or reductic acid, when present in quantities which are small in relation to that of the ascorbic acid present. When larger quantities of these compounds are present, additional corrections must be introduced.

SUMMARY

1. The kinetics of the reaction between HCHO and *l*-ascorbic acid or chemically related substances have been studied.

2. Indirect evidence is produced for the possible structure of the HCHO-*l*-ascorbic acid complex.

3. Lugg's method for determination of *l*-ascorbic

acid has been examined from the point of view of the above experiments, and necessary precautions are stressed.

4. Suggestions for other methods for the determination of *l*-ascorbic acid in the presence of reductic acid and reductone are advanced.

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Studies in Immunochemistry

7. THE ISOLATION FROM HOG GASTRIC MUCIN OF THE POLYSACCHARIDE-AMINO ACID COMPLEX POSSESSING BLOOD GROUP A SPECIFICITY

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Investigations on the nature of the blood group antigens present in human tissues and secretions are greatly restricted owing to the small amounts of active factors present and to the difficulty of obtaining the tissues and secretions in large quantities. An observation of Schiff [1930], however, revealed that commercial peptone contained a considerable quantity of a blood group A factor, and subsequently Schiff & Weiler [1931], Brahn, Schiff & Weinmann [1932] and Schiff [1933] showed that commercial pepsin likewise could serve as a readily available source of this blood group substance. A rich supply of the A-factor was discovered later in hog gastric mucin by Landsteiner & Chase [1936] and by Meyer, Smyth & Palmer [1937]. As a result of their work on the glyco-proteins occurring in gastric mucosa, Meyer *et al.* [1937] isolated from this tissue a polysaccharide that possessed intense A activity and showed that it contained N-acetylglucosamine, and galactose in equimolecular quan-

ties. On the basis of the glucosamine content the product was considered to be only 75 % pure, and it was observed that the material gave a positive Ehrlich diazo-reaction. Landsteiner & Harte [1940] confirmed these observations and extended the investigation to include a closer examination of the non-hexosamine nitrogenous constituent. They concluded that the A-substance consisted largely of carbohydrate but contained also a component rich in amino-acids.

The A-substance in hog gastric mucin is closely related serologically to the human blood group A factor. In view of the ease with which considerable amounts of hog gastric mucin could be obtained, it seemed a particularly suitable material for experimental study, the results of which could be employed subsequently as a basis for the elaboration of a method for the isolation of the blood group substances from human erythrocytes, tissues and tissue fluids. Cellular and tissue antigens, however,

are usually found to be labile complexes and it is becoming increasingly evident that if their isolation is to be achieved with the minimum of change in the physical, chemical and biological properties, it is essential to avoid conditions that involve extreme pH values, and to work with the material, as far as practicable, at low temperatures. The employment of these restricted conditions for the manipulation of molecular species of biological origin enables certain irreversible changes to be avoided or reduced to a minimum. With the above considerations in mind two methods have been elaborated for the isolation of the specific A-substance from commercial gastric mucin.

washed four times in this manner, and finally dried *in vacuo* (fraction 1, Table 1). The material showed no appreciable A activity as measured by either the haemolysis inhibition or isoagglutination inhibition test. The main aqueous extract, together with the washings after they had been concentrated to small volume, was warmed to about 35°, and 30% by weight of anhydrous Na₂SO₄ was added, in small portions at a time, to the vigorously stirred solution. The A-substance separated sharply between 27 and 30% of Na₂SO₄, as a sticky gelatinous mass, and was collected on the centrifuge. The material was suspended in water and after solution was reprecipitated with Na₂SO₄. In some preparations the A-substance was treated with Na₂SO₄ a third time. The final precipitate was dissolved in water, dialyzed at 0° until free from SO₄²⁻, spun to remove any insoluble residue, and dried *in vacuo* from the frozen state (fraction 2, Table 1).

Table 1. *The fractionation of an aqueous solution of crude gastric mucin by sodium sulphate precipitation*

Nature of fraction	Amount of fraction* (g.)	[α] ₅₄₆₁ (c, 0.5%)	Relative viscosity η	Total N (Kjeldahl) (%)	Ash (%)	Approximate minimum quantity (μ g.) inhibiting completely	
						(a) Isoagglutination	(b) A-haemolysis
(1) Insoluble in water, soluble in alkali	8.6	-45°	1.14†	11.2	3.8	100-200	—
(2) Precipitated from aqueous solution between 27-30% Na ₂ SO ₄	16.0	+ 8°	2.67	6.1	1.5	0.1	0.01
(3) Soluble in 30% Na ₂ SO ₄	3.0	-22°	1.22	3.8	3.9	25	2

* From 40 g. of a specimen of crude gastric mucin, of which 23% was diffusible through a cellophane membrane.

† In 0.05N NaOH.

EXPERIMENTAL

Isolation of the specific A-substance from gastric mucin

Method 1. Commercial gastric mucin, obtained from the Wilson Laboratories, Chicago, was suspended in saline or in water saturated with CO₂. The material, at a concentration of about 2%, was vigorously shaken and kept saturated with CO₂, until a thick viscous solution was obtained. Usually one or two days were required for the small hard particles of the crude dry gastric mucin to soften, swell and finally pass into solution. The thick,

Measured by its power to inhibit isoagglutination, fraction 2 is the most active A-substance yet obtained by us. Other constituents (fraction 3, Table 1) of the gastric mucin which were recovered from the Na₂SO₄ supernatant fluids showed much reduced capacity to inhibit the isoagglutination or haemolysis of A-erythrocytes by anti-A serum. The results of fractionating 14.5 g. of fraction 3 from aqueous solution by ethanol are set out in Table 2. The fractions show only slight A-activity, which amounts at the most to only a few per cent of the

Table 2. *The fractionation by ethanol precipitation of the 30% Na₂SO₄ soluble material (14.5 g.) obtained from crude gastric mucin*

Fraction no.	Limits of ethanol concentration for precipitation (% (v/v))	Weight of fraction (g.)	[α] ₅₄₆₁ (c, 0.5%)	Relative viscosity η	Total N (%)	Approximate dilution showing complete inhibition of	
						Isoagglutination	A-haemolysis
1	—	1.2	+36°	1.07	11.1	1:10 ⁴	1:10 ⁵
2	0-20	1.7	+ 4°	1.08	9.4	1:10 ⁴	1:2 × 10 ⁵
3	20-33	2.7	-14°	1.16	3.8	1:4 × 10 ⁴	1:6 × 10 ⁵
4	33-50	3.2	-15°	1.30	4.5	1:4 × 10 ⁴	1:10 ⁵
5	50-66	4.5	-32°	1.19	4.0	1:8 × 10 ⁴	1:2 × 10 ⁵

viscous suspension, at a concentration of about 1%, was centrifuged in the closed bowl of a Sharples centrifuge for 1 hr., and the slightly opalescent supernatant solution was then carefully withdrawn from the bowl by gentle suction. About a third of the original crude gastric mucin remained undissolved. The insoluble material was washed by suspending in saline or in water saturated with CO₂, being

activity of the purified A-substance. This material closely resembles the acid polysaccharide described by Meyer *et al.* [1937]. Further fractionation gave a main fraction with the following properties, [α]₅₄₆₁, -34°; N, 3.9%; limit of inhibition of isoagglutination, 1:20,000.

Method 2. Crude gastric mucin was ground in a mortar with 90% phenol. The solvent was added gradually, the material being worked up first to a paste, then to a thick suspension. A total volume of about 2 l. of phenol was used for each 100 g. of gastric mucin, and the final suspension was allowed to stand, with frequent shaking at room temperature, for 24–48 hr. The crude mucin was partly soluble, the insoluble portion being separated from the viscous solution by spinning in the Sharples centrifuge. The insoluble material from each 100 g. of crude mucin was resuspended in about 500 ml. of 90% phenol, allowed to stand for 12 hr., and again recovered from the phenol solution. After a further treatment with phenol this residue was washed with ethanol to remove phenol and dried *in vacuo* (fraction 1, Table 3). The main phenol supernatant fluid, together with the phenol washings from the phenol-insoluble residue, was treated with ethanol to precipitate the A-substance. The ethanol was added as a 1:1 phenol-ethanol mixture in order to avoid local precipitation of the A-substance, which was thrown out of solution as a gelatinous mass when the ethanol concentration reached 10% (v/v). The precipitate was collected, redissolved in half the original volume of 90% phenol, centrifuged to remove any undissolved material and reprecipitated with ethanol. Solution in phenol and reprecipitation with ethanol were both repeated a third time. The final clear, almost colourless jelly was immediately dialyzed at 0°, or was triturated with ethanol to remove excess phenol, and the resulting friable mass then suspended in water and dialyzed at 0° for several days to eliminate the last traces of phenol and

or the haemolysis of sheep cells by anti-A red cell serum, is much less than for the main A-substance fraction (fraction 2), which separated from the 90% phenol solution between 0 and 10% ethanol.

After the preliminary experiments had been completed, two preparations of the A-substance were made by method 1 and three large preparations by method 2. The yields of the final product were in each instance about 33% of the crude gastric mucin and in all the experiments described later in this paper it has not been possible to distinguish between the purified materials isolated by either of the two methods.

A preparation of mucin purchased in this country and purified by the two methods described above yielded purified A-substances that were very similar in properties to the A-substance described in this paper. The yield from the crude mucin was about 50%, the specific rotation of the purified material was, however, slightly less dextrorotatory, $+2^\circ \pm 2^\circ$ and the N was 4.9% or 5.1% according to the method of preparation used. The specimens gave rise to inhibition of isoagglutination at a dilution of $1:1.28 \times 10^6$. The relative viscosity (η , 2.45) was rather less than the value given for the A-substance derived from Wilson's gastric mucin.

Table 3. *The fractionation of crude gastric mucin from 90% phenol by ethanol*

Details of fraction	Amount of fraction* (g.)	[α] ₅₄₆₁	Relative viscosity in 0.9% NaCl†	Total N (%)	Ash (%)	Approximate minimum quantity (μ g.) inhibiting completely	
						(a) Isoagglutination	(b) A-haemolysis
(1) Insoluble in 90% phenol	36.9	+27°§	1.25	6.9	14.7	10–100	10–100
(2) Precipitated from 90% phenol by ethanol between 0–10% concentration	38.2	+10°	2.86	5.5	0.9	0.1	0.01
(3) Precipitated from 90% phenol by ethanol between 10–25% ethanol	20.7	–4°	1.24	8.0	2.0	1.0	0.1
(4) Precipitated from 90% phenol by ethanol between 25–50% ethanol	13.5	–67°	1.14	12.2	2.1	10	20
(5) Soluble in 50% ethanol phenol	14.7	–82°	1.05	10.4	5.2	No appreciable activity	

* From 145 g. of crude gastric mucin, of which, on dialysis, about 20% passed through a cellophane membrane.

† Relative viscosity of 0.3% NaCl taken as 1.00.

§ In 0.1N NaOH.

‡ Determined by Kjeldahl's method.

|| Uncorrected for ash.

ethanol. The clear viscous solution was then frozen at -20° and dried *in vacuo* from the frozen state (fraction 2, Table 3).

The properties of the other fractions obtained by the addition of ethanol to the pooled 10% ethanol solutions are also given in Table 3. The material precipitated from the 10% ethanol-phenol supernatant fluid on raising the ethanol concentration to 25% (v/v) contains an appreciable amount of A-substance which can, however, be readily recovered by repeating the fractionation process. It will be seen that the specific activity, as measured by the capacity of the fractions to inhibit isoagglutination

RESULTS

Physical and chemical properties of the A-substance

The material obtained by drying a neutral aqueous solution of the A-substance from the frozen state is of a white, silky, almost crystalline appearance. It dissolves readily in water or saline, and a 1% solution shows considerable viscosity and is faintly opalescent. A 2–3% solution behaves as a viscous solution with pronounced elastic properties, and at a concentration of 4–5% the viscous solution fails to flow out of an inverted test-tube.

Several preparations were examined after solution in buffer acetate (pH 4.0) or phosphate (pH 8.0) buffer (μ , 0.1), in the Tiselius electrophoresis apparatus, by our colleague Dr R. A. Kekwick, who reported (see Addendum) that specimens obtained by either method were essentially homogeneous. Electrometric titration of an aqueous solution of the A-substance failed to reveal any group ionizing between pH 2.0 and 10.5.

The analytical figures for these preparations are recorded in Table 4. The amount of organically bound P was between 0.05 and 0.1 % in all preparations. Sulphur was absent. The acetyl estimations

Harte [1940], reduced the relative viscosity of the A-substance to 1.09, a figure only slightly higher than that for 0.90 % saline. In view of the ease with which the viscosity of the A-preparations was destroyed it was considered of interest to investigate the influence, on viscosity and serological activity, of heating the material dissolved in buffered saline at pH 3.0, 5.0, 6.9 and 8.0. The change in viscosity with time of heating an 0.5 % solution at different pH values is shown in Fig. 1. The solutions were sealed in ampoules before heating. The rapidity with which the viscosity is lost when the A-substance is heated on the alkaline side of neutrality

Table 4. *The analytical data for preparations of the A-substance isolated from gastric mucin*

Method of preparation and no.	Weight of preparation (g.)	[α] _{D461} (0.5% in H ₂ O)	Relative viscosity (0.5% in saline)	Content (%) of				
				C	H	N*	Ash	Acetyl
Phenol extraction								
GM. 201	30	+11°	2.80	—	—	5.7	1.4	9.6
GM. 210	33	+14°	2.86	45.2	6.6	5.9	0.9	10.7
GM. 240	38	+10°	2.83	45.1	6.7	6.0	0.9	10.3
Aqueous extraction								
GM. 280	9	+ 8°	2.78	44.8	6.4	6.4	0.8	9.2
GM. 321	20	+12°	2.35	45.9	6.4	6.2	0.6	11.5

* By Dumas's method.

were carried out by the method of Elek & Harte [1936], but, although accurate and reproducible results were obtained with N-acetylglucosamine, the results with A-substance differed by as much as 15 %. The source of error was not detected, although the possibility of acyl residues other than acetyl was considered. Acetic acid was isolated as Ag salt (found, Ag 64.6; calc. 64.7 %) from an acid distillate of A-substance. The yield was of the same order (75 %) as was obtained in similar estimations on acetic acid.

Viscosity. The relative viscosity values of 0.5 % solutions of the preparations in 0.9 % NaCl were determined at 37° by means of a modified Ostwald viscosimeter. For the three large preparations of A-substance extracted by means of phenol, the figures were 2.80, 2.86, 2.83, whereas two preparations, isolated by means of aqueous extraction, gave values of 2.78 and 2.35; the viscosity of 0.90 % NaCl being taken as 1.00. After heating with 0.1N acetic acid at 95° for 10 min., with removal of the acid by dialysis, and recovery of the product by drying *in vacuo* from the frozen state, the A-substance, dissolved in 0.90 % NaCl, showed a relative viscosity of 2.07. Material which had been treated with 2 % Na₂CO₃ at 70° for 15 min. [Meyer *et al.* 1937], and likewise recovered after dialysis, also showed a considerably decreased viscosity (η , 1.27) and treatment of the undegraded high viscosity A-substance with anhydrous formamide at 150° for 1 hr., according to the technique used by Landsteiner &

(pH 8.0) is of interest and indicates that the A-substance should on no account be heated at

Fig. 1. Curves showing the fall in viscosity of undegraded A-substance on heating at 100° in buffered saline at pH 3.0, A; pH 5.0, B; pH 6.9, C; pH 8.0, D.

alkaline reaction. It is clear that on the acid side of neutrality the A-substance is definitely more stable, although considerable loss in viscosity takes

place on prolonged heating, even at pH 5.0 or 6.9. It is evident that such treatments induce a greater or lesser degree of physical degradation according to the conditions selected. The profound influence of some of these methods of treatment on the capacity of the A-substance to inhibit isoagglutination is shown in Table 5 and is discussed later.

An aqueous solution of the A-substance does not precipitate when treated with an equal volume of 2% solutions of the following reagents: picric acid, trichloroacetic acid (10%), aluminium sulphate, phosphotungstic acid (with or without 5% H_2SO_4), lead acetate, ferric chloride, potash alum, Fehling's solution and silver nitrate. The addition of 0.01M mercuric acetate, however, causes immediate gelation. Similarly, the addition of an equal volume of 0.05M borate buffer (pH 8.5) to a 1.0% solution of the A-substance gives rise to gel formation; the system, however, still retains pronounced elastic properties. The elastic quality may be readily shown by rotating a thin gelatinous solution in a suitable vessel and observing the return motion that follows immediately the original rotational motion has stopped. Air bubbles rising through a gel prepared in this manner from a 1% solution assume a pear shape, the tail of each bubble remaining attached to the bottom of the container and becoming long and thin as the bubble slowly rises to the surface. The sol-gel change is reversible. Dialysis of the gel at 0° against distilled water leads to the formation of the original viscous solution; the addition of borate buffer will again give rise to a firm, elastic gel. These reversible sol-gel changes do not occur with degraded preparations of the A-substance.

Chemical composition. The A-substance gives no positive colour test when treated directly with *p*-dimethylamino-benzaldehyde but gives rise to a strong purple colour if previously heated at 100° with 0.05N Na_2CO_3 for 6 min., according to the method of Morgan & Elson [1934]. The colour developed is equivalent to that given by a content of 12–13% of N-acetylglucosamine. This high value was difficult to explain in view of the fact that the preparations of A-substance showed only 0.4–0.6% of reduction by Somogyi's [1926] copper reduction method. In order to determine the N-acetylhexosamine colorimetrically, however, the A-substance must be heated with 0.05N Na_2CO_3 and it was found that during this treatment the reducing power of the substance increased rapidly to a value between 3.0 and 3.5% for different preparations. Heating for 6–30 min. gave rise to a maximum figure of 3.6% reduction. The reducing power of N-acetylglucosamine, determined by the above method, is equivalent to 27% of an equal weight of glucose; therefore, if we assume the whole of the reduction (3.3% average value) is due to N-acetyl-

glucosamine, then the N-acetylglucosamine colour should be equivalent to 12.2% N-acetylglucosamine, whereas values between 12 and 13% were found for different preparations. Several complex polysaccharide substances that were known to contain hexosamine units, such as hyaluronic acid, chondroitinsulphuric acid and the O somatic antigens of *Shigella dysenteriae* and *Eberth. typhosa*, as well as the simple methylhexosides, N-acetyl- and N-benzoylmethyl glucosamides, failed to give any colour when tested with Ehrlich's reagent after heating with 0.05N Na_2CO_3 . These results suggest that within the A-polysaccharide complex there exists an alkali-labile glycoside linkage involving the first carbon atom of the N-acetylhexosamine component. We have observed that the reddish purple colour develops with the A-polysaccharide rather more slowly than with N-acetylglucosamine; colorimetric readings should, therefore, be taken at the end of 90 min., rather than after 45 min. as originally suggested. An identical colour reaction, representing 10–11% N-acetylglucosamine, is given by the formamide-degraded A-substance.

The immunological properties of the A-substance

A measure of the distribution of the blood group A-substance throughout the different fractions arising during the process of isolation was obtained by means of the ability of the A-factor to (1) inhibit isoagglutination and (2) prevent the haemolysis of sheep erythrocytes by an anti-A rabbit immune serum.

The isoagglutination inhibition test was carried out as follows. Diminishing concentrations of the preparation under test (0.1 ml.) were mixed with 0.1 ml. of a human anti-A (α)-serum, representing in amount 3–4 completely agglutinating doses, and allowed to stand at room temperature for 1 hr. Freshly drawn A-erythrocytes (0.5% in 0.9% NaCl) were then added (0.1 ml.). The mixtures were thoroughly shaken, set aside for 2 hr. at room temperature and then examined for isoagglutination. The dilution of the A-preparation allowing minimal agglutination—that is, a few groups of 2–3 cells present in an otherwise unagglutinated microscopic field—was taken as the end-point of inhibition. The sterile anti-A serum, used as a standard α -agglutinin reagent throughout the investigation, was stored without antiseptic in sealed ampoules at -20° . The serum was tested from time to time against the erythrocytes of four group A₁ persons of average sensitivity, working in the laboratory; the agglutination end-point remained constant, throughout the investigation, at a dilution of 1:192. An additional check on the inhibition test was obtained by including a 'standard' purified gastric mucin preparation in each batch of tests; the inhibition titre obtained with this material remained unchanged over a period of 2 years.

The haemolysis test was made as follows. Dilutions of the gastric mucin (0.5 ml.) were mixed with 2–3 minimal haemolytic doses (M.H.D.) of anti-A rabbit immune serum (0.5 ml.), and 0.5 ml. of 1:10 dilution of fresh guinea-pig complement was added to each tube. The mixture was incubated at 37° for 1 hr. and 0.5 ml. of a 4% suspension

Table 5. *The inhibition of A-isoagglutination by different preparations of A-substance obtained from gastric mucin*

A-substance prepared by	Subsequent treatment	Degree of isoagglutination										
		Dilution* of A-preparation used in test										
		1:10 ⁴	1:2 × 10 ⁴	1:4 × 10 ⁴	1:8 × 10 ⁴	1:1.6 × 10 ⁵	1:3.2 × 10 ⁵	1:6.4 × 10 ⁵	1:1.28 × 10 ⁶	1:2.56 × 10 ⁶	Con- trol	
Method 1 (water or saline extraction and Na ₂ SO ₄ fractionation)	Untreated	0	0	0	0	0	0	1	1	2	4	
	Autoclaved (121°) 15 min. at 15 lb./sq. in.	0	0	0	0	0	1	2	2	3		
	Heated to 100° for 2 hr. in aqueous solution	0	0	0	0	0	1	2	3	3		
	Fractionated between 90 and 92% acetic acid	0	0	0	0	0	0	1	2	2		
Method 2 (phenol-extraction)	Heated in 2% Na ₂ CO ₃ at 70° for 15 min.	0	0	1	1	2	2	3	4	4		
	Untreated	0	0	0	0	0	0	0	1	2	4	
	Autoclaved (121°) 15 min. at 15 lb./sq. in.	0	0	0	0	0	0	1	2	2		
	Heated to 100° for 2 hr. in aqueous solution	0	0	0	0	0	1	1	2	3		
Material from crude gastric mucin in-soluble in 90% phenol	Heated in 2% Na ₂ CO ₃ at 70° for 15 min.	0	0	0	1	1	2	3	3	—		
	Heated in formamide, 1 hr. at 150°	0	1	2	2	3	3	3	3	—		
	None	1	2	4	4	4	4	4	—	—		
	None	4	4	4	4	4	4	4	—	—		

* Final dilution in the tubes is three times the dilution given in the table.

Table 6. *The inhibition of the haemolysis of sheep cells in 2-3 M.H.D. of anti-A rabbit serum by preparations of A-substance*

A-substance prepared by	Subsequent treatment	Degree of haemolysis										
		Dilution* of A-material used in test										
		1:10 ⁵	1:10 ⁶	1:2 × 10 ⁶	1:4 × 10 ⁶	1:8 × 10 ⁶	1:1.6 × 10 ⁷	1:3.2 × 10 ⁷	1:6.4 × 10 ⁷	1:1.28 × 10 ⁸	Con- trol	
Method 1 (water or saline extraction and Na ₂ SO ₄ fractionation)	Untreated	0	0	0	0	0	0	1	2	4	4	
	Autoclaved (121°) 15 min. at 15 lb./sq. in.	0	0	0	0	0	0	1	1	2		
	Heated to 100° for 2 hr. in aqueous solution	0	0	0	0	0	0	1	2	4		
	Fractionated between 90 and 92% acetic acid	0	0	0	0	0	0	0	1	3		
Method 2 (phenol extraction)	Heated in 2% Na ₂ CO ₃ at 70° for 15 min.	0	0	0	0	0	0	1	2	3		
	Untreated	0	0	0	0	0	0	1	2	3	4	
	Autoclaved (121°) 15 min. at 15 lb./sq. in.	0	0	0	0	0	0	1	2	2		
	Heated to 100° for 2 hr. in aqueous solution	0	0	0	0	0	0	0	1	2		
Material from crude gastric mucin in-soluble in 90% phenol	Heated in 2% Na ₂ CO ₃ at 70° for 15 min.	0	0	0	0	0	0	1	2	3		
	Heated in formamide, 1 hr. at 150°	0	0	0	0	0	0	1	2	4		
	None	4	4	4	4	4	4	4	4	4		
	None	4	4	4	4	4	4	4	4	4		

* Final dilution in the tubes is four times the dilution given in the table.

of washed sheep red cells was added. The greatest dilution of the A-preparation allowing just visible haemolysis to occur was taken as the end-point of the haemolysis inhibition. The control tube containing saline in place of the gastric mucin dilution showed complete haemolysis in 30 min. Because of the variable nature of the haemolytic system all the results given in Table 6 were obtained in a single experiment and are therefore strictly comparable.

The results of titrations by both serological tests are given in Tables 5 and 6. The end-point of inhibition of isoagglutination given by preparation GM. 280, which was prepared by aqueous extraction of the crude mucin (method 1), is not significantly different from that given by preparation GM. 240, which was obtained by the use of 90% phenol (method 2). The first traces of isoagglutination appear in the tube containing 0.1 ml. of a 1.64×10^5 (GM. 240) or 1.128×10^6 (GM. 280) dilution of the A-preparation. The absolute amount of the A-substance present is about $0.1 \mu\text{g}$. It will be observed from the results (Table 5), however, that heating these preparations in neutral saline at 100° for 120 min. causes a decrease (50–75%) in the power of the preparations to inhibit isoagglutination. Similarly, treatment with 2% Na_2CO_3 at 70° for 15 min. reduces considerably the activity of the preparation, and treatment with formamide at 150° for 1 hr. destroys more than 95% of the power of the preparation to inhibit isoagglutination. A 1% solution of the A-substance in 0.05N Na_2CO_3 shows less than 1% of the original activity (inhibition of isoagglutination) after heating at 100° for 15 min. After heating at pH 3.0, 6.9, 5.0 and 8.0 in sealed ampoules for 5 hr. at 100° , the capacity of the A-polysaccharide to inhibit isoagglutination fell to 12, 25, 25 and 2% respectively of the original value.

When the serological activity of the A-preparation is measured by the haemolysis inhibition test, however, a different picture is presented (Table 6). Again the serological activity of the undegraded material prepared by either method appears to be the same, and the absolute amount of A-substance, giving rise to almost complete inhibition of haemolysis when mixed with 2–3 M.H.D. of an anti-A rabbit serum prepared against human erythrocytes, is about $0.01 \mu\text{g}$. Treatment with 2% Na_2CO_3 at 70° for 15 min., with formamide at 150° for 1 hr. or with neutral saline at 100° for several hours, however, causes no destruction of the capacity of the preparation to inhibit haemolysis. Indeed, it will be seen that a slight increase in activity occurs on heating the A-substance at 100° for 2 hr. An increase in activity of this order for formamide-treated material has already been reported by Landsteiner & Harte [1940]. The use of an anti-serum (rabbit) to gastric mucin (2–3 M.H.D.), in place of the anti-A erythrocyte serum gave similar results [Morgan, 1943].

A specimen of the purest preparation of A-substance was tested for antigenicity in rabbits, 0.1 or 1.0 mg. being injected intravenously at 3–4 day intervals. After three or six doses, serum samples were collected 7 days after the last dose and titrated for anti-A immune body by means of the agglutination reaction with fresh human A-erythrocytes. In no instance was any significant rise in anti-A (α)-agglutinins recorded. An account of the immunological properties of the purified A-substance is given in greater detail elsewhere. It will suffice here to state that the conversion of the A-substance into a full antigen possessing A specificity has already been accomplished [Morgan, 1941; 1943].

Attempts to remove the amino-acid-containing components from the A-substance

In some respects the chemical nature of the A-substance resembles that of the O antigens of certain gram-negative bacteria. These biologically important complexes contain a large proportion of polysaccharide, a conjugated protein component making up the remaining part of the structure essential for the manifestation of antigenicity. Earlier work [Morgan & Partridge, 1940; 1941; 1942; Partridge & Morgan, 1940] on the nature of these substances has shown that in certain instances the bacterial antigenic complex can be dissociated into its component molecules by special means, and this knowledge suggested that the A-substance should be submitted to similar procedures with the hope of attaining a complete or partial dissociation of the component molecules. The methods employed and the results obtained are briefly described.

Fractionation from phenol solution. A-substance (2.0 g.) was treated with 250 ml. of 90% phenol, allowed to stand for 24 hr. and spun for 1 hr. in a Sharples centrifuge. The thin gelatinous substance which collected on the walls of the centrifuge bowl, and which represented the portion of the A-substance that was less readily soluble in phenol, was washed with ethanol to remove phenol, and dried. It weighed 0.83 g., possessed $[\alpha]_{\text{D}}^{25} + 13^\circ$, contained 5.8% N and showed a relative viscosity in saline of 2.85. The dilution that showed almost complete inhibition of isoagglutination was 1.64×10^5 . The phenol supernatant fluid was dialyzed until free from phenol and evaporated *in vacuo* to dryness. The material recovered weighed 0.56 g., showed $[\alpha]_{\text{D}}^{25} + 8^\circ$, contained 5.4% N and was only slightly less active than the original material in inhibiting isoagglutination. Relative viscosity, η , 1.59.

It has been observed that dry preparations of the purified A-substance prepared by either of the methods described are never completely soluble in 90% phenol, although originally the material was readily soluble in liquid phenol of this strength and could be repeatedly dissolved in the solvent and precipitated by the addition of 10% ethanol. The fractional extraction of the purified and dry A-

substance with 90% phenol indicates that the preparation is most probably chemically homogeneous, but is certainly not homogeneous with regard to particle size. The high viscosity of the phenol-insoluble portion when dissolved in neutral saline suggests that the A-substance is present as long chain aggregates of considerable molecular size, whereas the low viscosity of the phenol soluble portion indicates that this material consists of depolymerized aggregates having smaller chain lengths in aqueous solution.

Fractionation from formamide solution. The A-preparation (3.0 g.) was dissolved in 125 ml. of anhydrous formamide, cooled to 0° and treated with an equal volume of ethanol previously cooled to -20°. No precipitate was formed up to 45% (v/v) ethanol but a considerable amount of material separated when the ethanol concentration was increased to 50%. The preparation was collected and the material remaining in the ethanol-formamide was recovered by dialysis and evaporation. The substance thrown out of solution by ethanol was again dissolved in formamide, and the precipitation repeated. The thrice precipitated material weighed 1.1 g., showed $[\alpha]_{5461} + 16^\circ$, contained 5.4% N and was active in inhibiting isoagglutination at a dilution of $1:6.4 \times 10^5$; η , 2.87; ash, 0.4%. The material recovered from the combined ethanol-formamide solutions weighed 1.07 g., showed $[\alpha]_{5461} + 18^\circ$; N, 5.7%; η , 1.57; and was, by both serological tests, as active as the original material.

Fractionation from aqueous solution by organic solvents. A 1.6% solution of the A-preparation was used in each experiment. Treatment with acetone gave rise to two fractions, collected over the ranges 39–41% (v/v) and 41–50% (v/v) of acetone. Practically the whole of the material was precipitated by ethanol between the levels 47–50% (v/v) and by acetic acid between 90 and 92% (v/v). In each instance, however, the precipitated products possessed the same specific rotation, N content and serological titre as the original material.

Fractionation with ammonium sulphate. A-substance (5 g.), dissolved in 300 ml. water, was fractionated by addition of solid ammonium sulphate. Up to 72% of saturation, only a small amount of material separated, and this appeared to be similar to the original preparation. The main bulk (2.74 g.) of the A-substance was precipitated on raising the ammonium sulphate concentration to 81% of saturation. The material was collected, dialyzed free from the salt, precipitated with ethanol and dried *in vacuo*. The properties, $[\alpha]_{5461} + 13^\circ$; N, 5.4%, of this fraction were substantially the same as those of the original material. Removal of the salt from the 81% saturated supernatant fluid by dialysis and recovery of the material, however, gave rise to 0.62 g. of material, which was of low serological activity as measured by the isoagglutination inhibition test. The substance showed, $[\alpha]_{5461} - 14^\circ$, and possessed a relatively low N content (4.6%). The main fraction (2.74 g.) was redissolved in water and again fractionated as above, yielding a further 0.12 g. of similar material showing *laevo* rotation and low N. A third fractionation of the main fraction gave an additional small quantity of material showing similar properties, $[\alpha]_{5461} - 5^\circ$ N, 4.3%, and possessing very low activity measured according to the isoagglutination and isohaemolysis inhibition tests. At first it was thought that these fractions, which were readily soluble in high concentrations of ammonium sulphate, and

differed sharply from the original A-substance in chemical and serological properties, might represent the partly separated polysaccharide component of the A-substance. No definite evidence for this conclusion has, however, been obtained.

The above experiment yielded a highly purified sample of the A-substance. This material, thrice fractionated with ammonium sulphate, showed the following properties; $[\alpha]_{5461} + 12^\circ$; N, 5.63%; η , 2.59: maximum dilutions showing almost complete inhibition of isoagglutination, $1:6.4 \times 10^5$, and of haemolysis $1:32 \times 10^6$, respectively.

Fractionation with ammonium sulphate from alkaline solution. The technique was similar to that described above, but after each addition of the salt NaOH was added to maintain alkalinity. Only a single fractionation was performed. The results were similar to those obtained with the neutral solution of the material described above. The small fraction (5%) soluble in high concentrations of the salt showed the following properties $[\alpha]_{5461} - 27^\circ$; N, 4.6%. The activity of this material, as measured by both serological tests, showed only a few per cent of the original value.

Fractionation after heating with acetic acid. The possibility of separating the amino-acid component from the A-substance after degradation of the complex by heating with dilute acetic acid was investigated. A 1.6% solution (100 ml.) was heated at 100° with 1% acetic acid for 2 hr. The opalescence of the solution increased until it became quite opaque, but no material separated from solution. Without neutralization of the acid the solution was treated with ammonium sulphate. The first and main fraction was thrown out of solution between 70 and 85% saturation with the salt. This material separated, during dialysis, into a water-soluble and a water-insoluble part; the latter fraction, however, dissolved readily on the addition of a little Na_2CO_3 . The water insoluble-material weighed 0.55 g., showed $[\alpha]_{5461} + 9^\circ$, contained 5.9% N and was as active in the inhibition of haemolysis as the original material. The water-soluble fraction showed a lower relative viscosity (η , 1.37) compared with the original unheated material (η , 2.83), the rotation was somewhat higher, $[\alpha]_{5461} + 20^\circ$, and the N content was unchanged (5.6%). This material, apart from being degraded by treatment with hot acetic acid, was essentially unchanged chemically, but the capacity to inhibit isoagglutination was only about one-quarter of the original value. Once again the small fraction of the original material soluble in high concentrations of ammonium sulphate (above 85% saturation) showed a *laevo* rotation, $[\alpha]_{5461} - 20^\circ$, a somewhat lower N (4.9%) and was only weakly active in the isoagglutination and haemolysis inhibition tests.

The action of trypsin. A purified preparation of gastric mucin A-substance (1 g.) was dissolved in 100 ml. of 15% urea solution and the pH was adjusted to about 9.0 with Na_2CO_3 . Commercial trypsin (100 mg.) was then added and the mixture incubated at 37° for 6 hr. The solution was then dialyzed at 0° to remove urea, and concentrated to about 10 ml. Solid ammonium sulphate was added with thorough mixing and three fractions were removed. A small amount of material (45 mg.) separated on the addition of ammonium sulphate up to 72% of saturation. The material contained 5.5% N but was not clear enough for polarimetric examination even after the addition of alkali. The second and main fraction (0.62 g.), separating between 72 and 79% saturation, contained 5.6% N and showed $[\alpha]_{5461} + 12^\circ$. A small amount of material (60 mg. N, 4.7%) separated between 79% and full saturation at 18°, and a

further 65 mg. of material was finally recovered from the saturated ammonium sulphate solution. All fractions, with the exception of the last (not tested), showed no very significant change in serological activity. The treated material, however, had a lower viscosity (2.17) than the original preparation.

Treatment with chloroform-amyl alcohol. A 2% solution of A-substance ($[\alpha]_{5461} + 12^\circ$; N, 5.70%) was shaken with an equal volume of CHCl_3 -amyl alcohol mixture according to the method of Sevag [1934]. The aqueous layer, after several treatments with the CHCl_3 -amyl alcohol mixture, was fractionated with ammonium sulphate. The main fraction, 63% of the original material, separated between 73 and 82% of salt, showed a specific rotation $[\alpha]_{5461} + 12^\circ$ and contained 5.25% N. An additional 10% was recovered from the supernatant fluid. This fraction showed a lower N content but was otherwise similar to the main fraction. Both materials gave inhibition of isoagglutination at a dilution of $1:6.4 \times 10^5$.

It would appear that the application of the techniques described have in no instance succeeded in removing the amino-acid components from the polysaccharide moiety of the A-substance. The application of certain of these procedures, however, removes about 5% of material which shows a *laevo* rotation and possesses a lower N and a considerably diminished specific activity when compared with the purified A-substance.

Acid hydrolysis of the A-substance

The A-substance is readily hydrolyzed by heating in N HCl at 100° . Complete liberation of the component amino-acids, however, is only attained after hydrolysis with 6N HCl. The hydrolysis was carried out in sealed ampoules, samples being removed from time to time and immediately neutralized.

during hydrolysis was followed by Van Slyke's method [1929] and it is to be noted that any free amino-groups liberated through the elimination of the acetyl groups from N-acetylhexosamine are included in the results. The amino-acids set free during the hydrolysis were estimated by the method of Van Slyke & Dillon [1938] as modified by Van Slyke, Dillon, MacFadyen & Hamilton [1941] and the results are expressed as 'carboxyl-N'. Glucosamine is not determined under these conditions. 'Hexosamine' was determined according to the method of Elson & Morgan [1933] and is expressed as glucosamine. N-acetylglucosamine was found to be converted almost completely to glucosamine by heating with N HCl at 100° for 2 hr. Reducing substances reach a maximum value after about 4 hr. heating, whereas the optical rotation, the 'hexosamine' and the amino-N reach maximum values between 6 and 12 hr. hydrolysis. The maximum figure for amino-acid is reached only after several hours' heating with 6N HCl.

The maximum figures for amino-acid N (2.2%) and for hexosamine-N (2.3%) are together approximately equal to the maximum amino-N value (4.5%). During the early stages of the hydrolysis, however, the total amino-N value (Van Slyke's method) is greater than the sum of the amino-acid and hexosamine figures. This is most probably due to the fact that the Van Slyke method assays the terminal NH_2 groups of polypeptides, whilst the amino-acid determination (ninhydrin method) is specific for free amino-acids only. There remains a considerable discrepancy between the maximum amino-N value (4.5%) and the total N of the preparation (5.7%). If, however, all the N (3.5%) not

Table 7. *The hydrolysis of A-substance by HCl at 100°*

Acid strength	Time of heating (hr.)	$[\alpha]_{5461}$ ($c=1\%$, $l=1$)	Reducing power (glucose = 100)	Hexosamine* (%)	Nitrogen (%)			
					Total	α -Amino	Amino-acid	Hexosamine
N	0	+0.11°	0.5	0.0	5.72	0.19	0.0	0.0
	1	+0.35°	45	24.3	—	3.14	0.19	1.90
	6	+0.52°	50	26.9	—	4.02	0.46	2.05
	12	+0.51°	45	27.3	—	4.20	0.80	2.17
	24	+0.50°	—	26.6	—	4.29	1.32	2.24
6N	1	+0.44°	42	27.0	—	4.27	0.99	2.19
	6	+0.32°	43	24.0	—	4.55	2.17	1.88
	12	+0.24°	—	—	—	3.66	1.75	—

* Estimated by the colorimetric method.

The analytical figures obtained during the course of the hydrolysis of a typical preparation are given in Table 7. The figures largely confirm the results obtained earlier by Landsteiner & Harte [1940]. The reducing power was determined by Somogyi's modification [1926] of the Shaffer & Hartman method [1921] and the results are expressed in terms of glucose. The liberation of α -amino groups

accounted for as hexosamine is assumed to be in the amino-acid component, it follows that the latter comprises about 3.5×6.4 or 22% of the total complex. The maximum figure obtained for reducing substances is 52%, calculated as glucose. Differentiation of the reducing substances into fermentable and non-fermentable components by the rapid yeast 'absorption' method of Harding & Selby [1931]

revealed that the fermentable sugar did not exceed 3-4 % of the maximum reducing substances at any stage of the hydrolysis. If the hexosamine present is considered as glucosamine, which has quantitatively the same reducing power as glucose under the conditions of estimation employed, then the hexosamine accounts for 27 % of the reduction. If the remaining reduction (25 %) is assumed to be due to galactose, then this sugar is present to the extent of 32 %, since its reducing power, relative to glucose, is only 72 %. Qualitative tests for the presence of individual amino-acids show that tyrosine and cysteine are absent or present in very small amounts.

DISCUSSION

The isolation of the specific A-polysaccharide substance from crude gastric mucin by methods that avoid the use of mineral acid or of alkali, and enable the isolation procedures to be carried out at normal temperatures, is described.

The methods give an excellent yield of a serologically active polysaccharide-amino-acid complex, which possesses in full the property shown by crude gastric mucin of inhibiting the isoagglutination of A-erythrocytes by natural anti-A (α)-agglutinin (human), retains completely the high viscosity of the original mucin, and is essentially homogeneous when examined at pH 4.0 or 8.0 in the Tiselius electrophoresis apparatus. The material can be considered as the blood group A-substance largely in its undegraded state and it is noteworthy that these two radically different methods for the isolation of the active substance yield apparently identical products.

The A-substance prepared by either method is similar in composition to that described by Landsteiner & Harte [1940]. There are, however, a number of important differences in the physical and serological properties of our preparations of A-substance and those described by other workers. Preparations of A-substance made by either of the methods described in this paper show not only high serological activity, as determined by the inhibition of isoagglutination, but also considerable viscosity, and the property of forming an elastic gel on the addition of borate buffer at pH 8.5. The latter property is readily lost and it has been found that even slightly degraded preparations of the A-substance fail to form an elastic gel on the addition of the borate buffer. Thus, the addition of this reagent to a solution of A-substance will reveal at once whether the material has been degraded during the course of its preparation. In addition to the rapid decrease in the viscosity of the A-substance that arises from heating solutions of the substance, there is a steady fall in the capacity of the preparation to inhibit the isoagglutination of A-cells by either

natural human anti-A (α)-agglutinin or by the anti-A agglutinin of rabbit immune serum. The insensitive nature of the agglutination reaction as usually carried out, with progressively doubled dilutions of the A-substance, does not allow small changes in the capacity of an A-preparation to inhibit isoagglutination to be observed. For this reason it is not possible to detect a decrease in the power of a preparation to inhibit isoagglutination when the destruction is much less than 50 % of the original value. Nevertheless, it is apparent that heating at 100° or simple sterilization at 120° in neutral solution causes a loss in isoagglutination inhibiting activity, whereas almost complete destruction of this particular serological activity is brought about by heating with formamide or warming with dilute sodium carbonate. Although exposure to these conditions leads to drastic loss in viscosity and almost complete destruction of the power to inhibit isoagglutination, it is of interest to find that the resulting substance in each instance shows unchanged the property of inhibiting the haemolysis of sheep cells by anti-A rabbit immune serum. Indeed, there is evidence that heating with formamide may even increase this particular activity, as was originally observed by Landsteiner & Harte [1940]. These workers stated that the increase in anti-lytic activity is most probably due to a structural change, since 95 % of the starting material with about twice the potency could be recovered after this treatment. Jorpes & Norlin [1933; 1934] and Jorpes [1934] reported that the action of trypsin on the blood group A factor in human urine considerably decreased the anti-agglutinating activity but that the anti-lytic activity was not destroyed. They found that anti-agglutinating and anti-lytic factors could be separated by precipitating the anti-agglutinating factor from solution by tannin. Jorpes & Norlin consider that the anti-agglutinating factor is in this instance protein in nature, the anti-lytic substance being essentially polysaccharide. With material of high anti-lytic activity, we have not so far detected any chemical difference between preparations of the A-substance that show high or low activity in inhibiting isoagglutination and Landsteiner & Harte [1940] reported that they were unable to effect a separation of the A-substance into material showing different chemical and serological properties. Degradation of the native A-substance under the conditions devised by earlier workers for its isolation and purification almost certainly accounts for the low viscosity and much decreased power to inhibit isoagglutination, both of which are recorded in the literature for purified preparations, whereas the stability of the haemolysin-inhibiting property, when exposed to similar conditions, offers an explanation for the preservation of the intense anti-

haemolytic activity of these preparations. It must be emphasized that up to the present the preparations of A-substance, used by different workers, have been obtained almost exclusively from one source. This material, Wilson's gastric mucin (Type 1701-W), is the one most commonly used by bacteriologists for enhancing the virulence of bacterial cultures. We have examined one specimen of gastric mucin obtained in this country from Light and Co. and have found it to be very similar to the Wilson product. It is not known to what extent the A-factor in these commercial materials is degraded during their preparation. Our observations on the ease with which certain properties of the A-substance are destroyed suggest that further investigation of the properties of native gastric mucin should be undertaken with respect to its power to inhibit isoagglutination and A specific haemolysis and the relation of these activities to viscosity and power to increase the virulence of bacterial cultures. It is of interest that we have isolated from the fluid of pseudo-mucinous ovarian cysts a polysaccharide complex that is two or three times as active in inhibiting A-isoagglutination as the most active material isolated from hog gastric mucin. This material is, however, less viscous than hog gastric mucin A-substance.

It is not possible to compare accurately the activity of our preparations with those of other workers, because in many instances little or no indication is given of the strength of the anti-A (α)-agglutinin or of the anti-A rabbit haemolysin used in the tests. By replacing our test dose of anti-A (α)-serum by a quantity of serum only just sufficient to give complete agglutination of the A-cells used in the test, we found the amount of A-substance giving rise to a significant although incomplete inhibition of isoagglutination to be about 0.01 μ g. Similarly, haemolysis-inhibition tests in which 1 M.H.D. only of the lytic agent was employed showed that as little as 0.001 μ g. of the undegraded A-substance could be detected. Smaller absolute values which are, however, of doubtful value, can readily be obtained if the same pipette is used throughout to make the serial dilutions of the A-substance.

The question arises as to why the A-substance, which contains amino-acids as an integral part of the molecular complex and is superficially analogous to the O antigens of certain bacteria, is not itself antigenic. Closer examination shows, however, that the A-substance is not truly analogous to these bacterial antigens, which appear to be large poly-molecular complexes built by the combination of polysaccharide and protein molecules through secondary valency bonds. Some of these biologically important aggregates can be dissociated into their component molecules with complete loss of the

original specific antigenic property. The A-substance, however, is not separable into its component molecules by these procedures and it would appear, therefore, that the amino-acid components are in this instance more firmly linked, most probably by normal primary valency bonds, to the carbohydrate units of the polysaccharide molecules. Furthermore, it seems probable that the amino-acids are present either as single molecules or as short polypeptide chains and that the A-substance does not in fact contain an intact protein component. In support of this suggestion it has been shown that the A-substance can be converted into a full antigen that gives rise to specific A immune-body, by combination with a suitable protein molecule. The conditions employed to synthesize this functional poly-molecular aggregate suggest that the component molecules—A-substance and conjugated protein—are held together by secondary valency bonds only. An additional factor, however, that may well be responsible for the non-antigenicity of the A-substance, is its lack of tyrosine. The absence of this amino-acid has generally been considered to render a protein devoid of antigenic properties, although some evidence against this view has recently been given.

The preparation of the purified A-substance in large amounts has permitted an investigation into the nature of the constituent in commercial gastric mucin that is responsible for its 'virulence enhancing' power, a property which bacteriologists and immunologists have put to much practical use during the last few years. The undegraded A-substance appears to possess 'virulence enhancing' properties, whereas all other substances in gastric mucin, including the phenol-soluble protein component, are inactive in this respect. Any treatment, however, such as sterilization by heat, which destroys the viscosity of the A-substance and reduces the capacity of the preparation to inhibit isoagglutination (even when the power of the resulting material to inhibit the haemolysis of sheep cells by anti-A serum is completely retained) leads to destruction of the 'virulence enhancing' property.

SUMMARY

1. Two methods are described for the isolation from crude gastric mucin of a polysaccharide-amino-acid complex showing intense blood group A specificity.

2. The undegraded A-substance, which is essentially homogeneous, gives rise to slightly opalescent 1% aqueous solutions that show considerable viscosity. The substance inhibits, in extremely small amounts, specific isoagglutination and haemolysis of sheep cells by specific anti-A rabbit immune serum.

3. The native A-substance is readily degraded by heat treatment and gives rise to a substance which shows a lower viscosity in aqueous solution and which possesses a reduced power to inhibit isoagglutination. The partially degraded A-sub-

stance, however, shows a slightly increased activity in inhibiting A specific haemolysis.

4. The relationship of the purified undegraded A-substance to the 'virulence enhancing' agent in crude gastric mucin is discussed.

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ADDENDUM

Electrophoretic Examination of A-substance from Gastric Mucin

By R. A. KEKWICK

Three preparations of A-substance from gastric mucin (210, 240, 321) were examined in the Tiselius U-tube at 0° with the diagonal schlieren system for optical observation. Samples of the dried preparations were dissolved in buffers to give 2% solutions and dialyzed in cellophane membranes at 2° against several changes of the same buffer. Buffers used were acetate, pH 4.0, $\mu=0.1$, phosphate, pH 8.0, $\mu=0.1$ and phosphate-NaCl buffer, pH 8.0 (phosphate $\mu=0.1$ + NaCl $\mu=0.15$). In all these buffers very viscous solutions were obtained, which gave a considerable light scattering.

In all samples, both at pH 8.0 and pH 4.0, a single component accounted for 90% of the total refraction, the remaining 10% being associated with a somewhat ill-defined contaminant.

In pH 8.0 phosphate buffer $\mu=0.1$, the migration was anodic, $\mu=0.4 \times 10^{-5}$ cm.² sec.⁻¹ V.⁻¹, and in pH 4.0 acetate buffer $\mu=0.1$ a very slight cathodic migration occurred. Under each of these conditions the descending boundary remained sharper than the ascending, the reverse of what is usually observed, and this presumably was due to the high viscosity of the solution.