SUMMARY

1. A rapid and simple method for the estimation of *N*¹-methylnicotinamide is presented. It has been modified from the procedure described by Huff *et al.* (1945) by using methyl ethyl ketone instead of acetone, and by the adoption of two alternative blank procedures.

2. The conditions of the reaction and the specificity of the method have been studied.

3. With the blank procedures described the fluorescence due to coenzyme I and to *N*¹-methylnicotinuric acid betaine could be differentiated from that of *N*¹-methylnicotinamide.

4. Results of *N*¹-methylnicotinamide estimations have been reported for rat and human urines.

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Studies in Immunochemistry

8. THE ISOLATION AND PROPERTIES OF THE HUMAN BLOOD-GROUP A SUBSTANCE

By D. AMINOFF, W. T. J. MORGAN AND WINIFRED M. WATKINS

The Lister Institute of Preventive Medicine, London, S.W. 1

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The earliest attempts to isolate and identify the substances responsible for the human specific blood-group characters were made by extracting erythrocytes with ethanol (Brahn & Schiff, 1926; Dold & Rosenberg, 1928; Hallauer, 1929, 1932, 1934; Ottensosser, 1932; Stepanov, Kuzin, Markageva & Kosyakov, 1940). The group materials obtained were inhomogeneous, but the results demonstrated that the group substances could be isolated from red cells and were most probably of carbohydrate nature. There are special difficulties associated with the isolation of group substances from erythrocytes and it is now known that these materials are in some way bound up with the lipid constituents of the red-cell surface and are thus rendered water insoluble. Furthermore, the amount of the group substance present in the erythrocyte stroma is exceedingly small. For these and other reasons, methods for the

isolation of the specific substances from human red cells are largely undeveloped, and little is known concerning the exact chemical nature of the group-specific complexes on the erythrocyte surface.

The occurrence of the human blood-group substances in a water-soluble form in the tissue fluids and secretions of some individuals has been known for many years and our present knowledge of the human group substances has been obtained largely through a study of these water-soluble components. Landsteiner & Harte (1941) isolated from saliva substances of mucoid nature which possessed group-specific serological characters, and similar materials were obtained from human urine by Freudenberg & Eichel (1934, 1935) and by Freudenberg & Molter (1939). Witebsky & Klendshoj (1940, 1941) likewise isolated carbohydrate materials from the gastric juice of persons belonging to groups B and O, but they were unable to obtain the group substances free from contaminating materials owing to the small amount of the specific factors available and the absence, at that time,

of methods suitable for the separation of mucoids from proteins and other tissue constituents occurring in the secretions.

A careful study by Meyer, Smyth & Palmer (1937) of the glycoproteins present in pig gastric mucin revealed the presence of a neutral mucopolysaccharide which possessed an intense serological activity corresponding in specificity to that shown by the human group A substance, and a similar material was subsequently isolated from commercial peptone by Goebel (1938). Freudenberg & Westphal (1938) also obtained from this source a polysaccharide which possessed group A character, and it is now known that the tissues and secretions of certain animals, more especially of pig, horse and cattle, contain substances which carry serological specificities very similar to, or identical with, those shown by the human group substances.

Meyer *et al.* (1937) and Landsteiner & Harte (1940) considered that the neutral polysaccharide obtained from pig gastric mucin which possessed group A specificity was composed of equimolecular quantities of *N*-acetylglucosamine and galactose. The material gave a positive Ehrlich diazo reaction. As it was not possible to remove completely the components giving this reaction, Meyer *et al.* (1937) believed that, in the original complex, the glucosamine was firmly bound to a polypeptide chain. Other workers reported the presence of amino-acids in their most active preparations, but were uncertain whether these should be considered as true components of the group-specific material. Landsteiner & Harte (1940) recorded that the purified A substance isolated from pig gastric mucin contained rather more than a third of its total N in the form of amino-acids. Subsequently, Morgan & King (1943) obtained preparations of A substance from pig mucin by entirely different methods and established that a serologically active substance is obtained which contains 35–40% of its N as α -amino-acid N and that the substance is indeed a polysaccharide-amino-acid complex. It is now known that none of these earlier preparations was homogeneous and that the A substance isolated from commercial preparations of pig gastric mucin, peptone or pepsin is contaminated with at least one other mucoid. Examination by Bendich, Kabat & Bezer (1946) of the mucoid materials obtained from individual hog-stomach linings demonstrated that, whereas some materials showed intense A activity, others lacked this serological character and were in consequence designated as 'inactive'. Aminoff, Morgan & Watkins (1946) obtained similar results, but identified the 'inactive' mucoid as the so-called O substance, i.e. a material found in the secretions and body fluids of the majority of secretors, irrespective of their ABO group, but in greatest quantity in group O secretors (Witebsky & Klendshoj, 1941; Morgan & van Heyningen, 1944; Morgan & Waddell, 1945). This mucoid was designated H substance by Morgan & Watkins (1948) to indicate its ubiquitous nature and to differentiate it as a heterogenetic substance which is not a product of the O gene.

The investigations of Putkonen (1930) and Hartmann (1941) showed that, of a large number of human fluids and secretions examined, gastric juice and saliva were the most potent source of the group-specific substances. More recently, Kabat, Bendich, Bezer & Beiser (1947) have shown the latter secretion to contain less than 100 mg. of the group substance per litre. The small yield of the group material from these sources prompted Morgan & van Heyningen (1944) to examine pseudomucinous ovarian cyst fluids for their content of blood-group factors and it was

found that these pathological growths are frequently a potent source of the specific substances. The volume of individual cyst fluids varies from a few hundred millilitres to several litres, and cysts are sometimes found to contain several grams of the group-active mucoid which, furthermore, is occasionally present in a relatively pure state. A preliminary account of the isolation of human blood-group A substance from this source has been given (King & Morgan, 1944) and the immunological properties of the partially purified group material have also been described (Morgan & Watkins, 1944, 1945, 1948; Rainsford & Morgan, 1946).

The present paper describes in detail the isolation and properties of the human blood-group A substance obtained from pseudomucinous ovarian cyst fluids and establishes the material, on the basis of physical, chemical and immunological measurements, as being an essentially homogeneous mucoid or polysaccharide-amino-acid complex.

EXPERIMENTAL

A number of methods for the isolation of the group A substance have been employed and modified as additional experience demanded. The very variable quantity of the specific substance present and the unknown nature and amount of non-specific materials in the native cyst fluids, necessitates changing the details of the technique of isolation to suit the individual character of the cyst fluid investigated.

Materials and methods

General. The pseudomucinous ovarian cyst fluid, together with a specimen of the patient's blood and saliva, was collected from the hospital as soon as possible after removal of the cyst. The fluid was preserved at 0° in the presence of toluene or dried *in vacuo* from the frozen state. The blood group of the patient was established as A₁ or A₂ according to the reactions of the cells with human or animal α_1 (α_1) agglutinin. The saliva specimen was diluted with an equal volume of saline and boiled for 10 min. immediately after collection. Bacterial contamination was prevented either by the addition of toluene or by keeping the specimen at -10°. The concentration of the A substance in the cyst fluid and in the saliva was determined by means of the iso-agglutination inhibition test. The patient's erythrocytes, cyst fluid and saliva were also examined for the so-called O character (H specificity) in similar agglutination or inhibition tests (Morgan & Waddell, 1945; Morgan & Watkins, 1948). About 130 cyst fluids and saliva specimens have been examined and in no instance has the A substance been present in the cyst fluid when it was absent from the saliva.

Determination of serological activity. The iso-agglutination and sheep-cell haemolysis tests were carried out as described by Morgan & King (1943). A 'standard' preparation of A substance was included in all tests.

Total nitrogen. This was determined by the Kjeldahl method using the apparatus of Markham (1942) and a mixed bromocresol green and methyl red indicator (Ma & Zuazaga, 1942). The N content of the purest preparations was also determined by the Dumas micromethod (Pregl, see Grant, 1945).

Acetyl content. This was determined by the method of Kuhn & Roth (1933) as described by Grant (1945). The percentage recovery of acetyl from *N*-acetylglucosamine after hydrolysis with *p*-toluenesulphonic acid at 100° for increasing periods of hydrolysis is given in Fig. 1, which indicates that the release of acetyl groups is not complete until hydrolysis has proceeded for at least 4 hr., and the results of similar distillation experiments indicated that a heating period of 5 hr. was necessary to obtain the maximum acetyl value for the A substance.

Fig. 1. The liberation of acetyl groups from *N*-acetylglucosamine. The times refer to the times of hydrolysis under reflux; during the subsequent distillation the products are heated for an additional 20 min.

Estimation of the carbohydrate components. *N*-acetylhexosamine was assayed by the method of Morgan & Elson (1934) employing a photoelectric colorimeter. Acetylglucosamine concentrations between 10 and 40 $\mu\text{g./ml.}$ were used as standards. Amino sugar was determined by a modification of the method of Elson & Morgan (1933) using glucosamine concentrations of 10–40 $\mu\text{g.}$ as standards, a photoelectric colorimeter and a green light filter (λ , 540 m μ). Reducing power was determined as glucose by the copper-reduction method of Somogyi (1937).

The fucose content of the A substance was determined by oxidation of a partial acid hydrolysis product with periodate in Conway units. The method is based on the technique elaborated by Winnick (1942) for the determination of threonine. Thus NaHSO_3 (1.5 ml. of a 0.25M-solution) was pipetted into the central chamber of a Conway unit. The material to be analysed, contained in 0.5 ml. of water, was placed in the outer chamber and 1 ml. of 0.1M- K_3PO_4 added. The concentration of K_3PO_4 was adjusted so that 1 ml. exactly neutralized, to pH 7.0, the 0.5 ml. of 0.2M- HIO_4 which was then added. The unit was closed, rotated carefully to mix the solutions in the outer chamber, and allowed to stand at room temperature for 5 hr. A drop of 1% starch was then added to the inner chamber and the excess bisulphite oxidized with I_2 to a faint blue tint. The aldehyde-bound bisulphite was liberated by the addition of powdered Na_2HPO_4 or NaHCO_3 and titrated with 0.005N- I_2 . As controls, known amounts of fucose were oxidized in Conway units in a similar manner and the acetaldehyde

liberated determined. About 90% of the theoretical amount of acetaldehyde was recovered in the controls.

Estimation of amino-acids. α -Amino-N was determined by measuring the N liberated on treatment with HNO_3 in the Van Slyke-Neill manometric apparatus (Peters & Van Slyke, 1932). α -Amino-acid-N was determined by the ninhydrin method of Van Slyke, Dillon, MacFadyen & Hamilton (1941) at pH 2.5 and a reaction time of 8 min.

Chromatographic methods. Paper chromatography was used for the qualitative examination of the amino-acids, hexosamines and sugars and was employed as described by Consden, Gordon & Martin (1944) using as solvents collidine, phenol and *n*-butanol and Whatman no. 1 (57 $\times$ 46.5 cm.) paper. The amino-acids were detected by spraying the papers with ninhydrin. The sugar spots were developed by spraying with $\text{AgNO}_3/\text{NH}_3$ and heating to 105° for 5 min. (Partridge, 1946). The hexosamines were detected on the paper sheets as cherry red areas, by spraying with a modified alkaline acetylacetone reagent followed by *p*-dimethylaminobenzaldehyde in HCl-*n*-butanol (Partridge, 1948).

The isolation of group A substance

Several methods involving the fractionation of the crude cyst material were thoroughly examined. The procedures employed included: (1) Coagulation by heat and removal of the accompanying protein. (2) Fractional precipitation by addition of acetone or ethanol from a solution of the dry cyst material in (a) water, (b) ethylene glycol, (c) diethyleneglycol, (d) glycerol and (e) formamide. (3) Extraction of the dry cyst material with 90% phenol and subsequent fractionation of the phenol-soluble material by precipitation with increasing amounts of ethanol. (4) Fractional precipitation of an aqueous solution of material insoluble in 90% phenol by sodium sulphate. (5) The removal of free protein by means of trichloroacetic acid. None of these methods alone gives rise to a homogeneous material, but by employing a number of these procedures in a carefully selected order a technique was evolved which gave a material that was essentially homogeneous.

The procedure finally employed for the isolation of A substance, as applied to the cyst fluid from patient no. 84 (group A₁) was as follows: 2 l. of the fluid, which contained about 250 g. of dry substance ($[\alpha]_{5461} - 55^\circ$; N, 12.9%) were cooled to 0° and, under conditions of vigorous stirring, mixed with an equal volume of 10% trichloroacetic acid. After the material had stood for a short while in a cold room at -10° it was centrifuged at 0° and the precipitate washed at this temperature with about half the original volume of 10% (w/v) trichloroacetic acid and again collected by centrifugation at 0°. The opalescent supernatant fluid and washings were combined, the solution was centrifuged to remove a small amount of insoluble matter and immediately neutralized at 0° with 10N-NaOH, care being taken not to exceed pH 7.5. The solution was made slightly acid with acetic acid and vigorously stirred during the addition of an equal volume of ethanol at -10°. The opalescent solution was then poured into a further two volumes of ethanol at this temperature. The flocculent precipitate that formed was collected after standing at -10° for

some hours, dissolved in water, dialysed at 0° and dried from the frozen state. Treatment with trichloroacetic acid removed most of the free protein which accompanied the A substance in the native cyst fluid and enabled the subsequent steps in the isolation procedure to be carried out more effectively.

The dry material (10.1 g. $[\alpha]_{5461} + 10^\circ$; N, 7.9%) was suspended in 90% (w/v) phenol (200 ml.) and shaken mechanically at intervals during a period of several hours. The material was then centrifuged and the remaining solid extracted with half the original volume of 90% phenol. A third extraction of the residue was necessary to remove the A substance completely. The insoluble material showed little specific serological activity and was rejected. It was observed, however, that with cyst fluids which showed definite H activity (the so-called O character) the material insoluble or very sparingly soluble in phenol contained an enhanced concentration of this mucoid. The combined phenol extracts were then vigorously stirred and fractionally precipitated with ethanol, added in the form of a 1:1 (v/v) 90% liquid phenol-ethanol mixture to avoid local precipitation of the material first thrown out of solution. The colourless, gelatinous fractions which separated from the phenol solution were collected between the ethanol (v/v) levels 0 and 4.5, 4.5 and 8.5, 8.5 and 11, 11 and 17, 17 and 25, 25 and 75%. The largest and most active fraction separated between, 4.5 and 8.5%, weighed 4.07 g. and showed a dextrorotation, $[\alpha]_{5461} + 8 \pm 2^\circ$, and contained 6.3% N. This material was again dissolved in 90% phenol and the fractionation process repeated at the same levels of ethanol concentration. The major part of the material (N, 5.5%) precipitated again between 4.5 and 9.5% ethanol. The substance inhibited iso-agglutination under the test conditions in amounts of the order of 0.4 μ g. The material (2.5 g.) was again dissolved in 90% phenol and fractionated from solution by the addition of ethanol. A small fraction (0.022 g. $[\alpha]_{5461} + 19^\circ$, N, 4.7%) remained insoluble. The bulk of the material precipitated between the levels 4.5 and 7.5% ethanol and weighed 2.2 g. At this stage in the purification process no further material separated on increasing the ethanol concentration a few per cent, but over the range of ethanol concentration 10–75% (v/v) a small amount (0.09 g.) of material precipitated. This small fraction showed a laevorotation, $[\alpha]_{5461} - 3 \pm 2^\circ$, contained 8.7% N and showed a serological activity equivalent to about 2% of the most active material.

A portion of the main fraction (1.73 g.) was dissolved in 34 ml. of cold anhydrous ethylene glycol and precipitated from solution by the cautious addition of ethanol. No precipitate appeared until a concentration of 42% (v/v) ethanol was reached, and between this level and 47% ethanol the first fraction (0.3 g. N, 6.0%) was collected. Other fractions were removed over the ranges of ethanol concentration 47–50% (0.14 g. $[\alpha]_{5461} + 16^\circ$, N, 5.5%); 50–62% (0.60 g. $[\alpha]_{5461} + 14^\circ$, N, 5.4%), and 62–75% (0.07 g. $[\alpha]_{5461} + 14^\circ$, N, 5.0%). The material remaining in solution (20 mg.) contained 1.4% N.

Another specimen of A substance (3.2 g.) obtained from the same cyst fluid and isolated by means of trichloroacetic acid, phenol and ethylene glycol fractionation as described above was dissolved in neutral anhydrous formamide (50 ml.) and divided into three fractions by precipitation with ethanol. Very little material separated up to 50% (v/v) ethanol concentration. Thereafter material was collected between the ethanol levels 50 and 60% (5.7% N), 60 and

66% (5.8% N) and 66 and 75% (5.8% N). The first fraction contained the major part of the starting material and showed the highest serological activity in the iso-agglutination inhibition test.

To obtain a useful amount of the A substance for detailed examination, materials obtained by the above procedures which showed a N content of about 5.7%, an optical rotation, $[\alpha]_{5461} + 15 \pm 5^\circ$ and a maximum specific serological activity, were pooled and fractionated from aqueous solution by ethanol. The A substance separated almost entirely as a single main fraction within quite narrow limits of ethanol concentration. The materials obtained were dialysed, centrifuged to remove traces of insoluble matter and dried from the frozen state. The analytical figures for the materials which separated during the fractionation of 3.2 g. of A substance are given in Table 1.

Table 1. *The fractionation of A substance from aqueous solution by ethanol*

Precipitation levels (% (v/v) ethanol)	Yield (g.)	$[\alpha]_{5461}$	N content (%)
0→52.5	0.02	†	6.08
52.5→57.5	2.90	+ 15 ± 2°	5.76
57.5→excess*	0.14	+ 9 ± 1°	5.74

* About 4 vol.

† Too opalescent for accurate polarimetric observation.

A preparation of A substance, which was composed of material obtained by the above procedures from several different cysts, was fractionated by the addition of glacial acetic acid to a concentrated aqueous solution of the material and gave a main fraction which separated between the acetic acid levels 92–94% (v/v). The material contained 5.8% N and showed full capacity to inhibit iso-agglutination and the haemolytic activity of rabbit anti-A serum, but was nevertheless found to be somewhat inhomogeneous when examined in the Tiselius electrophoresis apparatus. This preparation, no. III, was used exclusively for the preliminary analytical determinations, the final values given being derived from the best material.

Tests for homogeneity

The following examinations were carried out to establish the homogeneity of the main preparation.

(1) *Fractional solubility test.* Attempts to apply the classical equilibrium method of solubility test failed owing to the ready and extreme solubility of the A substance in water which resulted in a solution of high concentration and considerable viscosity being obtained. For this reason a modified technique was used in which a known weight of A substance was shaken vigorously for a few minutes with successive small amounts of aqueous 30% (v/v) ethanol until all the material had dissolved. After each addition of aqueous ethanol and subsequent shaking, the suspension was centrifuged, the supernatant fluid completely removed and the material in solution recovered by drying *in vacuo* from the frozen state. The fractions so obtained were dissolved to give solutions of known concentration and analysed for total N and for optical rotation. A sample of each fraction was then hydrolysed by heating with

Table 2. *Fractional solubility test for homogeneity: pure A substance*

Fraction	I	II	III	IV	V	VI
$[\alpha]_{5461}$	$+14 \pm 6^\circ$	—	—	$+15 \pm 2^\circ$	$+13 \pm 2^\circ$	$+12 \pm 5^\circ$
Percentage N (Kjeldahl), <i>a</i>	5.67	5.52	5.61	5.58	5.71	5.71
Reduction (% calculated as glucose), <i>b</i>	53.7	53.3	53.0	52.6	54.0	53.7
Hexosamine (% calculated as base), <i>c</i>	36.7	36.7	37.4	37.4	36.7	37.5
<i>b/a</i>	9.48	9.65	9.44	9.44	9.45	9.41
<i>c/a</i>	6.47	6.64	6.66	6.71	6.43	6.56
<i>b/c</i>	1.46	1.45	1.42	1.41	1.47	1.43

Table 3. *Fractional solubility test for homogeneity: partially purified preparation of A substance from hog gastric mucin*

Fraction	I	II	III	IV	V
Percentage N (Kjeldahl), <i>a</i>	6.05	6.51	6.43	6.54	5.48
Reduction (% calculated as glucose), <i>b</i>	51.2	46.8	45.8	46.2	24.2
Hexosamine (% calculated as base), <i>c</i>	30.7	32.5	30.2	30.7	19.4
<i>b/a</i>	8.46	7.18	7.12	7.06	4.41
<i>c/a</i>	5.07	4.99	4.70	4.70	3.54
<i>b/c</i>	1.67	1.44	1.52	1.50	1.25

0.5N-hydrochloric acid at 100° for 16 hr. and the reducing power and hexosamine content determined. The values for the reducing power are not the maximum values owing to some decomposition after 16 hr. hydrolysis (Fig. 4). The results (Table 2) show that the analytical figures for each of the six fractions were very similar and that there is no evidence that material differing in composition from the A substance is present in the preparation. The results of the application of this technique to a preparation of A substance obtained from commercial hog mucin and known to be heterogeneous are set out in Table 3 and reveal at once the different composition of the fractions obtained.

(2) *Electrophoresis*. An electrophoretic analysis of the A substance was carried out at pH 4 and 8 and gave no evidence of inhomogeneity. Details are given by Dr R. A. Kekwick in an addendum to this paper.

(3) *Ultracentrifugal examination*. The A substance was also examined by Dr Kekwick using the Svedberg oil-turbine centrifuge and the 'diagonal schlieren' optical system. Full experimental details are given in the addendum.

(4) *Quantitative immunochemical analysis*. Through the kindness of Dr E. A. Kabat the group A substance was examined by the quantitative precipitation technique. All the hexosamine and fucose present in the A substance was specifically precipitated by human anti-A serum and thus indicated the absence of significant amounts of other mucoids which contain these carbohydrate constituents (Kabat, Baer & Knaub, 1949).

Properties of the A substance

Immunological. The serological activity of the A substance was measured by determining the minimum amount necessary to inhibit (a) the aggluti-

nating action of a standard anti-A agglutinin on A_1 cells, and (b) the haemolysis of sheep red cells by a rabbit anti-A immune serum in the presence of complement. Under strictly controlled conditions both of these tests are reliable and will detect an increase or decrease in activity of the order of 33 %. The minimum amount of A substance which will inhibit completely three to four agglutinating doses of anti-A agglutinin under the standard test conditions is of the order $0.2 \mu\text{g}$. The haemolysis test is considered to measure the 'Forssman' or heterophile component of the A agglutinin and is accepted as measuring a different, although closely related, serological property from that determined by the isoagglutination inhibition technique. The minimum amount of the material which will inhibit haemolysis under the test conditions is $0.05 \mu\text{g}$.

The A substance was examined for its capacity to inhibit: (1) anti-B agglutinin (human); (2) the so-called 'anti-O' agglutinins, as exemplified by (a) certain normal cattle (Schiff, 1927; Greenfield, 1928) and human sera (Morgan & Watkins, 1948), (b) the sera of goats immunized with *Shigella shigae* (Eisler, 1930, 1931) and (c) immune rabbit anti-'O' sera (Morgan & Waddell, 1945); (3) anti-M and anti-N sera; (4) anti-Rhesus sera, using anti-D, anti-C, anti-c and anti-E sera as test reagents; and (5) the 'Lewis' blood-group reagents, anti-Le^a and anti-Le^b sera (Grubb & Morgan, 1949). Tested at a concentration of 5 mg./ml. the A substance contained no detectable amounts of material able to neutralize the agglutinins B, M, N and Rh. Weak inhibition of H agglutinin (the so-called O agglutinin) by the A substance was first observed at a concentration of 0.2 mg./ml. Under identical test conditions the H mucoid isolated from the pseudomucinous ovarian cyst fluids of women belonging to group O, inhibited H agglutinin at a concentration of $0.1 \mu\text{g./ml.}$

Similarly, the A substance at a dilution of 1:10,000 showed some activity against the 'Lewis' anti-sera Le^a and Le^b, but this activity represented less than 1% of the activity shown by the purified Le^a- and Le^b-substances. The A substance does not precipitate with horse pneumococcus Type XIV anti-serum.

The capacity of the purified A substance to induce the formation of anti-A immune body in the rabbit was investigated. A group of six rabbits was given a series of six intravenous doses each of 0.05 mg. of the A substance dissolved in saline. The injections were made at 3-4-day intervals. The average anti-A agglutinin titre of the normal bleedings was 104. After immunization the average titre was 208. Three of the rabbits were given a further three intravenous doses each of 0.1 mg. of the A substance and were bled 1 week after the last dose. The average anti-A agglutinin titre increased to 429. The remaining three rabbits were given under the same conditions three doses of an artificial antigen (0.05 mg.) made from the same specimen of A substance and the conjugated protein component of the O antigen of *Shigella shigae* as described in an earlier communication (Morgan, 1943). The average anti-A agglutinin titre increased to 5400, a 50-fold increase over the average normal titre and rather more than ten times the value of the average titre after nine doses of the A substance alone. The results indicate that under the conditions of test the A substance possesses weak antigenicity only, but that it can be converted to a powerful antigen by combination with the conjugated protein component of the O antigen of *S. shigae*.

Physical. The relative viscosity, η , of a 0.5% solution of the A substance in 0.85% saline is 1.14. An elastic gel is not formed on the addition of borate buffer, pH 8.5, to a 1% solution of the A substance. The specific rotation, the mean value obtained from the examination of several preparations, is $[\alpha]_{5461} + 15 \pm 5^\circ$ in water (c , 1.0).

The ultraviolet absorption of the A substance was examined in a Beckmann Model D.U. quartz spectrophotometer. The concentration of the solution was 0.1% in 0.85% NaCl and the observations were made in a cell 1 cm. deep. The absorption curve is given in Fig. 2. There is no evidence of absorption between the wavelengths 220 and 260 m μ . It is doubtful if the very weak absorption between 260 and 300 m μ is significant.

Chemical. An aqueous (2%) solution of the purified A substance gives no precipitate when treated with an equal volume of 2% solutions of the following reagents: aluminium sulphate, potassium aluminium alum, lead acetate in dilute acetic acid or alkali, mercuric acetate, uranium acetate, silver nitrate, mercuric sulphate in 5N-H₂SO₄, phosphotungstic acid, neutral and acid copper sulphate, hydroferrocyanic acid, picric acid, trichloroacetic acid (10%), tannic

acid (10%) and salicylsulphonic acid (20%). The addition of an equal volume of a saturated solution of magnesium or ammonium sulphate gives no precipitate, but these reagents at full saturation give rise to some turbidity in the solution on warming. An aqueous solution of the A substance alone gives no precipitate on boiling. The biuret, ninhydrin, xanthoproteic, Millon, Hopkins-Cole, Selivanoff and Bial tests are negative. Ehrlich's diazosulphanilic acid test is faintly positive as is Sakaguchi's tests for arginine. The addition of alkaline copper sulphate to a solution of the A substance, as in the biuret test, gives rise to a voluminous precipitate.

Fig. 2. The ultraviolet absorption spectrum of A substance (0.1% solution in 0.85% NaCl).

The analytical figures for a specimen of A substance are: C, 44.2; H, 6.96; N, 5.72% (Dumas). The material contains less than 0.05% P and S. The ash content varies somewhat for different preparations, but is usually less than 1%. The acetyl content falls within the range 8.8-9.1% for different preparations.

Under the conditions for the determination of *N*-acetylglucosamine (Morgan & Elson, 1934) the A substance readily gives a colour with Ehrlich's reagent. The maximum colour given by *N*-acetylglucosamine, however, is obtained after 4 min. heating at 100° with the 0.05N-sodium carbonate, whereas the A substance requires 14 min. heating under the same conditions and the colour obtained is equivalent to 10.4% of *N*-acetylglucosamine. Further heating with the sodium carbonate results in a decreased colour production as a consequence of the decomposition of the very labile chromophoric group. Correcting for this decomposition, by extrapolation to zero time, the colour given by the A substance is equivalent to an *N*-acetylglucosamine content of 13.5%.

Acid hydrolysis of the A substance

The A substance was hydrolysed with 0.5 N- and 6 N-hydrochloric acid, and N-acetic acid. The hydrolyses were carried out in small (2–3 ml.) sealed glass ampoules which were heated for the appropriate length of time in a boiling water bath. The ampoules were then cooled and the contents evaporated to dryness *in vacuo* in small glass dishes over pellets of sodium hydroxide and conc. sulphuric acid. Small amounts of sodium chloride do not interfere with the hexosamine or reduction determinations and the 0.5 N-hydrochloric acid hydrolysates were therefore not evaporated to dryness but neutralized with an equal volume of 0.5 N-sodium hydroxide.

Fig. 3. Hydrolysis of A substance by 6N-HCl at 100° as determined by the liberation of (A) α -amino groups, and (B) α -amino-acids.

Hydrolysis with 6 N-hydrochloric acid gave rise to extensive decomposition with the formation of a precipitate of humin particles after 2 hr. heating. The course of the hydrolysis was followed by measuring the α -amino-N and the α -amino acid-N liberated. The results (Fig. 3) show that the liberation of α -amino-N reaches its maximum value in 4 hr. and represents about 91 % of the total N of the substance, whereas the α -amino acid-N reaches its maximum after 16 hr. heating and is equivalent to about 38 % of the total N. The difference between the analytical values for the two forms of nitrogen is due to the hexosamines which react quantitatively with nitrous acid, but do not liberate carbon dioxide on heating with ninhydrin.

The hydrolysis with 0.5 N-hydrochloric acid results in the ready liberation of reducing substances and hexosamines. The analytical results (Fig. 4) indicate that the maximum liberation of hexosamines, 37 % (as base), and of reducing sugars, 57 % (expressed as glucose), occur at approximately the same time

(4–5 hr.). Fucose is readily liberated and the maximum value 13.5 %, determined as acetaldehyde after periodate oxidation, is obtained after the first hour of hydrolysis. More prolonged hydrolysis results in some decomposition of the fucose, but the apparent increase in the fucose content in the later stages of hydrolysis could be attributed to the liberation of threonine in a form susceptible to oxidation.

Fig. 4. Hydrolysis of A substance by 0.5 N-HCl at 100° as determined by (A) reducing power, expressed as glucose, (B) hexosamine, expressed as glucosamine base, and (C) fucose, determined as acetaldehyde after oxidation with periodate.

Fig. 5. Hydrolysis of the A substance by N-acetic acid at 100° as determined by (A) reducing power, expressed as glucose, (B) N-acetylhexosamine, and (C) fucose, determined as acetaldehyde after oxidation with periodate.

Hydrolysis of the A substance with N-acetic acid at 100° allowed the rate and extent of liberation of fucose, reducing substances and N-acetylhexosamine residues to be determined and the results are set out in Fig. 5. The rate of liberation of fucose indicates that a maximum value 12.9 % is obtained after about 48 hr. hydrolysis and the maximum figure for reducing substances is reached at about the

same time. The colour given with Ehrlich reagent, after the hydrolysis products have been treated with dilute sodium carbonate, rises to a maximum value of about 15 % expressed in terms of *N*-acetylglucosamine. Simultaneous with these changes there is a steady loss of A activity as measured by the ability to inhibit iso-agglutination, but the capacity to inhibit the haemolysis of sheep cells by rabbit anti-A serum is not only retained but enhanced. Moreover, whereas the undegraded A substance gives no precipitate with horse anti-pneumococcal Type XIV serum the mild acid hydrolysis products readily yield a precipitate. The results of further examination of the products obtained after simple dialysis of a 16 hr. *N*-acetic acid hydrolysate have already been reported (Aminoff, Morgan & Watkins, 1948).

The products of complete hydrolysis with 6*N*-hydrochloric acid (16 hr.) were examined by partition chromatography using 1 mg. of the material. The chromatogram, run in two directions at right angles with collidine and phenol, gave rise to thirteen 'spots' on treatment with ninhydrin reagent containing 1 % acetic acid (Consden & Gordon, 1948), and subsequent heating of the chromatogram at 80–90° for 5 min. revealed no further coloured areas, thus indicating the absence of polypeptides in the hydrolysis products. The distribution of the 'spots' on the chromatogram is set out diagrammatically in Fig. 6*A*. Eleven of the 'spots' corresponded closely in position with those which were given by the following amino-acids: Lysine (1), arginine (2), aspartic acid (3), glutamic acid (4), glycine (5), serine (6), alanine (7), threonine (8), proline (9), valine (10), leucine and isoleucine (11) as shown in the chromatogram, Fig. 6*B*. An additional spot corresponding in position and colour with cysteic acid (*Cy*) appeared only occasionally and was then extremely weak. Two spots (12 and 13), not normally found in protein hydrolysis products, appeared on the chromatogram of the hydrolysate of the A substance (Fig. 6*A*).

In view of the known reactions of glucosamine and chondrosamine with ninhydrin, both of which give a greyish mauve coloured area on paper after heating at 90° similar to those developed by amino-acids, and the known presence of hexosamine in the A substance, evidence for the identity or non-identity of the unknown 'spots' with those that would arise from glucosamine or chondrosamine was sought. The experiments carried out to establish the presence of the two amino sugars have already been described (Aminoff & Morgan, 1948), and the chromatographic evidence that two amino sugars are present may be summarized as follows: (1) two greyish mauve spots, which do not occupy exactly the same position as any of the known amino-acids, develop on treatment with ninhydrin. (2) The same areas (12 and 13) give rise to cherry-red spots on treatment with acetyl-

acetone and Ehrlich reagents as described by Partridge (1948), and reduce the silver nitrate-ammonia reagent (Partridge, 1946). (3) The position of the two spots correspond exactly with those which develop on paper when chondrosamine and glucosamine (12 and 13 in Fig. 6*B*) are used as test substances and are treated with solvents under the same conditions; that is, the unknown substances have the same R_F values as glucosamine and chondrosamine.

(A)

(B)

Fig. 6. Two-dimensional chromatograms of (A) the products of the A substance obtained by hydrolysis with 6*N*-HCl for 16 hr. and (B) an artificial mixture of amino-acids and hexosamines. Developed in collidine for 48 hr. followed by 24 hr. in phenol/coal gas/ammonia 0.6 % (w/v).

Chromatograms run with collidine and phenol have been used to detect the presence of reducing sugars using the silver nitrate-ammonia reagent, but in our experience the traces of phenol which remain in the paper interfere with the reaction. Although collidine gives a useful separation of most of the constituents, the chloride ion has the same R_F value as

galactose in this solvent and there is in consequence a masking of the diffuse brownish spot given by this sugar when the silver nitrate-ammonia reagent is employed. The replacement of collidine with butanol gave rise to chromatograms which after spraying with silver nitrate-ammonia developed three brownish coloured areas, one of which was identified as hexosamine (glucosamine and chondrosamine have almost identical R_f values in this solvent). It was observed, however, that the hexosamine reagents also detected galactose and fucose as faint transient greyish blue spots when butanol was used as solvent. A white precipitate of silver chloride no longer interfered with the development of the brown area due to galactose but the similar mobilities of galactose and hexosamines in butanol now caused these two sugars to be poorly separated from each other. In an atmosphere of acetic acid (3 %) the mobilities of the hexosamines are reduced and the galactose is in consequence more easily separated and detected. The distinctive colours given by the hexosamines (pink) and fucose and galactose (greyish blue) on development with the hexosamine reagents serve to distinguish the glucosamine from the galactose when these sugars occur close together on the chromatogram.

Some preliminary observations have been made using partial hydrolysis products of the A substances obtained during the early stages (2, 4 and 8 hr.) of hydrolysis with 6N-hydrochloric acid. The chromatograms indicated that after 2 hr. hydrolysis a small amount of each amino-acid had been liberated. The colour intensity of the spots was much less than that observed with the 16 and 32 hr. hydrolysis products, but nevertheless the chromatograms were qualitatively similar. Heating the chromatograms at 90° for 5 min. caused no further well-defined coloured areas to develop, but induced the formation of a weak and diffuse coloration as a background suggesting the presence of many polypeptide units. The results obtained with the 16 hr. 0.5N-hydrochloric acid hydrolysis products indicated the absence of the leucine/isoleucine spot whilst serine, threonine, proline and valine were present in small quantities only.

The diffusate from the 16 hr. N-acetic acid hydrolysis product of the A substance when run in collidine and sprayed with ninhydrin, silver nitrate-ammonia and the hexosamine reagents, gave five distinct spots of which only that due to fucose persisted in the 0.5N-hydrochloric acid hydrolysate of the material, thus indicating the complex nature of the other components in the diffusate. The results of the chromatographic and quantitative analysis of the diffusible and indiffusible fractions from 16 hr. N-acetic acid hydrolysate after further hydrolysis with 0.5 and 6N-hydrochloric acid can be summarized as follows. The diffusate contains free fucose, and a

small amount of both hexosamines and of the amino-acids except the leucines, proline and traces of the hydroxyamino-acids. The indiffusible material contains galactose and about 80 % of the total hexosamines, but fucose was not detected. All the amino-acids initially present in the A substance are represented in this material which contains at least 85 % of the total amino-acid N in the A substance.

DISCUSSION

The procedure described for the isolation of the specific blood-group A substance from human ovarian cyst fluids has enabled useful amounts of this important tissue component to be obtained and certain of its physical, chemical and immunological properties to be recorded. Owing to the very variable nature of the ovarian cyst fluids an exact description of an isolation procedure which will be completely successful with all fluids cannot be given, but in our experience it has only been difficult to isolate A substance of high specific activity from fluids which contain relatively low concentrations of the group substance and high concentrations of contaminating protein or other mucoid. No attempt has been made to recover group material from paramucinous cyst fluids, the 'trembling jelly' type of cyst contents first described by Mitjukoff (1895).

In some instances the preliminary treatment of the diluted cyst fluid with trichloroacetic acid to remove excess protein is unnecessary, and direct extraction of the freeze-dried cyst fluid with 90 % phenol allows the specific material to be readily recovered in a relatively pure condition. It is to be noted, however, that extraction of the freeze-dried native cyst fluid with 90 % phenol sometimes yields the A substance in a form in which it is sparingly soluble in this solvent. Treatment of this material with trichloroacetic acid at 0° is then necessary and converts the A substance to a form which is soluble in phenol and which can then be fractionally precipitated by the addition of ethanol in the usual manner. In our experience, if homogeneous material is to be obtained, fractionation of the A substance from phenol solution by ethanol must be carried out within narrower limits of ethanol concentration than 0 and 10 % as described in an earlier paper (Morgan & King, 1943) and employed by Bendich *et al.* (1946) and by Kabat *et al.* (1947).

Preparations of A substance which have been repeatedly fractionated from solution in 90 % phenol with ethanol and which have remained unchanged in their physical, chemical and serological properties, on further fractionation from solution in anhydrous ethylene glycol or formamide by ethanol have likewise shown no change in composition and specific activity. Material obtained in this way shows only one component when examined in the Tiselius

electrophoresis apparatus at pH 4 and 8. Examination in the Svedberg ultra-centrifuge reveals that the material is polydisperse and that at a concentration of 0.5% the mean sedimentation constant of the material is S_{20} , 6.7×10^{13} .

Quantitative immunochemical analysis indicates that the material is essentially free from other materials which contain hexosamines or fucose. This conclusion is supported by the absence of the capacity of the A substance to inhibit anti-sera for the other blood-group factors.

The constancy of the physical, chemical and serological data obtained for material purified by the above methods indicates that the A substance described is most probably a homogeneous molecular complex. As such the material is suitable for detailed chemical and immunological study, including step-wise or complete degradation to its constituent residues. If any significance is to be attached to the isolation of certain substances, present in small amounts only, the homogeneity of the material investigated is of prime importance, a fact which is frequently overlooked. The material is, therefore, being examined further by adsorption frontal analysis according to the general procedure described by Claesson (1946) and by the counter-current distribution technique of Craig (1944).

The properties of the A substance reveal that the group A complex is a more stable molecular species than is the chemically similar O antigenic complex of *Shigella shigae* or *Salmonella typhi*, for it is observed that solvent systems which bring about dissociation of these complexes into their component residues fail completely to accomplish any separation of the A complex into its polysaccharide and amino-acid moieties. The A substance must therefore be considered as a complex mucoid which contains a polysaccharide component firmly linked to an amino-acid containing residue.

The undegraded A substance shows two serological properties; the capacity to inhibit the agglutination of group A red cells by human anti-A (α) serum and the power to inhibit the haemolysis of sheep red cells by rabbit immune anti-A serum, and these have been used to determine the amount of the A substance in a given preparation. Since the heterophile activity shows a large increase during the early stages of hydrolysis with N-acetic acid or after treatment with formamide at 150° (Landsteiner & Harte, 1940) the enhancement is most probably due, as suggested by these authors, to a structural change in the complex rather than to the presence of an acid-stable impurity. Contrary to the observation of Jorpes & Norlin (1933, 1934), Jorpes (1934), Jorpes & Thaning (1945) it has not been found possible to fractionate the A substance into two components, each showing one only of the two serological properties associated with the A substance. In one ex-

periment a small fraction of the A substance was soluble in glacial acetic acid and, determined by the two serological methods, the ratio of the activities of the soluble and insoluble fractions was the same as for the original A substance. Furthermore, the ratio of the two serological activities for the most highly purified specimen of A substance, obtained after the application of the various purification procedures described, was the same as for the A substance in the native cyst fluid. Although the inhibition of haemolysis is a more sensitive means of measuring A activity than is inhibition of iso-agglutination, the latter determines a more labile serological character and hence in order to detect any degradation it is advisable to follow the course of purification of the A substance by both methods.

Our preparations contain neither phosphorus nor sulphur in significant amounts and thus differ in this respect from those isolated from pooled hog stomachs by Kabat, Bendich & Bezer (1946) and described by them as containing up to 1.0% P and 0.4% S. It is also to be noted that the A substance obtained from human saliva by Kabat *et al.* (1947) likewise differs from our purified material in showing a laevorotation whereas the A substance we have obtained from human ovarian cyst fluids is dextrorotatory, when obtained free from impurities.

The amino-acid-containing moiety of the A complex contains at least eleven amino-acids. The aromatic and sulphur-containing amino-acids are absent or are present in extremely small amounts, whereas threonine occurs in a higher concentration than has been recorded for most proteins. Threonine has been isolated in crystalline form from a purified preparation of human A substance by Freudenberg, Walch & Molter (1942).

Complete hydrolysis of the A substance with mineral acid at 100° liberates reducing sugars and amino-acids. The maximum values found indicate that about 38% of the total N is present as α -amino-acid N, 51% as hexosamine N and 91% as α -amino N.

The polysaccharide component contains D-galactose, L-fucose—first identified as a constituent of the blood-group A substance obtained from pepsin by Bray, Henry & Stacey (1946)—and two hexosamines, D-glucosamine and most probably D-chondrosamine. The recognition that two different hexosamine molecules are present in a molecular species, which is apparently homogeneous, is of considerable interest, and suggests that a re-examination of the amino sugar components of other homogeneous mucopolysaccharides and mucoids is desirable, especially where the recovery of glucosamine is poor in terms of the amount of hexosamine believed to be present on the basis of the colorimetric determination.

The fucose content of the A substance, based on the yield of acetaldehyde liberated on oxidation with

periodate after mild acid hydrolysis is found to be about 13 %. Kabat *et al.* (1949), however, using the colorimetric method of Dische & Shettles (1948) give a considerably higher figure, 18 %, for our preparation of A substance. Assuming that this higher figure represents the total fucose content of the A substance these results indicate that only a part of the total fucose is in the form of acid-labile end groups.

The hexosamine content of the A substance is 37 %, but a slightly higher figure than this is frequently obtained after hydrolysis with 6N-hydrochloric acid. The increased colour (2–3 %) which arises after treatment with strong acid is believed to be due to the formation of chromogenic complexes between sugars or their degradation products and certain amino-acids, e.g. lysine. The hexosamine value, 37 %, expressed as *N*-acetylhexosamine represents 46 % of the A substance and thus would account for the total *N*-acetyl groups, 9 %, found to be present. Unhydrolysed A substance gives rise to a colour equivalent to about 13 % *N*-acetylglucosamine which represents only a quarter of the total *N*-acetylhexosamine residues calculated to be present, thus indicating that the *N*-acetylhexosamine units are combined by means of at least two different types of linkage.

The galactose content of the A substance was not determined, but an approximate value for the amount present can be obtained indirectly by subtracting the contribution of the other carbohydrate constituents, chondrosamine, glucosamine and fucose, from the total reducing value of the hydrolysate. The galactose is found to be present to the extent of a maximum of about 17 % and hence it may be considered that, contrary to the observations of Meyer *et al.* (1937) and Landsteiner & Harte (1940), *N*-acetylhexosamine and galactose are not present in equimolecular proportions.

The results of the hydrolysis of the human blood-group A substance with 0.5 N- and 6N-hydrochloric acid and subsequent qualitative and quantitative analysis of the hydrolysis products have given an indication of the nature and amount of the constituents present. Additional information as to the stability of the linkages and mode of combination of the components has been obtained from a study of the partial acid hydrolysis products of the material. The results, which have been briefly reported by Aminoff *et al.* (1948) demonstrate that the A substance shows no initial reactivity with horse anti-pneumococcal, type XIV, serum, but develops this serological property during the course of hydrolysis with acetic acid. Kabat, Bendich, Bezer & Knaub (1948) and Kabat, Baer, Bezer & Knaub (1948), who independently studied the same problem, state that all their preparations of A substance show pneumococcal type XIV specificity. In this important respect, therefore, the A substance

described by Kabat and his colleagues (1948) differs from our material. These workers also showed that the so-called O substance likewise develops increased pneumococcal type XIV specificity as a result of controlled acid hydrolysis (Kabat, Bendich, Bezer & Knaub, 1948) and it would appear that the A and H (so-called 'O') substances possess a structure common to both and to the pneumococcus type XIV polysaccharide. The serological differences in the A and H substances are, therefore, presumably associated with the acid-labile moiety.

The complete interpretation of the results of the action of *N*-acetic acid on the A substance is difficult on account of the complexity of the hydrolysis products. The unhydrolysed diffusate contains at least five components, four of which are still composed of several constituents. Fucose is present as such. The diffusate, which shows only one ninhydrin reacting spot, gives rise to several amino-acids after hydrolysis with 6N-hydrochloric acid. The polypeptide component contains small amounts of the individual amino-acids present in the original A substance, with the exception of the leucines and proline, whereas the hydroxyamino-acids, serine and threonine, are present in minute amounts only. It may be assumed, therefore, that these amino-acids are more firmly bound than the others in the A substance.

The A substance, after hydrolysis with acetic acid, shows an increased capacity to react with Ehrlich reagent under the conditions used for the determination of *N*-acetylglucosamine and 82 % of the *N*-acetylhexosamine units which give this reaction are present in the indiffusible material (Aminoff *et al.* 1948). There is, therefore, an increase in the number of *N*-acetylhexosamine residues present in the reactive form, and these *N*-acetylhexosamine units are attached to the more stable moiety of the A substance. Mild alkaline hydrolysis of the A substance (Morgan, 1946), on the other hand, renders most of the *N*-acetylhexosamine residues which give immediate colour with Ehrlich reagent readily diffusible and therefore alkaline hydrolysis would seem to involve the rupture of different linkages in the A substance from those attacked by acetic acid.

In contrast it is found that after treatment with *N*-acetic acid, the greater part of the fucose in the A substance is present in the diffusate as a free sugar. Since most of the fucose is readily liberated without extensive splitting of other glycosidic bonds, it seems reasonable to conclude that these labile fucose units are present as terminal residues and are probably attached to the *N*-acetylhexosamine molecules which are exposed as a result of the mild acid hydrolysis.

The results of the qualitative and quantitative examination of the acetic acid hydrolysis products of A substance show that (a) about three-quarters of the total fucose is liberated, (b) there is a rapid and

complete destruction of the capacity of the A substance to inhibit iso-agglutination, (c) there is an increase in its power to prevent the haemolysis of sheep cells by rabbit anti-A serum, and (d) a new serological character, that of reacting with horse anti-pneumococcus, type XIV, serum develops. In view of these observations, the possibility that degradation has occurred cannot be readily excluded for preparations of A substance showing any properties that could be associated with the above changes, e.g. a low fucose content and a cross-reactivity with horse anti-pneumococcal type XIV serum.

The analytical data indicate the presence of 46 % *N*-acetylhexosamine, 18 % fucose and a maximum of about 17 % galactose and account for the major part of the total complex. The remainder is made up of amino-acids, of which threonine represents the largest component. On the basis of these preliminary analytical figures, it would appear that the monosaccharide units are present in the following proportions: *N*-acetylhexosamine, 2; galactose, 1; and fucose, 1. The weight of the A substance which contains the minimum number of sugar units is 2×484 or 968 (where 484 is the weight of the A substance which contains one hexosamine unit). This is the weight of the repeating unit. From the ultracentri-

fugal and diffusion data the molecular weight of the A substance is of the order 260,000, and the molecule therefore contains 280 such units.

SUMMARY

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

2. The A substance is a dextrorotatory mucoid or polysaccharide-amino-acid complex. The material, which is polydisperse, is essentially homogeneous with respect to chemical, immunochemical and serological properties. The acid hydrolysis products contain 57 % reducing substances, expressed as glucose, and 37 % hexosamine. The α -amino-acid nitrogen represents about 38 %, and the α -amino nitrogen, 91 %, of the total nitrogen (5.7 %).

3. From the analytical results it would appear that the simplest unit of the carbohydrate moiety of the A substance contains one fucose, one galactose and two *N*-acetylhexosamine residues, and that there are about 280 such units in the molecule of A substance.

4. The significance of certain changes in the properties of the A substance which are associated with degradation are discussed.

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ADDENDUM

Physico-Chemical Examination of Blood-Group A Substance

By R. A. KEKWICK, *The Lister Institute, London, S.W. 1*

The A 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, $\lambda = 546 \text{ m}\mu$, isolated from a mercury arc by a suitable filter. As the main object of this examination was to detect any traces of contaminating high molecular substances, 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).

The 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, $I = 0.1$ and in phosphate buffer pH 8, $I = 0.2$, after prolonged electrophoresis only one component was demonstrable. At pH 4 the

boundaries were symmetrical, but at pH 8 both the ascending and descending boundaries were asymmetrical. Migration in each instance was anodic, but at pH 4 was so slight as to be barely detectable.

In the ultracentrifuge only one component was present at pH 4 and 8. At pH 8 the sedimentation constant was determined at three concentrations and the data are recorded in Table 1. Owing to the spreading of the boundary at low concentrations, it was not possible to make satisfactory measurements below a concentration of 0.5 g./100 ml. It will be observed that there is a marked dependence of sedimentation constant on concentration.

Table 1. *Sedimentation and diffusion constants of group A 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.)
0.42	—	1.70
0.50	6.7 ₀	—
0.75	5.9 ₈	—
0.81	—	1.60
1.00	5.4 ₂	—

In Table 1 are included two values for the diffusion constant kindly measured by Dr A. G. Ogston. The derived diffusion curves showed a departure from the Gaussian distribution.

The sedimentation constant was plotted against concentration, and values interpolated corresponding

to the concentrations employed in the diffusion experiments. From these and the experimentally determined value of the partial specific volume, 0.635, the molecular weight was determined from the expression

$$M = \frac{RTS}{D(1 - V\rho)},$$

where M = molecular weight, R = gas constant, S = sedimentation constant, D = diffusion constant, T = absolute temperature, V = partial specific volume,

Fig. 1. Electrophoresis patterns of a 2% solution of A substance in (a) acetate buffer, pH 4.0; $I=0.1$; current, 10.4 ma. Exposure 4 hr. after starting current. (b) Phosphate buffer, pH 8.0; $I=0.2$; current 14.5 ma. Exposure 5.25 hr. after starting current.

Fig. 2. Sedimentation diagrams of 1% solution of the A substance, 60 min. after reaching full speed, 60,000 rev./min. (a) Acetate buffer, pH 4.0; $I=0.1 + \text{NaCl}$ (0.15M); (b) phosphate buffer, pH 8.0; $I=0.1 + \text{NaCl}$ (0.15M).

ρ = density of solvent, resulting in a mean value of 260,000 (Svedberg & Pedersen, 1940). From the

same data the frictional ratio f/f_0 was computed, a value of 3.2 being obtained. This corresponds to an axial ratio of 60 (Svedberg & Pedersen, 1940), or may be interpreted as indicating a high degree of hydration.

DISCUSSION

The data obtained show that electrophoretically the substance is essentially homogeneous. This is an important finding as it indicates that the nitrogen in the preparation is not attributable to protein contamination, but is an integral part of the molecule.

For a substance having a molecular size of 260,000 associated with the observed large frictional ratio, which is reflected in the concentration dependence of the sedimentation constant, a rather sharp boundary would be expected to be maintained throughout sedimentation, provided that the substance was monodisperse.

The conclusion that the substance is moderately polydisperse must probably be drawn, but the data presented are insufficient to give a precise expression to the range and distribution of molecular size present in the preparation. Another possible explanation of the data might be that the molecules are uniform in mass, but as a result of preparative manipulation have become configurationally deformed to a varying extent.

SUMMARY

1. The blood-group A substance has been found to be essentially homogeneous electrophoretically.
2. The mean molecular weight of the substance calculated from diffusion and sedimentation data is 260,000, and the frictional ratio 3.2.
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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