12.6
Hexosamine (%)	26.6	27.0	27.8	29.4	22.3	18.7
Reducing sugar (%)	49.5	48.1	53.1	53.2	42.3	43.2
Viscosity (rel. c, 1% in water)	4.18	—	2.33	1.42	1.14	—
A activity* (μg.)	0.03	0.25	0.25	1.0	15	30
B activity† (μg.)	2.0	4.0	30	250	500	500

* See footnote to Table 1.

† End point of inhibition against natural human anti-B serum.

Table 7. *Analytical values of materials recovered after H substance no. 277 had been repeatedly incubated with papain*

Substrate was incubated in 0.1M-citrate-phosphate buffer, pH 4, with 2% of papain for 16 hr. at 37°. The material was recovered and digested twice more with papain under the same conditions.

Concn. of ethanol (% v/v) ...	Original substance	Fractions precipitated				
		47-50	50-55	55-60	60-75	Above 75
Material (mg.)	1500	530	240	100	110	20
Nitrogen (%)	5.3	5.0	5.3	5.0	5.1	5.0
Fucose (%)	18.5	19.1	17.0	15.4	15.6	—
Hexosamine (%)	28.0	28.1	28.2	25.9	25.3	—
Reducing sugar (%)	50.3	53.5	50.0	49.5	50.3	—
Viscosity (rel. c, 1% in water)	5.08	1.90	1.49	1.30	1.14	—
$[\alpha]_D$ (c, 1% in water)	-39°	-18°	-13°	-16°	-21°	—
$S_{20,w}$ values at concn. (g./100 ml.):						
1.0	4.09	3.72	3.74	3.05	2.05	—
0.5	5.61	4.84	—	—	—	—
0.38	5.63	—	—	—	—	—
0.25	6.77	6.19	—	—	—	—
H activity* (μ g.)	0.05	0.4	0.4	0.8	3.0	6.0

* End point of inhibition against rabbit immune anti-H serum.

Table 8. *Digestion of mucopolysaccharides of different size with papain*

Materials were obtained from mucopolysaccharide preparation (no. 350) by fractionation with ethanol. Each material was incubated separately with papain in citrate-phosphate buffer (pH 4) for 24 hr. Each digest was then fractionated within the same range of ethanol concentrations.

Limits (% v/v) of ethanol fractionation after incubation with papain	Ethanol (% v/v) fractionation limits of mucopolysaccharide materials before incubation with papain					
	48-55		55-57		57-65	
	Yield after incubation with papain and fractionation with ethanol					
	g.	%	g.	%	g.	%
48-55	1.087	64.0	—	—	—	—
55-58	0.133	7.8	1.225	73.0	0.286	16.8
59-65	0.432	25.4	0.400	23.0	1.300	76.4
68-75	0.040	2.2	0.064	3.4	0.106	6.2
Soluble in 75	0.011	0.6	0.011	0.6	0.012	0.6

experiment. A large preparation of Le^a-specific substance which was obtained from cyst no. 350 (Pusztai & Morgan, 1961a, c) and was insoluble in 90% phenol and in saturated ammonium sulphate at 60° was separated into three fractions by precipitation from water with ethanol concentrations of 48-55, 55-57 and 57-65% (v/v) respectively. Portions (1.8 g.) of each of these materials were separately incubated with papain, dialysed and recovered. Each material was dissolved in water (1%, v/v) and fractionated within the same arbitrarily chosen range of ethanol concentrations, and the yield of each fraction was recorded (Table 8).

Molecular size of the specific substances before and after incubation with ficin or papain

The specific mucopolysaccharides after incubation with ficin or papain possess a reduced vis-

cosity, an increased solubility in water-ethanol mixtures and become completely soluble in saturated ammonium sulphate at 60°, but nevertheless show no gross change in chemical composition. These changes suggest that the original materials are broken down to mucopolysaccharide entities of smaller molecular size. The analytical values obtained for H substance no. 277 and for the materials obtained from it after incubation with papain are given in Table 7. To obtain some evidence for the molecular size of the materials obtained, the sedimentation coefficient of each fraction was examined at 1% (w/v) concentration. In some instances values at lower concentrations were also obtained (Table 7). The preparations showed a good degree of homogeneity, but were somewhat polydisperse. The last fraction obtained, which was soluble in 75% (v/v) of ethanol, gave unsatisfactory results

because of its rapid diffusion in the ultracentrifuge cell. The sedimentation coefficients obtained for the other materials are given in Table 7, and the values obtained support the view that all the fractions recovered after incubation with papain are smaller than the original H substance. The intrinsic viscosity (0.62) of the H substance and of its largest fragment (0.445) obtained after incubation with papain were determined by a linear extrapolation to zero concentration of $\eta_{\text{spec.}}/c$ values plotted against concentration. The sedimentation coefficients obtained at the lowest dilution examined (0.25%), together with the $\eta_{\text{spec.}}/c$ values at the same concentration, the partial specific volume (0.63) and the densities of the solutions were substituted in the formula:

$$M = \frac{4690 \times (S_{20,w})^{\frac{1}{2}} \times [\eta]^{\frac{1}{2}}}{(1 - \bar{V}\rho)^{\frac{1}{2}}}$$

(cf. Schachman, 1957), to obtain molecular weights. The values obtained were 340 000 and 271 000 respectively for the original H substance and the largest of the decomposition products obtained from it after incubation with papain.

In a similar experiment H substance (no. 277) was incubated with ficin at pH 4 and the molecular weights of the original material and of the main decomposition product were determined. The examination was made by Mr E. Caspary, who determined the diffusion coefficients and used these in place of the intrinsic-viscosity values to calculate the molecular weight. The results are given in Table 9. The diffusion coefficients could not be extrapolated to zero concentration in a linear manner and the molecular weights are therefore calculated from the Svedberg formula

$$\frac{RTS_{20}^0}{D_{20}^0(1 - \bar{V}\rho_{20})}$$

with diffusion coefficients obtained for the lowest concentration and the appropriate sedimentation constant. The molecular weights obtained are 332 000 and 265 000 for the original H substance and the main decomposition product. The frictional values are 3.23 and 2.85 respectively. The results of these two experiments, in which either papain or ficin was used to bring about the degradation, showed that the largest of the products had a molecular weight which was smaller than that of the original specific substance and that the molecular size of the largest fragment was closely similar whichever enzyme was used.

A similar examination was made with a group A substance no. 346 (Table 1) and its main ficin and papain decomposition product (Table 3, b). In this instance the extent of the degradation, as determined from the measurements given in Table 10, appears to be greater than was found for

Table 9. *Physical properties of H-specific mucopolysaccharide and the main degradation product formed on incubation with ficin*

(1) and (2) Sedimentation coefficients. (3) Diffusion coefficients. (4) Frictional ratios. (5) Molecular weights of the (a) original and (b) the ficin-degraded H substance obtained from cyst fluid no. 277.

Concn. (mg./ml.)	(a)	(b)
(1) Sedimentation coefficients at pH 8.0, <i>I</i> 0.35		
10.0	4.89	4.64
7.5	5.49	5.14
5.0	6.25	6.05
2.5	6.87	6.50
Infinite dilution	7.51	7.30
(2) Sedimentation coefficients at pH 4.0, <i>I</i> 0.35		
10.0	4.81	4.81
5.0	6.24	6.01
2.5	7.54	6.75
(3) Diffusion coefficients at pH 8.0, <i>I</i> 0.35		
4.6	1.22	—
3.3	1.32	—
1.5	1.51	—
4.1	—	1.52
3.1	—	1.61
1.5	—	1.84
(4) Frictional ratio	3.23	2.85
(5) Molecular weights	332 000	265 000

Table 10. *Physical properties of A-specific mucopolysaccharide and the main degradation product formed on incubation with papain*

(1) Sedimentation coefficients. (2) $\eta_{\text{spec.}}/c$. (3) Partial specific volumes. (4) Calculated molecular weights of (a) original and (b) ficin-degraded A substance obtained from cyst fluid no. 346.

	(a)	(b)
(1) Sedimentation coefficients at pH 8.0, <i>I</i> 0.35		
Concn. (in refractive increments)		
0.330	3.65	—
0.200	4.70	4.20
0.150	5.20	4.57
0.100	—	5.06
Infinite dilution	6.71	5.93
(2) $\eta_{\text{spec.}}/c$ values at pH 8.0, <i>I</i> 0.35		
Concn. (mg./ml.)		
10.0	1.240	0.296
5.0	1.018	0.254
2.5	0.896	0.228
1.25	0.840	0.208
0.625	0.800	—
Infinite dilution	0.777	0.198
(3) Partial specific volumes at pH 8.0, <i>I</i> 0.35		
Concn. (mg./ml.)		
10.0	0.69	0.69
(4) Molecular weights	416 000	173 000

H substance no. 277, the molecular weight of the main decomposition product (173 000) being only about 40 % of the value (416 000) obtained for the original substance. The molecular weight of the decomposition product precipitated between 60 and 75 % of ethanol was examined in the Spinco analytical ultracentrifuge and from its behaviour was considered to be close to 25 000.

Amino acid composition of the fragments obtained from the specific mucopolysaccharides

Group A substance no. 346 was repeatedly treated with ficin and finally with papain, and separated into two main fractions: a material precipitating between 50 % and 55 % (v/v) of ethanol and a smaller component separating at a higher (60–75 %) ethanol concentration (Tables 1–3). The original substance and the two decomposition products were separately hydrolysed in 6*N*-hydrochloric acid at 116–120° for 24 hr. and each was subjected to quantitative amino acid analysis (Moore *et al.* 1958). The results (Table 11), as expected from the lower nitrogen content of the two decomposition products, show that these each contain less total amino acid on a weight basis than does the original material and show, moreover, significant differences in amino acid distribution. Thus in the original A substance, the proportion of the amino acids in the group serine, threonine and proline, is 45 %, whereas acidic amino acids and

basic amino acids amount to 14 % and 19 % respectively. The corresponding groups in the main decomposition product are 53 %, 12 % and 14 %, and the distribution in the smallest fragment, 35 %, 20 % and 22 %. The amino acid composition of A substance no. 401 and its two fragments is given in Table 11. The proportion of the total amino acids in the serine, threonine and proline group is 46 %; the acidic amino acid and the basic amino acids account for 11 % and 20 % respectively. The first and largest breakdown product contains 61 % of the amino acids of the former group and 6 % and 11 % of acidic and basic amino acids. The smallest of the decomposition products contains 47 % of the total amino acid in the serine, threonine and proline group, whereas acidic and basic amino acids represent 12 % and 17 % respectively of the total amino acids.

Group A substance obtained from cyst fluid no. 332 was separated into material (a) insoluble and (b) soluble in saturated ammonium sulphate at 60° (Pusztai & Morgan, 1961*b*; Table 1). Each material was incubated with ficin and fractionated into substances precipitating at different ethanol concentrations, as described throughout this paper. The original substances (a) and (b) and the main decomposition product from each, which precipitated between 50 % and 55 % (w/v) of ethanol, were subjected to quantitative amino acid analysis. The main decomposition product examined ac-

Table 11. *Amino acid analysis of A substances no. 346 and no. 401 and each of their two main decomposition products obtained after repeated incubation with ficin at pH 4.0*

Results are expressed as g. of amino acid/100 g. of blood-group substance.

Concn. of ethanol (% v/v) ...	No. 346			No. 401		
	Original substance*	Decomposition products*		Original substance†	Decomposition products*	
		50–55	60–75		48–53	58–66
Amino acid						
Aspartic acid	0.95	0.62	0.94	0.71	0.25	0.49
Threonine	3.38	2.98	2.03	3.57	3.33	2.07
Serine	2.07	1.66	1.14	2.33	1.95	0.87
Glutamic acid	1.33	0.93	1.54	0.94	0.36	0.58
Proline	1.64	2.01	1.15	1.64	1.45	1.74
Glycine	0.50	0.45	0.37	0.46	0.33	0.42
Alanine	0.92	0.92	1.06	0.89	0.78	0.92
Valine	0.66	0.42	0.42	0.73	0.48	0.65
Methionine	0.05	<0.05	0.09	<0.05	<0.05	<0.05
Leucine	0.56	0.34	0.38	0.58	0.24	0.26
Isoleucine	0.40	0.19	0.20	0.46	0.32	0.18
Tyrosine	0.20	0.10	<0.10	0.12	<0.05	<0.05
Phenylalanine	0.29	0.18	0.16	0.33	0.09	0.10
Lysine	0.86	0.51	0.50	1.11	0.24	0.29
Histidine	0.82	0.48	0.67	1.01	0.48	0.61
Arginine	0.82	0.52	1.31	0.72	0.30	0.64
Unknown basic‡	0.43	0.30	0.28	0.38	0.25	0.16
	15.88	12.66	12.34	16.03	10.95	10.08

* Values are not corrected for losses on hydrolysis.

† Corrected and extrapolated values determined from 24 and 48 hr. hydrolysis.

‡ Expressed as leucine.

Table 12. *Amino acid composition of the largest degradation product obtained from A substance no. 332 after incubation with ficin*

The group A mucopolysaccharide (a) insoluble and (b) soluble in saturated $(\text{NH}_4)_2\text{SO}_4$ at 60° was incubated with papain at pH 4 in citrate-phosphate buffer for 24 hr. The products were fractionated with ethanol and material was precipitated between 50% and 55% of ethanol from (a) and (b) and analysed for amino acid composition. Results are expressed as g. of amino acid/100 g. of substance.

Amino acid	(a)		(b)	
	Without ficin	With ficin	Without ficin	With ficin
Aspartic acid	0.31	0.23	0.46	0.39
Threonine	4.05	3.46	2.90	2.83
Serine	2.16	1.98	1.57	1.58
Glutamic acid	0.72	0.45	0.82	0.71
Proline	2.04	1.60	1.62	1.58
Glycine	0.43	0.35	0.37	0.35
Alanine	0.92	0.73	0.83	0.72
Valine	0.59	0.44	0.41	0.40
Methionine	0.11	0.13	0.12	0.08
Isoleucine	0.41	0.29	0.31	0.31
Leucine	0.35	0.24	0.35	0.35
Tyrosine	0.15	0.07	0.08	0.08
Lysine	0.29	0.14	0.26	0.22
Phenylalanine	0.35	0.27	0.27	0.27
Histidine	0.70	0.52	0.78	0.81
Arginine	0.55	0.32	0.44	0.35

counted, in each instance, for about 60% of the original material. The results (Table 12) show again that, as expected from the lower nitrogen content found for the decomposition products, these materials contain less amino acid than does the original substance. The results also indicate that the amount of certain amino acids in the main decomposition products is considerably reduced compared with that in the parent mucopolysaccharide. The changes are most pronounced in the material originally insoluble in hot ammonium sulphate where it is seen that this material contains 0.5 g. of threonine, 0.2 g. of serine and 0.27 g. of glutamic acid (per 100 g. of total substance) more than is contained in the decomposition product. Some of the missing amino acids could be in the materials precipitating at higher ethanol concentration and not subjected to amino acid analysis, but presumably not all, as their content of amino acid is also lower than that of the original mucopolysaccharide. These analyses were made early in the investigation and figures for the content of the unidentified basic substance (Table 11) were not obtained.

DISCUSSION

The determination of the ultimate structure of biologically important macromolecules is more and more becoming a study of the products of the step-

wise breakdown of these substances whether they are obtained by acid, alkaline or enzymic hydrolysis. In the field of mucopolysaccharide structure, partial acid hydrolysis or the action of carbohydrases have been extensively employed to this end and especially to determine the precise nature of the carbohydrate units responsible for the primary specificity of the naturally occurring blood-group-specific macromolecules (cf. Morgan, 1960). The nature and function of the amino acid-containing part of the mucopolysaccharides has also been examined (Pusztai & Morgan, 1958) by studying the action of ficin and papain on the specific substances.

The carbohydrases split many and different glycosidic linkages in the macromolecules, mainly in the terminal parts of the sugar chains, and released sugar units. Ficin and papain, on the other hand, hydrolysed only a small and limited number of linkages, most probably those which are intimately associated with the internal structure of the specific substance, and therefore gave rise to only a small amount of diffusible material.

The mucopolysaccharides after thorough incubation with ficin or papain at pH 4 showed marked changes in certain of their properties and always possessed a lower viscosity and a much reduced serological activity (Pusztai & Morgan, 1958, 1961c). In order to study these and other changes in greater detail, preparations of specific mucopolysaccharides which were free from gross heterogeneity (Pusztai & Morgan, 1961b) were exhaustively treated with ficin or papain or both and the products recovered and examined.

In most instances the mucopolysaccharides studied were insoluble in saturated ammonium sulphate at 60°. After incubation with ficin or papain they became soluble in ammonium sulphate and had a lower content of nitrogen, were less active serologically and were much reduced in viscosity. It was shown (Pusztai & Morgan, 1961b) that, besides a mucopolysaccharide that was insoluble in ammonium sulphate at 60°, there existed in many cyst fluids a mucopolysaccharide material which was soluble, and that such substances also contained less nitrogen than the substances which were insoluble in ammonium sulphate at 60°, and were obtained from the same cyst fluid. These materials were less active and had a lower viscosity than did the materials insoluble in hot ammonium sulphate. The soluble materials on treatment with ficin or papain behaved as did the corresponding mucopolysaccharide which was insoluble in ammonium sulphate, and the products of digestion likewise were less active and had a lower viscosity than did the starting material. It appears therefore that not only the structures responsible for making a material insoluble in

saturated ammonium sulphate are changed by ficin or papain, but that similar or other structural features which are associated with the entities soluble in ammonium sulphate are also modified. It is possible therefore that two types of structure are involved in the changes induced.

The specific substance, which exists in a form which is either insoluble or soluble in saturated ammonium sulphate at 60° (Pusztai & Morgan, 1961*b*), is precipitated by ethanol from solution in water within quite narrow limits of ethanol concentration. After thorough and repeated incubation with ficin or papain, the products obtained from either material precipitate over a much wider ethanol range and, in order to examine the products, they were divided by ethanol concentration into four or five arbitrarily chosen fractions and each material was recovered, analysed and the relative viscosity and the serological activity were determined. The yields of the different fractions recovered showed that two main materials were obtained. One, which usually accounted for about two-thirds of the original substance, was precipitated at a somewhat higher ethanol concentration than was needed to precipitate the original substance. The second fraction precipitated at a considerably higher concentration of ethanol, usually between 60% and 75% ethanol. This material makes up about 20% of the starting substance. Frequently material was precipitated at ethanol concentrations in between those required to obtain the two major fractions, but the amount was usually small and, together with any material remaining soluble in 75% of ethanol, seldom amounted to more than 20% or so of the total substance involved. Refractionation of the material collected over the arbitrarily selected range of 60% and 75% of ethanol gave a material which precipitated between quite narrow limits, usually 59% and 65%.

It seems reasonable to believe therefore that as a result of thorough proteolytic decomposition two kinds of mucopolysaccharide fragments are formed and that the occurrence of any additional components can be explained as follows. First, the fractionation with ethanol is not a precise procedure and some carry-over from fraction to fraction is certain to occur. This error can be largely eliminated by refractionating the materials over the same ranges of ethanol concentration. Secondly, it has already been stressed (Pusztai & Morgan, 1961*a, b*) that the blood-group mucopolysaccharides obtained from ovarian-cyst fluids give preparations which contain chemically similar or identical molecules but of very different size. With improved fractionation procedures, such as that employing saturated ammonium sulphate at 60° and high-speed centrifuging, the polydispersity of a

starting material can be reduced to a level where a preparation has 80–90% of its molecules closely similar in size (Pusztai & Morgan, 1961*a*, Fig. 2; Pusztai & Morgan, 1961*b*, Fig. 3); the remaining material, however, has a very different molecular size and contains both larger and smaller units. It might be expected that the enzymic degradation products of these different-sized mucopolysaccharides are of different size and will, in consequence, show different precipitation behaviour with ethanol. This suggestion is supported by the data obtained (Table 8) from an experiment in which three mucopolysaccharide preparations, each obtained from the same cyst fluid, but judged from their viscosity values to be of different average molecular size, were examined separately after incubation with papain. The amount of material (6.2%) precipitated within the ethanol range 68–75%, which was obtained from an original fraction which itself was precipitated within the range 57–65% (Table 8), can be compared with the amount of material (2.2%) precipitated over the same ethanol range (68–75%) but obtained from a mucopolysaccharide, which was precipitated from the original mucopolysaccharide at a lower ethanol concentration (48–55%) (Table 8). It appears that the smaller the molecular size of the starting material, the larger is the yield obtained of the smaller and most soluble (in ethanol) material. Similar conclusions can be drawn from the results given in Table 5, where after treatment with papain the yield of the 60–75% ethanol fraction (62 mg.) obtained from material insoluble in saturated ammonium sulphate at 60° is seen to be less than half that isolated from the corresponding ethanol fraction (130 mg.) derived from the same amount of material that was originally soluble in ammonium sulphate and which was most probably of smaller molecular size (Pusztai & Morgan, 1961*b*). Here again the mucopolysaccharide believed to be of smaller size was degraded by papain to give a larger yield of what is almost certainly a product of smaller size, as judged by solubility in ethanol. The importance of the narrowness of the molecular size range of the mucopolysaccharide entities contained in the starting preparation of the specific substance is well shown in the experiments made with A substance no. 401. According to the physical examination, this material, after very careful and thorough fractionation with both saturated ammonium sulphate at 60° and ethanol precipitation, contained a relatively narrow range of molecular sizes and, after exhaustive treatment with ficin and dialysis, could be separated quite sharply into mucopolysaccharide entities which were precipitated within the ethanol concentrations 48–53% and 58–66% respectively. No material precipi-

tated between 53% and 58% ethanol. The small amount (13 mg.) recovered from the 66% ethanol supernatant fluid contained lipid and other impurities from the original substance or from the reagents employed or from both.

The analytical figures in Tables 1-7 make it evident that as a result of the decomposition of the serologically specific mucopolysaccharides with ficin or papain, fragments are formed which are smaller in size and less active serologically. There is no evidence of any gross change in chemical composition when the amounts of the individual sugar components are determined in the decomposition products. There is one change, however, which is always found: the nitrogen content of the fragments is significantly lower than that of the material before incubation with the enzyme. In the preceding paper (Pusztai & Morgan, 1961c) it was shown that a limited number of peptide or ester bonds or both are broken during the digestion with ficin or papain. The results obtained in the present work substantiate the conclusion that a scission of peptide or ester bonds or both occurs, for it has been found that the fragments contain quantitatively less amino acid and thus account satisfactorily for the lower nitrogen value shown by these materials.

The results of the quantitative amino acid analyses given in Tables 11 and 12 indicate that the amino acid-containing part of the mucopolysaccharide macromolecule has a composition quite unlike that of any protein so far described. The high content of proline, 10-15% of the total amino acids, resembles that found in collagen, but otherwise the two materials are quite distinct. Similarly, the large amount of threonine and serine, which accounts for at least a third of the total amino acid in the two group A substances examined, is to be found in γ -globulin, but in few other proteins. The nearest in amino acid composition to the group mucopolysaccharides is the ovine salivary mucoprotein recently described by Gottschalk & Simmonds (1960).

The amino acid composition of the two main decomposition products shows significant differences from the original mucopolysaccharide when individual amino acids or groups of amino acids are compared. There is a net loss in amino acids and the heaviest loss for both decomposition products is on threonine and serine. The amount of individual amino acids found in the two main products makes it probable that the amino acids are not evenly distributed throughout the amino acid-containing structures in the original mucopolysaccharide. The bifunctional acidic or basic amino acids occur more frequently in the smaller of the decomposition products and therefore in that part of the amino acid-containing moiety in the original

mucopolysaccharide which gives rise to this particular fragment. The results of some experiments made on columns of Dowex 50X2 to determine the nature and amount of any small decomposition products released during digestion that is materials not precipitated by 75% of ethanol, showed that nitrogenous materials, mostly peptides, but only small amounts of free serine, threonine and glutamic acid, were present. These free amino acids were probably secondary decomposition products of the peptides released during the initial phase of the decomposition of the mucopolysaccharide.

In conclusion, it would appear that the specific mucopolysaccharides are, as a result of the scission of a few peptide or ester bonds in the internal structure of the intact mucopolysaccharide macromolecule, degraded by ficin or papain into two main kinds of mucopolysaccharide fragments.

SUMMARY

1. The specific blood-group mucopolysaccharides have been incubated with crystalline ficin or papain and the products of proteolysis examined.

2. The mucopolysaccharides are broken down essentially into two kinds of macromolecular fragments and a small amount of diffusible peptide material.

3. The larger of the decomposition products possesses a few per cent only of the serological activity of the original substance when the activity is measured by the haemagglutination-inhibition test. The smaller of the two fragments has considerably less activity than that of the larger one.

4. The qualitative composition of the carbohydrate moiety of the decomposition products is identical with, and the quantitative composition close to, that of the original substance.

5. The quantitative amino acid composition of the products of the proteolytic decomposition of the specific mucopolysaccharides shows they are not identical with those of the original substance, or with each other. The evidence suggests that the amino acids are not evenly distributed throughout the amino acid-containing moiety in the original specific mucopolysaccharides.

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Macroglobulins

1. STUDIES ON THE ISOLATION AND PHYSICAL PROPERTIES OF PATHOLOGICAL MACROGLOBULINS

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On examination in the ultracentrifuge, human sera are shown to possess small concentrations of protein which sediment some two or three times faster (i.e. sedimentation coefficients in excess of 15s) than the more usual globulin components. These macroglobulin components normally account for up to 5% of the total protein, but Waldenström (1944) pointed out that certain pathological sera contain much larger quantities of a protein (or group of proteins) whose sedimentation behaviour is very similar to that of the normally occurring macroglobulin. More recent work (Wallenius, Trautman, Kunkel & Franklin, 1957; Deutsch & Morton, 1958; Kunkel, 1960) has confirmed that a group of differently sedimenting components rather than a single species is involved, and that electrophoretically both α - and γ -globulin types occur, though not necessarily in a single case. The comparison of the properties, both physicochemical and immunological, of normal and pathological macroglobulins presents a problem of some complexity, but additional difficulty is introduced when only imperfect separations of the macroglobulins from normal serum components are available.

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At the time the present work began, the great resolution obtainable through the use of chromatographic columns of ion-exchange cellulose materials had become apparent (Sober, Gutter, Wyckoff & Peterson, 1956; Sober & Peterson, 1958; A. Feinstein, P. Johnson & C. B. Richards, 1958, unpublished work). Such columns have been used to isolate from a macroglobulinaemic serum the macroglobulins and other globulins for detailed physicochemical characterization and comparison with other serum proteins. The immunological properties of the various protein fractions have been compared and are described in the next paper.

EXPERIMENTAL

Blood was collected by venepuncture and allowed to clot at room temperature. After 3½ hr. the serum was separated and had a protein content of 10.4 g./100 ml. No precipitation occurred on cooling it to 2°, but dilution with water (Sia test: see Martin, 1960) caused precipitation.

Isolation of macroglobulins

Fig. 1 shows the sedimentation patterns of the serum from the case of macroglobulinaemia (b) alongside normal serum sedimentation patterns (a). In the pathological serum it is clear that a group of macroglobulins (approx. 32% of total protein) rather than a single component is