Studies in Immunochemistry

10. THE ISOLATION AND PROPERTIES OF LEWIS (Le^a) HUMAN BLOOD-GROUP SUBSTANCE

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During the last few years a considerable body of evidence has accumulated which indicates that mucoids, or complexes consisting of polysaccharides and amino-acids, are responsible for certain of the serological blood-group characters of human erythrocytes, tissue fluids and secretions. There are as yet, however, many blood-group properties which, although firmly established on serological and genetical grounds, remain, nevertheless, unassociated with any chemically defined substance.

Mourant (1946) discovered a new human blood-group character called Lewis and found that the red blood cells of about 25% of English people are agglutinated by anti-Lewis sera. The agglutination is independent of the other known blood-group characters, such as those shown by ABO, MN, *Rhesus*, P, Lutheran, and Kell systems. A further Lewis character was subsequently identified by Andresen (1948) and the two Lewis genes responsible for the serological and genetical behaviour observed up to that time were designated Le^a and Le^b, the corresponding antibodies as anti-Le^a and anti-Le^b and the gene products as Le^a substance and Le^b substance (Andresen, Callender, Fisher, Grubb, Morgan, Mourant, Pickles & Race, 1949).

The examination of a large number of bloods and salivas by Grubb (1948) and by Grubb & Morgan (1949) showed that the secretions of individuals who are 'Lewis positive', i.e. persons whose erythrocytes are agglutinated with anti-Le^a serum, do not contain the A, B and H substances which are to be expected according to the ABO blood group of the individual. It was also observed that the secretions from Lewis-positive persons always contain Le^a substance (cf. Brendemoen, 1949) and that, therefore, this gene product would be most easily isolated from the tissue fluids and secretions of individuals with Lewis-positive erythrocytes.

The isolation and characterization of the mucoid which is responsible for the Le^a character of human erythrocytes and secretions forms the subject matter of the present paper.

EXPERIMENTAL

The methods employed for the isolation and purification of the Lewis Le^a substance from ovarian cyst fluids were similar to those described earlier for the

group A and H substances (King & Morgan, 1944; Morgan & Waddell, 1945; Aminoff, Morgan & Watkins, 1950). The importance of using gentle procedures for the isolation of blood-group substances is now well known, and accordingly methods designed to bring about a minimum of change in these labile complexes have been employed.

Materials and methods

General. In earlier experiments ovarian cyst fluids were selected which contained the Le^a substance, irrespective of whether the more common group factors A, B, H and Lewis Le^b were also present. The discovery that Lewis-positive persons secreted Le^a substance without the other group substances indicated that for the isolation of the Le^a substance the cyst fluids obtained from Lewis-positive persons were the materials of choice.

Determination of serological activity. The iso-agglutination inhibition tests used throughout the investigation were as described by Grubb & Morgan (1949) and employed an anti-Le^a serum, 'Hughes', which was obtained from an individual with the following group characters: A₂, Le(a-), secretor of A and H substances, CDe/CDe, Kell negative, Lu(a-). The test cells used were always obtained from the same person, were group O Lewis-positive and were agglutinated to a titre of 16 by the serum 'Hughes'. A 'standard' preparation of Le^a substance was included in all tests, which were read after standing for 2-3 hr. at 14-16°. Tests used to determine the presence or absence of A and B substances in the cyst fluids were performed as described by Morgan & King (1943). Cyst fluids derived from persons belonging to group O were examined for H substance according to the procedure of Morgan & Waddell (1945).

Total nitrogen. N content was determined by the Kjeldahl method, using the apparatus of Markham (1942) and the bromocresol green-methyl red indicator of Ma & Zuazaga (1942). Occasionally the N content of the final preparations was checked 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) (see Aminoff *et al.* 1950). A heating period of 6 hr. is necessary to obtain the maximum acetyl value for the Le^a substance.

Estimation of carbohydrate components. The total fucose was determined by the method of Dische & Shettles (1948) employing a Hilger Uvispek spectrophotometer. The fucose content was calculated on the basis of the difference in the extinctions at 400 and 430 mμ. The acid-labile fucose was determined, after hydrolysis of the Le^a substance with *n*-acetic acid at 100° for 48 hr., by measuring the acetaldehyde liberated on treatment of the hydrolysis products with

periodate (Aminoff *et al.* 1950). The method was slightly modified by carrying out the oxidation in a stronger phosphate buffer (0.4 M) at pH 7.5 instead of pH 7.0 as used earlier. Under these conditions 95–100% of the theoretical amount of acetaldehyde was recovered from fucose.

Reducing power was determined as glucose by the copper-reduction method of Somogyi (1937).

N-Acetylhexosamine was determined according to Morgan & Elson (1934), using a photoelectric colorimeter. Acetylglucosamine concentrations between 10 and 40 µg./ml. were employed as standards.

Glucosamine and chondrosamine were assayed by a modification of the method described by Elson & Morgan (1933), using glucosamine hydrochloride concentrations of 10–40 µg. as standards, a photoelectric colorimeter and a green light filter (maximum transmission, 540 mµ.).

Chromatographic methods. Amino-acid analyses were carried out by the two-dimensional paper-partition chromatographic technique of Consden, Gordon & Martin (1944), using as solvents *s*-collidine and phenol, and, as supporting medium, Whatman no. 1 paper. Carbohydrate-containing areas were revealed by spraying the paper with AgNO₃-NH₃ (Partridge, 1946) or with aniline hydrogen phthalate (Partridge, 1949). The areas on the paper which contained amino sugars were indicated by treatment with alkaline acetylacetone followed by *p*-dimethylaminobenzaldehyde-HCl in *n*-butanol (Partridge, 1948).

α-Amino N and α-amino-acid N. *α*-Amino N was determined by measuring the N₂ liberated on treatment with HNO₃ 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.

The isolation of the Le^a substance

The methods used for the isolation and purification of the Le^a substance were very similar to those described for the human blood-group A substance (Aminoff *et al.* 1950).

A typical procedure, applied to cyst fluid 'UN', was as follows: the cyst fluid (2.5 l.) was dried from the frozen state, yielding 83 g. of material. Previous experience had shown that extraction of the dried cyst material with 90% phenol was usually the most effective first step in the isolation of the specific substance, and the results of a preliminary experiment in this instance also showed that the active material was concentrated largely in the phenol-insoluble residue, whereas most of the contaminating protein dissolved in the phenol. The whole of the dried cyst material was therefore treated repeatedly with 90% (w/v) phenol, and a phenol-insoluble material (10.6 g.; N, 7.4%) was obtained. This material was only partially soluble in water. The water-

insoluble residue (5.1 g.; N, 9.9%) dissolved in dilute NaOH at 0°, but the resulting solution showed little Le^a activity. The main water-soluble component was thoroughly dialysed and dried from the frozen state. The material (5.3 g.; [α]₅₄₆₁ – 35°; N, 6.0%) was highly active in iso-agglutination inhibition tests against Le^a agglutinin and a part of this material was set aside and subsequently employed as the laboratory 'standard' Le^a substance.

The N content of the water-soluble material indicated that the preparation was substantially uncontaminated with free protein, but nevertheless the material was put through the deproteinization technique described by Sevag (1934). The results of preliminary experiments indicated that the deproteinization proceeded most effectively at pH 4.6. The whole of the main preparation of Le^a substance was therefore dissolved to yield a 0.5% solution in 0.1 N-Na acetate buffer, pH 4.6, and was vigorously stirred with an equal volume of a 1:1 mixture of CHCl₃ and amyl alcohol in 250 ml. centrifuge pots for 20–30 min. The two liquid phases were then separated by centrifugation, the supernatant fluid was siphoned off and the denatured protein in the organic solvent layer washed with an equal volume of fresh buffer. The aqueous supernatant fluid and the buffer washings were pooled and treated again at pH 4.6 with the organic solvent mixture. The aqueous supernatant fluid was given a third treatment with CHCl₃-amyl alcohol, dialysed and dried, from the frozen state. The product (3.1 g.; [α]₅₄₆₁ – 50°; N, 5.25%) showed high Le^a activity. The insoluble material which remained suspended in the organic phase and at the solvent interface was collected by pouring the suspension into excess ethanol and separating the solid by centrifugation. An appreciable amount of Le^a substance separated with the denatured protein, but this material could be largely recovered by repeating the Sevag deproteinization procedure. The substance separating at the solvent interface yielded a final product (2.0 g.; [α]₅₄₆₁ – 46°; N, 7.9%) which showed about half the serological activity of the water-soluble material.

The highly active material obtained after deproteinization by Sevag's technique was separated into six fractions by the addition of ethanol to a 1.5% aqueous solution of the substance. The fractionation was carried out at 0° in the presence of 1% of potassium acetate. After the adjustment of the ethanol concentration to a new level the suspension was allowed to stand for 30–40 min. at 0° before the material was collected by centrifugation. The analytical figures obtained for the six fractions (Table 1) suggest that the original material was not grossly inhomogeneous chemically. All fractions were equally active serologically when expressed in terms of the standard Le^a substance. Some 80% of the total material was thrown out of solution over the range 50–54% (v/v) ethanol, but electrophoretic examination at pH 8.0 in

Table 1. *The fractionation of Le^a substance from aqueous solution by the addition of ethanol at 0°*

Fraction no.	Precipitation level (% (v/v) ethanol)	Yield (g.)	Specific rotation [α] ₅₄₆₁	N content (%)
1	40–44	0.01	—*	5.5
2	44–47	0.06	—	5.2
3	47–50	0.17	—	5.4
4	50–54	2.19	–38°	5.3
5	54–57	0.13	–28°	5.2
6	57–Excess	0.18	–36°	5.4

* Too opalescent for accurate observation.

phosphate ($I, 0.2$) revealed the presence of two components in almost equal amounts. The low mobilities of both substances and their fairly rapid diffusion under the conditions of electrophoresis rendered any attempt at electrophoretic separation impracticable. The possibility of separating the mixture by fractional centrifugation was considered, but examination in the ultracentrifuge showed that the material sedimented as a single component (Fig. 1). The area of the peak given by the diagonal schlieren optical system accounted for almost the whole of the material and suggested that the components of the mixture were of similar particle weights or possessed compensating shapes.

Fig. 1. Sedimentation diagrams of a 1% solution of Le^a substance in phosphate (pH 8.0, $I = 0.2$): (a) 50 min. and (b) 80 min. after reaching full speed (270,000 g).

In view of the success which had attended the isolation of the group A substance following treatment of the native cyst fluid material with trichloroacetic acid at 0° , the possibility of applying this method to the purification of Le^a substance was examined. The possible degradation of the Le^a complex with loss of specific serological properties had to be considered, however, and a preliminary experiment with a side fraction was therefore first undertaken. Without giving details of the analytical figures of the materials obtained on subsequent fractionation of material rendered soluble in phenol by treatment with trichloroacetic acid, it may be stated that an appreciable increase in specific serological activity was shown by the material precipitated from solution in 90% phenol by the addition of ethanol up to 10% (v/v) concentration. An electrophoretic examination of this material at pH 8.0 showed the presence of a fast-moving substance, presumably protein, which accounted for about 10% of the preparation, and two other components of low mobility which from the areas of their peaks in the diagonal schlieren diagrams were present to the extent of approximately 70 and 20%. Electrophoretic analysis of the Le^a substance prior to the application of the trichloroacetic acid-phenol technique showed that the material contained these two components in nearly equal amounts. It seemed probable, therefore, that this treatment had preferentially rendered phenol-soluble one of the two major components of the inhomogeneous specimen of Le^a substance used.

The remaining material from cyst fluid 'UN' which was insoluble in 90% phenol was treated with trichloroacetic acid and subsequently fractionated from phenol solution by ethanol. An electrophoretically homogeneous specimen of Le^a substance was not isolated; the analytical figures for the most active materials obtained from this cyst fluid fell within the range: $N, 5.4-5.6\%$. $[\alpha]_{5461} - 28$ to -36° .

A second cyst fluid, no. 123, obtained from a Lewis-positive person belonging to group A_2 , was dried from the frozen state. The material weighed 58 g. and possessed 6% of the serological activity of the 'standard' Le^a substance. Extraction of the material with 90% liquid phenol yielded 6.5 g. of phenol-insoluble residue which was found to contain almost the whole of the Le^a specific substance. This material was only partially soluble in water; the aqueous extract after dialysis gave 1.6 g. of water-soluble material ($[\alpha]_{5461} - 40^\circ$; $N, 5.7\%$) which was serologically as active as the 'standard' preparation. The material was deproteinized by the Sevag procedure as described above, and gave a product (1.2 g.; $[\alpha]_{5461} - 40^\circ$; $N, 4.8\%$) which showed no enhancement of serological activity.

It was observed that the active material, although insoluble in 90% phenol, was largely soluble in a solution of 90% phenol which contained 20% (v/v) ethylene glycol, and could be recovered from this solvent mixture by addition of excess phenol. This solution and precipitation procedure was repeated twice on the active material with the object of removing any non-dialysable peptide of low molecular weight which was not removed by the Sevag procedure. The analytical figures of the product recovered, however, were identical with those of the starting material.

About 1 g. of the partially purified Le^a substance was dissolved in phenol-glycol mixture and was recovered by the gradual addition of increasing quantities of ethanol. The results of the fractionation are given in Table 2. The active substance was precipitated over a wide range of ethanol concentration, but no material was obtained which differed markedly in properties from the starting material. In a further effort to purify the material, the fractions *a, b, c* and *d* (Table 2) were mixed together and treated with 5% (v/v) trichloroacetic acid at 0° . The solution remained clear and, after neutralization, concentration *in vacuo*, dialysis and drying from the frozen state, gave a product which weighed 0.92 g. and contained 5.2% N . About a third of the material passed into solution on treatment with 90% phenol and was recovered by addition of ethanol to 10% (v/v) concentration. The precipitate was dissolved in a further quantity of phenol and again precipitated with ethanol at the same concentration. The material was finally dissolved in 90% phenol and fractionated from solution by ethanol within the concentration levels given in Table 3. The analytical figures and serological activities of the three middle fractions showed no significant variation (Table 3) and the materials were recombined. Electrophoretic examination of the material at pH 8.0 and 4.0 and ultracentrifugal analysis at

Table 2. The fractionation of Le^a substance from phenol-glycol solution by ethanol

Fraction	Precipitation level (% (v/v) ethanol)	Yield (g.)	Specific rotation $[\alpha]_{5461}$	N content (%)
<i>a</i>	0-8	0.05	-46°	4.3
<i>b</i>	8-11	0.28	-42°	5.2
<i>c</i>	11-14	0.33	-45°	4.9
<i>d</i>	14-20	0.27	-40°	5.1
<i>e</i>	20-excess	0.11	-38°	5.0

Table 3. *The fractionation from phenol by ethanol of Le^a substance after treatment with trichloroacetic acid*

Fraction	Precipitation level (% (v/v) ethanol)	Yield (g.)	Specific rotation [α] ₅₄₆₁	N content (%)	Serological activity (% of standard preparation)
a	Phenol insoluble	0.58	-38°	5.3	100
b	0-5.0	0.08	-42°	5.0	150
c	5.0-7.5	0.11	-45°	5.0	150
d	7.5-10.0	0.13	-44°	5.0	150
e	10.0-excess	0.01	—	5.6	100

pH 8.0 failed to demonstrate inhomogeneity. The details of these analytical procedures are given by Dr R. A. Kekwick in an addendum to this paper.

A third ovarian cyst fluid, no. 157, obtained from a Lewis-positive, Le(a+), patient, measured about 8 l. and gave 320 g. of dry material, which, after extraction with 90% phenol by the usual technique, yielded a phenol-insoluble residue (29 g.; N, 7.1%) which was only partially soluble in water. The water-soluble component (14 g.; N, 6.1%) showed half the serological activity of the 'standard' Le^a preparation. This material was again extracted with 90% phenol and was now found to be appreciably soluble. The insolubility of the material in phenol at the first stage of the isolation procedure is presumably due to the presence of the fraction which is eliminated as a water-insoluble jelly. The phenol-soluble substance was fractionally precipitated from this solvent by gradual addition of ethanol. The material which separated from solution between the levels 0-10% ethanol was twice precipitated from phenol over the same range of ethanol concentration to give a product (1.4 g.; N, 5.8%; [α]₅₄₆₁ -40±4°) which was as active as the standard Le^a preparation. A solution of the substance in 0.2M-acetate, pH 4.6, was further deproteinized by repeated treatment with 1:1 (v/v) amyl alcohol/CHCl₃ as described earlier and gave a final product (1.1 g.; N, 5.6%; fucose, 11.5%; hexosamine, 33%; [α]₅₄₆₁ -40±4°) which showed only a single peak when examined in the electrophoresis apparatus at pH 8.0. This material has not yet been studied in greater detail.

Properties of the Le^a substance

Immunological. The two most active preparations of Le^a substance inhibited the serum 'Hughes' at a dilution of 1:10⁶. They were examined for their capacity to inhibit the following heterologous blood-group reagents: (1) Anti-H agglutinins (so-called anti-O) of animal and human origin (Schiff, 1927; Greenfield, 1928; Morgan & Waddell, 1945; Annison & Morgan, 1950). (2) Human anti-O serum (Boorman, Dodd & Gilbey, 1948; Morgan & Watkins, 1948). (3) Human anti-A and anti-B agglutinins. (4) Rabbit anti-M and N agglutinins. (5) Anti-P agglutinins. (6) Anti-*Rhesus* sera, representing anti-D, anti-C, anti-c and anti-E test reagents, and (7) anti-Le^b agglutinins. Tested at a concentration of 5 mg./ml., the best preparations showed no capacity to neutralize any of the test agglutinins.

The Le^a substance failed to induce in rabbits the formation of anti-Le^a agglutinins when given intra-

venously in several doses each of 0.1 mg. The substance showed no capacity to inhibit the haemolysis of sheep red cells by rabbit anti-A serum in the presence of complement and, in some tests made by Dr J. McCrea, failed to inhibit the haemagglutinating activity of heated LEE influenza virus. The material gave a precipitate with horse antipneumococcal type XIV serum and in giving this reaction differed in behaviour from the purified human A, B and H substances, which failed to precipitate with the type XIV reagent. The crude cyst fluid from a Lewis-positive patient gave rise to a precipitate with type XIV anti-serum.

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

Physical. The mean value of the specific rotation of the two most active preparations is [α]₅₄₆₁ -41±4° (c, 1.0) in water. Relative viscosity determinations were made on the same two specimens dissolved in 0.85% sodium chloride, using a modified Ostwald viscosimeter at 37°; the values obtained, expressed in terms of the solvent, were: η , 1.36 and 1.38. These values are somewhat higher than those reported for A substance.

Solutions of the Le^a substances (0.10% in 0.85% NaCl) were examined in a Hilger quartz spectrophotometer (Uvispek) using a 2 cm. cell. The absorption curve for one specimen is given in Fig. 2. No significant amount of absorption is shown by the material between 260 and 300 m μ . and the absence of appreciable amounts of aromatic amino-acids and nucleic acid is indicated.

Chemical. A precipitate was not obtained when a 2% aqueous solution of the Le^a substance was treated with an equal volume of the following protein precipitants: trichloroacetic acid (10%, v/v), salicylosulphonic acid (20%, v/v), tannic acid, or picric acid. The Le^a substance likewise gave no precipitate on addition of solutions of lead acetate (in dilute acetic acid), mercuric acetate, aluminium sulphate in 5N-sulphuric acid, phosphotungstic acid, acid or neutral copper sulphate, or an acetic acid solution of potassium ferrocyanide.

The biuret, ninhydrin, xanthoproteic, Millon, Hopkins-Cole, Seliwanoff and Bial tests were negative. The Sakaguchi test for arginine, as modified by Keyser (1948), and the diazobenzene-sulphonic acid test for tyrosine and histidine gave only a trace of colour when applied to a 2% solution of the Le^a substance.

The analytical figures obtained for the most active specimen of Le^a substance were: C, 41.5; H, 7.1; N, 5.3% (average Kjeldahl value). The phosphorus content was less than 0.02% and the sodium fusion test for sulphur indicated that this element was not present in appreciable amounts, a conclusion which is supported by the finding that cysteic acid and methionine were absent from the partition chromatograms of the total acid hydrolysate of the Le^a substance and that the unhydrolysed material failed to react with toluidine blue, a reagent used by Jorpes (1946) for the detection of polysulphate groups in polysaccharides.

The acetyl content of the Le^a substance was about 10% (6 hr. hydrolysis) and was in excess of that required for the *N*-acetylhexosamine calculated to be present from the total hexosamine value. The distillates obtained from the *N*-acetyl determinations were neutralized and were used to identify the volatile organic acid by means of an *S*-benzylthiuronium salt (Donleavy, 1936). The *S*-benzylthiuronium salt obtained in low yield after recrystallization from 80% ethanol melted at 134° and gave a mixed melting point of 135° with an authentic specimen of *S*-benzylthiuronium acetate.

The fucose content of the Le^a substance was about 12%. The maximum colour developed by the Le^a substance, after heating at 100° for 14 min. with dilute sodium carbonate (pH 10.8) and treatment with *p*-dimethylaminobenzaldehyde (Morgan & Elson, 1934), was equivalent to about 9% of *N*-

acetylglucosamine. *N*-Acetylglucosamine required heating for 4 min. to develop its maximum colour under the same conditions.

The Le^a substance, after heating with dilute sodium carbonate for 14 min. at 100°, was dialysed against distilled water at 0°. The diffusate gave an immediate colour with the *p*-dimethylaminobenzaldehyde reagent, whereas the indiffusible residue gave no appreciable colour (cf. King & Morgan, 1944; Morgan, 1946). These results again demonstrate how closely in certain of its properties the Le^a substance resembles the human A substance.

Oxidation of the Le^a substance with periodate

The rate of reduction of periodate by the Le^a substance at pH 5.0 and 7.3 was measured by the techniques employed by Aminoff & Morgan (1951).

Oxidation at pH 5.0. A solution (0.1%) of the Le^a substance in 0.1M-acetate, pH 5.0, was treated with 0.005M-HIO₄. The rate of reduction of periodate and the loss of biological activity of the substance were determined. The results, shown graphically in Fig. 3, are closely similar to those obtained for the A substance under the same conditions. The analytical results, corrected for over-oxidation by extrapolating the uptake curve to zero time, showed that 1 mg. of Le^a substance reduces 0.273×10^{-5} g.mol. of HIO₄, and hence 365 g. of Le^a substance reduces 1 g.mol. of HIO₄.

Fig. 3. Rate of reduction of periodate by Le^a substance at pH 5.0 (○) and pH 7.0 (□). The resulting inactivation at pH 5.0 as determined by the capacity of the oxidized material to inhibit the agglutination of Le (a+) cells by Le^a agglutinin (×).

Oxidation at pH 7.3. The rate of periodate uptake was greater at pH 7.3 than at pH 5.0 (Fig. 3). The uptake curve showed a less definite inflexion after 2 hr. and the periodate consumption continued steadily for several days. The serological activity of the substance was lost at approximately the same rate (Fig. 3) as at pH 5.0.

Acid hydrolysis of the Le^a substance

The Le^a substance was hydrolysed in sealed glass ampoules at 100° with (a) 0.5N-HCl, (b) 6N-HCl and (c) N-acetic acid. After heating under conditions b and c for the appropriate time the ampoules were cooled, opened and the contents evaporated to dryness *in vacuo* in small glass dishes over conc. H₂SO₄ and pellets of KOH. The products of hydrolysis with 0.5N-HCl (a) were neutralized with an equal volume of 0.5N-NaOH and not evaporated to dryness. The products of hydrolysis with 6N-HCl (b) were dark brown in colour and contained a precipitate of humin.

The course of hydrolysis with 6N-HCl was followed by measuring the α -amino N liberated. The results (Fig. 4) show that the liberation of α -amino N reached its maximum value in about 4 hr. and then represented 92% of the total N of the Le^a substance. The α -amino-acid N after 16 hr. hydrolysis was equivalent to about 37% of the total N. The α -amino N value includes the N liberated from the amino sugars set free during the hydrolysis.

Fig. 4. Hydrolysis of Le^a substance by 6N-HCl at 100° as determined by the liberation of α -amino groups.

The liberation of reducing sugars and hexosamines is best followed by an examination of the products of 0.5N-HCl hydrolysis. The analytical results (Fig. 5) show that the maximum liberation of reducing sugars, 57% expressed as glucose, and of hexosamines, 32% expressed as glucosamine base, occurred at about the same time (6 hr.).

Hydrolysis of the Le^a substance with N-acetic acid at 100° allowed the rate and extent of liberation of fucose, reducing sugars and N-acetylhexosamine residues to be determined. The results (Fig. 6) indicate that a maximum value of about 13% fucose, determined as acetaldehyde after oxidation with periodate, is obtained after 24 hr. hydrolysis and a maximum value under these conditions for reducing sugars is reached at about the same time. There is an appreciable increase in the amount of 'N-acetylglucosamine' colour given by the Le^a substance during the first 2 hr. of hydrolysis after which time the original value of 8.9%, expressed as

N-acetylglucosamine, reaches a maximum value of 10.3% and then decreases.

During treatment with N-acetic acid at 100° the Le^a substance rapidly lost its capacity to inhibit iso-agglutination brought about by Le^a serum. At the same time there was a considerable enhancement of the pneumococcus type XIV character of the product. There was, however, no development in the products of a capacity to prevent the haemolysis of sheep cells by rabbit anti-A serum and complement.

Fig. 5. Hydrolysis of Le^a substance by 0.5N-HCl at 100° as determined by (A) reducing power, expressed as glucose and (B) hexosamine liberated, expressed as glucosamine base.

Fig. 6. Hydrolysis of the Le^a substance by N-acetic acid at 100° as determined by (○) reducing power, expressed as glucose, (Δ) 'N-acetylhexosamine' colour, and (□) fucose, determined as acetaldehyde after oxidation with periodate.

Chromatographic analysis of the Le^a substance

A detailed investigation of the nature of the constituents of the Le^a substance was made by partition chromatography.

Amino-acids. The products of the complete hydrolysis (16 hr.) of the material (1 mg.) with 6N-HCl were put as a spot at one corner of a sheet of Whatman no. 1 paper. The

spot was exposed to NH_3 vapour and allowed to dry. The paper was then placed in the development tank together with a duplicate paper containing a similarly placed spot composed of an artificial mixture of those amino-acids believed as a result of a preliminary experiment to be present in the Le^a substance. *s*-Collidine saturated with water was used for the first run, which lasted 48–72 hr. The papers were then removed from the tank, dried at 37° , turned through a right-angle and irrigated for 24 hr. with a solution of water-saturated phenol (A.R.) in an atmosphere of coal gas containing NH_3 . The positions of the individual amino-acids were revealed by spraying the papers with 0.1% solution of ninhydrin in butanol containing 1% (v/v) acetic acid (Consden & Gordon, 1948) and keeping the sheets at 37° for 1 hr. The papers were subsequently heated at 100° for 5 min. to detect the presence of peptides, but no additional coloured areas appeared. The chromatogram given by the complete hydrolysis products of Le^a substance gave rise to thirteen mauve spots on treatment with ninhydrin. Eleven of the spots on the chromatogram corresponded closely in position with those which were given by the following amino-acids: lysine, arginine, aspartic acid, glutamic acid, glycine, serine, alanine, threonine, proline, valine and leucine (isoleucine). The remaining greyish-mauve spots are not normally found in protein hydrolysis products and were subsequently identified as arising from glucosamine and chondrosamine.

Reducing sugars. The Le^a substance was hydrolysed with 0.5N-HCl at 100° for 16 hr. and evaporated to dryness, and the chromatographic analyses of the sugar constituents were made by techniques essentially the same as those described by Partridge (1948). Strips of Whatman no. 1 paper (18×34 cm.) were employed as the inert supporting medium and the chromatograms were developed by irrigation with solvents run under gravity.

Five positions were marked across the width of each of eight filter paper strips and the following mixtures were put on each paper: (a) fucose, galactose and rhamnose; (b) the acid hydrolysate of Le^a substance; (c) the acid hydrolysate of Le^a substance together with fucose and galactose; (d) fucose, galactose and the two amino sugar hydrochlorides. Different sugars were introduced at position (e) as a further check on the resolving power of the solvent systems and in order to compare the colours produced by these sugars after treatment with aniline hydrogen phthalate with those produced by the sugar components of the acid hydrolysates of the Le^a substance. The eight paper strips were then irrigated in pairs with the following four solvent systems: (1) phenol-water, in an atmosphere of coal gas and HCN; (2) collidine-water; (3) *n*-butanol (40%), acetic acid (10%) and water (50%); (4) *n*-butanol-water, in an atmosphere of NH_3 . The chromatograms were developed for 16–24 hr. with solvent systems (1) and (2), and for 36–48 hr. for the solvents (3) and (4). The period employed depended on the temperature of the laboratory during the irrigation. The strips were dried and one member of each pair was sprayed with $\text{AgNO}_3\text{-NH}_3$ reagent and the other with aniline hydrogen phthalate. No evidence was obtained for the presence in the Le^a substance of sugars other than fucose, galactose and the two hexosamines. A chromatogram of the hydrolysate of the Le^a substance was also sprayed with the naphthoresorcinol-trichloroacetic acid reagent (Partridge, 1948), when the absence of ketoses and uronic acids from the material was established.

Galactose (R_F , 0.44) can be readily distinguished from glucosamine (R_F , 0.62) and chondrosamine (R_F , 0.65) by virtue of its lower mobility in phenol, but fucose (R_F , 0.62) is not identifiable with this solvent, if a mixture of the four sugars is examined; this is so despite the fact that fucose reacts much more intensely than the hexosamines with aniline hydrogen phthalate. The use of *s*-collidine as the moving organic phase, however, allows fucose (R_F , 0.44) to be easily distinguished from the other three sugars. Galactose (R_F , 0.34) moves at almost the same rate as glucosamine (R_F , 0.32) in collidine, but can be readily detected (R_F , 0.16) between fucose (R_F , 0.27) and the amino sugars (R_F , 0.12–0.13) on chromatograms irrigated with butanol-acetic acid-water.

The $\text{AgNO}_3\text{-NH}_3$ reagent for the detection of sugars is somewhat unsatisfactory, since it is applied in the form of an aqueous solution and some migration of the sugar-containing areas may occur if the chromatogram is not lightly and evenly sprayed. Furthermore, Cl^- migrates at speeds comparable to those of the sugars in some solvents and under these circumstances reacts with the reagent to give a heavy white precipitate of AgCl which masks the brown-coloured areas given by the reducing sugars. Fortunately, the aniline hydrogen phthalate reagent is applied to the chromatogram in butanol and does not give rise to noticeable migration of the sugar-containing areas, and furthermore the presence of Cl^- does not interfere with the brown coloration given by reducing sugars.

The amino sugars. The investigations of Aminoff & Morgan (1948) showed that a good separation of the amino sugars on paper is facilitated by the use of alkaline solvents, such as *s*-collidine and *n*-butanol- NH_3 . Chromatograms were therefore run with the hydrolysis products of Le^a substance, alone and admixed with glucosamine and chondrosamine, using these solvents for irrigation. Five positions were marked out along the width of each of two filter paper strips (18×34 cm.) and the following substances were put on each paper in the order given: (a) glucosamine hydrochloride, (b) glucosamine and chondrosamine hydrochlorides, (c) acid hydrolysis products of Le^a substance, (d) acid hydrolysis products of Le^a substance with (b) added, and (e) chondrosamine hydrochloride alone. The chromatograms were developed in parallel with *s*-collidine as solvent for 48 hr. and then dried. One strip of each pair was sprayed with the ninhydrin reagent and the other successively with the two hexosamine reagents. The chromatograms indicated that glucosamine and chondrosamine are components of the Le^a substance.

The two-dimensional chromatograms of the hydrolysis products of the A substance and the Le^a substance revealed that, whereas the glucosamine- and chondrosamine-containing areas derived from the A substance were almost identical in size and colour intensity, the glucosamine spot given by the hydrolysis products of the Le^a substance was two to three times as large as the chondrosamine area.

The possibility that the second amino sugar spot was due to *N*-methyl-L-glucosamine was rendered improbable when it was shown that the latter substance possesses a slightly greater mobility in *s*-collidine than glucosamine and gives colours with the ninhydrin and hexosamine reagents which differ from those produced by the two unsubstituted amino sugars, glucosamine and chondrosamine.

The isolation of the component sugars

The sugars were isolated by partition chromatography on cellulose columns according to the general procedure described by Hough, Jones & Wadman (1949). The results of preliminary experiments with paper chromatograms showed that fucose and galactose could be separated from each other and from the amino sugars also present in the acid hydrolysates of the Le^a substance, if the solvent system 1:1 *n*-butanol/2% aqueous acetic acid was employed.

All melting points given below are uncorrected.

Le^a substance (500 mg.) was hydrolysed in sealed tubes with 0.5*N*-H₂SO₄ at 100° for 8 hr. The hydrolysate was neutralized to pH 7 with saturated Ba(OH)₂ and the precipitated BaSO₄ removed by centrifugation and thoroughly washed with water. The solution and washings were combined and taken to dryness *in vacuo*. The residue was dissolved in a few drops of water and placed on the top of a cellulose column (35 × 2.5 cm.) which had been previously washed by the passage of 500 ml. of the organic phase of the *n*-butanol/2% aqueous acetic acid system. The chromatogram was allowed to develop under gravity and 4 ml. portions of the solvent issuing from the column were collected. The sugars were detected and identified as described by Hough *et al.* (1949). The fucose was readily obtained completely free from galactose and the amino sugars and most, but not all, of the galactose emerged from the column uncontaminated with the amino sugars.

L-Fucose as diphenylhydrazone. The fractions issuing from the column which contained fucose were evaporated to dryness *in vacuo* at 30–40°. The residue was dissolved in water (2 ml.), and glacial acetic acid (0.2 ml.) and freshly distilled diphenylhydrazine (60 mg.) were added. The solution was allowed to stand overnight in the dark and at room temperature, and was then cooled, when 35 mg. of needle-shaped crystals separated. The derivative was twice recrystallized from 95% (v/v) ethanol when 19.5 mg. of material melting at 201° was obtained. Mixed melting point with authentic fucose diphenylhydrazone remained unchanged. (Found: C, 65.1; H, 6.5; N, 8.7. Calc. for C₁₈H₂₂O₄N₂: C, 64.7; H, 6.7; N, 8.5%. [α] – 13° in pyridine (c, 1).)

D-Galactose α-methylphenylhydrazone. The fractions collected which contained galactose were mixed and evaporated to dryness *in vacuo*. The crystalline residue was dissolved in water (2 ml.), and freshly distilled α-methylphenylhydrazine (0.1 ml.), ethanol (2 ml.) and 10% acetic acid (0.1 ml.) were added. The mixture was kept at 37° for 2 hr. and overnight at 0°. D-Galactose α-methylphenylhydrazone (59 mg.) separated and after recrystallization from 30% (v/v) ethanol gave 48 mg. of needle-shaped crystals m.p. 191°. Mixed melting point with authentic D-galactose α-methylphenylhydrazone 191°. Found: C, 53.8; H, 7.4; N, 10.2. Calc. for C₁₃H₂₀O₅N₂: C, 54.9; H, 7.0; N, 9.9%.)

D-Galactose α-methylphenylhydrazone pentaacetate. Galactose α-methylphenylhydrazone (20 mg.) was suspended in 1 ml. of dry pyridine and 0.2 ml. of acetic anhydride added at 0° (Wolfson & 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 was collected and well washed with water. The material after recrystallization from 50% ethanol gave 29 mg. of D-galactose α-methylphenylhydrazone pentaacetate. [α]_D + 36 ± 2° in 95% ethanol (c, 1); m.p. 139–140° without decomposition. Mixed melting point with an authentic specimen of the pentaacetate remained unchanged.

N-2,4-Dinitrophenylamino sugars. A method for the separation and identification of small amounts of a mixture of glucosamine and chondrosamine as dinitrophenyl (DNP) derivatives has been described by Annison, James & Morgan (1950, 1951). The yields of the DNP derivatives obtained from known amounts (10–20 mg.) of the amino sugar hydrochlorides and recovered from the kieselguhr column were of the order of 80%. The results of qualitative paper chromatography had shown that the Le^a substance contained the amino sugars, glucosamine and chondrosamine, in the ratio of about 3:1. In order, therefore, to isolate sufficient DNP-chondrosamine to allow the material to be purified by crystallization and analysed, it was necessary to hydrolyse 500 mg. of the Le^a substance. The yield of the mixed DNP-amino sugars represented 69% of the total hexosamine present in the Le^a substance. The DNP-glucosamine weighed 97.4 mg. and the DNP-chondrosamine 36.3 mg. The ratio of glucosamine to chondrosamine was 1:2.7. The isolated DNP-glucosamine had melting point 197–200° and mixed melting point with authentic DNP-glucosamine 199–201°. [α]_D + 68 ± 4° in 80% ethanol (c, 1). (Found: C, 42.0; H, 4.6; N, 12.0. Calc. for C₁₂H₁₈O₉N₃: C, 41.7; H, 4.4; N, 12.2%.) Isolated DNP-chondrosamine had melting point 177–180° and mixed melting point with authentic DNP-chondrosamine 178–180°. [α]_D + 82 ± 4° in 80% ethanol (c, 1). (Found: C, 41.5; H, 4.4; N, 11.9. Calc. for C₁₂H₁₈O₉N₃: C, 41.7; H, 4.4; N, 12.2%.)

The examination of the products of partial hydrolysis of Le^a substance

The products of the hydrolysis of 50 mg. of Le^a substance with *N*-acetic acid at 100° for 48 hr. were dialysed in a thoroughly washed cellophane 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 CHCl₃ until the dialysis was complete. The total diffusates were then mixed together, concentrated to a small volume and dried from the frozen state. The indiffusible contents of the cellophane bag were likewise dried. The diffusible material (16 mg.) was obtained as a hygroscopic glass, the indiffusible material (17.1 mg.) as a light-brown friable solid. Fucose, galactose, an *N*-acetylhexosamine and traces of glucosamine and chondrosamine were detected by chromatography in the diffusate. The solvents employed, however, were not chosen to resolve a mixture of the *N*-acetylhexosamines. The developed chromatogram of the diffusate when sprayed with aniline hydrogen phthalate indicated the positions of fucose and galactose only. AgNO₃·NH₃ gave in addition three other brown spots associated with slowly moving substances which were presumably due to the presence in the diffusate of partial hydrolysis products of the polysaccharide moiety of the Le^a substance. Two of these unidentified spots reacted with the hexosamine reagents.

The presence in the diffusate of a small amount 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 which appeared on treatment with $\text{AgNO}_3\text{-NH}_3$ reagent were no longer present after the diffusate was hydrolysed further with 0.5N-HCl for 16 hr. at 100°. Fucose, galactose and the two amino sugars were then detected. The ratio of glucosamine to chondrosamine in the diffusate, as judged by the areas of the spots produced, was about 5:1. The chromatographic analyses of the diffusate after hydrolysis with 6N-HCl showed that traces of all the amino-acids except proline, leucine and arginine were present.

The non-diffusible material was hydrolysed with 0.5N-HCl at 100° for 16 hr. and examined chromatographically for sugars. Fucose, which is stable under the conditions of hydrolysis employed, was not detected in these analyses, but galactose, glucosamine and chondrosamine were identified. The ratio of glucosamine to chondrosamine was estimated from the areas of the spots produced to be about 3:2.

The amino-acid distribution in the indiffusible material was investigated after further hydrolysis with 6N-HCl for 16 hr. at 100° and compared with that given by the original, fully hydrolysed material. No significant difference could be detected.

DISCUSSION

The results of the investigations described show that the Lewis Le^a substance is a mucoid material which possesses physical and chemical properties very similar to those recorded for the other water-soluble products of the human blood-group genes. Whereas one might expect that the products of allelomorphous genes would closely resemble each other chemically, it is perhaps surprising that the products of genes genetically independent should be so similar. This emphasizes the importance of determining the precise differences in the nature of these gene products for our understanding of the mechanism of gene action.

Ovarian cyst fluids have been used as a source of Le^a substance and by selecting cyst fluids obtained from Lewis-positive, $\text{Le}(a+)$, women it is possible to avoid the presence of A, B, H and Le^b substances in the starting material. Once again it has been found that the variable amount of the Le^a substance present and the unknown nature of the accompanying unspecific materials in the native cyst fluids leads to an unpredictable behaviour of the crude material and an exact description of an isolation procedure which leads to an homogeneous product and can be applied successfully to all cyst fluids cannot be given.

The blood-group substances in dried, native ovarian cyst fluids are usually found to be insoluble in 90% phenol. A preliminary treatment of the material with this solvent, however, readily removes a considerable part of the unspecific protein and leaves a mucoid material which possesses almost the whole of the blood-group activity of the native fluid. It has been found that the phenol-insoluble mucoid still contains a small amount of material which from its nitrogen content appears to be

largely protein and this impurity can be most easily and completely removed, if the substance is fractionally precipitated from solution in 90% phenol. The problem of getting the serologically active mucoid into solution in 90% phenol has been only partially solved. It has been observed, however, that after the removal of accompanying unspecific material the specific mucoid frequently becomes appreciably soluble in 90% phenol and can be recovered from solution in this solvent by addition of ethanol. Two other procedures have also been found which will bring about the solution of the specific mucoid in 90% phenol. The first consists in treating the native material with pepsin at pH 2.3 and 37° for several days. This method has been used extensively by Kabat and his co-workers (see Kabat, 1949), but for reasons which will be given elsewhere we have not used the method, believing that prolonged peptic hydrolysis brings about a change in the blood-group substance. The second method involves treatment of an aqueous solution of the phenol-insoluble material with 5% trichloroacetic acid at 0° for a short time. A part of the substance is subsequently found to be soluble in 90% phenol and can be recovered from this solvent by addition of ethanol to a low final concentration. Irrespective of group specificity the phenol-insoluble mucoid materials so far investigated are found to dissolve, in part and in the cold, in 90% phenol which contains 20% ethyleneglycol. The soluble component, which exhibits a much increased serological activity, can be recovered from this and other similar organic solvent systems by addition of excess phenol or ethanol.

The application of the different techniques for the isolation of the blood-group mucoid gives a material of high Le^a activity which appears to be essentially homogeneous on the basis of its behaviour during electrophoresis. The electrophoretic diagrams obtained for periods of observation up to nearly 5 hr. at pH 4.0 and 8.0 fail to reveal the presence of more than a single component. No appreciable migration of the Le^a substance takes place at pH 4.0, but a slow movement towards the anode is detected in buffer solution at pH 8.0. Examination in the ultracentrifuge at pH 8.0 showed that only one main component was present, but the material was nevertheless moderately polydisperse. The mean sedimentation constant at a concentration of 0.5% is S_{20} , 6.7×10^{13} . From the ultracentrifugal and diffusion data the particle weight of the Le^a substance is of the order 270,000.

The application of the usual techniques of paper chromatography to the products of acid hydrolysis of the Le^a substance demonstrates that fucose, galactose, glucosamine and chondrosamine are component sugars and that at least eleven amino-acids are also present in the native complex. The

chromatograms are indistinguishable qualitatively from those given by the group A substance. The aromatic acids, phenylalanine and tyrosine, are absent, whereas threonine occurs in a higher concentration than any of the other amino-acids. Sulphur and phosphorus are not present in appreciable amounts, and the Le^a substance shows no characteristic absorption bands in the ultraviolet region (210–300 m μ).

The results of the quantitative determination of the component sugars in the different blood-group substances indicates that the amounts of the sugar residues are different in the different gene products. A substance, for example, contains about 18% fucose, three-quarters of which is liberated as free sugar after hydrolysis with acetic acid (pH 2.8) at 100°, whereas Le^a substance contains only some 13% fucose, almost the whole of which is split off under the same conditions of hydrolysis. The ratio of D-glucosamine to D-chondrosamine in the group A substance is very close to one, whereas these two amino sugars are found together in the Le^a substance in the ratio of about 3:1. The low yield of *S*-benzylthiouronium acetate obtained from the distillate collected during the quantitative determination of acetic acid liberated by the Le^a substance does not allow the presence of other volatile organic acids to be excluded.

Kabat, Bendich, Bezer & Knaub (1948) reported that the A, B and O (H) substances obtained from hog and human sources precipitate with anti-pneumococcal type XIV horse serum. Our preparations of these specific substances obtained from human ovarian cysts fail to react with this antibody (Aminoff *et al.* 1950 and unpublished observations). In view of the finding now reported that Le^a substance reacts with antipneumococcal type XIV serum it seems probable that human erythrocytes belonging to all groups within the ABO classification are agglutinated by this antiserum, as first reported by Finland & Curnen (1938), Weil & Sherman (1939) and Goebel, Beeson & Hoagland (1939), not by virtue of their A, B and H receptors but rather through the reactivity of other components of the red cell surface, such as through the presence of the product of the Le^a gene. Specific substances responsible for other serological group characters, and especially that derived from the closely related 'Lewis' Le^b gene, might well react with pneumococcal type XIV antibody. In most instances, however, the chemical nature of the substances associated with these other group characters, such as M, N, P, *Rhesus*, etc., is unknown. Some indirect evidence from a study of the action of periodate on the erythrocyte surface (Morgan & Watkins, 1951) suggests that these receptors might be due to carbohydrate-containing materials, and therefore could possibly contain the basic glucosamine-galactose

structure which, according to Kabat, Baer, Bezer & Knaub (1948), is responsible for the serological cross-reactivity observed. The hypothesis, however, cannot be substantiated until a more detailed examination of the reactivity of a much larger number of undegraded blood-group substances with the type XIV antiserum has been undertaken.

The examination of the genetical schemes (see Grubb & Morgan, 1949; Race & Sanger, 1950; Andresen, Andersen, Jordal & Henningsen, 1950; Grubb, 1951) elaborated to explain the serological behaviour of erythrocytes, of tissue fluids and secretions indicates the complex nature of the Lewis group system and it must be admitted that the genetics of this system are not completely understood. According to Grubb (1951) it seems probable that the serological observations can be accounted for, if four genes, existing as two allelomorphous pairs, Le^a-Le^a and Le^b-Le^b, are each responsible for inducing the formation of a gene product which contributes to the ultimate serological 'Lewis' character of the individual's erythrocytes and secretions. It is not known if the gene products Le^a and Le^b actually exist, but if they do Le^a substance will be encountered in rather less than half the fluids obtained from 'Lewis' positive persons, whereas all such fluids will contain Le^b substance. Since it is generally believed that the products of allelomorphous genes are basically similar, it would seem probable that the product of the gene Le^a is closely related chemically to that of the gene Le^b which is now known to be a mucoid. In the absence of serological reagents for the detection of Le^a and Le^b substances these materials would remain unidentified serologically if they are present in preparations of Le^a substance.

The establishment of homogeneity of material of large particle size is a matter of considerable difficulty, and in spite of the apparently satisfactory nature of the results of a number of homogeneity tests which have been applied to the Le^a preparations involving the use of physical, chemical and immunological measurements, it is evident that the similarity of the products of allelomorphous genes and the absence in some instances of specific serological reagents for their detection and complete characterization suggest caution in concluding that the Le^a preparations described are completely homogeneous. It is to be observed that the quantitative immunochemical technique developed by Kabat *et al.* (see Kabat, 1949), although a valuable procedure for the measurement of homogeneity in many instances, is not necessarily immediately applicable to every problem of the kind. Sera suitable for quantitative precipitation tests in the blood-group field have to be made largely by the immunization of man or animals. The immunizing antigens, whether they are red cells or native secre-

tions, such as saliva or partially purified group specific substances, almost certainly contain antigens in addition to the one under investigation. The immune serum formed would most probably possess antibodies specific for the antigens injected and precipitate with all or some of the components of the original 'antigen' preparation. A serum with these properties is not a suitable reagent for use in the quantitative immunochemical technique.

The particle sizes of the human blood-group substances which have been isolated so far are such that one might expect that a strictly limited variation in composition or structure would be encountered in group materials obtained from individuals whose erythrocytes and secretions ostensibly show the same serological specificity. Indeed, it seems probable that only a part of a group substance whose particle size is as large as 250,000 need possess the specific configuration necessary to bring about its combination with homologous antibody. The remaining part of the group substance could presumably vary to some extent as long as the change brought about no demonstrable influence on the closeness of fit of the substance with the antibody concerned. At this early stage in the development of our knowledge of the human blood-group substances, however, every effort should be made to obtain these materials uncontaminated with other substances of closely similar composition and origin, as far as it is possible to do so by employing in a critical

manner the physical, chemical and immunological techniques at present available, before it is concluded that similar group characters in different individuals are due to materials possessing a different chemical composition.

SUMMARY

1. The isolation and purification of the human blood-group Le^a substance is described.
2. The substance is a laevorotatory mucoid. The mucoid is slightly polydisperse, but appears to be essentially homogeneous on the basis of a number of physical, chemical and immunological properties.
3. L-Fucose, D-galactose, D-glucosamine, D-chondrosamine and eleven amino-acids have been identified as components of the Le^a substance.
4. The products of acid hydrolysis of the Le^a substance contain 57 % of reducing substances, expressed as glucose, 13 % of L-fucose and 32 % of hexosamine. The α -amino-acid nitrogen represents about 37 %, and the α -amino nitrogen 92 %, of the total nitrogen (5.0 %).
5. Evidence for the homogeneity of the Le^a substance is discussed and the possible presence of other mucoids is recognized.

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ADDENDUM

Physicochemical Examination of Lewis (Le^a) Blood-group Substance

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The Lewis (Le^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 was 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).

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, pH 4, $I=0.2$ and in phosphate 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 ascending and descending boundaries were asymmetrical, and the peaks were partially obscured by

Fig. 1. Ultracentrifuge and electrophoresis diagrams of Lewis (Le^a) substance. (a) Ultracentrifuge diagram of 1% (w/v) solution in phosphate-NaCl (phosphate pH 8, $I=0.2$, 0.15 M-NaCl) after 50 min. at 270,000 *g*. (b) Electrophoresis diagram of 1.6% (w/v) solution in phosphate (pH 8, $I=0.2$) after 270 min. at 5 V./cm. (c) Electrophoresis diagram of 2% (w/v) solution in acetate (pH 4.0, $I=0.2$) after 300 min. at 5 V./cm. Migration direction in (b) and (c) shown by arrows.

a small amount of material associated with marked light scattering (Fig. 1). Migration at both pH 8 and 4 was anodic, but very slight at pH 4.

In the ultracentrifuge only one component was present. The sedimentation constant was determined at three concentrations (Table 1). Owing to the spreading of the boundary it was not possible to make satisfactory measurements below a concentration of 0.5 g./100 ml. There is a marked dependence of sedimentation constant on concentration.

In Table 1 is included a value for the diffusion constant, kindly measured by Dr A. G. Ogston, at a concentration of 1 g./100 ml. The partial specific volume was found to be 0.643.

Table 1. *Sedimentation and diffusion constants of Lewis blood-group substance*

(Sedimentation and diffusion constants corrected to water at 20°.)

Concentration (g./100 ml.)	$S_{20} \times 10^{13}$ (corr.)	$D_{20} \times 10^7$ (corr.)
0.50	6.71	—
0.75	6.27	—
1.00	5.44	1.37

From the sedimentation and diffusion constants determined at a concentration of 1 g./100 ml., and the partial specific volume, a molecular weight of 270,000 was obtained from the equation

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

where M = molecular weight, R = gas constant, S = sedimentation constant, D = diffusion constant, T = absolute temperature, V = partial specific volume, and ρ = density of solvent (Svedberg & Pederson, 1940).

From the same data the frictional ratio f/f_0 was computed, a value of 3.8 being obtained. This

corresponds to an axial ratio of about 90 (Svedberg & Pederson, 1940); alternatively this value may be interpreted as indicating a high degree of hydration.

DISCUSSION

The characteristics of the Lewis blood-group substance are very similar to those previously reported for the blood-group A substance (Kekwick, 1950). Electrophoretically the substance is essentially homogeneous, which indicates that the protein nitrogen of the material is not due to contamination with extraneous protein, but is an integral part of the molecule. The ultracentrifuge data suggest a moderate degree of polydispersity, since the spread of the sedimenting boundary is greater than would be expected for a homogeneous substance of molecular weight 270,000 also showing the rather marked dependence of sedimentation constant on concentration found in this instance. The data are insufficient to give a precise expression of the range and distribution of molecular size. The molecule is probably either highly asymmetrical or highly hydrated.

SUMMARY

1. The Lewis Le^a blood-group substance is essentially homogeneous electrophoretically.
2. The mean molecular weight of the substance calculated from diffusion and sedimentation data is 270,000, and the frictional ratio 3.8.
3. The substance is probably moderately polydisperse and the molecular shape highly asymmetrical, or alternatively the molecule is highly hydrated.

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