α -amino N, 13 % trimethylamine oxide N, 12 % glycine betaine N, 2 % volatile base N, 11 % nitrogen atoms other than α -amino in the α -amino acids, and 4 % taurine-N.

3. The water-soluble non-protein nitrogen of the hepatopancreas was partially accounted for as 38 % α -amino N, 3 % trimethylamine oxide N, 14 % glycine betaine N, and 5 % volatile base N.

4. The non-protein α -amino N in muscle and hepatopancreas lies between 200 and 400 mg./100 g. wet tissue; in blood the value is less than 10 mg./100 ml. Fifteen free amino acids have been detected in the muscle, seven in the hepatopancreas and six in the blood. Free glycine is present in relatively large amounts in all three tissues as is proline in muscle and taurine in hepatopancreas.

5. The reducing power of lobster blood is equivalent to 20 mg. glucose/100 ml. and the reducing substance has been demonstrated chromatographically to be glucose.

6. The changes occurring in water-soluble non-protein N of lobster muscle and hepatopancreas during incubation at 30° have been studied.

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Studies in Immunochemistry

16. THE ISOLATION OF BLOOD-GROUP ACTIVE MUCOIDS FROM OVARIAN CYST FLUIDS*

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The isolation and characterization of human blood groups A, B, H and Le^a specific substances from ovarian cyst fluid have been described in considerable detail by Morgan & Waddell (1945), Aminoff, Morgan & Watkins (1950), Kekwick (1950), Annison & Morgan (1952*a, b*), Kekwick (1952*a, b*), Gibbons & Morgan (1954) and Caspary (1954). The group materials, which sedimented and migrated as single substances during ultracentrifuging and electrophoresis, were characterized as mucoids and were found to be chemically similar, though not identical. Before attaching definite genetical significance to the differences in the analytical figures found, one might consider whether these differences can be correlated with the serological specificity observed, or whether the nature of the final product depends on the methods of fractionation employed. The results of the examination of nine cyst materials derived from 'secretors'

belonging to different phenotypes within the ABO system are summarized in this paper. Cyst fluids obtained from the so-called 'non-secretors', which almost always contain Le^a substance only (Grubb, 1948; Grubb & Morgan, 1949; Morgan, 1950) are not included. The cyst contents were fractionated until they yielded at least one mucoid material which showed no evidence of inhomogeneity when examined in the ultracentrifuge or by electrophoresis.

MATERIALS AND METHODS

Cyst fluids. The fluids were kept at 0–4° and dried from the frozen state as soon as possible after removal from the cystadenoma. In general the cyst fluids were more than 1 l. in volume, but fluids of smaller volume were used when they showed some special serological character.

Fractionation procedures. The methods of purification employed are described by Aminoff *et al.* (1950), Annison & Morgan (1952*a, b*), and Gibbons & Morgan (1954). Alkaline conditions were not used in any stage of the isolation

* Part 15 of this series: Davies, Morgan & Record (1955).

procedure and, except in two instances, acid conditions were also avoided. The dried crude cyst fluid was initially extracted with 90% (w/w) phenol, and, though active material was occasionally recovered from solution in phenol, the major part of the materials associated with specific serological activity was always found to be insoluble in liquid phenol at this stage. Subsequent steps in the purification procedure were carried out at room temperature except that involving the fractionation of the mucoid material from water with Na_2SO_4 which was performed at 37° to obtain adequate concentration of the salt. The materials recovered in all instances were dissolved in water, dialysed for several days at 0° against frequent changes of distilled water, and finally dried from the frozen state.

Physical examinations. Ultracentrifugal and electrophoretic examinations were carried out at pH 8 as described by Kekwick (1950, 1952*a, b*) and Caspary (1954).

Serological assay. The serological activity of each fraction was determined by the technique of inhibition of agglutination (e.g. Morgan & King, 1943). When tests were made using sera available in small quantity only, the volumes of serum, test solution, and erythrocyte suspension were reduced to about one-tenth of those employed with the readily obtainable natural human α - and β -sera. The A substances were tested with natural human anti-A ($\alpha_1 + \alpha$) serum, using both A_1 and A_2 erythrocytes as test cells, and the B substances with natural human, immune rabbit and immune human anti-B sera as described by Gibbons & Morgan (1954). The H active substances were titrated against rabbit immune anti-H sera prepared as described by Annison & Morgan (1952*b*), and frequently with the human anti-H serum 'Warboys' (see Annison & Morgan, 1950; Watkins, 1952). An anti-Le^b serum was also used to characterize some of the preparations, particularly those derived from secretors belonging to group O.

Laboratory 'standards' of blood-group substances were included in all titrations. The activity of each preparation is expressed (Table 3) as a percentage of that of the most active material that we have available.

Analyses

In the following estimations those of reducing sugars, hexosamine and galactose were carried out on samples which had been hydrolysed at 100° for 16 hr. in 0.5 N-HCl; all other estimations were made on the intact materials.

Nitrogen. Kjeldahl nitrogen was determined using the apparatus of Markham (1942).

Methylpentose. The method of Dische & Shettles (1948) using the CyR 10 procedure was used. Fucose standards were included in each determination.

Hexosamine. A modification of the method of Elson & Morgan (1933) was used. Glucosamine hydrochloride standards in the range 10–50 μg . were included in each determination. Extinctions were read at 535 $m\mu$. with a spectrophotometer.

Reducing sugars. These were determined by the colorimetric technique of Nelson (1944). Reducing values are expressed in terms of glucose (Merck) which was used as standard.

Hexose. The diphenylamine reaction of Dische (1929) was employed since, of the many reagents available for carbohydrate estimation, this method appears the most specific for hexoses in the presence of methylpentose, hexosamine, and amino acids, substances which occur in the human

blood-group mucoids. To the hexose (100–400 μg .) in 1.5 ml. of water or dilute acid is added 3 ml. of 10% (w/v) ethanolic diphenylamine-acetic acid-conc. HCl (1:4:5, by vol.). The mixture is heated at 100° in a sealed ampoule for 30 min. and after cooling extinctions are read spectrophotometrically at 640 $m\mu$. Fucose, glucosamine, and a mixture of amino acids give colours equivalent to about 7, 4 and 3% respectively of an equal weight of galactose, and two series of galactose standards were therefore set up with each hexose estimation. Both series contained appropriate quantities of the substances known to be present in the hydrolysis products of blood-group substances such that the amount of galactose in each sample could be obtained by interpolation. It is necessary to allow the colour to develop in sealed ampoules to prevent loss of ethanol by evaporation.

'N-Acetylhexosamine' colour. N-Acetylhexosamine colour intensity/time of heating relationship was obtained using the standard conditions recommended by Aminoff, Morgan & Watkins (1952).

'Direct' Ehrlich reaction. The blood-group substances give a certain amount of colour with acid *p*-dimethylaminobenzaldehyde without prior treatment with alkali. The materials described were tested according to the method of Werner & Odin (1952), and the colour intensity developed expressed as a percentage of that given by an equal weight of sialic acid.

EXPERIMENTAL

Isolation of blood-group substances from

(a) group A_1 cyst fluids

Cyst 203. The phenol-insoluble water-soluble fraction (20 g.; N, 5.3; methylpentose, 19.8%) dissolved in 2 l. of water was fractionated by addition of ethanol. Two major fractions were obtained, one separating between the levels of 52.5 and 55% (v/v) ethanol (10 g.; N, 4.6; methylpentose, 19.7%) and another between the levels of 55–60% (v/v) ethanol (fraction 203/1; 8 g.; N, 4.8; methylpentose 20.0%). Fraction 203/1 moved during electrophoresis at pH 8 as a single substance, whereas the former material showed two components, the smaller of which moved slightly faster and appeared to be similar electrophoretically to fraction 203/1. The electrophoretically heterogeneous material was dissolved in ethylene glycol (2%, w/v) and fractionated by the addition of dioxan. A fraction (4.6 g.; N, 4.9; methylpentose, 20.7%) precipitated between the levels of 42 and 43.7% (v/v) dioxan and showed on electrophoresis a reduced amount of the faster-moving component. The material was dissolved in water (2%, w/v) and fractionated by addition of Na_2SO_4 . A fraction (203/2; N, 5.4; methylpentose, 19.7%) removed between levels of 23 and 25% (w/v) Na_2SO_4 was found to migrate as a single substance, the faster-moving component initially present had been removed and could be identified in increased amount in fractions adjacent to the main fraction in both the ethylene glycol-dioxan and water- Na_2SO_4 fractionations. A mixture of equal amounts of fractions 203/1 and 203/2, each of which sedimented as single substances in the ultracentrifuge, showed two components on electrophoresis.

A small amount (0.5 l.) of cyst fluid 203 was not dried initially but was fractionated as follows. The cyst fluid was saturated with ether and cooled to 0° and a small precipitate was removed. The fluid was then cooled to –8° and fractionated by the addition of ethanol at that temperature. A

fraction (16 g.; N, 11.4; methylpentose, 7.1%) precipitated between the levels of 23 and 30% (v/v) ethanol and contained almost all the serological activity and methylpentose originally present in the crude cyst material. About 10 g. of inactive, highly coloured material was eliminated. The active material after drying from the frozen state was stirred for about 30 min. with 400 ml. of anhydrous liquid NH_3 at -60° and the solid and liquid phases separated by brief centrifuging. The material in solution in NH_3 was recovered by pouring the liquid into 4 times its volume of dry acetone at room temperature. The ammonia was rapidly lost by evaporation. The acetone was distilled off and any residual ammonia removed by storing *in vacuo* over H_2SO_4 . The solid so obtained dissolved in water to give a neutral solution which was dialysed and dried.

The first NH_3 extract (1.6 g.; N, 6.0; methylpentose, 15.0%, serologically fully active) was dissolved in 200 ml. of water and fractionated by addition of ethanol. A precipitate was removed between the levels 54 and 59% (v/v) ethanol (0.6 g.; N, 5.7; methylpentose, 18.5%) and a material (0.4 g.; N, 5.8; methylpentose, 17.9%) remaining in solution at 59% (v/v) ethanol was also recovered. These materials were not examined for homogeneity by physical techniques.

Cyst 196. The phenol-insoluble water-soluble fraction (10.5 g.; N, 7.5; methylpentose, 16.5%) was dissolved in 200 ml. ethylene glycol and fractionated by addition of a pyridine-acetic acid mixture (5:1, by vol.). The largest and most active fraction (2.7 g.; N, 6.3; methylpentose, 17.3%) was removed between the levels of 65 and 70% (v/v) pyridine-acetic acid and another less active fraction (1.1 g.; N, 5.6; methylpentose, 18.0%) between the 70 and 75% (v/v) pyridine-acetic acid. The former material was fractionated from water (270 ml.) with ethanol, and a precipitate (2.4 g.; N, 5.8; methylpentose, 18.2%) was removed between 50 and 55% (v/v) ethanol concentrations. The precipitate was taken up in 150 ml. glycerol and fractionated by addition of ethanol. A precipitate was removed between 0 and 16% (v/v) ethanol (1.15 g.; N, 5.7; methylpentose, 16.9%) and another between 16 and 33% (0.77 g.; N, 5.7; methylpentose, 17.1%). These two fractions were mixed (material 196/1) and subsequently examined by electrophoresis and ultracentrifuging; no inhomogeneity was detected.

The material precipitated from ethylene glycol by pyridine-acetic acid mixture between levels of 70 and 75% (v/v) was again subjected to refractionation from ethylene glycol (100 ml.) with pyridine-acetic acid, the fraction precipitating between 70 and 75% being again collected (1.0 g.; N, 5.6; methylpentose, 17.7%). Electrophoresis of this material revealed the presence of a very small amount of rapidly migrating material, in addition to the major component. The fast-moving component was removed by precipitating the material twice from phosphate buffer by the addition of ethanol to 55% (v/v) concentration. The final material (fraction 196/2, 0.9 g.; N, 5.7; methylpentose, 17.4%) behaved as a single substance during electrophoresis and ultracentrifugation.

(b) a group A_2 cyst fluid

Cyst 194. The phenol-insoluble water-soluble fraction (4.7 g.; N, 5.6; methylpentose, 16.0%) dissolved in 500 ml. of water was fractionated by addition of ethanol. The material (fraction 194/1, 1.24 g.; N, 5.4; methylpentose, 16.3%) which precipitated above 57% (v/v) ethanol con-

centration was found to behave on electrophoresis and ultracentrifuging as a single substance.

(c) a group B cyst fluid

Cyst 192. The phenol-insoluble portion of this cyst material was entirely soluble in water. The material (about 20 g.) dissolved in 750 ml. of water gave no precipitate on the addition of 750 ml. of ethanol. The further addition of 200 ml. saturated aqueous NaCl solution gave a precipitate (14 g.; N, 5.4; methylpentose, 14.5%) which was removed. The supernatant fluid contained a material (5 g.; N, 4.3; methylpentose, 12.0%) of negligible activity against human β -serum, but moderately active against immune anti-B and anti-Le^b serum, but owing to the lack of adequate amounts of a reliable anti-Le^b serum, further work on this material was not undertaken. The precipitate, which was active against natural β -serum and of low activity against anti-Le^b serum was dissolved (1%, w/v) in 1% (w/v) aqueous NaCl and centrifuged in a 'Spinco' preparative ultracentrifuge at 35000 g, for 1 hr. The material (17.5 g.; N, 5.0; methylpentose, 15.0%) remaining in solution was recovered, dissolved in 500 ml. water and reprecipitated by addition of ethanol and saturated NaCl solution as previously described. The precipitate (6.8 g.; N, 5.2; methylpentose, 16.0%) behaved electrophoretically at pH 8 as a single substance, but a minor component sedimenting somewhat more rapidly than the main component was detected on ultracentrifugal examination. The material was further fractionated with ethanol from solution in 500 ml. 50% (w/v) aqueous urea; the fraction which separated between 52 and 55% (v/v) ethanol concentrations (4.0 g.; N, 5.3; methylpentose, 16.6%) being subsequently dissolved in 200 ml. anhydrous formamide and fractionated with ethanol. The material (3.1 g.; N, 5.4; methylpentose, 15.0%) which precipitated between levels of 50 and 55% (v/v) ethanol was collected, dissolved in 250 ml. of water and fractionated by addition of Na_2SO_4 to yield a material (fraction 192/1, 1.0 g.; N, 5.5; methylpentose, 15.7%) between 22 and 23% (w/v) Na_2SO_4 concentration, which behaved electrophoretically and ultracentrifugally as a single substance.

(d) group O cyst fluids

Cyst 193. The phenol-insoluble water-soluble material (4.6 g.; N, 4.0; methylpentose, 12.0%) dissolved in 500 ml. of water was fractionated by addition of ethanol, and the material (1.05 g.; N, 5.3; methylpentose, 19.3%) separating between 52.5 and 60% (v/v) ethanol levels was again fractionated from water with ethanol. The precipitate, (fraction 193/1, 0.82 g.; N, 5.0; methylpentose, 20.8%) which formed between 47 and 53% (v/v) ethanol concentrations, migrated electrophoretically and sedimented ultracentrifugally as a single substance.

The phenol-insoluble water-insoluble material (5 g.; N, 7.1; methylpentose, 16.5%) obtained from the crude cyst substance was shaken with phenol-water mixture (300 ml. of each phase) as described by Westphal, Lüderitz & Bister (1952). A small amount of material was recovered from the aqueous phase, the bulk of the preparation remained insoluble in both phases. Formamide (300 ml.) was then added with stirring to the phenolic phase when part of the insoluble precipitate, which is almost entirely in the lower (phenol) phase, passed into solution. Ethanol was then added to the phenol-formamide solution and the

precipitate (0.86 g.; N, 6.7; methylpentose, 13.1%) formed between 7 and 11% (v/v) ethanol concentration was collected. The precipitate was dissolved in 80 ml. of water and was fractionated by addition of Na_2SO_4 . The fraction obtained between the levels of 20 and 23% (w/v) Na_2SO_4 concentration was found to be partly insoluble in water, but the water-soluble part (fraction 193/2, 0.38 g.; N, 6.2; methylpentose, 15.4%) behaved electrophoretically and ultracentrifugally as a single substance.

Cyst 116. The phenol-insoluble, water-soluble material (14.5 g.; N, 5.0; methylpentose, 21%) was thoroughly re-extracted with 90% (w/w) phenol when the major part of it dissolved. This behaviour is occasionally observed with materials initially insoluble in phenol (Annisson & Morgan, 1952a). Ethanol was added to the phenol solution and the precipitate (fraction 116/1, 11.5 g.; N, 4.8; methylpentose, 26.2%) formed between the ethanol concentrations of 0 and 5% (v/v) showed no evidence of heterogeneity when examined by electrophoresis or ultracentrifuging.

Cyst 172. The water-soluble part of the phenol-insoluble material (1.6 g.; N, 5.1; methylpentose, 18.5%) was dissolved in ethylene glycol (70 ml.) and fractionated by addition of pyridine-acetic acid mixture (5:1, by vol.). The material (0.85 g.; N, 5.2; methylpentose, 22.3%) which precipitated between 83.3 and 85.7% (v/v) pyridine-acetic acid mixture was found to be inhomogeneous electrophoretically and was dissolved in 70 ml. anhydrous formamide and fractionated by addition of isopropanol. A material (fraction 172/1, 0.312 g.; N, 4.4; methylpentose, 25.9%) which precipitated between 20 and 30% (v/v) isopropanol was found to behave electrophoretically and ultracentrifugally as a single substance.

Cyst 126. The crude cyst material was worked up in five batches and with each portion of the phenol-insoluble, water-soluble material a somewhat different procedure was used for further purification. About one-third of the material was treated with 10% (w/v) trichloroacetic acid at 0° (e.g. Annisson & Morgan, 1952a) and after neutralization, dialysis, and drying from the frozen state, was subsequently re-extracted with 90% (w/w) phenol; most of the material remained insoluble. Other portions of the phenol-insoluble water-soluble material were dissolved in phenol-ethylene glycol mixtures and fractionally precipitated with ethanol (e.g. Annisson & Morgan, 1952a) and/or fractionated from water with Na_2SO_4 . Five fractions so obtained which were similar serologically and chemically, were mixed together and fractionally precipitated from water with ethanol. The material (fraction 126/1, 5.4 g.; N, 4.2; methylpentose, 25.1%) precipitated between 55 and 60% (v/v) ethanol concentration was found to migrate electrophoretically and sediment ultracentrifugally as a single polydisperse substance.

(e) a group A_1B cyst fluid

Cyst 111. The phenol-insoluble water-soluble material was fractionally extracted with ethylene glycol-phenol mixtures, increasing the amount of glycol by 5% (v/v) with each extraction. The fraction insoluble in 20% but soluble in 25% (v/v) ethylene glycol-phenol was subsequently dissolved in sodium acetate buffer at pH 4.6 and shaken repeatedly with amyl alcohol- CHCl_3 mixtures as described by Annisson & Morgan (1952a). The material (fraction 111/1, 8.5 g.; N, 4.9; methylpentose, 17.7%) remaining in the

final supernatant liquid showed no evidence of heterogeneity when examined by electrophoresis or ultracentrifuging.

Hydrolysis of mucoids with acetic acid

The effect of *N* acetic acid hydrolysis on all these materials was studied. In each instance a sample (30–35 mg.) was hydrolysed at a concentration of 1% (w/v) in *N* acetic acid in a sealed ampoule for 16 hr. at 100°. The material was then dialysed for 6 days at 0° against water saturated with chloroform which was changed daily, the total diffusate being collected and finally made up to 200 ml. The indiffusible material was recovered quantitatively, made up to 10 ml., and then analysed for *N*, methylpentose, and after further hydrolysis for 16 hr. in 0.5*N*-HCl, for hexosamine and reducing sugars. The diffusate was analysed for methylpentose, the overall recovery of which was for each material not less than 95%. The results are included in Table 1.

RESULTS

The chemical analyses of each of the materials whose isolation has been described are recorded in Table 1. The galactose content was calculated from the total reducing value by subtracting the reduction due to the known amounts of fucose and hexosamine. It can be seen that agreement between the analytical figure for galactose arrived at in this way, and that obtained directly by means of diphenylamine reaction is not good, the diphenylamine reaction giving with all materials a galactose value higher than that obtained from the reducing value.

The amount of methylpentose, hexosamine, galactose (calc. from reducing value) and non-hexosamine nitrogen (calc. from total *N* and hexosamine *N*) remaining indiffusible after 16 hr. at 100° in *N* acetic acid is recorded (Table 1) as a percentage of the weight of material taken for hydrolysis. The diffusible methylpentose is expressed as a percentage of the total methylpentose.

Paper chromatography

The mucoids were hydrolysed in 0.5*N*-HCl at 100° for 16 hr. and the hydrolysates examined for sugars by paper chromatography. Pyridine-ethyl acetate-water was used as solvent system (Jermyn & Isherwood, 1949) and the papers were sprayed with aniline hydrogen phthalate (Partridge, 1949) and hexosamine reagents (Partridge, 1948). Fucose, galactose, glucosamine and galactosamine only were detected; the presence of galactosamine in fractions 126/1 and 172/1 was doubtful.

The 'direct' Ehrlich reaction

The range of values (1–8%, expressed as sialic acid, cf. Table 1) encountered in this reaction is rather wide and shows no obvious correlation with specificity. Rather large amounts of material (5 mg. of substance; 0.1–0.5 mg. of sialic acid) are required

Table 1. *The composition of the blood-group mucoids*

Fraction	203/1	203/2	196/2	196/1	194/1	192/1	193/1	193/2	116/1	172/1	126/1	111/1
Specificity	...	...	...	...	...	...	...	...	...	...	...	...
Total N (%)	4.8	5.4	5.7	5.7	5.4	5.5	5.0	6.2	4.8	4.4	4.2	4.9
Fucose (%)	20.0	19.7	17.4	16.9	16.3	15.7	20.8	15.4	26.2	25.9	25.1	17.7
Hexosamine (%)	33.5	33.4	30.1	34.0	29.7	24.0	28.1	22.5	25.6	29.1	25.3	27.0
Reducing sugars (% as glucose)	58.0	56.9	52.1	58.0	55.7	57.0	60.9	50.6	61.6	66.6	58.5	55.0
Galactose (direct) (%)	26.9	27.6	26.2	27.6	29.8	38.4	34.0	34.1	36.1	35.2	35.4	28.8
Galactose (calculated) (%)	21.0	20.5	19.0	22.0	24.8	35.6	31.3	25.6	33.6	33.7	29.2	26.6
Non-hexosamine N (%)†	2.2	2.8	3.3	3.0	3.1	3.6	2.8	4.4	2.8	2.1	2.2	2.8
N-Acetylhexosamine reaction												
Time (min.)‡	14	15	11.5	14	14	16.5	13.5	15	12	14	14	13
As N-acetylglucosamine (%)§	7.1	8.1	8.0	7.8	6.1	7.2	7.8	10.3	10.5	5.3	4.3	9.7
'Direct' Ehrlich reaction (%)	5.9	3.9	4.8	5.3	8.2	7.5	1.9	2.1	1.1	2.5	3.2	2.9
Acid-labile fucose (%)¶	82	64	61	65	80	88	87	77	86	84	95	73
Material indiffusible after hydrolysis in N acetic acid for 16 hr.**												
Hexosamine (%)	21.4	27.9	26.9	27.5	23.1	16.9	22.3	20.9	21.0	20.7	9.9	22.2
Galactose (%)*	10.0	16.9	14.4	15.3	16.7	15.7	18.3	22.0	21.6	22.1	11.6	15.4
Fucose (%)	3.8	7.1	6.9	6.2	3.5	1.8	3.3	3.5	3.9	3.9	1.2	5.0
Non-hexosamine N (%)†	1.7	2.5	2.4	2.6	2.4	2.4	2.6	3.5	2.2	1.7	1.1	2.2

* Calculated from reducing values.

† Time of heating in 0.05N Na₂CO₃ to give maximum colour in terms of N-acetylglucosamine.

‡ As % of colour given by an equal weight of N-acetylglucosamine.

§ As % of colour given by an equal weight of sialic acid.

¶ Diffusible after hydrolysis in N acetic acid for 16 hr. at 100°. Expressed as % of total fucose.

** As % of starting material.

to give adequate colour for estimation. Some experiments were carried out to determine whether enzymic treatment or oxidation with periodate destroys the chromogen, and also a search was made among a limited number of 1-amino and 2-amino sugar derivatives for a compound of known structure giving a similar colour.

Preparation 203/1 (50 mg.) in 5 ml. citrate-HCl buffer, pH 2.5, was treated with crystalline pepsin (Armour) (5 mg.) at 37°, for 48 hr.; but the direct Ehrlich-reacting structure was not destroyed or rendered diffusible. Similarly, when a specimen of mucoid, which showed A and H activity and was derived from crude pepsin, was oxidized with periodate at pH 7.5 for 20 hr. under the conditions described by O'Dea & Gibbons (1953) the material showed no destruction of the chromogenic structure. The following substances (1 mg.) fail to give significant colour when tested according to the procedure for the detection of sialic acid (Werner & Odin, 1952): 1-Amino-1-deoxy-D-glucose and its N-acetyl, N-glycyl (oxalate salt) and N-(N-formylglycyl) derivatives, N-p-tolyl-1-amino-1-deoxy-D-fructose, methyl N-acetyl-3:4:6-tri-O-methyl-2-amino-2-deoxy-β-D-galactopyranoside, methyl N-acetyl-4:6-di-O-methyl-2-amino-2-deoxy-α-D-glucopyranoside, N-acetyl-3-O-methyl-2-amino-2-deoxyglucopyranoside and (N-anilino)-N-acetyl-2-amino-2-deoxyglucopyranoside.

This last compound gives a strong direct Ehrlich-reaction if acetic acid replaces water as solvent; sialic acid itself, however, gives little colour under these conditions.

DISCUSSION

Twelve preparations of mucoid material each possessing A, B, H or AB specificity have been isolated from ovarian cyst fluids. These preparations are not so exhaustively characterized as are the single specimens of blood-group substance reported earlier (Aminoff *et al.* 1950; Annison & Morgan, 1952*a, b*; Gibbons & Morgan, 1954), but were, nevertheless, subjected to electrophoretic and ultracentrifugal examination at pH 8 and were found to behave essentially as single molecular species under these conditions. The composition of native ovarian cyst material is very variable and the behaviour of such material on fractionation is in consequence difficult to predict. No purification procedure or sequence of procedures can be described which invariably yields homogeneous material, but all the methods employed have effected a useful measure of purification. The cyst materials described are those from which it proved possible to isolate substances which are believed to be substantially homogeneous. A large number of other cyst mucins were investigated but they yielded

material which, though serologically active and closely similar in chemical composition to certain of the preparations described here, could nevertheless be shown by physical examination to contain two or more components.

Part of one cyst material was purified by extraction with liquid ammonia; this procedure has little to recommend it since it offers no advantages over the phenol method and is technically more difficult to accomplish. It is of considerable importance, however, to know that by means of an entirely different procedure material chemically and serologically similar to that isolated by phenol extraction can be obtained.

The chemical, physical and serological data for the materials examined are given in Tables 1 and 2 and show that the analytical figures differ for different preparations even when these are of the same serological specificity within the ABO system. Such variations are considered to be beyond the limits of analytical error.

strong acid, might be expected to estimate such reversion products as though they were free galactose.

The significance of the 'direct' Ehrlich reaction given by the group mucoids is not clear. It may be due to an impurity in the preparations or to the presence of a chromogenic structure which is an integral part of the blood-group mucoids. A number of substances of low molecular weight, for example, sialic acid (Blix, 1936; Blix, Svennerholm & Werner, 1952), neuraminic acid (Klenk, 1941; Klenk & Lauenstein, 1952), lactaminic acid (Kuhn & Brossmer, 1954) and neuramine-lactose (Trucco & Caputto, 1954; Heyworth & Bacon, 1954), which have the property of reacting with Ehrlich's reagent without prior treatment with alkali, in contrast to the *N*-acetyl amino sugars, have been isolated from natural sources. In no instance, however, is the structure known, although Gottschalk (1951, 1954) has advanced some suggestions as to the nature of an Ehrlich-positive chromophore.

Table 2. *Serological and physical properties of the blood-group substances*
Activities expressed as % of the most active material available.

Fraction	Sedimentation constants* (Svedberg units)	Natural human anti-A serum and A ₁ cells	Natural human anti-A serum and A ₂ cells	Natural human β serum	Rabbit immune anti-B serum	Rabbit immune anti-H serum	Serum 'Warboys' (anti-H)	Serum 'Hughes' (anti-Le ^b)
203/1	5.02	50	50	0	—	<1	—	<1
203/2	5.64	100	100	0	—	<1	—	<1
196/2	—	25	25	0	—	<1	—	—
196/1	5.25	100	100	0	—	<1	—	<1
194/1	6.79	50	100	0	—	50	—	6
192/1	7.00	0	0	100	100	1.5	—	1.5
193/1	5.41	0	0	0	—	100	50	50
193/2	6.29	0	0	0	—	100	—	12
116/1	—	0	0	0	—	100	100	100
172/1	7.41	0	0	0	—	100	—	100
126/1	6.08	0	0	0	—	1.5	100	<1
111/1	5.46	1.5	50	1.5	100	1.5	—	—

* Corrected to water at 20°.
 0 Indicates no inhibition at 1:1000 concentration of the test material.
 — Not tested.

The values for galactose obtained directly by the diphenylamine reaction range from 104 to 143 % of the corresponding values calculated from the reducing power of the hydrolysates, and the differences are generally higher in the A substances than in the B or H substances. While it is known that mixtures of galactose and amino acids may be heated to 100° in 0.5 N hydrochloric acid for several hours with only slight reduction in reducing value, it is not unlikely that sugars and amino acids may be liberated during hydrolysis of a mucoid in a reactive form and condense to give substances with a smaller reducing value. The diphenylamine reaction, which involves heating the material for 30 min. with

Hiyama (1949) isolated 2-carboxypyrrole as a degradation product of sialic acid, and Gottschalk (1953) has obtained the same compound by the direct alkaline hydrolysis of mucin. Blix *et al.* (1952) report that sialic acid is usually associated with hexosamine, though the substance itself does not give rise to hexosamine on acid hydrolysis. We have examined a number of *N*-glycosides and *N*-acetylhexosamine derivatives for a 'direct' Ehrlich reaction, but none were found to give a colour reaction similar to that obtained with sialic acid or lactaminic acid.

From Table 1 it appears that there is some correlation between serological specificity and chemical

composition. This becomes more obvious when the amounts of the individual carbohydrate components are expressed as a ratio (Table 3). In spite of there being some uncertainty in the galactose figure used, which is calculated from the reducing value, the correlation between galactose:hexosamine ratio and specificity is quite striking. A similar conclusion can be drawn if the galactose value obtained by the diphenylamine reaction is used, and it seems that the galactose:hexosamine ratio is about 0.6 for A substances, 1.2 for H substances and, for the single specimen of B substance, about 1.5. The ratio fucose:hexosamine is also generally found to be lower for the group A preparations than it is for

that within the series of preparations of H substance examined the highest and the lowest values for equivalent *N*-acetylglucosamine are found. The H substances, however, are serologically much more heterogeneous and in certain instances can be sharply differentiated. Watkins (1952) and Watkins & Morgan (1954 and unpublished results) have shown that there are a number of sera of the same general anti-H specificity which nevertheless on closer examination show sharp differences in their reactivity with individual preparations of H substance. It must be emphasized therefore that the serology of the H substances and their position in the ABO genetic system is far from clear.

Table 3. *Ratios of the carbohydrate components of the blood-group mucoids*

Ratios are expressed relative to divisor = 1. Galactose calculated from reducing values.

Fraction	Specificity	Fucose: hexosamine	Galactose: hexosamine	Fucose: galactose	Material indiffusible after hydrolysis with <i>N</i> acetic acid (16 hr. at 100°)	
					Fucose: hexosamine	Galactose: hexosamine
203/1	A ₁	0.60	0.63	0.95	0.18	0.47
203/2	A ₁	0.59	0.61	0.96	0.26	0.61
196/1	A ₁	0.58	0.63	0.92	0.26	0.54
196/2	A ₁	0.50	0.65	0.77	0.23	0.55
194/1	A ₂	0.55	0.84	0.66	0.16	0.67
192/1	B	0.65	1.49	0.44	0.11	0.93
193/1	H	0.74	1.12	0.66	0.15	0.82
193/2	H	0.69	1.14	0.60	0.17	1.00
116/1	H	1.02	1.31	0.80	0.19	1.03
172/1	H	0.90	1.16	0.77	0.19	1.07
126/1	H	0.99	1.15	0.86	0.12	1.17
111/1	A ₁ B	0.66	0.99	0.67	0.23	0.69

the H substances but the difference is less striking. The galactose:hexosamine ratio of the indiffusible material which remains after the A and H substances are heated with *N* acetic acid and dialysed is not greatly altered from that of the original group substance, but this treatment considerably alters the fucose:hexosamine ratio as much of the fucose is very acid-labile and can be recovered in the diffusate. Some of the galactose in the B substance, however, seems to be released preferentially by mild acid hydrolysis (see Tables 1 and 3). The most variable analytical figure is the non-hexosamine nitrogen which suggests that the carbohydrate moieties of the various preparations which possess the same group specificity are closely similar, one preparation differing from another chiefly in amount of amino acid it contains and in molecular size.

The variation in chemical composition encountered among the H substances is rather greater than that met with among the A substances, particularly in the value given in the *N*-acetyl-hexosamine reaction. It is of interest to observe

The amount of fucose present relative to other carbohydrate components (Table 3) shows slight variation among different preparations of the same group specificity, a fact possibly connected with the acid lability of this constituent. The H substances generally contain rather more fucose than the A or B substances.

Leskowitz & Kabat (1954) find some differences in the glucosamine:galactosamine ratio between the A, B and H substances and, independently, Rondle (1954) and Rondle & Morgan (1955) also report some correlation between serological specificity and hexosamine ratio. Group A mucoids usually give a glucosamine:galactosamine ratio which varies from rather less than 1.0 to about 1.5, whereas the ratio is closer to 3 for B and Le^a substances and varies from 3 to 12 for H substances.

The reactivity of the various mucoid preparations with anti-Le^b serum is in nearly every instance much higher among those possessing H activity alone than among those showing A, B or AB specificity. This might suggest that Le^b and H specificities are identical, but this is clearly not so,

since, in contrast to anti-H sera, anti-Le^b sera do not react with erythrocytes of group O Le(a+) individuals (by definition) and the ratio H activity:Le^b activity is not constant in different preparations of H substance. Quantitatively, therefore, it would appear that the reactivity of the products from group O secretor individuals with anti-Le^b sera is far greater than is the reactivity of materials derived from secretors belonging to groups A, B or AB. The H-mucoid described by Annison & Morgan (1952*b*), however, reacts only weakly with anti-Le^b serum; this material has, however, a low (13%) fucose content.

The genes *A* and *B*, defined by Bernstein (1924), behave as single genetical entities (cf. Wiener, 1943). If there exist unique *A* and *B* substances of fixed chemical structure which are the products of the action of these genes, then the differences in chemical composition encountered between different preparations of the same blood-group specificity imply that all the specimens so far isolated and characterized as essentially homogeneous are nevertheless heterogeneous. This possibility cannot be completely excluded since physical homogeneity does not establish chemical purity. Furthermore, all preparations of blood-group substances have shown some degree of polydispersity when subjected to ultracentrifugal analysis. The genes *A* and *B*, may, however, give rise, not to a single product, but to several, each of which differs to some extent in composition and structure, but all of which nevertheless possess the configuration necessary for specific combination with the corresponding antibody. Two demonstrably different, apparently homogeneous *B* substances were isolated from one cyst fluid by Gibbons & Morgan (1954), who considered that their findings offered evidence for the existence of multiple gene products. In the present work it has been found possible in three instances to isolate two apparently homogeneous materials from the same cyst fluid (cyst materials 193, 196 and 203, Table 1). In two of the three instances the two relevant preparations possess significantly different sedimentation constants (Table 2) and the two materials isolated from cyst 203 (group *A*) showed different electrophoretic mobilities. While it is not yet possible to be certain that these preparations are free from contamination or that degradation of an originally homogeneous substance has not occurred *in vivo* or during isolation, taken as a whole the evidence suggests that different and distinguishable molecular species may carry the same blood-group specificity. Kabat, Baer & Knaub (1949) on the basis of results of the application of a quantitative precipitation reaction put forward the idea that blood-group substances are to be regarded as a family of similar materials rather than as a single substance. Our results certainly suggest

that the secretions of any one individual contain a range of substances each with a common and dominant serological specificity. Differences in physical and chemical properties between different preparations of blood-group substances, each possessing the same specificity within the ABO system, are, therefore, to be anticipated. Gibbons & Morgan (1954) have suggested that the ABO and Lewis genes should not be regarded as directly and solely responsible for the production of the appropriate blood-group antigen, but are rather to be considered as genes which control a stage, or series of stages, in the building up of certain substances by the body, modifying them so that the substances finally produced possess the configuration which is associated with *A*, *B* or *AB* specificity according to the genotype of the individual. If this suggestion is accepted, then substances which have undergone modification during biosynthesis will be found as neutral mucoids in the secretions, and as constituents of the red-cell envelope and other tissue cells. Witebsky & Klendshoj (1941) have suggested that the O (*H*) substance is a basic mucoid on which genes *A* and *B* superimpose their reactive structures, and the view that the *A* and *B* genes act by bringing about a modification of the mucoids which are, in the absence of specific gene action, common to all human beings has been put forward by Wiener & Wexler (1952). The latter authors believe that their suggested modifications are relatively minute, whereas we consider that earlier results and those now presented make it highly probable that they are not inconsiderable.

The properties of the material obtained from a group A₁B 'secretor' are of interest; firstly, the ratios of the analytical figures for the carbohydrate portion of the mucoid are very close to the mean of the values found for the *A* and *B* substances; secondly, it possesses restricted serological properties, it being reactive with natural anti-*A* ($\alpha_1 + \alpha$) when *A*₂ cells are employed as test cells, but is only poorly active when *A*₁ cells are so employed; similarly, it is active when measured against immune rabbit anti-*B* serum but against natural human β -serum it shows a much lower order of activity. One might conclude that when both *A* and *B* genes are present there is a competitive influence between them, and as a result both genes reproduce their serologically reactive groupings less precisely, or alternatively that the active structures are situated together on the same mucoid macromolecule so that they inhibit each other's sphere of action owing to steric interference.

SUMMARY

1. The isolation and characterization of substantially homogeneous mucoids which are responsible for the group specificity of human bloods

and secretions within the ABO blood-group system are described.

2. The ratios of certain of the carbohydrate components appear to show some correlation with specificity.

3. Evidence is presented which suggests that the blood-group genes do not control the formation of a single product but of a multiplicity of similar but not identical materials, each of which possesses the same dominant specificity within the ABO system.

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Comparative Detoxication

4. ETHEREAL SULPHATE AND GLUCOSIDE CONJUGATIONS IN INSECTS

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The conjugation of phenols with sulphuric acid in mammals is well known but we have little information concerning its occurrence in other organisms (cf. Williams, 1947). It occurs in hens (Sperber, 1949) and their eggs (Takahashi, 1928; Layton, Frankel, Scapa & Barrows, 1951), in frogs (de Meio, 1945; Christiani, 1878) and in the slow worm

(Smith, 1954, unpublished). An ethereal sulphate of fusarubin has also been found as a metabolic product of the mould *Fusarium solani* (Ruelius & Gauhe, 1950). No study has been made of this detoxication mechanism in insects, but recently Myers & Smith (1954) reported that in addition to β -glucosides, a second conjugated phenol, possibly