nected only to its reservoir. In this way either bases or acids may be titrated without having to empty and refill the burette. The indicator used was methyl red in saturated aqueous solution. For further experimental details the two earlier papers should be consulted.

SUMMARY

1. The separation and micro-estimation of a series of aliphatic primary, secondary and tertiary amines is described.

2. A straight-line relationship is obtained when the logarithms of the retention volumes are plotted against the number of carbon atoms for each type of primary, secondary and tertiary amine. By means of this relationship the relative retention volume of any amine may be predicted provided the retention volume of any other member in the correct homologous series is known.

3. The utilization of columns having polar and non-polar liquid phases to determine whether an unknown amine is primary, secondary or tertiary is described. A plot of the relative retention volumes of primary, secondary and tertiary amines on paraffin against those obtained with 'Lubrol MO' liquid phases shows the values for each type of amine to fall on straight lines of different slope.

4. Out of 27 amines containing from 1 to 12 carbon atoms only two pairs (*iso*- and *sec.*-butylamine; di*iso*- and di-*sec.*-butylamine) cannot be separated on 4 ft. columns.

5. The separation of the homologues of pyridine is described as a suitable method for the approximate standardization of pyridine bases for chromatographic use.

Thanks are due to Dr A. J. P. Martin, F.R.S., for much valuable advice and discussion. Appreciation is also expressed to the National Coal Board (Eastern Region) and Messrs Yorkshire Tar Disillers Ltd. for gifts of pyridine homologues.

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Studies in Immunochemistry

11. THE ISOLATION AND PROPERTIES OF THE HUMAN BLOOD-GROUP H SUBSTANCE

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The different sera of human and animal origin which bring about the agglutination of human O erythrocytes strongly, whilst agglutinating cells belonging to other blood groups less readily or not at all, are generally referred to as 'anti-O' sera. The isolation from human red cells of a material possessing the capacity to neutralize the agglutinating action of these sera on O cells was first reported by Hallauer (1934) and subsequently by Stepanov, Kuzin, Markageva & Kosyakov (1940). Witebsky & Klendshoj (1941) described the isolation of a small quantity of serologically active material from the gastric juice of group O persons. The homogeneity of the substances was in no instance established, but the results were important in that they demonstrated that serologically specific materials of carbohydrate nature could be obtained from the erythrocytes and gastric juice of group O persons. Landsteiner & Harte (1941) likewise isolated the so-called O substance from the saliva of individuals belonging to group O and showed that the material was largely carbohydrate in nature and could not be distinguished chemically from that obtained from the saliva of persons belonging to groups A and B.

Following the demonstration by Morgan & Van Heyningen (1944), that ovarian cyst fluids obtained from women secreting their specific blood-group substances in a water-soluble form are a convenient and potent source of these materials, Morgan & Waddell (1945) isolated from this source a polysaccharide-amino-acid complex which possessed the so-called O specific character. The material obtained was not electrophoretically homogeneous, but nevertheless a preliminary examination of the substance was undertaken and it was found to possess a laevorotation with $[\alpha]_{5461} - 22°$, to contain 5·9 % nitrogen and to yield, after acid hydrolysis, 43 % of reducing sugars, expressed as glucose, and 33 % of hexosamine, as glucosamine base. Hydro-

lysis of the material with strong acid revealed that 43 % of the total nitrogen was present as amino-acid nitrogen and that the nitrogen of the liberated α-amino groups accounted for at least 81 % of the total nitrogen of the O substance. The sera of rabbits immunized with this so-called O substance were found to contain, after absorption with A_1B cells, quite potent antibody reactive against O and A_2 cells.

It is now recognized that the so-called anti-O sera detect a substance which is not a specific product of the activity of Bernstein's *O* gene, but that they react with a material which is present to a variable extent on the surface of the erythrocytes of the majority of human subjects. A substance which is probably identical serologically with that which occurs on the red-cell surface is found in the secretions of about 75 % of all persons and, as was clearly demonstrated by Schiff & Sasaki (1932), is present in the secretions of individuals who belong to blood group A_1B, i.e. in the secretions of persons who do not possess an *O* gene. These and other observations would seem to indicate that the so-called O substance described in the literature is not a specific product of the *O* gene, and to avoid confusion Morgan & Watkins (1948) proposed that the material be called H substance in place of O substance.

Detailed procedures for the isolation of certain of the human blood-group factors from ovarian cyst fluids have already been reported (Morgan & Waddell, 1945; Aminoff, Morgan & Watkins, 1950; Annison & Morgan, 1952), and essentially similar methods are now described for the isolation and characterization of the H substance from the same source.

EXPERIMENTAL

The cyst fluids studied were obtained from group O individuals and those fluids were selected which were rich in H substance. The fluids chosen usually possessed a relatively low concentration of the Lewis blood-group substance Le^b and, in certain specimens, of Le^a substance also (Grubb & Morgan, 1949; Grubb, 1951).

Materials and methods

Cyst fluids. These materials were usually dried from the frozen state and stored at 0° or −10° until required. Cyst fluids preserved in their native state were kept under toluene at 0°.

Serological activity. The agglutination tests employed to measure the activity of the preparations of H substance were carried out as described by Morgan & Van Heyningen (1944), and all the different anti-H sera available (Annison & Morgan, 1950) were also used at one time or another as control reagents. When human anti-H sera were used, much smaller volumes were taken and a technique similar to that described by Grubb & Morgan (1949) for Le^a inhibition tests was employed. A 'standard' preparation of H substance of human origin was included in all tests.

Analytical methods. The procedures employed to examine the H substance were the same as those used in similar studies with the blood-group A and Le^a substances (Aminoff & Morgan, 1948; Aminoff *et al.* 1950; Annison & Morgan, 1952). C, H and N analyses were made by Weiler and Strauss, Oxford. Optical rotations were measured at room temperature, approx. 20°.

The isolation of the H *substance*

Preliminary experiments using cyst fluid no. 93 indicated that when the dried fluid was extracted with 90 % (w/v) phenol-water solution the serologically active substance remained insoluble in the liquid phenol. In view of the ease with which this procedure eliminated most of the contaminating protein in one step, the remainder of the dried cyst fluid (92 g.; N, 12·9 %), which possessed about 5 % of the specific activity of the 'standard' H preparation, was similarly extracted. The phenol-insoluble residue was suspended in water and dialysed for several days at 0–2° and the water-insoluble component thoroughly extracted with water. The aqueous solution and the combined washings were concentrated *in vacuo* and dried from the frozen state to yield a product (8·56 g.; N, 5·9 %) which was as active as the laboratory 'standard' H substance. A sample of the water-insoluble residue was dissolved by adding a little alkali to an aqueous suspension of the material maintained at 0°. Serological tests indicated that this material (6·6 g.; N, 7·2 %) possessed rather less than half the activity of the 'standard' H solution. The remainder of the water-insoluble material was subsequently rendered water-soluble by pepsin treatment as described later in this paper.

The absence of a precipitate on the addition of 10 % (w/v) trichloroacetic acid to the water-soluble material (N, 5·9 %) indicated that the preparation was substantially free from extraneous protein, but in order to remove the remaining traces of protein, deproteinization (Sevag, 1934) was applied. The best conditions for the removal of protein were found by preliminary experiments and applied as follows: a solution (0·5 %) of the substance in 0·1 M-sodium acetate, pH 4·6, was vigorously stirred in 250 ml. centrifuge pots with an equal volume of a 1:1 mixture of $CHCl_3$ and amyl alcohol for about 30 min. The two liquid phases were then separated by centrifugation, and the supernatant fluid removed. The denatured protein which collected at the solvent interface was treated with an equal volume of fresh buffer, the aqueous layer recovered and added to the first aqueous phase. The denatured material was collected by pouring the $CHCl_3$-amyl alcohol layer and the material suspended in it into excess ethanol, and separating the precipitated material by centrifugation. The specific substance was treated three times with the organic solvent mixture. The results shown in Table 1 indicate that this treatment was effective in deproteinizing the mucoid solution, although a good deal of the specific substance separated with the denatured protein.

The deproteinized material (Table 1, fraction *e*) was examined electrophoretically at pH 8·0, and was found to show some inhomogeneity. This partially purified H substance was subsequently fractionated from aqueous solution at 37° with Na_2SO_4. Powdered anhydrous Na_2SO_4 was slowly added, with stirring, to a 2 % solution of the H substance (4·1 g.) to yield a concentration of 22 % (w/v) Na_2SO_4. In order to maintain more precise control over the precipitation, further Na_2SO_4 beyond this amount was added as a saturated solution. Four fractions were collected,

the last of these being the material remaining in solution after saturation with Na_2SO_4 had been reached. The largest fraction was redissolved in water and the fractional precipitation with Na_2SO_4 repeated. A large active fraction was again thrown down over a narrow range of Na_2SO_4 concentration. The results of these fractionations are shown in Table 2. A sample of the purified substance (Table 2, fraction 2*b*) dissolved in water to give a fairly viscous opalescent solution. Electrophoretic analysis of this material at pH 8·0 again demonstrated some inhomogeneity. The subsequent isolation of electrophoretically homogeneous blood-group substances from these materials suggested the use of the same technique with the H substance.

A small-scale experiment revealed that some 30–40% of the partially purified product obtained by Na_2SO_4 fractionation was rendered phenol-soluble by the trichloroacetic acid treatment, and this material, after precipitation from phenol solution with ethanol, was found to possess a somewhat higher activity than the standard H substance. The whole of the available substance was therefore treated with

Table 1. *Removal of protein from partially purified* H *substance by treatment with* $CHCl_3$*-amyl alcohol mixture*

(The serological activity of a material in this table and in the following tables is expressed in terms of the activity of a standard solution of the H substance.)

Nature of fraction	Yield (g.)	Specific rotation $[\alpha]_{5461}$	N content (%)	Serological activity (% of standard preparation)
(*a*) 1st	1·92	*	6·35	100
(*b*) 2nd Material separating at interface	1·10	−47°	6·3	100
(*c*) 3rd	0·35	−35°	5·85	100
(*d*) 1st Material soluble in aqueous phase†	—	−35°	5·4	100
(*e*) 3rd	4·53	−32°	5·2	100

* Too turbid for accurate polarimetric observation. † Small sample only examined.

Table 2. *Fractionation of partially purified* H *substance with* Na_2SO_4

Fraction	Precipitation level (% (w/v) Na_2SO_4)	Yield (g.)	Specific rotation $[\alpha]_{5461}$	N content (%)	Serological activity (% of standard preparation)
1 (*a*)	0–24·6	0·10	*	4·45	100
(*b*)	24·6–25·8	3·05	−20°	5·6	100
(*c*)	25·8–27·0	0·32	−35°	4·45	100
(*d*)	27·0–Excess	0·24	−30°	4·4	50
(*e*)	Material in final supernatant fluid	0·17	−29°	4·4	50
	Further fractionation of 1 (*b*)				
2 (*a*)	0–24·5	0·07	*	5·0	100
(*b*)	24·5–26·0	2·83	−24°	5·65	100
(*c*)	Material soluble above 26·0	0·09	−32°	4·9	100

* Too turbid for polarimetric observation.

Table 3. *Fractionation of partially purified* H *substance from* 90% *phenol with ethanol*

Fraction	Precipitation level (% (v/v) EtOH)	Yield (g.)	Specific rotation $[\alpha]_{5461}$	N content (%)	Serological activity (% of standard solution)
1 (*a*)	Phenol-insoluble material	1·41	−48°	5·05	100
(*b*)	0–10·0	1·15	−37°	5·3	150
(*c*)	10–excess	0·15	−30°	6·3	100
	Further fractionation of 1 (*b*)				
2 (*a*)	Phenol-insoluble	0·29	−43°	5·2	100
(*b*)	0–3·5	0·37	−33°	5·3	150
(*c*)	3·5–7·0	0·23	−29°	5·3	150
(*d*)	7·0–10·0	0·20	−30°	5·3	150
(*e*)	10–excess	0·01	−36°	5·4	150

The specific substance at this stage of purification was insoluble in phenol, but earlier work showed that both the A substance (Aminoff *et al.* 1950) and the Le^a substance (Annison & Morgan, 1952) became in part phenol-soluble on treatment with 5% trichloroacetic acid at 0° for a short time. 5% (w/v) trichloroacetic acid. A precipitate did not form, showing the absence of appreciable quantities of free protein. The solution was neutralized, dialysed, and the material was recovered by drying from the frozen state. The dry substance was then exhaustively extracted with 90%

phenol, and the soluble material divided into two fractions by the addition of ethanol to the phenol solution. The ethanol level was adjusted to 10% (v/v), by the addition of 1:1 phenol-ethanol mixture and the material thrown out of solution was collected by centrifugation. The supernatant solution was treated with several times its volume of ethanol, and the precipitate formed was collected. The phenol-insoluble residue and the two phenol-soluble products were washed free from phenol with ethanol, dialysed at 0° against water and dried from the frozen state. The analytical figures and serological activities of these fractions are shown in Table 3.

The fraction precipitated over the range 0–10% ethanol (1*b*) was resuspended in liquid phenol, when a small amount of it failed to dissolve after shaking for some hours with successive portions of fresh phenol. The soluble substance was fractionally precipitated from solution by slowly adding increasing amounts of a 1:1 (v/v) 90% phenol-ethanol mixture. Three major fractions separated between the levels 0 and 10% (v/v) ethanol: only a trace of material was precipitated by the addition of excess ethanol. The results of the final fractionation are included in Table 3. The analytical figures of fractions *b*, *c* and *d* suggested that the phenol-soluble material was not grossly inhomogeneous chemically and these fractions were therefore united. Electrophoretic and ultracentrifugal analysis of this product at pH 4·0 and 8·0 showed the presence of only one component (see Addendum to this paper).

half the activity of the standard H substance, was extracted with 90% phenol when rather more than half of the material dissolved. The phenol-insoluble residue (Fig. 1, 1*b*) dissolved in water to form a highly viscous, turbid solution, which possessed not more than 25% of the activity of the standard H solution. The phenol-soluble material was fractionally precipitated from solution with ethanol, and the fractionation repeated several times (Fig. 1). Each fraction collected was washed free from phenol with ethanol, dissolved in water, dialysed at 0°, and dried from the frozen state.

The final product precipitated from phenol over the range 0–10% ethanol (Fig. 1, 4*b*) was split into four parts by further fractionation from phenol between narrower limits of ethanol concentration. The hexosamine content and reducing-sugar value relative to glucose of each fraction after hydrolysis with 0·5 N-HCl at 100° for 16 hr. was estimated, in addition to the analytical figures usually determined, as shown in Table 4. The analytical results suggested that the starting material 4*b* was essentially homogeneous, and in consequence fractions 5*a*, *b*, *c* and *d* (Table 4) were combined. The product was examined in the Tiselius electrophoresis apparatus at pH's 8·0 and 4·0, when the presence of only one component was demonstrated. Ultracentrifugal analysis also failed to show that the preparation was inhomogeneous.

The fucose content (13·1%) of this preparation, which was isolated by means of peptic digestion, was found to be slightly lower than that of the first preparation (14·2%) (Table 3, 2*b*, *c* and *d*). The former preparation was also shown

Table 4. *Fractionation of* H *substance from solution in* 90% *phenol by ethanol*

(H substance used was material derived from fraction 4*b* in Fig. 1.)

Fraction	Precipitation level	Yield (g.)	$[\alpha]_{5461}$	N (%)	Hexosamine (%)	Reduction (%)	Serological activity (% of standard solution)
5 (*a*)	0–3·0	0·38	−26°	5·3	29·6	53	150
(*b*)	3·0–5·5	0·73	−31°	5·25	30·0	53	150
(*c*)	5·5–7·5	0·57	−32°	5·3	29·1	54	150
(*d*)	7·5–10·0	0·11	−28°	5·35	29·7	53	150

Pepsin treatment of the H *substance*

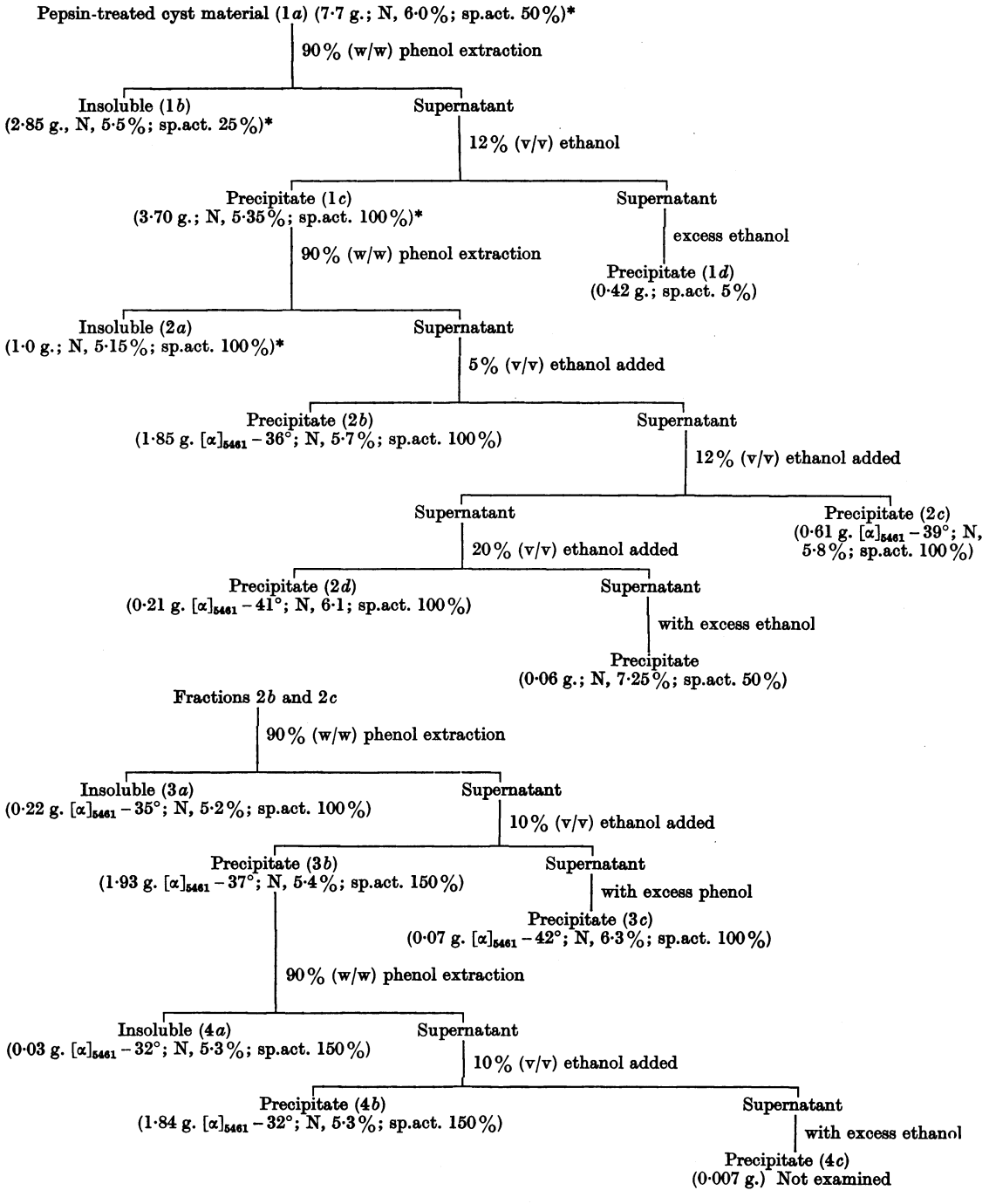
A considerable portion of the specific material present in the crude cyst, the whole of which was originally soluble in water, was found, after drying from the frozen state and treatment with phenol, to be insoluble in water. A possible interpretation of this phenomenon is that part of the group substance present in the cyst was bound to protein which was denatured and thus made insoluble. An attempt was therefore made to liberate this non-available group substance by digesting the insoluble material with pepsin. A small amount of the water-insoluble material was suspended with a trace of crystalline pepsin in 0·2 M-HCl-citrate, pH 2·6, at 37° for 48 hr. The substance passed completely into solution and after dialysis at 0° was dried from the frozen state to yield a water-soluble product possessing about half the serological activity of the standard H preparation. The whole of the water-insoluble material, together with a number of specimens similarly contaminated with protein (11·6 g.) which arose as side fractions in other purification procedures, was digested with 2 mg. of crystalline pepsin under similar conditions. The solution obtained was thoroughly dialysed at 0°, and dried from the frozen state. The main product (Fig. 1, 1*a*, 7·7 g.; N, 6·0%) which possessed about to possess a slight capacity to precipitate with anti-pneumococcal type XIV serum, a property not shown by the other material. These results suggest that a slight degradation had taken place during peptic digestion and the material was accordingly used as the starting material from which the sugar components, after they had been identified in the best H preparation, were isolated.

Test for homogeneity

The homogeneity of the main preparation (Table 3, fractions 2*b*, *c* and *d*) was studied by the following procedures.

(1) *Fractional solubility test.* The ready and extreme solubility of the H substance in water, with the formation of highly viscous solutions, prevented the application of the usual equilibrium method of solubility test. A fractional solubility test of the type employed for the human A substance (Aminoff *et al.* 1950) was therefore used.

The preparation (650 mg.) was shaken in a 50 ml. centrifuge pot with successive portions (about 20 ml.) of 32% (v/v) ethanol-water. The suspension was shaken for about



* Aqueous solutions too turbid for polarimetric observation.

Fig. 1. The fractionation of pepsin-treated H substance from solution in 90% phenol by ethanol. (The serological activities of the materials given in this figure are expressed in terms of the activity of a standard solution of this H substance, shown as sp.act., x%.)

1 min., centrifuged to separate the liquid and solid phases, the supernatant fluid decanted, fresh solvent added, and the procedure repeated. Five fractions, including the final solid residue, were thus obtained. The serological activity, specific rotation, N content, reducing power and hexosamine content of each fraction are recorded in Table 5.

The results obtained indicated that the material was probably homogeneous chemically and contained no material of very different composition from that of the most active material.

(2) *Electrophoresis.* An electrophoretic analysis of the H substance at pH's 4·0 and 8·0 gave no evidence of inhomogeneity. Details are given in the Addendum to this paper.

Table 5. *Fractional solubility test for homogeneity on* H *substance*

(For details of fractionation see Table 3, 2*b*, *c* and *d*, and Text.)

Fraction	I	II	III	IV	V
Specific rotation $(\alpha)_{5461}$ (±5)	−26°	−30°	−29°	−31°	−27°
(*a*) N (Kjeldahl) (%)	5·2	5·2	5·1	5·2	5·2
(*b*) Reduction (%) (calculated as glucose)	52·2	52·7	52·5	53·1	52·4
(*c*) Hexosamine (%) (calculated as base)	30·3	30·8	30·9	30·3	31·0
(*d*) Serological activity (% of standard preparation)	150	150	150	150	150
b/*a*	10·04	10·13	10·29	10·21	10·08
c/*a*	5·82	5·92	6·06	5·83	5·96
b/*c*	1·72	1·71	1·70	1·72	1·69

(3) *Ultracentrifugal examination.* The H substance was also examined in the ultracentrifuge. Details are given in the Addendum.

(4) *Quantitative immunochemical analysis.* Through the kindness of Prof. J. R. Marrack and Mr R. G. S. Johns the H substance from cyst 93 was examined by a quantitative precipitation technique similar to that described for A substance by Kabat, Baer & Knaub (1949). The antiserum employed was prepared by us in rabbits by means of an artificial antigenic complex made from the H substance and the conjugated protein component of the O somatic antigen of *Shigella shigae* (Morgan, 1943). The percentage of the glucosamine in the added H substance that was precipitated was calculated with allowance for solubility of the precipitate and for the apparent glucosamine content of the antibody, as done by Bendich, Kabat & Bezer (1946); allowance was also made for an effect of protein on estimates of glucosamine (Johns & Marrack, 1952). In the region of antibody excess the percentage of glucosamine precipitated, calculated in this way, was from 78 to 110. The full results of these experiments will be published in a separate paper.

Properties of the H *substance*

Immunological properties. The serological activity of the H substance was measured by determining the minimum amount necessary to inhibit the agglutinating action on O cells of anti-H sera of human and animal origin. The inhibition of two to three completely agglutinating doses of human anti-H serum was observed to occur with an equal volume of a dilution of 1 in 5×10^5 of H substance, i.e. with about 1 μg. of the material.

The H substance, when tested at a concentration of 5 mg./ml., showed no capacity to inhibit two or three completely agglutinating doses of the following heterologous blood-group reagents: (1) human anti-A and human anti-B agglutinins; (2) rabbit anti-M and anti-N agglutinins; (3) anti-P agglutinins; (4) anti-*Rhesus* sera, consisting of anti-D, anti-C, anti-c and anti-E reagents, and (5) anti-Le^a and anti-Le^b agglutinins. Some inhibition of human anti-O serum (Morgan & Watkins, 1948) was observed, however, although comparative tests with an inhomogeneous preparation of O substance obtained from human erythrocytes of group O suggested that the O substance content of the H preparation was equivalent to not more than a few per cent of the preparation from O stroma. The H preparation showed no capacity to inhibit the haemolysis of sheep red cells by rabbit anti-A erythrocyte serum in the presence of complement. Samples of the material were tested after treatment with N-acetic acid at 100° for 1–6 hr., but in no instance was there any evidence for the development of 'Forssman' activity.

Earlier investigations suggested that a partially purified preparation of the H substance isolated from human ovarian-cyst fluid was antigenic when injected into rabbits (Morgan & Waddell, 1945). This conclusion does not seem to hold for the preparations of H substance isolated during the present work, judging from the results of similar experiments with the more highly purified material.

A group of six rabbits, selected for the low reactivity of their sera with A and B cells, was given, at 3- to 4-day intervals, three intravenous doses each of 0·1 mg. of H substance dissolved in saline. The rabbits were bled 1 week after the last dose. No significant increase in the agglutination titre of the sera against O cells with respect to that of the normal bleedings was found. The rabbits were now given a further three intravenous doses, each of 0·1 mg. of the H substance, under the same conditions as before. The anti-O cell titre of each of the resulting sera, however, was not appreciably higher than that of the normal bleedings.

A similar group of six rabbits was given, under the same conditions, three doses, each of 0·05 mg., of an artificial

antigen made by coupling the same specimen of H substance with the conjugated protein component of the O somatic antigen of *S. shigae*. This antigen was prepared by the method described for the corresponding artificial A antigen (Morgan, 1943). The average anti-O cell agglutinin titre increased to about 2000, a 100-fold enhancement above the normal titre. The results indicated that under the conditions of test in rabbits the H substance is not antigenic, or only very weakly so, whereas it is readily converted into a powerful antigen by combination with the conjugated protein component of *S. shigae*.

The preparation of the H substance which had not been treated with pepsin failed to give a precipitate when tested with horse anti-pneumococcal type XIV serum, and in this respect differed from the H-active materials isolated by Kabat and his co-workers (see Kabat, 1949). The H preparation isolated by the use of pepsin, however, showed a weak reactivity when tested at a dilution of 1:1000 with the pneumococcus type XIV antiserum. The type XIV polysaccharide gave a heavy precipitate when mixed with its homologous serum under similar conditions.

The H substance was examined by Dr J. McCrea for its capacity to inhibit the agglutination of fowl cells by the heated Lee (type B) influenza virus. The materials were found to be inactive. The high activity of a specimen of purified human blood-group H (so-called 'O') substance reported by Burnet, McCrea & Anderson (1947) must be attributed to an active material contaminating the preparation examined.

Physical properties. The relative viscosity (η) of a 0·5 % solution of the H substance in 0·85 % saline was 1·31. The mean value of the specific rotation of the most active preparations of the H substance, was $[\alpha]_{5461} - 30 \pm 3°$ in water (c, 1·0).

The ultraviolet absorption of the H substance (0·1 % in 0·85 % sodium chloride) was examined in a Hilger quartz spectrophotometer (Uvispek) using optical cells 2 cm. deep. The absorption curve (Fig. 2) shows that no absorption maxima occur between 220 and 300 mμ.

Chemical properties. A precipitate was not formed when a 2 % aqueous solution of the H substance is treated with any of the usual protein precipitants. The biuret, ninhydrin, xanthoproteic, Millon, Hopkins-Cole, Seliwanoff and Bial tests were negative. Ehrlich's diazosulphanilic acid test was faintly positive as was Sakaguchi's test for arginine. The addition of alkaline copper sulphate to a solution of the H substance, as in the biuret test, gave rise to a voluminous precipitate.

The analytical figures for the pooled specimen of H substance (Table 3, fractions 2*b*, *c* and *d*) isolated from cyst no. 93 are: C, 41·4; H, 6·9; N, 5·3 % (Kjeldahl). The phosphorus content is less than 0·02 %, and the sodium fusion test for sulphur indicates that this element is not present in appreciable amounts.

The acetyl content of the H substance is 8·7 % (6 hr. hydrolysis) and is in excess of that required for the *N*-acetylhexosamine calculated to be present from the total hexosamine value. The distillates obtained from the acetyl determinations were neutralized and were used to identify the volatile organic acid as an *S*-benzylthiouronium salt (Donleavy, 1936). The *S*-benzylthiouronium salt obtained after recrystallization from 80 % ethanol melted at 134° and gave a mixed m.p. of 136° with an authentic specimen of *S*-benzylthiouronium acetate (m.p. 136°).

The total fucose content of the H substance was rather more than 14 %. Under the conditions used for the determination of *N*-acetylglucosamine (Morgan & Elson, 1934; Aminoff, Morgan & Watkins, 1952) the H substance gave a colour with Ehrlich's reagent. The maximum colour developed

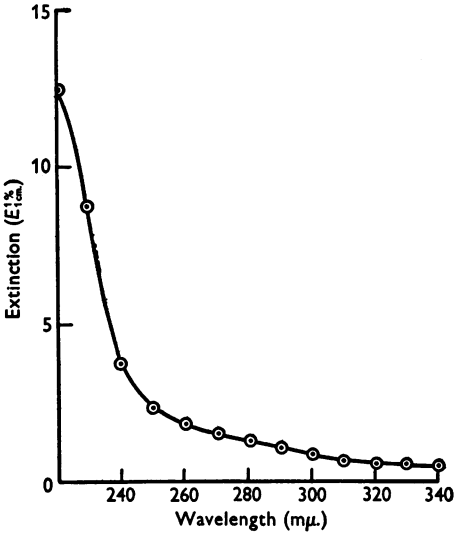


Fig. 2. The ultraviolet absorption spectrum of H substance (0·1 % solution in 0·85 % NaCl).

by the H substance after heating at 100° for 12 min. with dilute sodium carbonate at pH 10·8 (optimal heating conditions), was equivalent to about 7·5 % (unextrapolated value) of the colour given by an equal weight of *N*-acetylglucosamine. *N*-acetylglucosamine requires 4 min. heating to develop its maximum colour under the same conditions.

The H substance after heating with dilute sodium carbonate for 16 min. at 100° was dialysed through well washed cellophan against distilled water at 0°. The diffusate gave an immediate colour with Ehrlich's reagent, whereas the indiffusible residue gave no appreciable colour (cf. King & Morgan, 1944; Morgan, 1946 Annison & Morgan, 1952).

Oxidation of the H *substance with periodate*

The rate of reduction of periodate by the H substance at pH's 5·0 and 7·3 was measured by the

techniques employed by Aminoff & Morgan (1951) in similar experiments with the human A substance.

Oxidation at pH 5·0. A solution (0·1 %) of the H substance in 0·1 M-acetate, pH 5·0, was treated with 0·005 M-HIO_4. The rate of reduction of periodate and the loss of biological activity of the substance were determined. The results (Fig. 3) indicate that the rate of reduction of periodate at pH 5·0 by H substance is similar to that found for the A and Le^a substances. The biological activity of the H substance, however, is destroyed much more rapidly than the activity of the A and Le^a substances under similar conditions. The analytical results, corrected for over-oxidation by extrapolating the uptake curve to zero time, show that 1 mg. of the H substance reduces $0{\cdot}277 \times 10^{-5}$ mole of HIO_4, and hence about 362 g. of H substance reduces 1 mole of HIO_4.

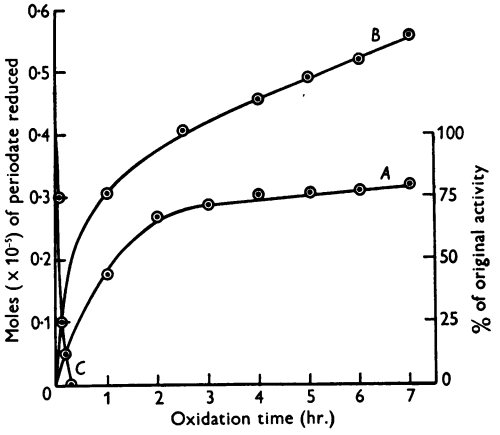


Fig. 3. The rate of reduction of periodate by H substance at pH 5·0 (curve *A*) and pH 7·3 (curve *B*). The resulting inactivation at pH 5·0 as determined by the capacity of the oxidized material to inhibit the agglutination of O cells by anti-H agglutinin (curve *C*).

Oxidation at pH 7·3. The rate of reduction of periodate is greater at pH 7·3 than it is at pH 5·0 (Fig. 3). The uptake curve shows a less definite inflexion after 2 hr. and the periodate consumption continues steadily for several days. The serological activity of the substance is lost at approximately the same rate as at pH 5·0 (Fig. 3).

Hydrolysis of the H *substance by acid*

The H substance was hydrolysed in sealed glass ampoules at 100° with (*a*) 0·5 N-HCl, (*b*) 6 N-HCl and (*c*) N-acetic acid, as described for the Le^a substance (Annison & Morgan, 1952).

The course of hydrolysis with 6 N-HCl was followed by measuring the α-amino N and the α-amino-acid N liberated. The results (Fig. 4) show that the liberation of α-amino N reaches a maximum in about 4 hr. and then represents 88 % of the total N of the H substance, whereas the α-amino-acid N after 16 hr. hydrolysis is equivalent to about 41 % of the total N. The α-amino N value includes the N liberated from the amino sugars set free during hydrolysis.

The liberation of reducing sugars and hexosamines was followed by an examination of the products of hydrolysis with 0·5 N-HCl. The results (Fig. 5) show that the maximum liberation of reducing sugars, 54 % expressed as glucose, and of hexosamines, 31 % expressed as glucosamine base, occur at about the same time (6 hr.).

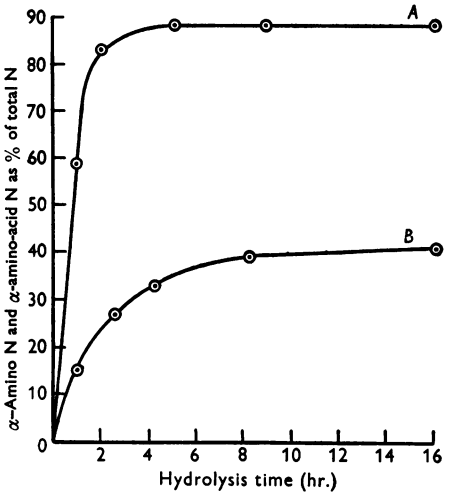


Fig. 4. Hydrolysis of H substance by 6 N-HCl at 100° as determined by the liberation of α-amino groups (curve *A*), and α-amino-acids (curve *B*).

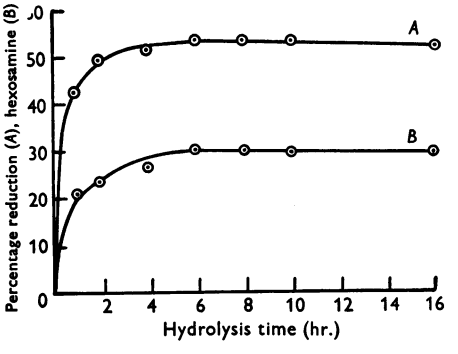


Fig. 5. Hydrolysis of H substance by 0·5 N-HCl at 100° as determined by (*A*) reducing power, expressed as glucose and (*B*) hexosamine liberated, expressed as glucosamine base.

The H substance was hydrolysed with N-acetic acid at 100°, and the rate and extent of liberation of fucose, reducing sugars and *N*-acetylhexosamine residues determined. The results (Fig. 6) show that a maximum value of about 13 % fucose determined as acetaldehyde after oxidation with periodate is obtained after 20 hr. hydrolysis and a maximum value for reducing sugars is reached at about the same time. There is an appreciable increase in the amount of '*N*-acetylhexosamine' colour given by the H substance during the first 2 hr. of the hydrolysis period. After this time the original value of 7·5 % expressed as *N*-acetylglucosamine, reaches a maximum value of 8·8 % and then decreases.

Treatment with N-acetic acid at 100° rapidly inactivates the H substance and at the same time induces the formation of a product which possesses the property of reacting with horse anti-pneumococcal type XIV serum.

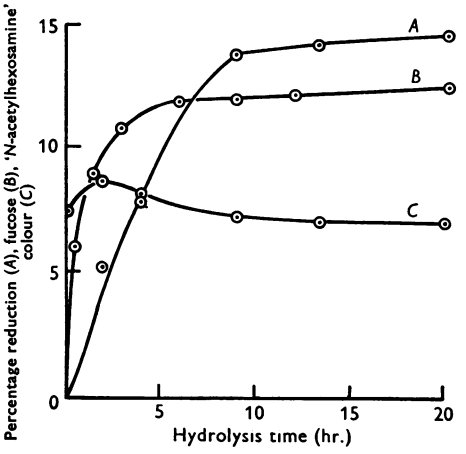


Fig. 6. Hydrolysis of the H substance by N-acetic acid at 100° as determined by (*A*) reducing power, expressed as glucose, (*B*) fucose, determined as acetaldehyde after oxidation with periodate and (*C*) '*N*-acetylhexosamine' colour.

Chromatographic analysis of the H *substance*

A detailed investigation of the sugar and amino-acid components of the H substance was made by the usual techniques of partition chromatography on paper. The experimental procedures employed were the same as those described for similar work with the Le^a substance (Annison & Morgan, 1952).

Amino-acids. Two-dimensional paper chromatographic analysis of 6N-HCl hydrolysates of the H substance revealed that the amino-acid content of the material was indistinguishable from that of the A and Le^a substances (Aminoff *et al.* 1950; Annison & Morgan, 1952). The amino-acids identified on paper were as follows: lysine, arginine, aspartic acid, glutamic acid, glycine, serine, alanine, threonine, proline, valine and leucine (isoleucine).

Amino sugars. The presence of chondrosamine, in addition to glucosamine in the A substance, was first shown by Aminoff & Morgan (1948). Similar experiments established that both amino sugars were components of the H substance; similar findings have been reported for the Le^a substance (Annison & Morgan, 1952).

Sugars. D-Galactose and L-fucose were shown to be components of the H substance as described by Annison & Morgan (1952) for the Le^a substance.

Isolation of the component sugars and amino sugars

Sugars. The H substance (500 mg.) was hydrolysed with 0·5N-HCl and the product, after neutralization, run on a cellulose column according to the general procedure of Hough, Jones & Wadman (1949), using the solvent system 1:1 *n*-butanol/2% (v/v) acetic acid-water.

L-*Fucose diphenylhydrazone.* The fucose obtained from the column was dissolved in water (2 ml.), and glacial acetic acid (0·2 ml.) and freshly distilled diphenylhydrazine (60 mg.) added. The solution was allowed to stand overnight in the dark and at room temperature and was then cooled, when 30 mg. of needle-shaped crystals separated. The derivative was twice recrystallized from 95% (v/v) ethanol and 17 mg. of material melting at 200° were finally obtained. Mixed m.p. with authentic fucose diphenylhydrazone (Muther & Tollens, 1904) remained unchanged. (Found: C, 66·1; H, 6·8; N, 8·6. Calc. for $C_{18}H_{22}O_4N_2$: C, 64·7; H, 6·7; N, 8·5%.) Optical rotation $[\alpha]_D$ − 14° in pyridine (*c*, 1).

D-*Galactose α-methylphenylhydrazone.* The fractions issuing from the column which contained galactose were evaporated to dryness *in vacuo* at about 30°. The residue was dissolved in water (2 ml.), and freshly distilled α-methylphenylhydrazine (0·1 ml.), ethanol (2 ml.) and 10% (v/v) acetic acid (0·1 ml.) were added. The mixture was kept at 37° for 2 hr. and overnight at 0°. D-Galactose α-methylphenylhydrazone (54 mg.) separated, and after recrystallization from 30% ethanol gave 48 mg. of needle-shaped crystals m.p. 191°. Mixed m.p. with authentic D-galactose α-methylphenylhydrazone 190°. (Found: C, 52·2; H, 6·9; N, 9·7. Calc. for $C_{13}H_{20}O_3N_2$: C, 54·9; H, 7·0; N, 9·9%.)

D-*Galactose α-methylphenylhydrazone pentaacetate.* Galactose α-methylphenylhydrazone (19 mg.) was suspended in 1 ml. of dry pyridine, and 0·2 ml. of acetic anhydride added at 0° according to the method of Wolfrom & Christman (1931). The mixture was allowed to stand overnight at 0°, and, with frequent shaking, at room temperature for a further 24 hr. The solution was then poured into excess chilled water and the white crystalline material which was thrown out of solution was collected and washed thoroughly with water. The material after recrystallization from 50% ethanol gave 29 mg. of D-galactose α-methylphenylhydrazone pentaacetate, m.p. 138–140° without decomposition. Mixed m.p. with authentic specimen of the pentaacetate remained unchanged. Specific rotation $[\alpha]_D + 38 \pm 3°$ in 95% (v/v) ethanol (*c*, 1.)

2:4-*Dinitrophenyl* (*DNP*) *amino sugars.* The amino sugar components of the H substance were separated and identified as their 2:4-dinitrophenyl derivatives by partition chromatography on kieselguhr columns using the solvent system 30% amyl alcohol-$CHCl_3$/0·2M-potassium borate, pH 9·7 (Annison, James & Morgan, 1951). Two 100 mg. samples of the H substance isolated by the use of pepsin were hydrolysed for 16 hr. at 100° with 0·5N-HCl in an atmosphere of N_2. The products were condensed with 1-fluoro-2:4-dinitrobenzene and the resulting amino sugar derivatives separated and isolated. The results obtained with the two samples were: (1) Total yield of DNP amino sugars, 79%. DNP-glucosamine, 23·4 mg.; DNP-chondrosamine, 20·7 mg. Ratio, glucosamine:chondrosamine, 1:0·89. (2) Total yield of DNP amino sugars, 71%. DNP-glucosamine, 22·3 mg.: DNP-chondrosamine, 20·2 mg. Ratio, glucosamine:chondrosamine, 1:0·91. The yields of DNP amino-sugars were estimated colorimetrically.

N-2:4-*Dinitrophenyl*-D-*chondrosamine.* A part of the DNP-chondrosamine, obtained in Exps. 1 and 2 above, was pooled and recrystallized from acetone-water and then acetone. Yield, 9·2 mg., m.p. 178–180° (uncorr.), mixed m.p. with an authentic sample of DNP-chondrosamine, 179–181° (uncorr.). Specific rotation observed: $[\alpha]_{5461}$ $+80 \pm 4°$ in 80% (v/v) ethanol (*c*, 1). (Found: C, 41·9; H, 4·7; N, 11·9. Calc. for $C_{12}H_{15}O_9N_3$: C, 41·7; H, 4·4; N,

12·2%.) $[\alpha]_{5461}$ +84±3° in 80% (v/v) ethanol (Annison *et al.* 1951).

N-2:4-*Dinitrophenyl*-D-*glucosamine*. A mixed specimen of DNP-glucosamine (61 mg.) obtained from a number of preparations of H substance was crystallized from acetone-water and from acetone-light petroleum (b.p. 69–80° range). Yield 12·2 mg., m.p. 197–200° (uncorr.). Optical rotation $[\alpha]_{5461}$ +66±4° (*c*, 1% in 80% (v/v) ethanol). (Found: C, 41·9; H, 4·3; N, 11·9. Calc. for $C_{12}H_{15}O_9N_3$: C, 41·7; H, 4·4; N, 12·2%.) $[\alpha]_{5461}$ +65±3° in 80% (v/v) ethanol (Annison *et al.* 1951).

Partial hydrolysis of the H *substance*

H substance (50 mg.) was hydrolysed with N-acetic acid at 100° for 48 hr. in sealed glass ampoules and the products were dialysed in a thoroughly washed cellophan bag for 7 days at 0° against frequent changes of distilled water. The diffusates were stored at 0° in the presence of a few drops of $CHCl_3$ until the dialysis was complete. The total diffusates were then mixed together, concentrated to small volume and dried from the frozen state. The non-diffusible contents of the cellophan bag were likewise dried. The diffusible material (about 19 mg.) was obtained as a hygroscopic glass, the non-diffusible material (16 mg.) as a light brown friable solid. Fucose, galactose, an *N*-acetylhexosamine and traces of glucosamine and chondrosamine were detected in the diffusate by paper chromatography. The solvents employed in this experiment did not resolve a mixture of *N*-acetylhexosamines and the precise identity of the material giving the *N*-acetylhexosamine spot was not elucidated. The developed chromatogram of the diffusate when sprayed with aniline hydrogen phthalate revealed the positions of fucose and galactose only. The $AgNO_3$-NH_3 reagent gave, in addition, three other spots due to slowly moving substances which presumably comprised partial hydrolysis products of the polysaccharide moiety of the H substance. Two of these three unidentified spots reacted also with the hexosamine reagent. The presence in the diffusate of an amino-acid or peptide which moved slowly in all the solvents used was revealed when the chromatograms were sprayed with ninhydrin. This spot and the other three unidentified spots were no longer present after the diffusate was hydrolysed further with 0·5N-HCl at 100° for 16 hr. Only fucose, galactose and glucosamine and chondrosamine, in what appeared to be approximately equal amounts, were detected in this hydrolysate. A part of the diffusate was hydrolysed further with 6N-HCl at 100° for 16 hr., and examined for amino-acids by two-dimensional paper chromatography. Traces of all the amino-acids present in the original material except leucine, proline and arginine were detected. The contents of the dialysis bag were examined chromatographically for sugars after hydrolysis with 0·5N-HCl. Fucose could not be detected in any of the chromatograms developed with the four solvent systems employed for sugar analysis. The presence of galactose and equal amounts of the two amino sugars was demonstrated. The qualitative composition of the indiffusible material in terms of amino-acids was found to be indistinguishable from that of the undegraded H substance.

DISCUSSION

The isolation of the blood-group H substance in an essentially homogeneous condition from human ovarian cyst fluids has allowed this immunologically important material to be studied and identified as a mucoid which possesses properties very similar to those of the other human blood-group substances already described.

The H substance sediments in the ultracentrifuge at a rate similar to that found for the blood-group A and Le^a substances under the same conditions (Kekwick, 1950, 1952) and the diffusion constants of the three materials are not grossly dissimilar. The particle weight of the H substance is of the order 320 000 and is thus somewhat larger than that found for the other two group substances. It may well be, however, that in their native state these materials have an even larger particle size. The specimens of H substance obtained possess a laevorotation of about −30°, and in this respect differ from the A substance prepared from cyst fluids which has a positive rotation. Kabat, Bendich, Bezer & Beiser (1947), however, record laevorotations for a large number of group A preparations isolated by them from human saliva, stomachs and amniotic fluids. The H substance contains the sugars L-fucose, D-galactose, D-glucosamine and D-chondrosamine and 11 amino-acids, and indeed is so far indistinguishable on qualitative grounds from the A and Le^a substances. Analysis of the H substance, however, reveals several differences in the quantitative make-up of the material compared with the other group substances. For example, the L-fucose content of the H substance appears to be not more than 14%, whereas this sugar is contained in the corresponding A mucoid to the extent of at least 18%. Similarly, the ratio of glucosamine to chondrosamine in the Le^a substance is probably of the order 3 to 1, whereas these two amino-sugars are present in the H substance in a ratio very close to 1. Differences in the quantitative composition of preparations of blood-group mucoids which show a single specificity, such as A or B or H, have been recorded in the literature, but it is obvious that significance cannot be attached to variations found in the amounts of a component sugar or amino-acid present in individual preparations unless it is known that these materials are essentially homogeneous on the basis of the results of physical, chemical, serological and immunochemical examination. There has been no opportunity so far of preparing from each of several cyst fluids specimens of group substance of a single specificity and established homogeneity and, until this has been accomplished, it would be inadvisable to express an opinion as to whether the recorded quantitative variations in the amounts of components do, in fact, establish that differences in composition occur in mucoids exhibiting similar blood-group specificity.

H substance isolated from cyst fluids by the methods described possesses no capacity to give a specific precipitate when mixed with pneumococcal

type XIV horse serum. In this respect, therefore, the H substance behaves as do the blood-group A and B substances and does not show the pneumococcal type XIV activity found to be associated with the Le^a substance prepared from cyst fluids under the same conditions. In our experience H substance isolated by a procedure which involves prolonged peptic digestion shows some reactivity with the pneumococcal type XIV antiserum.

Partial hydrolysis of the group substances with weak acetic acid causes them to react with pneumococcal type XIV horse antiserum and thus to develop an overlapping specificity with the specific polysaccharide of this organism. This pronounced cross-reactivity suggests that the *N*-acetylhexosamine-galactose chains which make up major structures within the polysaccharide moieties of the group substances are similar in pattern to each other and to the *N*-acetylglucosamine-galactose structures of the pneumococcus type XIV specific polysaccharide. According to Kabat, Baer, Bezer & Knaub (1948) and Kabat (1949), the fucose molecules in the undegraded group substances shield the *N*-acetylglucosamine-galactose structures common to these materials and prevent the cross-reactivity with the pneumococcal type XIV antiserum. The reactivity of the undegraded Le^a substance and the inactivity of the A and H substances with pneumococcal type XIV serum could perhaps be explained as a consequence of the higher glucosamine-chondrosamine ratio in the Le^a substance compared with the other group materials, with the result that the larger number of cross-reacting structures present cannot be adequately screened from the pneumococcal type XIV antibody by the fucose molecules, which presumably protrude from and in part dominate the essential surface configuration of the group substance.

Hydrolysis of the H substance with acetic acid rapidly liberates the whole of the fucose component, and it is of interest to record that to bring about a loss of half the original group specific activity shown by preparations of H, Le^a and A substances it is necessary to heat them with N-acetic acid for 15 min. 60 min. and about 150 min. respectively. The more rapid loss of group specific serological activity shown by the H substance is, therefore, probably correlated with the more ready liberation of fucose from the material.

The exact genetical relationship of the H substance described to the blood-group substances A and B is not yet clear. According to a theory proposed by Hirszfeld & Amzel (1940) and modified by Morgan & Watkins (1948) and by Boorman, Dodd & Gilbey (1948) the genes *A*, *B* and *O* are formed from a primary gene *H* by progressive mutations. Between the original gene form *H* and the completely mutated genes *A*, *B* and *O* there exists a series of transitional gene forms which give rise to A and H, B and H, and O and H substances on the surface of the A, B and O erythrocytes respectively. If a person possesses a single or double dose of Schiff's secretor gene, *S*, then the secretions will similarly contain A and H or B and H substances. As far as it is possible to determine at present there is no appreciable amount of a water-soluble substance which possesses group O character in the secretions of persons belonging to group O. The secretions from group O individuals are therefore found to possess H substance only. The hypothesis implies that when mutation is complete, gene forms designated A_c, B_c and O_c arise which are without an *H* gene component. It is in the serum of a person whose gene forms are devoid of H character that one would expect to find an anti-H agglutinin. This agglutinin, which is found very rarely, is neutralized in its action on group O cells by the human H substance. We have encountered two human sera which possessed useful anti-H agglutinins, one derived from a person belonging to group A and the second from a person belonging to group B. The cells of both individuals failed to react at room temperature with anti-H sera and therefore the erythrocytes seemed to be devoid of H substance as would be anticipated by the hypothesis. Evidence that the *H* gene was absent from the chromosome was sought by examining the donor's saliva for A and H, and B and H substances, respectively. Both persons were, however, found to be Lewis positive and non-secretors. Thus additional and essential evidence for establishing the absence of the *H* gene, by demonstrating the absence of a product of its activity, could not be obtained.

It is evident that if this hypothesis of the inheritance of the blood-group characters, based as it is on the activity of transitional gene forms, is correct then there are many far-reaching theoretical and practical consequences to consider, especially from the point of view of those engaged in the isolation and characterization of the blood-group substances. Perhaps the most important one at this stage in the development of the subject is to determine if the product of the activity of transitional genes—such as A_2 when present in homozygous form (A_2A_2)—is a single substance which possesses two distinct serological characters, A and H, or whether the products are two distinct substances each possessing a single serological specificity, A or H.

There remains the possibility that the H character is the result of the presence of a genetically independent blood-group system H–h. In the event of this being so it will be necessary to account for the undoubted influence of the *A*, *B* and *O* genes on the quantitative manifestation of the H character of erythrocytes, and on the amount of water-soluble H

substance in secretions. Predictions inherent in these hypotheses are open to a measure of experimental proof and attempts are now being made to obtain evidence for or against the ideas put forward.

SUMMARY

1. Methods for the isolation and purification of the human blood-group H substance from ovarian cyst fluids are described.

2. The H substance is a laevorotatory mucoid which contains about 5·3 % nitrogen and is similar in general composition and properties to the mucoids which are responsible for the human blood-group A, B and Lewis (Le^a) characters.

3. The H substance obtained is somewhat polydisperse, but is essentially homogeneous with respect to certain physical, chemical immunochemical and serological properties.

4. L-Fucose, D-galactose, D-glucosamine, D-chondrosamine and 11 amino-acids have been identified as components of the H substance.

5. The acid hydrolysis products contain 14 % fucose, 54 % reducing substances, expressed as glucose, and 31 % hexosamine. The α-amino-acid nitrogen is equivalent to about 41 %, and the α-amino nitrogen to 88 % of the total nitrogen.

6. Possible relationships between the products of the *A*, *B* and *O* genes and the H substance are discussed.

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ADDENDUM

Physicochemical Examination of Blood-Group H Substance

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The H substance was examined in the electrophoresis apparatus and ultracentrifuge, and in addition the partial specific volume and diffusion constant were determined.

METHODS

Electrophoresis. The material was examined in a Tiselius electrophoresis apparatus at 0°, using the diagonal schlieren optical system and monochromatic light, $\lambda = 546$ mμ.,

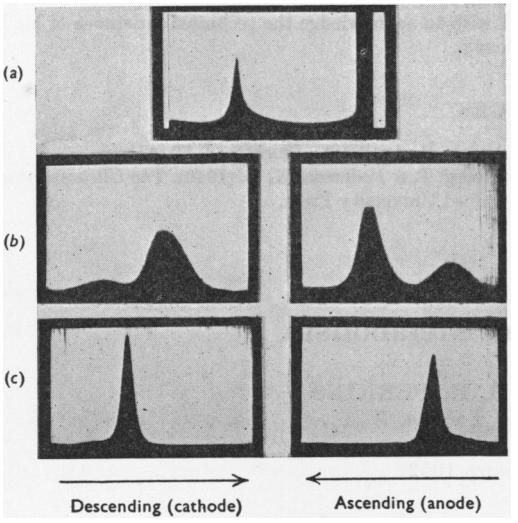

Fig. 1. Sedimentation diagram and electrophoresis patterns of blood-group H substance. (*a*) Sedimentation after 60 min. at 270 000 *g*. 1% (w/v) in phosphate-NaCl. (Phosphate, pH 8, $I = 0{\cdot}2$; NaCl, 0·15 M.) (*b*) Electrophoresis pattern after 180 min. at 5 V./cm. of 2% (w/v) solution in phosphate, pH 8, $I = 0{\cdot}2$. (*c*) Electrophoresis pattern after 220 min. at 5 V./cm. of 2% (w/v) solution in acetate, pH 4, $I = 0{\cdot}1$. Lower arrows show direction of migration in both (*b*) and (*c*).

isolated from a mercury arc by a suitable filter. As the main object of this examination was to detect any traces of contaminating substances of high molecular weight, solutions of salt-free material were made up in suitable buffers to give a concentration of 2 g./100 ml., and dialysed against buffer overnight.

Sedimentation. The ultracentrifugal examination was carried out in a Svedberg oil turbine machine at 60 000 rev./min. (270 000 *g*) using an optical system similar to that of the electrophoresis apparatus. The variation of sedimentation constant with concentration was examined over a limited concentration range. Solutions were made up in suitable buffers and sufficient NaCl added to suppress any charge effect on sedimentation.

Diffusion. The diffusion measurements were made by Dr A. G. Ogston, using the method of Coulson, Cox, Ogston & Philpot (1948). Values for the diffusion constant and sedimentation constant were corrected to water at 20° (Svedberg & Pedersen, 1940).

Partial specific volume. This was determined pyknometrically at 25°, the substance being dissolved in the buffer used for the majority of the sedimentation constant measurements.

RESULTS

In acetate buffer, pH 4, $I = 0{\cdot}1$, after prolonged electrophoresis only one component was demonstrable. At pH 4 the boundaries were symmetrical and showed very little spreading. At pH 8 the ascending boundary was symmetrical though the apex of the peak exhibited a slight flattening, and the descending boundary was asymmetrical (Fig. 1). The spreading of both boundaries at pH 8 was considerably greater than at pH 4. Migration in each instance was anodic, but very slight at pH 4.

Table 1. *Sedimentation and diffusion constants of* H *substance*

(Sedimentation and diffusion constants corrected to water at 20° (Svedberg & Pedersen, 1940).)

Concentration (g./100 ml.)	$S_{20} \times 10^{13}$ (corr.)	$D_{20} \times 10^{7}$ (corr.)
0·50	6·64	—
0·75	6·04	—
1·00	5·21	1·21

In the ultracentrifuge at pH 8 only one component was present, and the peak remained relatively sharp even after 90 min. at 270 000 *g* in a 1% (w/v) solution, and at 0·5% (w/v) a satisfactory peak was present after 60 min. at 270 000 *g*. The sedimentation constant was determined at three concentrations and the data of Table 1 show that there is a marked variation of sedimentation constant with concentration.

Included in Table 1 is a value for the diffusion constant kindly measured by Dr A. G. Ogston. The partial specific volume of the substance was 0·636.

From the sedimentation and diffusion constant values obtained in a solution containing 1 g./100 ml. H substance, and using the determined value of the partial specific volume, the molecular weight was calculated from the usual equation (Svedberg & Pedersen, 1940) giving a value of 320 000. From the same data the frictional ratio f/f_o obtained was 4·2. This corresponds to an axial ratio of > 100, or may be interpreted as indicating a high degree of hydration.

DISCUSSION

The data indicate that electrophoretically the substance is essentially homogeneous, which means that the nitrogen in the preparation is not attributable to a protein contaminant but is an integral part of the molecule.

For a substance of molecular size 320 000 associated with the observed large frictional ratio which is reflected in the concentration dependence of the sedimentation constant, a rather sharper boundary would be expected to be maintained throughout sedimentation if the substance was strictly monodisperse. The molecular size appears to be rather higher than that previously reported for blood-group A substance (Kekwick, 1950) and Le^a substance (Kekwick, 1952), and there is evidence of a higher degree of molecular asymmetry.

The conclusion must probably be drawn that the substance is moderately polydisperse, but the data are insufficient to give a precise expression of the range and distribution of molecular size in the preparation.

SUMMARY

1. The blood-group H substance has been found essentially homogeneous electrophoretically.

2. The mean molecular weight of the substance calculated from sedimentation and diffusion data is 320 000 and the frictional ratio 4·2.

3. The substance is probably moderately polydisperse and the molecular shape highly asymmetrical.

I wish to acknowledge the technical assistance of Mr H. Murray.

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Citric Acid and Bone Metabolism

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Since Dickens (1941) showed that 90 % or more of the citric acid of the body was located in the skeleton, and Class & Smith (1943) demonstrated its presence in various body concretions, evidence has been accumulating that this substance plays an important part in the metabolism of bone. A connexion between calcium and citric acid was established when Pincus, Peterson & Kramer (1926) found that these substances formed a soluble complex which is assumed to account for the non-ionizable part of the diffusible fraction of serum calcium. Gomori & Gulyas (1944) showed that citrate injections in dogs made serum calcium more ultrafiltrable so that a rapid urinary excretion of calcium took place, and similar results were obtained in dogs by Chang & Freeman (1950). A rise in serum calcium caused by parathormone was shown by Alwall (1944) to be accompanied by a rise in serum citrate, and in Gomori's experiments, in which dogs were injected with citrate, microscopic examination of the bone showed a picture similar to that observed after toxic doses of parathormone. The connexion between serum calcium and citrate was further established by Freeman & Chang (1950). These authors found that high doses of vitamin D administered to dogs raised not only the serum calcium but also the serum citrate, and suggested that this was due to increased liberation of these substances from the bone reservoir. A possible explanation for the presence of citrate in bone was indicated by the work of Kuyper (1938), who showed that the precipitate of calcium, phosphate and citrate formed *in vitro* in the presence of suitable concentrations of these substances is actually a complex such as may exist in bone. He also found that the solubility of the precipitate was increased