Studies in Immunochemistry

19. FURTHER OBSERVATIONS ON THE PREPARATION AND PROPERTIES OF HUMAN BLOOD-GROUP-SPECIFIC MUCOPOLYSACCHARIDES*

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The human blood-group-specific mucopolysaccharides are usually obtained by extracting freeze-dried secretions, such as saliva, gastric juice, ovarian-cyst fluids, meconium or tissue digests with liquid 90 or 95 % (w/v) phenol. The active substances are largely insoluble in this solvent. If soluble, however, as they frequently are after liberation from tissues by digestion with pepsin at pH 1-2, they can be readily recovered by the addition of low concentrations of ethanol. Mucopolysaccharides isolated in this way are free from gross contamination with unspecific protein and fatty substances which occur in the native secretions and tissue extracts, and after further purification by fractional precipitation from aqueous solution with suitable organic solvents are the materials which have been most frequently used for chemical and serological studies (cf. Kabat, 1956; Morgan, 1959*a*, 1960).

Until recently these materials were believed to be substantially homogeneous, but a more thorough examination has now shown that in most instances they can be separated into at least two kinds of mucopolysaccharide substance, which, although closely similar in many respects, differ from each other in certain important properties. The method used to bring about the separation depends on the solubility differences of the materials in saturated ammonium sulphate at 60°.

The behaviour of these substances in saturated ammonium sulphate at 60° has been used to develop a simple procedure which allows the specific-blood-group mucopolysaccharides to be isolated from native cyst fluids without the use of phenol and thus enables them to be obtained by a method which is different from that usually employed. Details of this work are given in the present paper.

EXPERIMENTAL

Cyst fluids obtained from pseudomucinous ovarian cystadenomata, were kept at 1-3° after the addition of toluene or were dried from the frozen state as soon as possible after removal from the patient. The materials were obtained from 'secretor' persons belonging to each of the groups, A₁, B, O, A₁B and A₂B and from 'non-secretor'

persons. The fluids from this latter class of individual were the source of Le^a-specific substance which was free from A, B or H serological character (Grubb & Morgan, 1949; Annison & Morgan, 1952*a*).

Chemical analyses. All preparations were thoroughly dialysed against distilled water at 1-3° and dried from the frozen state. The materials were further dried for 30 min. over P₂O₅ at 78° and 1-2 mm. Hg pressure before analysis. The methods used for the determination of N, fucose, hexosamine and reducing sugars after acid hydrolysis are as given by Pusztai & Morgan (1961). Quantitative amino acid analyses were made by the method of Moore & Stein (1954) on materials which had been refluxed with constant-boiling HCl on an oil bath for 24 hr. at 135-140°.

Physical examination. Ultracentrifugal and electrophoretic examinations were made as described by Kekwick (1950). The relative viscosity was determined at 20° at a concentration of 1 % (w/v) in water in an Ostwald-type viscosimeter. The intrinsic viscosities were determined from measurements made at 20° in phosphate buffer (I 0.2) containing 0.15 M-NaCl, pH 8.

Serological examination. The materials were examined for their capacity to inhibit specific haemagglutination as described by Morgan & King (1943). Naturally occurring anti-A (α) and anti-B (β) agglutinins in normal human sera were used for measuring A and B activity. Immune rabbit anti-A serum and A-specific plant-seed agglutinins (Morgan & Watkins, 1953) were also used as anti-A reagents in the inhibition tests. The H activity (Morgan & Watkins, 1948; Annison & Morgan, 1950; Watkins & Morgan, 1955) was measured by titration with rabbit immune anti-H sera prepared from H substance as described by Annison & Morgan (1952*b*). In certain instances a human anti-H agglutinin (Watkins & Morgan, 1954), an eel anti-H serum (Jonsson, 1944; Watkins & Morgan, 1952) or an H-specific plant-seed agglutinin (Morgan & Watkins, 1953) were also used. The Le^a activity of the materials was measured as described by Grubb & Morgan (1949) and Annison & Morgan (1952*a*) with a human anti-Le^a serum.

The A activity of certain of the A-specific substances was also determined according to the precipitin method of Heidelberger & MacPherson (1943), as developed by Kabat & Bezer (1945).

Group A preparations were examined for their capacity to inhibit the haemolysis of sheep erythrocytes by rabbit anti-A cell haemolytic serum and complement (Morgan & King, 1943).

Blood-group-specific mucopolysaccharides. The materials were prepared according to the method given by Gibbons, Morgan & Gibbons (1955), in which dried cyst fluid was extracted with 90 or 95 % (w/w) phenol and the phenol-

* Part 18: Pusztai & Morgan (1961).

insoluble residue freed from phenol by thorough dialysis against water. The thick viscous solution obtained was centrifuged to remove any water-insoluble gel and to the clear supernatant fluid was added 2% (w/v) potassium acetate and increasing amounts of ethanol. In most instances, the major part of the material contained in the extract was precipitated between 45 and 55% (w/v) ethanol concentration; the material was immediately dissolved in water to give a 2% (w/v) solution, reprecipitated within the same concentrations of ethanol, and the material obtained thoroughly dialysed against distilled water at 1–3° and dried. Preparations of group-specific mucopolysaccharides made in this way were used for the fractionation experiments described below. Unless stated otherwise, all materials used were insoluble in 90% phenol and soluble in water. The analytical figures for some of the materials studied are given in Table 1.

A typical example of the procedure used to separate these mucopolysaccharide preparations into two closely similar specific substances was as follows. An aqueous 1% (w/v) solution of the purified mucopolysaccharide (3 g.) obtained from cyst fluid no. 332 (group A) was saturated with $(\text{NH}_4)_2\text{SO}_4$, heated with a slight excess of $(\text{NH}_4)_2\text{SO}_4$ crystals to about 60° and held at this temperature for about 5 min. A flocculent precipitate formed and was collected after cooling the solution by skimming from the surface, with or without previous centrifuging. Any part of the precipitate detached during this operation was recovered by filtration. Reheating the supernatant solution to 70–80° in the presence of a slight excess of solid $(\text{NH}_4)_2\text{SO}_4$ gave no further precipitate. The precipitate was thoroughly washed with saturated $(\text{NH}_4)_2\text{SO}_4$, dissolved in water and dialysed to remove salt. The supernatant solution (or filtrate or both) and washings were combined, dialysed to remove $(\text{NH}_4)_2\text{SO}_4$ and concentrated to about 100 ml. Both materials were then dried from the frozen state, weighed and analysed. The preparations were dissolved separately in water to give about 1% (w/v) concentration, saturated with $(\text{NH}_4)_2\text{SO}_4$ at 60° and heated at this temperature for about 5 min. The material which was originally insoluble under these conditions again precipitated and the component which was earlier soluble in the hot saturated $(\text{NH}_4)_2\text{SO}_4$ remained in solution. The materials recovered after the second treatment with $(\text{NH}_4)_2\text{SO}_4$ showed no significant change in weight. The analytical figures for the two materials obtained are given in Table 1.

The fractionation experiment was repeated on the phenol-insoluble mucopolysaccharide obtained from another group A cyst fluid (no. 368). A similar separation into material which was insoluble or soluble in saturated $(\text{NH}_4)_2\text{SO}_4$ at 60° occurred. The analytical figures are given in Table 1.

Mucopolysaccharide materials obtained from two group O cyst fluids (nos. 338 and 324), from a group A₁B-active fluid (no. 117), a fluid obtained from a person belonging to group A₂B (no. 186) and from a Le* + 'non-secretor' person (cyst no. 238) were similarly separated into mucopolysaccharides which were either insoluble or soluble in saturated $(\text{NH}_4)_2\text{SO}_4$ at 60°. All substances were finally precipitated from water by ethanol, thoroughly dialysed at 1–3°, centrifuged at 30 000 g for 30 min. to remove traces of insoluble material, dried from the frozen state and analysed. The results are given in Table 1.

Polydispersity of mucopolysaccharide preparations. Material (about 1 g.), dissolved in phosphate buffer (I 0.2,

pH 8.0) containing 0.15M NaCl to yield a 1–2% (w/v) solution, was centrifuged for 5 hr. at 0–1° and 105 000 g (average value) in a Spinco (model L) preparative centrifuge. The contents of each of the centrifuge tubes were then divided into four approximately equal parts, starting from the top, and each consisting of the liquid contained in about a 1.8 cm. length of the centrifuge tube. The materials so recovered were separately dialysed to remove salts and dried from the frozen state. There was usually a small amount of the mucopolysaccharide deposited on the bottom of the centrifuge as a semi-transparent gel.

RESULTS

The results of the experiments described above, and of many similar ones not described here, show that highly active preparations of the blood-group-specific mucopolysaccharides, obtained from dried ovarian-cyst fluid by extraction with 90% (w/w) phenol, can be separated in most instances into at least two mucopolysaccharide components, one of which is insoluble in saturated ammonium sulphate at 60°, and the other completely soluble under these conditions. The relative amount of each material finally recovered from the phenol-insoluble, water-soluble mucopolysaccharide preparation varies considerably according to the cyst fluid. Four cyst fluids, the total so far available to us, derived from 'secretors' belonging to group B, were all found to be soluble in saturated ammonium sulphate at 60°. The analytical figures obtained for these materials were, however, closely similar to those obtained for the other group-specific substances and will be described later.

In three instances, cyst fluids no. 277 (group O), no. 346 (group A₁) and no. 350 (group Le*), the phenol-insoluble, water-soluble material recovered was found to be almost completely insoluble in saturated ammonium sulphate at 60°.

Physical properties. The mucopolysaccharides precipitated by hot saturated ammonium sulphate solution frequently separate as a thick visco-elastic mass which can be easily and completely recovered. Some mucopolysaccharides are, however, first thrown out of solution as light flocculent precipitates which rapidly become sticky, coalesce and are then readily collected from the surface of the saturated ammonium sulphate. The relative viscosity of the mucopolysaccharides recovered shows considerable variation (Table 1). Repeated treatment of the materials with hot ammonium sulphate under the conditions described, however, does not alter their viscosity. The values found for the relative viscosity of a 1% (w/v) solution of the substances in water at 20° show that the materials insoluble in hot ammonium sulphate possess, in every instance examined, a higher viscosity than that found for the materials soluble in saturated ammonium

Fig. 1. Electrophoretic patterns of H-active mucopolysaccharides. Ascending boundaries in phosphate buffer, pH 8, I 0.2, after 55 mA hr. Concn. 2% (w/v). Materials (a) (324) and (b) (277) are mucopolysaccharides insoluble in 90% phenol, soluble in water and insoluble in saturated $(\text{NH}_4)_2\text{SO}_4$ at 60° . Substance (c) is material from cyst substance 324, which is insoluble in 90% phenol and without further treatment. Material (d) is substance derived from 324, which was soluble in saturated $(\text{NH}_4)_2\text{SO}_4$ at 60° . Phenol-insoluble, water-insoluble mucopolysaccharide 324, was made soluble with pepsin at pH 1.9 and separated into materials insoluble (e) and soluble (f) in saturated $(\text{NH}_4)_2\text{SO}_4$ at 60° .

sulphate at 60° (Table 1), provided that the substances compared are obtained from the same cyst fluid.

Immediately after their precipitation from hot saturated ammonium sulphate, the mucopolysaccharides are insoluble in cold saturated ammonium sulphate solution, whereas after they have been freed from salt by dialysis and recovered they are soluble in cold saturated ammonium sulphate, as indeed they were before heating.

The optical rotation of the mucopolysaccharides insoluble in hot saturated ammonium sulphate are less laevorotatory than are the materials soluble under these conditions and obtained from the same phenol-insoluble, water-soluble cyst preparation (Table 1).

Electrophoretic examination of the materials obtained from the phenol-insoluble preparations shows that the materials insoluble in saturated ammonium sulphate at 60° are usually electrophoretically homogeneous (Fig. 1a, b), and this finding holds for the ammonium sulphate-insoluble mucopolysaccharides obtained from the phenol-insoluble water-soluble preparations which are themselves electrophoretically inhomogeneous (Fig. 1c). The materials soluble in hot saturated ammonium sulphate, however, frequently show some degree of electrophoretic heterogeneity (Fig. 1d), which is perhaps to be expected since the materials soluble in saturated ammonium sulphate at 60° might well contain, besides the serologically specific mucopolysaccharide, other materials, there in relatively small amounts only, which remain in the phenol-insoluble water-soluble preparations and are soluble under these conditions. In certain instances, however, both the ammonium sulphate-insoluble and -soluble materials are electrophoretically homogeneous.

Ultracentrifugal studies were made only with electrophoretically homogeneous mucopolysaccharide preparations and the materials proved to be, without exception, substantially homogeneous, although polydisperse (Fig. 2a-d).

The sedimentation coefficients of the ammonium sulphate-insoluble and -soluble components derived from cyst 368 (group A_1) were determined at various concentrations, and the values obtained were found to be strongly dependent on the concentration (Table 2). The intrinsic viscosity value for each material was determined by a linear extrapolation to zero concentration of the η spec./c values (Table 2) plotted against the concentration. The molecular weight was calculated from the sedimentation and viscosity data by means of the formula (cf. Schachman, 1957)

$$M = \frac{4690 \times (S_{20,w})^{\frac{1}{2}} \times (\eta)^{\frac{1}{2}}}{(1 - \bar{v}\delta)^{\frac{1}{2}}},$$

Fig. 2. Sedimentation diagrams of A- and H-active mucopolysaccharides. Substances (1%, w/v) in phosphate buffer at pH 8 (*I* 0.2) containing 0.15M-NaCl. Centrifuging at 270 000 *g*. Substances (a), (b) and (c) are materials insoluble in 90% phenol, soluble in water and insoluble in saturated $(\text{NH}_4)_2\text{SO}_4$ at 60°, obtained respectively from cyst fluids no. 277 (H-active), 346 and 368 (A-active). (a) and (b) 135 min. and (c) 140 min. after reaching 270 000 *g*. Material (d) is insoluble in 90% phenol, soluble in saturated $(\text{NH}_4)_2\text{SO}_4$, from cyst fluid no. 368. Pattern after 140 min. at 270 000 *g*.

Table 2. *Physical properties of group A mucopolysaccharide from cyst fluid no. 368*

(1) Sedimentation coefficients, (2) intrinsic viscosity (η spec./c) values at different concentrations, (3) partial specific volumes and (4) molecular weights for the mucopolysaccharide components (a) insoluble and (b) soluble in saturated ammonium sulphate at 60°. All measurements were made in phosphate buffer, pH 8.0 (*I* 0.2), containing 0.15M-NaCl.

(1) Sedimentation coefficients

Concn. (mg./ml.)	(a)	(b)
10.0	5.56	5.42
7.5	6.36	5.95
5.0	7.05	6.57
Infinite dilution	8.55	7.73

(2) η spec./c values

Concn. (mg./ml.)	(a)	(b)
10.0	1.540	0.700
5.0	1.270	0.610
2.5	1.135	0.572
1.25	1.080	—
Infinite dilution	1.002	0.530

(3) Partial specific volumes

Concn. (mg./ml.)	(a)	(b)
10.0	0.625	0.625

(4) Molecular weight

(a)	(b)
5.11×10^5	3.19×10^5

where M is the molecular weight, $S_{20,w}$ the sedimentation coefficient at infinite dilution, $[\eta]$ the intrinsic viscosity, $\bar{v}$ the partial specific volume and δ the density of the solution. The molecular weights obtained are given in Table 2.

The degree of polydispersity of a few mucopolysaccharide materials insoluble or soluble in saturated ammonium sulphate at 60° were determined, the simple procedure described in the Experimental section being used (see also Pusztai & Morgan, 1961). The results show that the material which was insoluble in saturated ammonium sulphate at 60°, and obtained from cyst fluid no. 368 (group A₁) was readily resolved into a range of molecular entities of different sizes in spite of the apparent homogeneity of the material when examined by the usual sedimentation procedure. The corresponding material which was soluble in saturated ammonium sulphate at 60°, and was obtained from the same cyst fluid, likewise yielded materials which showed a range of molecular sizes. The physical, chemical and serological data obtained for the different mucopolysaccharide fractions are given in Table 3. If the percentage recovery of each fraction obtained from these materials is plotted against the corresponding intrinsic viscosity value, a typical Gaussian curve for the molecular size distribution is obtained and shows that the majority of the mucopolysaccharide entities in each preparation are closely similar in size (Fig. 3).

Similar information on the approximate size distribution, in terms of intrinsic viscosity, in a preparation of group-specific mucopolysaccharide can also be obtained by careful fractionation with ethanol. Thus the component insoluble in saturated ammonium sulphate obtained from cyst fluid no. 368 was reprecipitated twice from 1% (w/v) solution between 50 and 55% ethanol concentration, and the resulting material was subjected to a similar fractionation in the preparative ultracentrifuge. The molecular size-distribution curve obtained showed a pronounced sharpening, resulting from the removal during fractionation of those mucopolysaccharide units having a smaller size than the average (Fig. 3).

Chemical properties. The analytical results given in Table 1 show that without exception the components insoluble in saturated ammonium sulphate at 60° contain more nitrogen than do the corresponding soluble materials, and that the latter, with the single exception of the substance recovered from the phenol-insoluble, water-soluble material obtained from cyst no. 117, A₁B, contain more carbohydrate than do the former. As the total amino sugar is closely similar for each pair of substances, it seems possible that components insoluble in saturated ammonium sulphate solution at 60° have a greater amount of amino acid per molecule of

Table 3. *Chemical, physical and serological properties of mucopolysaccharide preparations obtained from cyst fluid no. 368 (group A)*

Material insoluble in 90% phenol was separated into two mucopolysaccharide components: (a) insoluble and (b) soluble in saturated ammonium sulphate at 60°; each material was separated into five fractions by differential centrifuging at 105 000 g for 5 hr. at 0–2°.

	Recovery		N (%)	Fucose (%)	Amino sugar* (%)	Reducing sugar* (%)	Intrinsic viscosity η spec./c	Serological activity	
	(mg.)	(%)						Haemag- glutination- inhibition end point (μ g.)	Antibody N precipitated (μ g.)
					(a) Insoluble				
First fraction (top)	64	6.3	4.7	15.8	28.6	49.4	0.637	0.08	99.0
Second fraction	190	18.4	4.8	15.9	29.0	50.2	0.876	0.04	101.6
Third fraction	233	22.6	4.9	16.0	29.4	50.8	0.920	0.04	103.0
Fourth fraction (bottom)	496	48.2	5.1	16.3	29.8	51.2	1.064	0.02	104.4
Deposit	47	4.5	5.4	15.8	28.2	50.0	1.310	0.02	104.4
					(b) Soluble				
First fraction (top)	80	8.0	3.8	18.0	29.8	52.3	0.218	0.6	84.5
Second fraction	201	19.6	4.4	17.0	30.5	54.4	0.427	0.3	85.8
Third fraction	226	22.0	4.5	17.5	31.3	55.0	0.445	0.3	88.4
Fourth fraction (bottom)	503	48.8	4.4	17.4	31.8	55.6	0.535	0.15	89.8
Deposit	17	1.6	4.5	16.3	31.6	55.3	0.557	0.15	89.8

* After hydrolysis at 100° with 0.5N-HCl for 18 hr. expressed as glucosamine base or as glucose.

Fig. 3. Approximate size distribution of mucopolysaccharide preparations obtained from cyst fluid no. 368 (group A₁). The phenol-insoluble water-soluble material was separated into material soluble or insoluble in saturated (NH₄)₂SO₄ at 60°. Each material was centrifuged at 105 000 g and divided into five fractions (see text). A portion of the material insoluble in phenol, soluble in water, was also precipitated twice between 50 and 55% (v/v) of ethanol and similarly separated by centrifuging into five fractions. The percentage of total material centrifuged which was recovered in each fraction was plotted against the intrinsic viscosity of each fraction for material soluble (▲) or insoluble (●) in saturated (NH₄)₂SO₄ at 60° and for materials obtained from the ethanol-fractionated substance (■).

polysaccharide than have the soluble substances isolated from the same source. Some preliminary results for the amino acid composition of three pairs of substances are set out in Table 4.

Serological properties. Although the activity of individual preparations which possess the same specificity, for example, all of which belong to group A, show marked differences in quantitative serological behaviour, nevertheless the mucopolysaccharides insoluble in saturated ammonium sulphate which have A or H activity are found to be more active than the corresponding soluble substances obtained from the same cyst fluid (Table 1). However, the constant and significant difference found in the capacity of the two kinds of mucopolysaccharide material obtained from a single source to neutralize the natural human group-specific isoagglutinins is less pronounced when human or animal immune agglutinins, natural eel-serum or plant-seed agglutinins are employed as test reagents (Tables 5 and 6).

The amounts of antibody nitrogen precipitated from rabbit immune anti-A serum by the addition of increasing quantities of the original phenol-insoluble, water-soluble mucopolysaccharide and by the materials insoluble or soluble in ammonium sulphate separated from it, are shown in Figs. 4 (substance 332) and 5 (substance 186). In every instance examined so far, the material insoluble in saturated ammonium sulphate at 60° precipitates more antibody from the test serum than does the

Table 4. Quantitative amino acid analyses of group-specific mucopolysaccharides

Materials insoluble in 90% phenol isolated from cyst fluids were separated into mucopolysaccharides: (a) insoluble and (b) soluble in saturated (NH₄)₂SO₄ at 60°. Results are expressed as g. of amino acid/100 g. of mucopolysaccharide. Analysis of materials 332 (a) and (b) were kindly made by Dr Gabrielle Ellinger. Analyses of substances 324 (a) and (b), and 186 (a) and (b) were obtained by Dr K. Bailey to whom we wish to express our thanks.

Amino acid	A substance 332		H substance 324		Substance 186	
	(a)	(b)	(a)	(b)	(a)	(b)
Aspartic acid	0.31	0.46	0.62	0.25	0.45	0.30
Threonine	4.05	2.90	5.55	3.90	3.60	1.90
Serine	2.16	1.57	1.61	2.11	2.20	1.13
Glutamic acid	0.72	0.82	1.02	0.53	0.59	0.39
Proline	2.04	1.62	2.42	1.79	1.50	1.66
Glycine	0.43	0.37	0.49	0.30	0.33	0.30
Alanine	0.92	0.83	1.00	0.65	0.64	0.71
Valine	0.59	0.41	0.80	0.46	0.55	0.33
Methionine	0.11	0.12	0.11	—	—	—
Isoleucine	0.41	0.31	0.49	0.41	0.31	0.15
Leucine	0.35	0.35	0.54	0.46	0.36	0.19
Tyrosine	0.15	0.08	0.23	—	0.15	} 0.32
Phenylalanine	0.29	0.26	0.27	—	0.20	
Lysine	0.35	0.27	0.76	0.71	0.77	0.33
Histidine	0.70	0.78	0.53	0.70	0.49	0.44
Arginine	0.50	0.44	0.61	0.27	0.71	0.38
	14.08	11.59	17.05	12.54	12.85	8.53

Table 5. Haemagglutination-inhibition end points and activity ratios of blood-group A-specific mucopolysaccharides

Materials (a) insoluble and (b) soluble in saturated (NH₄)₂SO₄ at 60° were titrated in inhibition tests with three to four agglutination doses of anti-A reagents of different origin and group A cells. Results are given as minimum amount (μg.) of substance giving inhibition of agglutination.

Anti-A reagent	A substance from cyst fluid no. 332			A ₂ B substance from cyst fluid no. 186		
	(a)	(b)	(b)/(a)	(a)	(b)	(b)/(a)
Normal human serum (1:10)*	0.006	0.4	66	0.25	8	32
Immune human serum (1:50)*	0.4	6	15	62	500	8
Immune rabbit serum (1:200)*	0.4	1.6	4	1	8	8
<i>Vicia cracca</i> extract (1:30)*	0.4	1.6	4	1	4	4
<i>Dolichos biflorus</i> extract (1:20)*	3	6	2	4	16	4

* Dilution of serum or 10% seed extract used as test dose of agglutinin.

Table 6. Haemagglutination-inhibition end points and activity ratios of H-active mucopolysaccharides

Materials (a) insoluble and (b) soluble in saturated (NH₄)₂SO₄ at 60° were titrated in inhibition tests with three to four agglutination doses of anti-H reagents of different origin and group O cells. Results are given as minimum amount (μg.) of substance giving inhibition of agglutination.

Anti-H reagent*	Substance from cyst fluid no. 186 (A ₂ B)			H substance from cyst fluid no. 338		
	(a)	(b)	(b)/(a)	(a)	(b)	(b)/(a)
Immune human serum (1:100)	125	500	4	2	62	31
Rabbit immune serum (1:25)	0.05	0.2	4	0.25	0.50	2
Cattle serum (1:2)	0.2	0.2	1	2	4	2
Eel serum (1:50)	0.8	0.8	1	2	8	4

* See footnote Table 5.

ammonium sulphate-soluble component obtained from the same source.

The serological activity of each fraction obtained from the ammonium sulphate-insoluble or -soluble components of A substance (cyst no. 368) after differential sedimentation at 105 000 g for 5 hr. was plotted against the intrinsic viscosity value of each material, the estimate of the serological activity being obtained from the measurement of the minimum amount of material required (a) to inhibit isoagglutination (Fig. 6) or (b) precipitate antibody nitrogen (Fig. 7).

The so-called Forssman activity (Schiff & Adelsberger, 1924) of this group A substance, and of the materials separated from it by ammonium sulphate treatment, was found to be the same within the limits of sensitivity of the test system.

Action of pepsin on the mucopolysaccharides

The consistently higher nitrogen content of the ammonium sulphate-insoluble mucopolysaccharide compared with that of the corresponding soluble material obtained from the same preparation of phenol-insoluble, water-soluble mucopolysacchar-

ide made it expedient to establish that the additional nitrogen was a part of the macromolecule and not a co-existing impurity, and whether it could be removed without changing the physical and serological properties of the mucopolysaccharide. To obtain this evidence the mucopolysaccharide obtained from cyst fluid 186 (Table 1), which was insoluble in saturated ammonium sulphate at 60° after three treatments with this reagent, was digested with crystalline pepsin and the products were recovered. The mucopolysaccharide remaining insoluble in saturated ammonium sulphate at 60°, however, was found to be closely similar in chemical composition to the original material, and there was no significant decrease in the nitrogen content (Table 7). A number of changes in the starting material had taken place, however, the most obvious being the formation of a certain amount of mucopolysaccharide which was soluble in hot saturated ammonium sulphate (Table 7). The material was similar to, but not identical with, the ammonium sulphate-soluble material recovered from the original mucopolysaccharide preparation (Table 1, substance 186, c). As it was possible that some or all of the limited changes induced could

Fig. 4. Antibody N precipitated from antibody by the addition of increasing amounts of mucopolysaccharide. Substance no. 332 (group A), phenol-insoluble, water-soluble (○), and material derived from it which was insoluble (●) or soluble (▲) in saturated $(\text{NH}_4)_2\text{SO}_4$ at 60°.

Fig. 5. Antibody N precipitated from antibody by the addition of increasing amounts of mucopolysaccharide. Substance no. 186 (group A₂B), phenol-insoluble, water-soluble (○), and material derived from it which was insoluble (●) or soluble (▲) in saturated $(\text{NH}_4)_2\text{SO}_4$ at 60°.

have been due to hydrolysis brought about by the citrate-hydrochloric acid buffer (pH 1.9) acting for 3 days at 37°, the following control experiment was made. A portion (200 mg.) of the mucopolysaccharide was incubated under the same conditions but without pepsin. The major part of the material was unchanged and retained its insolubility in saturated ammonium sulphate at 60°. There was formed, however, about 25 % of a mucopolysaccharide material which was soluble in saturated ammonium sulphate at 60° and was closely similar in properties to the soluble component found after peptic digestion (Table 7).

The mucopolysaccharide soluble in saturated ammonium sulphate which was obtained from the phenol-insoluble, water-soluble material recovered from cyst fluid no. 186 (Table 1, substance 186, c) was similarly incubated with pepsin. The analytical figures for the recovered material indicated (Table 8) that little change had occurred. The particular preparation of rabbit anti-H serum used to determine the H activity (cf. Annison & Morgan, 1950) showed that there was some loss in capacity to neutralize H agglutinins. However, other rabbit anti-H reagents showed that it was not always possible to demonstrate loss in H activity after the H substance had been incubated with pepsin.

Fig. 6. Relation between serological activity and intrinsic viscosity of mucopolysaccharide preparations. Serological activity was determined as the minimum amount of substance ($\mu\text{g.}$) inhibiting three to four completely agglutinating doses of specific antibody. Titrations were made on phenol-insoluble, water-soluble substance obtained from cyst fluid no. 368 (group A_1) after the material had been separated into mucopolysaccharide which was (a) soluble or (b) insoluble in saturated $(\text{NH}_4)_2\text{SO}_4$ at 60°. Each substance was then separated into five fractions by centrifuging at 105 000 g (see text) and the intrinsic viscosity (η spec./c) value of each fraction is plotted against the reciprocal of the serological activity of the fraction.

Solubilization of a water-insoluble mucopolysaccharide gel with pepsin and treatment with saturated ammonium sulphate at 60°

It has been known for a considerable time (Aminoff, Morgan & Watkins, 1950) that extraction of the freeze-dried contents of ovarian-cyst fluids and other secretions with 90 % (w/w) phenol gives a mucopolysaccharide which is insoluble in this solvent and is, in part, insoluble in water. These water-insoluble components are readily solubilized by pepsin and are then obtained in a suitable condition for treatment with hot saturated ammonium sulphate solution.

The phenol-insoluble, water-insoluble component of cyst fluid no. 324 (group O) was incubated with crystalline pepsin under the conditions given in Table 9. The gel dissolved rapidly and, after a few days' digestion, the solubilized mucopolysaccharide was isolated and finally precipitated from solution in water by the addition of ethanol up to 55 % (v/v) concentration. The analytical results (Table 9) show that after the solubilization with pepsin and fractionation with ammonium sulphate the mucopolysaccharide preparation is resolved into two muco-

Fig. 7. Relation between serological activity and intrinsic viscosity of mucopolysaccharide preparations. Serological activity was determined as the maximum amount of antibody N ($\mu\text{g.}$) precipitated by the preparation in the presence of excess of antibody. Determinations were made on phenol-insoluble, water-soluble substance obtained from cyst fluid no. 368 (group A_1) after the material had been separated into mucopolysaccharide which was (a) soluble or (b) insoluble in saturated $(\text{NH}_4)_2\text{SO}_4$ at 60°. Each substance was then separated into five fractions by centrifuging at 105 000 g (see text) and the intrinsic viscosity (η spec./c) value of each fraction was plotted against the serological activity of the fraction.

Table 7. *Action of pepsin on the specific mucopolysaccharide obtained from cyst fluid no. 186 (group A₂B)*

Mucopolysaccharide (2 g.), insoluble in saturated (NH₄)₂SO₄ at 60°, was incubated with crystalline pepsin (40 mg.) in 0.1 M-citrate-HCl buffer, pH 1.9, for 3 days at 37°. Control experiment (200 mg.): same mucopolysaccharide and buffer but without pepsin. Toluene was added as preservative. Material after digestion was dialysed and separated into mucopolysaccharide, (a) insoluble and (b) soluble in saturated (NH₄)₂SO₄ at 60°.

	Original material	Substances recovered after incubation			
		With pepsin		Without pepsin	
		(a)	(b)	(a)	(b)
Amount (g.)	—	0.95	0.69	0.152	0.042
Nitrogen (%)	5.3	5.1	4.7	5.3	4.7
Fucose (%)	20.3	19.8	21.3	20.5	21.0
Amino sugar (%)*	25.1	25.7	27.2	25.8	26.9
Reducing sugar (%)*	50.0	50.6	54.2	49.6	53.8
Relative viscosity (η)	3.38	2.60	2.08	3.45	1.75
[α] _D in water (c, 1%)	-30°	-22°	-26°	-28°	-26°
A activity (μg.)†	1.0	1.0	4.0	1.0	4.0
H activity (μg.)‡	0.2	1.6	3.2	0.2	0.4

* After hydrolysis at 100° with 0.5 N-HCl for 18 hr.

† Inhibition end point with human anti-A (α) serum.

‡ End point of inhibition with rabbit immune anti-H serum.

Table 8. *Action of pepsin on group-specific mucopolysaccharide obtained from cyst fluid no. 186 (group A₂B)*

Mucopolysaccharide (0.65 g.), soluble in saturated ammonium sulphate at 60°, was incubated with pepsin as described in Table 6. Material remained completely soluble in saturated (NH₄)₂SO₄ at 60° and, before analysis, was precipitated twice from 1% (w/v) solution in water between 45 and 50% (w/v) ethanol concentration.

	Original material	Material after digestion
Nitrogen (%)	4.2	3.9
Fucose (%)	21.6	22.2
Amino sugar (%)*	27.8	25.2
Reducing sugar (%)*	56.2	57.8
Relative viscosity (η)	1.96	1.50
[α] _D in water (c, 1%)	-47°	-43°
A activity (μg.)†	16	16
H activity (μg.)‡	0.2	3

* See footnote Table 7.

† End point of inhibition with human anti-A (α) serum.

‡ End point of inhibition with rabbit immune anti-H serum.

polysaccharides, one of which is insoluble in hot saturated ammonium sulphate and the other soluble in this reagent. The substances are electrophoretically homogeneous at pH 8 (Fig. 1e, f) and are closely similar in those physical, chemical and serological properties examined to the mucopolysaccharides isolated from the material originally insoluble in 90% phenol, soluble in water and obtained from the same cyst fluid (Table 1, b and c). The procedure described has been applied, with similar results, to the paramucinous or 'trembling-jelly' type of ovarian-cyst contents.

Isolation of blood-group-specific mucopolysaccharides from ovarian-cyst fluids without the use of phenol

For reasons given by Morgan (1958) attempts were made to obtain blood-group-specific mucopolysaccharides from crude ovarian fluids without employing phenol at any stage in the isolation procedure. The large amount of unspecific protein in cyst fluids precludes the direct treatment with hot saturated ammonium sulphate, as described above, or indeed the use of methods which depend on the

Table 9. *Analytical figures for mucopolysaccharides obtained by the solubilization of water-insoluble mucopolysaccharide gel by pepsin*

Water-insoluble mucopolysaccharide gel from cyst fluid (no. 324) dissolved in 0.1 M-citrate-HCl buffer, pH 1.9, and incubated with 1% (w/w) crystalline pepsin for 3 days at 37°. Toluene was added as preservative. Digest was dialysed and separated into material (a) insoluble and (b) soluble in saturated (NH₄)₂SO₄ at 60°.

	Original mucopolysaccharide gel	Gel solubilized with pepsin and separated into	
		(a)	(b)
Amount (g.)	3.9†	1.9	1.8
Nitrogen (%)	7.7	6.1	5.0
Fucose (%)	9.7	16.0	17.8
Amino sugar (%)*	17.0	25.1	26.3
Reducing sugar (%)*	39.0	48.2	55.9
Relative viscosity (η)	—	3.52	1.79
[α] _D in water (c, 1%)	—	-29°	-38°
H activity (μg.)†	—	0.05	0.10

* See footnote Table 7.

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

‡ Dry weight.

simple denaturation of the protein by heat or organic solvents, because any procedure involving the removal of large amounts of protein by precipitation also removes by entrainment and unspecific binding considerable amounts of mucopolysaccharide. A small amount of mucopolysaccharide which is soluble in saturated ammonium sulphate can usually be recovered from cyst fluids which are suitably diluted and saturated with this salt, and heated to 60°, thus demonstrating that mucopolysaccharides soluble under these conditions and isolated by the procedures described earlier in this paper exist in native cyst fluids. The method is, however, of limited practical importance.

Table 10. *Digestion of native cyst fluid with pepsin to recover mucopolysaccharide without the use of 90 % phenol*

Native cyst fluid (no. 324, group O), 800 ml., was digested at pH 1.9 in 0.1M-citrate-HCl buffer with 50 mg. of crystalline pepsin. Toluene was added as preservative. Material was dialysed, saturated with $(\text{NH}_4)_2\text{SO}_4$ and heated at 60° for 5 min. Mucopolysaccharide materials (a) insoluble and (b) soluble, under these conditions, were recovered.

	Mucopolysaccharide	
	(a)	(b)
Amount (g.)	7.5	0.6
Nitrogen (%)	10.8	8.5
Fucose (%)	8.2	17.7
Amino sugar (%)*	12.0	25.8
Reducing sugar (%)*	24.4	50.2

* See footnote Table 7.

Table 11. *Fractionation of a pepsin digest of ovarian-cyst fluid*

(a) Material (6.8 g.) insoluble in saturated $(\text{NH}_4)_2\text{SO}_4$ (Table 10, substance a) was extracted with dimethylsulphoxide (800 ml.) and the resulting solution fractionated with ethanol. Material was recovered between 20 and 60 % of ethanol (4.6 g.). Re-extracted with dimethylsulphoxide and recovered between 30 and 50 % (w/v) ethanol concentration. Material was dissolved in water and fractionated with ethanol between 45 and 50 % concentrations.

(b) Material (0.6 g.) soluble in $(\text{NH}_4)_2\text{SO}_4$ at 60° (Table 10, substance b) was repeatedly fractionated with ethanol between 50 and 55 % concentrations.

	Mucopolysaccharide	
	(a)	(b)
Nitrogen (%)	6.0	5.0
Fucose (%)	17.4	17.5
Amino sugar (%)*	24.3	27.0
Reducing sugar (%)*	49.1	52.0
Relative viscosity (η)	5.24	1.79
$[\alpha]_D$ in water (c, 1 %)	-30°	-38°
H activity (μg)†	0.05	0.10

* See footnote Table 7.

† Titrated with rabbit immune anti-H serum.

Digestion of the cyst fluid with pepsin as a first stage in the isolation procedure will largely overcome the co-precipitation of mucopolysaccharide and protein. Thus native cyst fluid digested with pepsin, dialysed and the mucopolysaccharide components separated into materials insoluble and soluble in saturated ammonium sulphate at 60°, gave mucopolysaccharides which, in most instances, were contaminated with extraneous nitrogenous material (Table 10), most probably undigested protein and indiffusible hydrolysis products. The nitrogen values were, however, reduced by extracting the dry materials with dimethylsulphoxide and fractionation of the dissolved material with ethanol (Table 11).

The results show the materials obtained without the use of phenol are closely similar to those recovered from the same cyst fluid by the phenol-extraction procedure (Table 1).

DISCUSSION

Evidence that it is possible to obtain from the secretions of a single individual at least two blood-group-specific mucopolysaccharides which differ in their physical, chemical and serological properties was brought forward by Gibbons & Morgan (1954), who isolated from an ovarian-cyst fluid obtained from a group B person, two mucopolysaccharides, designated B and B' substances, that differed in their capacity to react with normal human anti-B (β) agglutinins, but which nevertheless were indistinguishable in their reactions with immune anti-B agglutinins of human or animal origin. The mucopolysaccharides differed in their nitrogen content and in their relative viscosities and optical rotation, but were in many other properties closely similar. These findings seemed to indicate that at least two forms of a blood-group-specific mucopolysaccharide could co-exist in the secretions of a single individual. Attempts to show at that time the presence of more than one kind of specific blood-group substance in the cyst fluids of other persons were not successful, and the results obtained with that particular B secretion were believed to be somewhat exceptional. It was considered unlikely, however, that the B' substance was an artifact arising from the method of preparation since it was observed that the B substance was not demonstrably altered and changed into B' substance under any of the conditions to which it was subjected during isolation.

It has now been found that if preparations of blood-group-specific mucopolysaccharide obtained by the extraction of freeze-dried ovarian-cyst fluids with 90 % phenol (Gibbons *et al.* 1955) are dissolved in water, saturated with ammonium sulphate and heated to 60° for a few minutes, a mucopolysaccharide material is frequently precipitated from solu-

tion. The amount of mucopolysaccharide insoluble under these conditions varies considerably from one cyst fluid to the next and whereas, in some instances, about 95 % of the starting material is found to be insoluble, with other preparations such as the phenol-insoluble, water-soluble materials from H-active cyst fluid no. 278 (Morgan, 1959*b*), and all the B-active materials so far examined, 95 % or more of the phenol-insoluble, water-soluble substance remains soluble in the hot saturated ammonium sulphate. Most preparations of mucopolysaccharide obtained by phenol extraction, however, yield between a half and two-thirds of their weight as material insoluble in saturated ammonium sulphate at 60°, the remaining part being completely soluble in this reagent. After the first treatment with ammonium sulphate there is no evidence of change in weight or demonstrable change in properties if the isolated mucopolysaccharides are repeatedly exposed to saturated ammonium sulphate under the conditions described. It seems probable therefore that each kind of mucopolysaccharide exists as such in the native secretion.

Apart from the property of being either soluble or insoluble in saturated ammonium sulphate solution at 60°, the two mucopolysaccharides can be clearly differentiated in other of their properties, physical, chemical and serological. A 1 % solution in water or sodium chloride solution of the substance insoluble in saturated ammonium sulphate at 60° usually has a higher viscosity than that shown by the ammonium sulphate-soluble material obtained from the same cyst fluid. The results of the experiments which separate mucopolysaccharides into sizes by centrifuging indicate that within a family of similar molecules obtained from the same cyst fluid, the smallest size molecules in the material insoluble in saturated ammonium sulphate are apparently larger than the largest molecules in the material soluble in this reagent. Similarly, the optical rotations of the ammonium sulphate-insoluble preparations are much less laevorotatory than are those of the soluble materials, a finding which is in agreement with the conclusions of Schellman (1958), who considers that for molecules of similar kind, the optical rotation is determined by the folding and compactness of the molecule: the greater the folding and compactness, the less the laevorotation.

The physical measurements, especially the sedimentation constants, found for the ammonium sulphate-insoluble and -soluble materials, indicate a considerable difference in the molecular size of the two mucopolysaccharide preparations, and this fact may account for the inhomogeneity observed earlier for some carefully purified specimens of the specific substances. It seemed improbable at that time that the contaminant was protein as, in most instances, the materials which showed unsatisfactory physical

behaviour possessed a lower nitrogen content than did the materials which were apparently homogeneous. The contaminant, however, could have been a substance of closely similar composition and structure which had an appreciably lower nitrogen content. Treatment of some of these early preparations with hot saturated ammonium sulphate has now shown that they can be resolved into at least two mucopolysaccharide components.

The molecular weights determined for the ammonium sulphate-insoluble and -soluble components of mucopolysaccharide no. 368 indicate that the average molecular size of the former component (511 000) is considerably larger than that found for the soluble component (319 000). The results obtained earlier (Pusztai & Morgan, 1960, 1961) for a similar pair of mucopolysaccharides, both of which, however, contained a sialic acid component, gave values of 237 000 and 107 000, which fall into line with the results now obtained with a neutral blood-group-specific mucopolysaccharide.

It seems possible that the separation of blood-group substances that are closely similar in chemical composition, into two materials, largely depends on the size of the component macromolecules. The molecules which have a size larger than a certain limiting value will be precipitated by hot ammonium sulphate, whereas all the smaller ones will remain in solution. This suggestion, however, although supported by the values found for the intrinsic viscosity of the two kinds of material obtained from the same cyst fluid, does not seem to hold when materials from different cysts are compared. The material soluble in saturated ammonium sulphate obtained from cyst fluid no. 368, for example, is larger (319 000) than is the material (237 000) insoluble in the same reagent, obtained from cyst fluid no. 350. A more extensive examination of further pairs of materials must be made before a final conclusion can be reached on the relationship between molecular size and the property of solubility or insolubility in saturated ammonium sulphate at 60°. The influence of the shape or compactness of the macromolecule on the solubility behaviour in saturated ammonium sulphate at 60° is not known.

Chemically, the two kinds of mucopolysaccharide preparation are similar, but not identical, and it is mostly found that the ammonium sulphate-insoluble component has a higher nitrogen content than has the soluble mucopolysaccharide. A quantitative determination of the individual amino acids in three pairs of specific mucopolysaccharides, each pair isolated from a single cyst fluid, showed that the components insoluble in saturated ammonium sulphate at 60° had appreciably more amino acid per 100 g. of mucopolysaccharide than did the corresponding soluble components, thus indicating that

the insoluble materials had more amino acid built into their macromolecular structure. If, however, the amount of an individual amino acid is considered, this conclusion does not necessarily hold. Thus the amount of the two acidic amino acids is larger (1.28 g./100 g.) in the ammonium sulphate-soluble component of A substance no. 332 than in the corresponding insoluble mucopolysaccharide (1.03 g./100 g.). Similarly, the amount of serine (1.61 g./100 g.) is significantly less in the ammonium sulphate-insoluble component of H substance from cyst fluid no. 324 than in the corresponding ammonium sulphate-soluble material (2.11 g./100 g.). The same applies to proline (1.50 and 1.66 g./100 g.) in the corresponding preparations from cyst fluid 186 (A₂B). These preliminary results indicate that although the general pattern of the amino acid-containing units in the mucopolysaccharide is similar, there are nevertheless apparently significant differences in the amounts of total and individual amino acids in each substance. This finding is being more closely examined with other highly purified materials. The analyses obtained on the materials, however, confirm the earlier findings (Aminoff *et al.* 1950; Morgan, 1954-55; Gibbons & Morgan, 1954; Carsten & Kabat, 1956) that there is a minimum of 11 or so amino acids present in the blood-group-specific mucopolysaccharides and that the hydroxyamino acids and proline make up rather more than half the total amino acids. It seems that phenylalanine, and perhaps other amino acids, must be included as components, although they are present in variable and in rather small amounts. Nevertheless, they occur in what are believed to be highly purified specimens of the specific mucopolysaccharides. The irregularities found in the amount and composition of the amino acids in the mucopolysaccharide preparations makes it advisable to consider whether such differences, if confirmed, can also contribute to the solubility behaviour of the blood-group-specific mucopolysaccharides in hot saturated ammonium sulphate.

Further and most interesting differences are also found in the serological behaviour of the two kinds of material. The mucopolysaccharides which are insoluble in saturated ammonium sulphate at 60° have a greater capacity to neutralize natural isoagglutinin than have the corresponding materials which are soluble, and this behaviour holds, irrespective of the group specificity within the ABO system; it also holds for the corresponding Le^a-specific mucopolysaccharides. The difference in the capacity of the two kinds of mucopolysaccharides to inhibit haemagglutinin is most pronounced when the naturally occurring antibodies in human serum are used as test reagents. Immune human and rabbit agglutinins are less satisfactory reagents to demonstrate this difference and plant-seed aggluti-

nins sometimes fail to distinguish serologically between materials which are insoluble or soluble in saturated ammonium sulphate at 60°.

Examination by the serological precipitation technique of Heidelberger & MacPherson (1943) of the two forms of A-specific mucopolysaccharide obtained from the same cyst fluid shows that the mucopolysaccharide insoluble in ammonium sulphate precipitates more antibody nitrogen from a given anti-A serum than does that which is soluble in this reagent. This confirms the conclusion drawn from the results obtained by the haemagglutination-inhibition test, that the material insoluble in hot saturated ammonium sulphate is more active than the soluble component. The behaviour of mucopolysaccharide components isolated from each of these materials by high-speed centrifuging and selected according to their intrinsic viscosity values further indicates that the larger macromolecules precipitate a greater amount of antibody nitrogen from an antiserum than do the smaller-sized units. The results taken together therefore suggest that the specifically reactive structures of the larger mucopolysaccharide molecules combine with the receptor sites on the antibody molecules more satisfactorily than do those of the smaller molecules, and that there is a fraction of antibody which is not precipitated from the serum by this latter mucopolysaccharide material. One might tentatively assume that the quantitative difference in serological behaviour is, to a considerable extent, also determined by a secondary macromolecular structure and that the ultimate conformation of the larger of the macromolecules is better suited for serological reaction. Evidence in agreement with this conclusion and based on the results of ultrasonic treatment (Morgan, 1959c) and the action of crystalline papain and ficin (Pusztai & Morgan, 1958; Pusztai, 1960) on the specific mucopolysaccharides, have already been obtained.

After prolonged peptic digestion at a moderately high H⁺ ion concentration, a considerable part of the mucopolysaccharide which is insoluble in saturated ammonium sulphate at 60° remains insoluble in this reagent and is, apparently, very resistant to modification by pepsin at pH 1.9. Nevertheless, after peptic digestion a part of the material becomes soluble in hot saturated ammonium sulphate. In the absence of pepsin, however, it is shown that the acid conditions alone bring about the formation of some material which is soluble, and it must be concluded that peptic digestion, under conditions of high H⁺ ion concentration, brings about a limited and possibly significant amount of degradation.

The relative resistance of the specific substance to peptic hydrolysis under the conditions described allows a method to be elaborated which permits the

group-specific mucopolysaccharides to be isolated from ovarian-cyst fluids, and from other similar secretions not described in this paper, such as saliva, gastric juice and tissue digests, without the use of phenol. The water-insoluble mucopolysaccharide which frequently remains when phenol is used to isolate and purify the group substance does not arise when digestion of the native materials with pepsin and subsequent treatment with hot saturated ammonium sulphate is employed. The results of this treatment support the conclusion, reached earlier, that the water-soluble mucopolysaccharide which is insoluble in 90 % phenol is not modified during isolation from the native cyst fluid. The materials recovered through the action of pepsin, followed by treatment with saturated ammonium sulphate at 60°, are closely similar to the phenol-insoluble, water-soluble materials originally described, provided that materials from the same source are used for each procedure employed. Of the two methods, extraction with 90 % phenol has the advantage of simplicity, but the disadvantage of giving a lower yield owing to the recovery of the larger molecules as water-insoluble gels. On the other hand, the preliminary incubation of the tissue fluid with pepsin is a longer process, and induces a certain limited amount of degradation of the mucopolysaccharide by the acid conditions of digestion, but water-insoluble materials are not obtained. The method can be used for the solubilization of paramucinous cyst contents and tissues which are difficult or impossible to handle successfully by treatment with liquid phenol alone. It is to be observed, however, that secretions which contain sialomucopolysaccharide should not be exposed to digestion with pepsin as, at the acid reaction employed, the sialic acid component is readily eliminated (Pusztai & Morgan, 1960, 1961).

SUMMARY

1. Blood-group A-, H- and Le^a-specific mucopolysaccharides, isolated from human ovarian-cyst fluids by extracting the dry cyst contents with 90 % phenol, can be separated in many instances into two closely similar mucopolysaccharide materials by heating in saturated ammonium sulphate solution at 60° for a few minutes. A part of the mucopolysaccharide preparation is precipitated and a part remains in solution. The group B-active mucopolysaccharides insoluble in 90 % phenol so far examined are almost completely soluble in saturated ammonium sulphate at 60°.

2. The mucopolysaccharides insoluble or soluble in saturated ammonium sulphate at 60° possess the same qualitative composition, but differ in certain physical and chemical properties. The materials insoluble under these conditions have a greater

content of amino acid, are more viscous and have a larger molecular weight than have the materials soluble in ammonium sulphate, provided that the materials compared are obtained from the same cyst fluid.

3. The mucopolysaccharides insoluble in saturated ammonium sulphate at 60° are more active in haemagglutination-inhibition tests than are the ammonium sulphate-soluble materials. Group A-specific mucopolysaccharides, which are insoluble under these conditions, precipitate more antibody nitrogen from a specific antiserum than do the corresponding materials which are soluble in saturated ammonium sulphate at 60°.

4. Digestion of native cyst fluids or water-insoluble paramucinous gels with pepsin at pH 1.9, followed by treatment with saturated ammonium sulphate at 60°, allows blood-group substances to be recovered without the use of phenol and provides an alternative method for isolating these materials.

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The Reaction between Elastase and Elastic Tissue

4. SOLUBLE ELASTINS*

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Hall (1951) reported that, if the ratio of solution to solid was raised sufficiently, elastin could be made to pass into solution by prolonged treatment with boiling 40% urea solution. Adair, Davis & Partridge (1951) also reported the dissolution of elastin by dilute oxalic acid. Before this, elastin was considered to be an exceptionally stable protein, in that the normal solvents were without effect on it. Horbaczewski (1882) described a material to which he gave the name hemielastin, derived from native elastic tissue by treatment with hydrochloric acid and pepsin, and further details of a similar material (elastose) were given by Richards & Gies (1902). These authors indicated that the soluble material obtained from elastin exhibited decreased solubility at elevated temperatures. This particular property was further studied by Adair *et al.* (1951), who showed that the phenomenon was due to the separation of the solution into protein-rich and protein-poor fractions, presumably by the production of coacervates between the molecules of the soluble elastin. Bowen (1953), by ultracentrifuge and diffusion studies, revealed that soluble elastin prepared with urea consisted of polydisperse protein of relatively high molecular weight. At least two molecular species appeared to be present and the results were consistent with the existence of an equilibrium between the two. Partridge, Davis &

Adair (1955) separated the products of oxalic acid action into two main components and characterized their physical and chemical properties (Partridge & Davis, 1955). The high-molecular-weight protein (α -elastin) was polydisperse and capable of forming coacervates resulting in a two-phase system similar to that observed by Bowen. Low-molecular-weight material (β -elastin) did not form coacervates on warming and was monodisperse. It is significant that the two proteins were reported as having almost identical amino acid analyses, and the terminal amino and carboxyl group content of the two species was consistent with the view that if α -elastin were built up from β -elastin residues, then the latter must be joined through unknown cross-linkages. These same authors showed that such linkages could not be disulphide in nature.

Wood (1958) obtained soluble elastin by dissolution in a variety of solvents and showed that a material, having many of the properties of native elastin, could be reconstituted by maintaining the soluble material in a coacervated state at an elevated temperature for long periods.

The present paper reports new methods of preparing α -elastin and additional observations on its properties. The effect of detergents throws light on the phenomenon of coacervation and the results of studies on the proteolytic action of elastase on α -elastin may contribute to the general theory of proteolysis.

* The paper by Hall & Czerkawski (1959) is to be regarded as Part 3.