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Studies in Immunochemistry

23. SOME OBSERVATIONS ON THE HETEROGENEITY OF PREPARATIONS OF HUMAN BLOOD-GROUP-SPECIFIC SUBSTANCES*

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In man, blood-group-specific substances occur in saliva, gastric juice, ovarian-cyst fluids, meconium etc. These sources of the blood-group substances, however, frequently contain considerable quantities of other constituents, mainly proteins, which are most readily removed by digestion with pepsin at pH 1.5–2.0, or by extracting the freeze-dried materials with 90 % or 95 % (w/v) phenol. After extraction with phenol, the group-specific substances, which have been characterized as mucopolysaccharides, remain as phenol-insoluble residues (Morgan & King, 1943), the water-soluble components of which, after further purification, yield the specific blood-group substances in a high state of purity (Gibbons, Morgan & Gibbons, 1955; Pusztai & Morgan, 1961*b*). The examination of a considerable number of blood-group substances prepared in this manner showed that they have a remarkably similar amino acid composition, irrespective of the serological specificity of the substances within the ABO and Lewis blood-group systems (Pusztai & Morgan, 1963). In a few instances, however, substances that had been similarly purified were found to differ significantly in amino acid composition, and it seemed possible that the specific mucopolysaccharides obtained from cyst fluids by the procedures described could sometimes be contaminated with extraneous pro-

tein or glycoprotein contained in the cyst fluid. Examination of the specific substances and some of these apparently abnormal substances by immuno-electrophoresis with an anti-(human plasma protein) serum, however, gave no evidence to support the suggestion that these mucopolysaccharides were contaminated with a protein or glycoprotein constituent derived from the plasma.

In an attempt to explain the different amino acid composition that was occasionally found in thoroughly purified preparations of group-specific mucopolysaccharides, a number of selected preparations were examined for their behaviour on diethylaminoethylcellulose columns, and the opportunity was taken to determine the amount and observe the distribution of sialic acid in a few of the substances. The results are given in this paper.

EXPERIMENTAL

Blood-group substances. These were obtained from ovarian-cyst fluids by the methods of Morgan & King (1943) and Gibbons *et al.* (1955), and were purified further by fractionation with saturated ammonium sulphate at 60°, thorough dialysis, fractionation from water by ethanol, centrifuging at 30000*g* for 1 hr. and drying from the frozen state (Pusztai & Morgan, 1961*b*). The substances obtained were examined in the Svedberg oil-driven ultracentrifuge and by moving-boundary electrophoresis, as described by Kekwick (1950). In some instances, materials obtained at different stages in the fractionation process were examined in greater detail by Dr J. M. Creeth, in a Spinco model E ultracentrifuge.

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Chemical analyses. The freeze-dried materials were further dried over phosphorus pentoxide for 30 min. at 78° and 1–2 mm. Hg pressure before analysis. The methods used for the determination of nitrogen and fucose, and for hexosamine and reducing sugars after acid hydrolysis, are those given by Pusztai & Morgan (1961*a*). The carbohydrate content of solutions obtained directly from DEAE-cellulose columns was determined by the phenol-sulphuric acid method of Dubois, Gilles, Hamilton, Rebers & Smith (1956). Sialic acid was determined by the method of Warren (1959). Quantitative amino acid analyses were made by the method of Moore, Spackman & Stein (1958), as described by Pusztai & Morgan (1963).

Serological examination. The materials were examined for their capacity to inhibit specific haemagglutination (cf. Pusztai & Morgan, 1961*b*; Watkins, 1962). The quantitative precipitation technique of Heidelberger & MacPherson (1943*a, b*), as developed by Kabat & Bezer (1945), was also employed when suitable precipitating sera were available.

Chromatography on diethylaminoethylcellulose. Whatman DEAE-cellulose (DE50) was purified by treatment with *N*-sodium hydroxide until the washings became colourless and, after removal of the excess of sodium hydroxide by repeated washing with distilled water, was equilibrated with 0.01 *M*-sodium chloride solution. The DEAE-cellulose columns (2.0 cm. × 150 cm. or 3.5 cm. × 50 cm.) were packed, small sections at a time, by pouring in a thin slurry of the DEAE-cellulose and occasionally applying slight pressure. The materials to be examined were dissolved in 0.01 *M*-sodium chloride, added to the column and recovered by gradient elution with solutions of sodium chloride of increasing concentration from 0.01 *M* to 1 *M*.

RESULTS

The behaviour of many different preparations of blood-group-specific substance on DEAE-cellulose chromatography were investigated, and four of these materials were selected to demonstrate the variability that was encountered in mucopolysaccharide preparations that had been through the isolation and purification procedures described.

(1) The mucopolysaccharide recovered from cyst fluid no. 350 (Pusztai & Morgan, 1961*a*), a Le^a-active substance obtained from a 'non-secretor' person, contained 18 % of sialic acid and had an amino acid composition which did not differ significantly from that found for the majority of purified blood-group-specific substances (Pusztai & Morgan, 1963). Elution of the substance from a DEAE-cellulose column (3.5 cm. × 50 cm.) gave only one peak (Fig. 1). This was arbitrarily divided into two parts by pooling the eluates collected during the rising part of the peak (fraction 1*a*, tubes 35–50), and from the material comprising the descending portion (fraction 1*b*, tubes 51–65). The eluates obtained from tubes 66–100 were collected and, after mixing together, gave an additional fraction (fraction 2). Analytical figures are given for each material (Table 1).

The experiment was repeated with a longer (2.0 cm. × 150 cm.) column, and there was some evidence that a slight resolution of the material occurred. However, the analytical figures for portions of the eluate, selected as in the first experiment, were closely similar to each other and to those given in Table 1, and it was evident that only small and probably insignificant differences existed in the sialic acid content, the nature or amount of the carbohydrate components, or in the qualitative and quantitative amino acid composition of the material comprising the arbitrarily divided major elution peak. A slight, but significant, increase in the content of the acidic amino acids in the last trailing part of the main fraction was observed, but this material comprised only a few per cent of the whole preparation.

Fractions 1*a* and 1*b* (Fig. 1) were examined serologically and found to be as active as was the original material, 0.03 μg. of which completely neutralized the test dose of agglutinin.

(2) The B-active preparation obtained from cyst fluid no. 376 that gave a sharp symmetrical peak in the ultracentrifuge at pH 4.6, with only a small amount of boundary-spreading, suggesting a relatively narrow range of molecular sizes, was readily resolved into fractions by chromatography and gave three well-defined elution peaks (Fig. 2). The materials comprising these were collected separately as fraction 1 (tubes 2–12), fraction 2 (tubes 28–50) and fraction 3 (tubes 62–92).

Fig. 1. Behaviour of blood-group Le^a substance (no. 350, 1.0 g.) on a DEAE-cellulose column (3.5 cm. × 50 cm.) equilibrated with 0.01 *M*-NaCl. Broken line, continuous gradient elution from 0.01 *M*- to 1 *M*-NaCl concentration. Contents of tubes, each containing 60 ml., were pooled as shown between arrows to give fractions 1*a*, 1*b* and 2. Elution was finally continued with saturated NaCl. The amount of mucopolysaccharide eluted in each tube was determined by phenol-sulphuric acid method (*E* at 486 mμ).

Table 1. *Composition of fractions obtained from blood-group substance no. 350 (Le^a substance) after chromatography on DEAE-cellulose*

Amino acids are expressed as: (a) g./100 g. of substance: (b) μ moles/100 μ moles of total amino acids.

	Original substance		Fraction 1a		Fraction 1b		Fraction 2	
Weight (g.)	1.0		0.38		0.41		0.045	
Nitrogen (%)	4.7		4.4		4.6		4.6	
Fucose (%)	8.7		8.9		8.8		8.7	
Hexosamine (%)	26.9		27.7		27.2		25.7	
Reducing sugar (%)	50.0		52.8		50.6		51.1	
Sialic acid (%)	18.0		17.0		19.0		18.6	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Aspartic acid	0.42	2.9	0.40	2.7	0.46	3.3	0.66	4.2
Threonine	3.70	27.6	3.66	28.0	3.74	27.2	3.75	26.7
Serine	1.70	14.4	1.69	14.7	1.76	14.4	1.65	13.3
Glutamic acid	0.62	3.8	0.59	3.6	0.70	4.2	0.80	4.6
Proline	1.69	12.9	1.66	13.1	1.70	12.6	1.68	12.3
Glycine	0.57	6.6	0.55	6.6	0.55	6.5	0.61	6.8
Alanine	1.10	10.5	1.05	10.6	1.03	10.3	1.12	10.6
Cystine (half)	0.12	0.8	0.11	0.8	0.10	0.8	0.14	1.0
Valine	0.70	5.4	0.69	5.4	0.71	5.5	0.69	5.0
Isoleucine	0.32	2.1	0.31	2.1	0.32	2.1	0.35	2.3
Leucine	0.69	4.7	0.69	4.8	0.71	4.8	0.70	4.2
Tyrosine	0.08	0.5	0.05	0.3	0.08	0.5	0.17	0.7
Phenylalanine	0.29	1.5	0.27	1.4	0.30	1.5	0.31	1.6
Lysine	0.20	1.3	0.14	0.9	0.22	1.4	0.29	1.7
Histidine	0.41	2.3	0.41	2.4	0.40	2.2	0.43	2.3
Arginine	0.50	2.7	0.48	2.6	0.52	2.7	0.55	2.7
	13.11		12.75		13.30		13.90

Fig. 2. Behaviour of blood-group B substance (no. 376, 204 mg.) on a 2 cm. x 150 cm. DEAE-cellulose column. Details were as given in Fig. 1. Contents of tubes, each containing 50 ml., were pooled as shown between arrows to give fractions 1, 2 and 3.

There was no significant difference in the amount or composition of the amino acids in each fraction (Table 2), but, whereas the first fraction had less than 1 % of sialic acid, fractions 2 and 3 contained 1.3 % and 4.3 % respectively. There was an increase in the total nitrogen in the fractions recovered with increasing concentration of sodium chloride.

Fig. 3. Antibody N precipitated from anti-B serum by the addition of increasing amounts of fractions 1, 2 and 3 (Table 2) obtained from B substance (no. 376) after separation on a DEAE-cellulose column. The amounts (μ g.) of antibody N precipitated by each fraction gave values that fell within the limits indicated by the vertical lines.

The fractions obtained were all as serologically active as the original substance, 0.05 μ g. of which completely neutralized the test dose of B-agglutinin, and each fraction had about the same activity,

Table 2. Composition of fractions obtained from blood-group substance no. 376 (B substance) after chromatography on DEAE-cellulose

(a) and (b): see Table 1.									
	Original substance		Fraction 1		Fraction 2		Fraction 3		
Weight (mg.)	204		74		47.5		41.6		
Nitrogen (%)	4.8		4.6		4.7		4.9		
Fucose (%)	18.8		19.0		19.1		18.6		
Hexosamine (%)	27.6		28.0		28.0		27.1		
Reducing sugar (%)	53.6		54.0		53.8		53.0		
Sialic acid (%)	2.5		0.8		1.3		4.3		
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	
Aspartic acid	0.71	3.8	0.55	3.0	0.70	3.7	0.75	3.8	
Threonine	4.72	28.0	4.65	28.6	4.60	27.6	4.82	27.6	
Serine	2.61	17.6	2.50	17.6	2.62	17.6	2.75	17.8	
Glutamic acid	0.90	4.3	0.80	4.0	0.88	4.3	0.93	4.3	
Proline	2.31	14.2	2.33	14.6	2.30	14.2	2.30	13.7	
Glycine	0.61	5.8	0.60	5.9	0.63	6.0	0.64	5.8	
Alanine	1.26	10.0	1.30	10.7	1.32	10.6	1.27	9.8	
Cystine (half)	—	—	—	—	—	—	0.10	0.5	
Valine	0.73	4.4	0.70	4.4	0.70	4.3	0.78	4.5	
Isoleucine	0.37	2.0	0.39	2.2	0.40	2.2	0.35	1.8	
Leucine	0.35	1.9	0.30	1.7	0.33	1.8	0.40	2.0	
Tyrosine	0.10	0.4	0.05	0.2	0.08	0.3	0.12	0.5	
Phenylalanine	0.18	0.8	0.15	0.7	0.20	0.8	0.23	1.0	
Lysine	0.39	1.9	0.30	1.5	0.35	1.7	0.44	2.0	
Histidine	0.44	2.0	0.42	2.0	0.45	2.1	0.44	1.9	
Arginine	0.67	2.8	0.68	2.9	0.70	2.8	0.76	3.0	
	16.35		15.72		16.26		17.08		

when measured by the quantitative precipitation test (Fig. 3).

(3) The mucopolysaccharide preparation obtained from the Le^a-active cyst fluid no. 444 contained a considerable amount of sialic acid (8.5 %), and had an amino acid composition (Table 3) which was unusual in that it contained much more aspartic acid, glutamic acid and cystine and less threonine, serine and proline than were most frequently encountered in preparations of blood-group substances. Elution of the material from a DEAE-cellulose column (3.5 cm. × 50 cm.) gave two peaks (Fig. 4). The first peak, fraction 1, was arbitrarily divided into two subfractions, comprising the rising (1a) and the descending (1b) portions of the peak. The amino acid composition of fractions 1a and 1b (Table 3) was found to approach more closely the amino acid composition of the Le^a mucopolysaccharide no. 350 (Table 1). The materials, fractions 1a and 1b, however, contained different amounts of sialic acid.

The original material and fractions 1a and 1b were examined in the ultracentrifuge. The sedimentation patterns obtained for fractions 1a and 1b (Figs. 5, 2 and 3) were sharper and less asymmetric than was the sedimentation diagram of the starting material [Fig. 5 (1)].

Fraction 1a was again passed through the same DEAE-cellulose column under similar conditions, and the material eluted as a single peak except for

Fig. 4. Behaviour of blood-group Le^a substance (no. 444, 0.80 g.) on a 3.5 cm. × 50 cm. DEAE-cellulose column. Details were as given in Fig. 1. Contents of tubes, each containing 70 ml., were pooled as shown between arrows to give fractions 1a, 1b and 2. Elution was finished with saturated NaCl.

a small tailing end, which was discarded. The material that made up the single peak was examined in the ultracentrifuge [Fig. 5 (4)] and was immediately rechromatographed on a longer (2.0 cm. × 150 cm.) column.

A partial resolution into two fractions, 1a₁ (tubes 27–60) and 1a₂ (tubes 61–85), was obtained (Fig. 6). A small amount of substance recovered in the effluent collected after tube 85 was discarded. Both fractions (1a₁ and 1a₂) now had an amino

acid composition (Table 4) which was close to that found for most blood-group-specific mucopolysaccharides. There was, however, a small difference in the sialic acid content of the two materials. Fractions 1a₁ and 1a₂ were examined in the ultracentrifuge and gave pictures (6) and (7) respectively (Fig. 5). Each substance gave an almost symmetrical diagram, and there was no evidence of gross heterogeneity.

Fig. 5. Ultracentrifuge patterns obtained with mucopolysaccharide fractions obtained from cyst fluid no. 444. All materials were approximately 1% total concentration in potassium acetate–potassium chloride–acetic acid buffer, pH 4.6, *I* 0.10. Speed 56100 rev./min.: patterns for (1)–(5) were obtained after 20–25 min. at speed, for (6) and (7) after 35–40 min. at speed. Phase-plate angles: 40°, 60°, 50°, 50°, 50°, 45° and 45° respectively. (1) Original blood-group substance. (2) Subfraction 1a (Fig. 4). (3) Subfraction 1b (Fig. 4). (4) 1a after rechromatography. (5) 1b after rechromatography. (6) and (7) Two subfractions (1a₁ and 1a₂) from main fraction (4) after further chromatography on a longer (150 cm.) column (Fig. 6).

Fig. 6. Further fractionation of Le^a substance no. 444 (Fig. 3, fraction 1a, 108 mg.) on 2 cm. × 150 cm. DEAE-cellulose column. Details were as given in Fig. 1. Contents of tubes, each containing 30 ml., were pooled as shown between arrows to give fractions 1a₁ and 1a₂.

Table 3. Composition of fractions obtained from blood-group substance no. 444 (Le^a substance) after chromatography on DEAE-cellulose

(a) and (b): see Table 1.

	Original substance		Fraction 1a		Fraction 1b		Fraction 2		Residue	
Weight (g.)	0.80		0.215		0.225		0.170		0.048	
Nitrogen (%)	4.5		4.2		4.0		5.7		4.0	
Fucose (%)	8.8		9.4		9.1		6.5		9.6	
Hexosamine (%)	30.6		33.3		31.2		24.1		31.1	
Reducing sugar (%)	53.5		59.1		56.1		45.5		52.0	
Sialic acid (%)	8.5		6.0		8.9		8.7		9.0	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Aspartic acid	1.18	7.8	0.56	5.0	0.50	5.3	2.05	9.5	0.55	6.2
Threonine	1.99	14.6	2.28	22.8	1.78	21.1	2.09	10.6	1.53	18.8
Serine	1.26	10.6	1.39	15.5	1.03	13.8	1.42	8.3	1.01	14.1
Glutamic acid	1.31	7.8	0.53	4.2	0.58	5.6	2.84	11.9	0.69	6.9
Proline	1.42	10.8	1.39	14.3	1.17	14.2	1.45	7.8	0.96	12.1
Glycine	0.44	5.2	0.45	7.1	0.42	7.8	0.56	4.6	0.33	6.5
Alanine	1.09	10.7	0.93	12.3	0.78	12.3	1.58	10.9	0.71	11.8
Cystine (half)	0.40	2.9	—	—	0.10	1.2	0.81	4.1	0.16	1.9
Valine	0.79	5.9	0.52	5.2	0.44	5.3	1.39	7.3	0.44	5.6
Isoleucine	0.31	2.1	0.20	1.8	0.15	1.5	0.40	1.8	0.17	1.9
Leucine	0.87	5.9	0.30	2.7	0.28	2.9	1.76	8.3	0.45	4.0
Tyrosine	0.32	1.6	—	—	—	—	—	—	—	—
Phenylalanine	0.52	2.8	0.15	1.0	0.05	0.4	0.58	2.1	0.16	1.5
Lysine	0.67	4.0	0.15	1.1	0.21	2.1	1.41	5.9	0.24	2.3
Histidine	0.59	3.3	0.46	3.5	0.38	3.3	0.72	2.8	0.34	3.2
Arginine	0.79	4.0	0.52	3.5	0.42	3.3	1.15	4.1	0.38	3.2
	13.95		9.48		8.29		20.21		8.02	

Fraction 1b (Fig. 4 and Table 3) was again subjected to chromatography on the same (3.5 cm. $\times$ 50 cm.) column and the single main fraction obtained was examined in the ultracentrifuge [Fig. 5 (5)], and was then subjected to further chromatography on a 2 cm. $\times$ 150 cm. DEAE-cellulose column. The material was partly resolved into two fractions, 1b₁ and 1b₂. The two fractions were similar in composition and closely resembled fractions 1a₁ and 1a₂ obtained from fraction 1a (Table 4). Fractions 1b₁ and 1b₂ also showed a slight difference in their contents of sialic acid, which were 7.9 % and 9.1 % respectively.

Attempts to isolate a substance containing an increased amount of acidic amino acids from the material comprising fraction 2 (Fig. 4 and Table 3) by rechromatography on DEAE-cellulose failed, as the material (150 mg.) added to a DEAE-cellulose column was, for some unexplained reason, poorly recovered. The small amount (30 mg.) of substance obtained, however, was closely similar in chemical composition to the main components (Table 3, fractions 1a and 1b).

Le^a-specific haemagglutination inhibition tests made with material comprising fractions 1 and 2 (Fig. 4) did not definitely establish that the former fraction was more active than the latter, although it was our impression that this was so. Suitable

sera were not available to determine the behaviour of these two fractions in quantitative precipitation tests.

(4) The material obtained from the B-active cyst fluid no. 435 separated into four fractions on a DEAE-cellulose column (Fig. 7), and it is evident from the results given in Table 5 that, whereas the

Fig. 7. Behaviour of blood-group B substance (no. 435, 250 mg.) on a 2 cm. $\times$ 150 cm. DEAE-cellulose column. Details were as given in Fig. 1. Contents of tubes, each containing 40 ml, were pooled as shown between arrows to give fractions 1, 2, 3 and 4.

Table 4. Composition of fractions obtained from fraction 1a (Table 3) after rechromatography on DEAE-cellulose

(a) and (b): see Table 1.

	Fraction 1a ₁		Fraction 1a ₂	
Weight	—		—	
Nitrogen (%)	4.1		4.2	
Fucose (%)	9.4		9.3	
Hexosamine (%)	33.0		33.5	
Reducing sugar (%)	58.0		58.5	
Sialic acid (%)	5.8		6.9	
	(a)	(b)	(a)	(b)
Aspartic acid	0.47	4.3	0.49	4.6
Threonine	2.56	27.0	2.48	26.6
Serine	1.33	15.6	1.30	15.6
Glutamic acid	0.58	4.8	0.60	5.0
Proline	1.33	13.3	1.34	14.5
Glycine	0.40	6.4	0.39	6.3
Alanine	0.76	10.5	0.70	10.1
Cystine (half)	0.09	0.9	0.07	0.8
Valine	0.43	4.6	0.45	4.7
Isoleucine	0.17	1.6	0.18	1.7
Leucine	0.25	2.4	0.22	2.1
Tyrosine	—	—	—	—
Phenylalanine	0.09	0.7	0.09	0.7
Lysine	0.18	1.5	0.19	1.7
Histidine	0.29	2.4	0.29	2.5
Arginine	0.42	3.0	0.43	3.1
	9.35		9.22

Fig. 8. Antibody N precipitated from anti-B serum by the addition of increasing amounts of fractions obtained from B substance (no. 435) after separation on a DEAE-cellulose column. A, Unfractionated material; B, fraction 1; C, fraction 2; D, fraction 3; E, fraction 4.

Table 5. Composition of fractions obtained from blood-group substance no. 435 (B substance) after chromatography on DEAE-cellulose

	(a) and (b): see Table 1.									
	Original substance		Fraction 1		Fraction 2		Fraction 3		Fraction 4	
Weight (mg.)	250		26		57		45		75	
Nitrogen (%)	4.5		4.3		4.1		3.8		5.9	
Fucose (%)	20.6		24.9		21.3		20.6		14.3	
Hexosamine (%)	23.9		24.5		23.4		25.8		21.0	
Reducing sugar (%)	51.4		53.6		52.1		61.0		48.8	
Sialic acid (%)	5.1		0.3		2.5		4.9		9.0	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Aspartic acid	1.06	6.7	0.56	4.1	0.47	3.7	0.40	3.7	2.30	10.5
Threonine	2.56	18.8	3.22	26.9	3.00	26.4	2.51	25.8	2.00	10.0
Serine	1.48	11.8	1.80	16.9	1.68	16.6	1.30	15.2	1.30	7.5
Glutamic acid	1.20	6.8	0.66	4.4	0.61	4.3	0.50	4.2	3.09	12.6
Proline	1.36	9.8	1.76	14.6	1.62	14.6	1.28	13.7	1.28	6.7
Glycine	0.50	5.6	0.45	5.9	0.48	6.6	0.40	6.5	0.60	4.8
Alanine	1.10	10.5	0.98	10.8	0.92	10.9	0.88	12.0	1.71	9.3
Cystine (half)	0.33	2.4	0.10	0.8	0.08	0.6	0.15	1.5	0.96	4.8
Valine	0.82	5.8	0.51	4.4	0.48	4.3	0.42	4.4	1.50	7.8
Isoleucine	0.33	2.1	0.20	1.5	0.18	1.9	0.16	1.5	0.30	1.9
Leucine	0.92	5.8	0.20	1.5	0.20	2.1	0.22	2.1	1.68	7.8
Tyrosine	0.28	1.2	0.10	0.5	—	—	0.12	0.8	0.35	1.2
Phenylalanine	0.44	2.3	0.15	0.9	0.12	0.7	0.18	1.3	0.54	2.0
Lysine	0.70	4.0	0.16	1.1	0.16	1.7	0.20	1.7	1.60	6.6
Histidine	0.50	2.7	0.42	2.7	0.40	2.6	0.32	2.4	0.60	2.3
Arginine	0.75	3.6	0.52	3.0	0.51	3.0	0.45	3.2	1.20	4.2
	14.33		11.73		10.91		9.49		21.01	

major part of the preparation comprising fractions 1, 2 and 3 had a closely similar amino acid composition, fraction 4 was different, both in the total quantity of the amino acid-containing moiety present and in its composition, which had an increased amount of aspartic acid, glutamic acid and cystine. No further separation or purification of the fractions was attempted, but they were examined in haemagglutination inhibition and precipitation tests, and the results showed that the materials having the highest blood-group activity had the expected amino acid composition. In precipitation tests (Fig. 8), fraction 4 behaved as if it was contaminated with a material of lower activity or with a substance that was serologically inactive, or with both.

Separation of an artificial mixture of mucopolysaccharides

Artificial mixtures of blood-group substances of different specificity and sialic acid content, but of closely similar amino acid composition, were examined to determine their separability on DEAE-cellulose columns, under the conditions described. In one experiment, a mixture comprising 50 mg. each of B-active material, no. 376 (Table 2, fraction 1), that contained less than 1.0 % of sialic acid, A-active substance no. 396, containing 1.8 % of sialic acid and Le^a-active material no. 350 (Table 1 and Fig. 1), containing 18 % of sialic acid,

Fig. 9. Elution pattern obtained from an artificial mixture of A-(no. 396), B-(no. 376) and Le^a-(no. 350) specific substances recovered from a 2 cm. x 150 cm. DEAE-cellulose column. Details were as given in Fig. 1. Contents of tubes, each containing 10 ml., were pooled as shown between arrows, to give fractions 1, 2 and 3.

were dissolved in 10 ml. of 0.01 M-sodium chloride and chromatographed on a DEAE-cellulose column (0.9 cm. x 150 cm.). The elution pattern obtained is given in Fig. 9, and in Table 6 the amount of the materials recovered, the sialic acid content of each fraction and their serological activities are recorded.

In another experiment, 50 mg. amounts of two B substances, nos. 376 (Table 2, fraction 1) and 435 (Table 5, fraction 3), containing 0.8 % and 4.9 % of sialic acid respectively, were mixed with 50 mg. of the Le^a-active mucopolysaccharide preparation no. 350 (Table 1). The elution pattern obtained for the mixture is given in Fig. 10. The two group-B-specific mucopolysaccharides were satisfactorily separated from each other. The Le^a activity of the fractions was not determined.

DISCUSSION

The amino acid compositions of more than 20 individual blood-group-specific mucopolysaccharides obtained from human ovarian-cyst fluids were found by Pusztai & Morgan (1963) to be closely similar, irrespective of the serological specificity of each substance within the ABO and Lewis blood-group systems. The amino acid composition conformed to a characteristic and rather original

pattern in which the four amino acids, threonine, serine, proline and alanine, predominated. The aromatic amino acids and sulphur-containing amino acids were present in small amounts only. Occasionally, however, there also occurred preparations of group-specific substances which, although similar in general chemical pattern, differed significantly in amino acid composition from that described by Pusztai & Morgan (1963). It was suspected that some other mucopolysaccharide or glycoprotein was present in the particular ovarian-cyst fluid from which the specific substance had been obtained and, because of its closely similar properties, was not separated from the specific substance during purification.

To obtain some direct evidence for this suggestion, experiments were made to determine whether, after further purification, the amino acid composition of these seemingly abnormal specific substances approached more closely, or became identical with, those preparations which comprised the majority of specific substances isolated and which were believed to be the blood-group substances essentially free from extraneous materials.

An examination of the preparations that did not have the expected amino acid pattern showed that they contained greater amounts of the acidic amino acids, aspartic acid and glutamic acid, and of cystine. It was possible, with some preparations, to demonstrate by electrophoresis that there was more than one kind of molecular species in the materials, and it seemed possible therefore that a fractionation of the substance could be made by chromatography on DEAE-cellulose columns. However, sialic acid is present to a variable extent in most preparations of the blood-group substances (Gibbons *et al.* 1955; Pusztai & Morgan, 1961*a*; Morgan, 1963, and unpublished work), and the occurrence of these acidic groups would most probably complicate the separation on DEAE-cellulose of a mucopolysaccharide from a similar material that was more acidic in character because of its enhanced content of aspartic acid and glutamic acid.

Fig. 10. Elution pattern obtained from an artificial mixture of two preparations of B substance (nos. 376 and 435) and Le^a substance (no. 350) recovered from a 2 cm. × 150 cm. DEAE-cellulose column. Details were as given in Fig. 1. Contents of tubes, each containing 10 ml., were pooled as shown between arrows, to give fractions 1, 2 and 3.

Table 6. Recovery and serological activity of materials obtained from an artificial mixture of three mucopolysaccharides with different specificity and sialic acid content after fractionation on DEAE-cellulose

	B substance (no. 376)		A substance (no. 396)		Le ^a substance (no. 350)	
	Added (fraction 1, Table 2)	Recovered (fraction 1, Fig. 9)	Added	Recovered (fraction 2, Fig. 9)	Added (fraction 1, Table 1)	Recovered (fraction 3, Fig. 9)
Amount on column (mg.)	50	—	50	—	50	—
Amount recovered (mg.)	—	42	—	39	—	41
Sialic acid (%)	0.8	0.4	1.8	2.0	17.9	17.8
A activity*	—	> 1000	0.1	0.1	—	> 1000
B activity*	0.05	0.05	—	> 1000	—	> 1000

* Minimum amount (μg./0.1 ml.) of material giving inhibition of haemagglutination.

The examination of blood-group substances on DEAE-cellulose columns nevertheless readily showed differences in behaviour, and demonstrated that a fractionation of some mucopolysaccharide preparations could be brought about, according to their content of aspartic acid and glutamic acid or of sialic acid and, in certain instances, through the occurrence of both these types of variation within the molecules in the preparation. On the basis of their behaviour on DEAE-cellulose, four selected mucopolysaccharide preparations are described and show the different types of material that can occur.

The mucopolysaccharide recovered from cyst fluid no. 350 (Pusztai & Morgan, 1961*a*) was the simplest kind of preparation examined; it was not resolved into more than one component by DEAE-cellulose (Fig. 1). It appeared, so far as could be demonstrated by the analytical procedures employed, that all molecular species in the preparation had a closely similar composition and the amino acid content of the arbitrarily selected fractions 1*a* and 1*b* (Table 1), obtained from the single elution peak, conformed closely to the pattern found earlier for the majority of highly purified preparations of blood-group-specific mucopolysaccharides (Pusztai & Morgan, 1963).

The B-active mucopolysaccharide preparation obtained from cyst no. 376 readily separated into three fractions on DEAE-cellulose chromatography (Fig. 2). Each fraction was closely similar to the amino acid composition expected (Table 2), but contained different amounts of sialic acid, which varied from less than 1.0 % to 1.3 % and 4.3 % respectively. This type of mucopolysaccharide preparation, that could be readily resolved into fractions having different amounts of sialic acid, is the one most frequently obtained from ovarian-cyst fluids.

Another kind of mucopolysaccharide preparation was obtained from cyst fluid no. 444. Here the preparation readily separated on a DEAE-cellulose column (Fig. 4) into two main fractions and some residual material. The main fractions differed only slightly in their content of sialic acid, but the amino acid composition of each was quite dissimilar (Table 3). The first component eluted resembled more closely the amino acid composition of a typical group-specific mucopolysaccharide and, after rechromatography (Table 4, fractions 1*a*₁ and 1*a*₂), became almost identical in amino acid composition with that of the most thoroughly purified specific mucopolysaccharides. However, the material was rather more inhomogeneous, in terms of distribution of sialic acid, than was preparation no. 350, the sialic acid content varying from 5.8 % for fraction 1*a*₁ (Table 4) to 9.1 % for fraction 1*b*₂. The sialic acid content is also rather higher than

that of most group-specific mucopolysaccharides but, so far as could be determined, there were no mucopolysaccharide molecules without sialic acid.

Attempts to isolate from fraction 2 (Fig. 4), by DEAE-cellulose chromatography, a substance containing an enhanced amount of aspartic acid and glutamic acid failed. The amount of sialic acid in fractions 1 and 2 (Fig. 4) would probably alone not have allowed them to be as well separated from each other on DEAE-cellulose as they were, and it seems probable therefore that the separation that did take place was due to the greater amount of acidic amino acids in fraction 2.

The mucopolysaccharide obtained from cyst fluid no. 435 showed an even more complex behaviour on DEAE-cellulose than did group substance no. 444. Four fractions were obtained (Fig. 7) which differed from each other in their content of sialic acid (Table 5), and the last fraction to be eluted contained much more aspartic acid, glutamic acid and cystine than did the first three fractions, which were closely similar in amino acid composition to the blood-group substances most frequently encountered. Fractions 1 and 2 had a B activity as high as the original material, determined by haemagglutination inhibition and precipitation tests (Fig. 8); fraction 4 had less activity.

The resolving power of DEAE-cellulose columns for artificial mixtures of selected blood-group substances was demonstrated in two experiments in which mixtures of three substances, each possessing a strictly specific and characteristic serological property and a similar amino acid composition, but which had different amounts of sialic acid, were satisfactorily separated from each other.

It is evident that quantitative amino acid analysis and chromatography on DEAE-cellulose columns of preparations of blood-group substances that have already been extensively purified, and are known to be free from gross heterogeneity, can reveal differences in individual preparations that are not readily detected by the methods generally employed. These additional techniques are of considerable value in helping to assess more accurately the extent of heterogeneity of the materials.

A naturally occurring blood-group-specific substance entirely without sialic acid has so far not been found in ovarian-cyst fluids. There is, however, no evidence that any of the steps in the purification procedures used remove sialic acid from the specific substances, but it is known that sialic acid is set free by the acid conditions employed when blood-group substances are isolated from tissues, tissue fluids and secretions by the prolonged action of pepsin at pH 1.5 and 37°.

Apart from the possibility that some preparations are still contaminated with relatively small

amounts of chemically different mucopolysaccharides, the blood-group substances appear to occur most frequently in at least two forms: those that have a high content of sialic acid, and those that have relatively little sialic acid. Examination of the former materials, as described in this paper, does not reveal any appreciable variation in the sialic acid content of the individual molecules within the preparation. In the second group, the mucopolysaccharide molecules are found to vary in their content of sialic acid, and it is always possible that in these latter preparations there are some mucopolysaccharide molecules that do not contain sialic acid. The mucopolysaccharide molecules having small, but nevertheless significant, amounts of sialic acid might arise from a sialic acid-rich material which is more or less stripped of its sialic acid by the action of a tissue sialidase (see Warren & Spearing, 1960; Carubelli, Trucco & Capulto, 1962; Morgan & Laurell, 1963), or perhaps these substances are the products of a metabolic step that adds sialic acid with varying efficiency.

The purified blood-group substances most thoroughly studied have been obtained from ovarian-cyst fluids by extracting the freeze-dried fluid with 90–95 % (w/w) phenol and removing the phenol by dialysis. The preparation at this stage can usually be separated into substances that are readily soluble in water, or are gel-like in their behaviour and have a very limited solubility in water. The mucopolysaccharides that are soluble in water, after further purification, are blood-group-specific substances, and are believed to be essentially free from impurities. These substances have been well characterized, chemically and serologically.

The gel-like materials that are very sparingly soluble in water, however, have only been subjected to limited study, the crucial problem being how to solubilize the materials without inducing significant chemical change. Their solubilization has recently been studied by Dr J. R. Dunstone, in this Laboratory, and many of the sparingly soluble materials can now be obtained as water-soluble, serologically active preparations that are free from gross heterogeneity. These preparations, however, while retaining the overall mucopolysaccharide composition and general pattern most frequently found for the most active blood-group substances, nevertheless contain proportionally more aspartic acid, glutamic acid and cystine. As a result of these investigations, it seems possible that the presence of varying amounts of these materials in the readily soluble mucopolysaccharide preparations is responsible for the greater amount of acidic amino acids and cystine that sometimes occurs, as described in this paper.

SUMMARY

1. The behaviour on columns of DEAE-cellulose of blood-group-specific mucopolysaccharides obtained from human ovarian-cyst fluids, and purified by the methods described, was investigated.

2. Some preparations were not resolved into more than one component, whereas others were readily fractionated into several materials. The heterogeneity of the specific substances was sometimes caused by the presence of a material that contained a greater amount of aspartic acid, glutamic acid and of cystine than was found in the majority of purified blood-group mucopolysaccharides. Other preparations having an apparently normal amino acid composition were heterogeneous with respect to the content of sialic acid in individual molecules in the preparation. Some preparations were heterogeneous for both these types of variation.

3. Possible reasons for the increased amounts of aspartic acid, glutamic acid and cystine, or the variability in the sialic acid content of the mucopolysaccharide preparations examined, or both, are briefly discussed.

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