α_1 -Acute-Phase Globulins of Rats

MICROHETEROGENEITY AFTER ISOELECTRIC FOCUSING

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1. Improved resolution of mixtures of α_1 -globulins was obtained by the use of isoelectric focusing. 2. Because material recovered after isoelectric focusing in polyacrylamide gels behaved in a manner which suggested interaction with components derived from the gel, isoelectric focusing when used for preparative purposes was done in a matrix of Sephadex G-75. 3. By this means material from the individual bands formed by isoelectric focusing in 6M-urea could be isolated. The stability of these substances was examined by further isoelectric focusing. 4. Analysis of material that had been shown to be homogeneous by isoelectric focusing in the absence of urea and of that from several individual bands derived from the same sample by isoelectric focusing in 6M-urea showed different proportions of sialic acid but no change in amino acid composition. 5. In the presence of 6M-urea the isoelectric points found were increased by 0.14-0.25 pH unit. After removal of most of the sialic acid with neuraminidase the increase was 0.36-0.72 pH unit. After treatment with 0.025M-H₂SO₄ at 80°C for 1 h, which removed all the sialic acid, the increase was 0.40-0.87 pH unit. 6. Because removal of all the sialic acid did not decrease the number of bands formed by isoelectric focusing the observed heterogeneity could not be caused entirely by the presence of various proportions of sialic acid.

Investigations of the α_1 -globulins present in rat plasma (Gordon & Louis, 1969; Gordon, 1971) indicated the probable existence of many such proteins with closely similar physicochemical properties. Because complete resolution of such mixtures by electrophoresis in polyacrylamide gel had proved difficult, in the present work isoelectric focusing was used as an additional technique. Employed on a preparative scale in a matrix of Sephadex G-75 this has made possible the isolation of small quantities of almost completely homogenous material. Subsequent analysis has revealed certain of the features which are responsible for the microheterogeneity of these mixtures. Blood from rats suffering from adjuvant arthritis proved to be an excellent source of the protein.

Methods

Pretreatment of rats

Serum was obtained from rats of the hooded and Sprague-Dawley strains, maintained at the National Institute for Medical Research, which had been pretreated in either one of the following ways. (a) Subcutaneous injection of turpentine (Gordon & Louis, 1969); (b) adjuvant arthritis induced by subcutaneous injection into a foot-pad of killed tuberculosis bacillus homogenized in paraffin oil (Glenn *et al.*, 1968). In

this case the rats were bled on the 18th. day after injection (Billingham *et al.*, 1971).

Isolation procedure

Stage 1, chromatography on Sephadex G-150. This was done as described by Gordon & Louis (1969) except that a larger column (200cm×5.5cm) was used.

Stage 2, electrophoresis in polyacrylamide gel. This was done as previously described except that the Quickfit (Stone, Staffs., U.K.) preparative polyacrylamide electrophoresis apparatus was used.

Stage 3, preparative isoelectric focusing. The technique was a modification of that described by Delincee & Radola (1970). A thick suspension of Sephadex G-75 superfine grade (approx. 15g) in 250ml of 1% pH5-7 Ampholine and AnalaR 6M-urea solution was poured into a glass trough (34cm×16cm×0.4cm). The gel bed was levelled and then excess of liquid removed by filter papers applied at the ends of the gel. When sufficiently dry, part of the bed (16cm×3cm×0.4cm) was removed from each end and replaced by filter paper (Whatman no. 31 extra-thick chromatography paper) previously soaked in either 0.4M-ethanolamine or 0.2M-orthophosphoric acid and partially dried. Contact between these strips and the electrode chambers containing the appropriate solution was made with Whatman no. 1 paper strips shielded by dialysis tubing.

The protein (20–30 mg) in 6 M-urea was loaded on to the surface of the gel by using a paper strip (16 cm × 1 cm) (Whatman no. 31).

Focusing was done with the trough resting on a cooled (5–7°C) copper block in a humid chamber, for 40 h at 300 V.

Whatman no. 1 filter paper was used for printing and, after drying and washing with 20% (w/v) trichloroacetic acid, staining was done with 0.05% Amido Black in aq. 7% (v/v) acetic acid followed by destaining in aq. 7% (v/v) acetic acid. By transferring marks (previously made on the paper) on to the gel during printing, protein bands could be located and cut out from the gel. These were then eluted from the Sephadex, thoroughly dialysed against water at 2°C and finally freeze-dried.

Extraction of protein from polyacrylamide gel

Each section of gel was homogenized by passage through a no. 18 syringe needle. The product, suspended in water to a total volume of 20 ml, was dialysed at 2°C against a large volume of water. Afterwards the suspension was filtered and the filtrate was concentrated by rotary evaporation in a warm water bath.

Treatment with neuraminidase

Sufficient neuraminidase [type VI from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.; chromatographically purified from *Clostridium perfringens*] was used to give a ratio of 20 munits of enzyme/mg of α_1 -globulin. These materials were dissolved in 1 M-sodium acetate buffer, pH 4.4, and dialysed at 37°C against the same buffer solution containing a drop of toluene, as bacteriostatic agent. After 24 h and again after 48 h further amounts (each 10 munits) of enzyme were added. After 72 h of enzyme treatment the sac was transferred and dialysis was continued at 2°C against water.

Treatment with acid

Samples of α_1 -globulin were dissolved in 0.025 M- H_2SO_4 and maintained at 80°C for 1 h.

Isoelectric focusing in polyacrylamide gel

Conditions were the same as those used by Awdeh *et al.* (1968). The gels which did not contain 6 M-urea were prepared on the day before because less-sharp bands were sometimes formed when the gels were used soon after setting. Gels were stained first by means of Bromophenol Blue (Gordon, 1971). After extensive washing in water adjusted to pH 4.0 with HCl the gels were restained with Amido Black [0.05% in aq. 7% (v/v) acetic acid].

Isoelectric focusing in two dimensions in polyacrylamide gel

Conditions were the same as used for separation in a single dimension except that after 20 h, only a small part of the gel was stained. With this as a guide the section containing bands of protein was cut out and placed between two glass plates. Next a further volume of the same acrylamide, Ampholine and urea solution was run into the remaining space between the plates. The gel thus obtained was subjected to isoelectric focusing so that the applied voltage was at 90° to that used for the first stage.

Determination of ammonia released at pH 11.5

To remove non-covalently bound ammonia, 5 mg of α_1 -globulin dissolved in 0.8 ml of 0.033 M-HCl was mixed with 10 ml of diethyl ether–propanol (1:1, v/v). The precipitate was removed by centrifugation and redissolved in water. After adjustment to pH 11.5 and 0.08 M with Na_2HPO_4 , the solution was kept at 37°C for 20 h in a Conway dish. Finally the amount of ammonia which had distilled into 0.025 M- H_2SO_4 in the centre compartment was measured by the ninhydrin method (Moore & Stein, 1948).

Measurement of pH in polyacrylamide gels

After isoelectric focusing, gels were marked and the combined surface electrode of a Pye–Unicam pH-meter was applied at a series of points. To allow for the effect of 6 M-urea present in some gels solutions of Ampholine were adjusted to a number of values of pH. After addition of solid urea to a final concentration of 6 M the values of pH were again measured.

Reduction and alkylation

The procedure of Salaman & Williamson (1971) was used, except that the reaction was carried out in the absence of 8 M-urea.

Results

Isoelectric focusing in polyacrylamide gel in the absence of urea

This was done on material prepared from sera from normal rats, from those at 3 days after subcutaneous injection of turpentine and from others suffering from adjuvant arthritis at the 18th day after injection of *Mycobacterium tuberculosis*. The results of isoelectric focusing on materials obtained after stage 1 (Plate 1a and 1b) indicate that the serum from rats suffering from adjuvant arthritis contained very much more α_1 -globulin of higher isoelectric point than did the serum from normal rats; the concentration of α_1 -globulin was also increased in the serum of

EXPLANATION OF PLATE I

Results of isoelectric focusing in a gel containing pH3-6 and pH5-8 Ampholine and no urea

(a) Fraction after chromatography on Sephadex G-150 of serum from normal rats and (b) and (c) from those at day 18 of adjuvant arthritis. (d), (e), (f) and (g) show successive fractions obtained by preparative electrophoresis in polyacrylamide of the same material as that used for (c) except that it had been refractionated on Sephadex G-150.

EXPLANATION OF PLATE 2

Comparison of results obtained by isoelectric focusing in polyacrylamide gel and by using Sephadex G-75 for stabilization

The α_1 -globulin in each was the same as that used for Plate 1(g). (a) In Sephadex G-75 superfine, 6M-urea and pH 5-7 Ampholine; (b) in polyacrylamide gel, 6M-urea and pH 3-6 and pH 5-8 Ampholine; (c) in polyacrylamide gel, no urea and pH 3-6 and pH 5-8 Ampholine.

EXPLANATION OF PLATE 3

Isoelectric focusing of material extracted from isoelectric focusing of α_1 -globulin in a polyacrylamide gel

Initially a sample of α_1 -globulin (stage 2) labelled with ^{125}I was subjected to isoelectric focusing in polyacrylamide gel containing 6M-urea and pH3-6 Ampholine. After this the bands were located by radioautography and a section of gel containing only one band was homogenized and eluted as described in the Methods section. The protein recovered from the area occupied by the single band was re-run (a) on its own and (b) mixed with some of the original material. The arrow shows the position corresponding to that of the band in the first isoelectric focusing from which the material was eluted.

EXPLANATION OF PLATE 4

Results of isoelectric focusing in two dimensions

(a) The same material as that used in Plate 2(b) was first subjected to isoelectric focusing in polyacrylamide gel containing 6M-urea and pH 3–6 and pH 5–8 Ampholine. The section of gel containing the bands (unstained) was then incorporated near one edge of a similar gel containing 6M-urea and pH 3–6 Ampholine. (b) A further amount of the same α_1 -globulin was applied near one edge and subjected to the second isoelectric focusing only.

EXPLANATION OF PLATE 5

Results of isoelectric focusing in a gel containing 6M-urea and pH3-6 and pH5-8 Ampholine of individual fractions obtained after isoelectric focusing in Sephadex G-75 as shown in Plate 2(a)

In (a) and (e) original material was used; in (b) material from bands 6+7 was used; in (c) material from band 4 was used; and in (d) material from band 2 was used.

EXPLANATION OF PLATE 6

Effects of treatment with acid and with neuraminidase

(a), (b) and (c) show results of isoelectric focusing in a gel containing pH 3–6 and pH 5–8 Ampholine and no urea. (a) Untreated material, the same material as in Plate 1(d), was used. (b) The same material as in (a) was used after treatment with 0.025M-H₂SO₄ at 80°C for 1 h. (c) The same material as in (a) was used after treatment with neuraminidase as described in the Methods section. (d), (e) and (f) show results of isoelectric focusing in a gel containing 6M-urea and pH 3–6 and pH 4–8 Ampholine. The material used in (d), (e) and (f) is the same as (a), (b) and (c) respectively. The values of pH in (d), (e) and (f) are corrected for the presence of urea.

rats after injection of turpentine. To make a valid comparison a small amount of ^{125}I -labelled α_1 -globulin was added initially to the sera used for Plates 1(a) and 1(b). After stage 1 the location of this radioactivity permitted materials with identical chromatographic properties to be selected.

After stage 2 partial separation of the mixture of α_1 -globulin was obtained as judged by isoelectric focusing in gels not containing urea (Plate 1d–1g). Comparison of the pI values of these bands indicated that those for bands A, B and C corresponded to those of the material investigated previously (see Gordon & Louis, 1969, Fig. 4).

Stability in 6M-urea

Material which had been dissolved in 6M-urea and kept at room temperature for periods of up to 18h gave an unaltered pattern when re-subjected to isoelectric focusing in 6M-urea. To see if the pattern of bands obtained in 6M-urea might be due, at least in part, to cyanate present in the urea, a preparation of α_1 -globulin, which showed one major and several minor bands when subjected to isoelectric focusing in the absence of urea, was dissolved in 0.02M-sodium cyanate, both at pH 8.1. After keeping the solutions for 1 h at room temperature and 50°C respectively, samples of each were subjected to isoelectric focusing both in the absence and the presence of 6M-urea. After the treatment in 0.02M-sodium cyanate the only change in pattern which occurred was an increase in strength of one of the minor bands. However, the treatment with 1M-sodium cyanate caused a shift of the whole pattern towards the anode. The bands obtained by isoelectric focusing of this material in 6M-urea with a mixture of pH 3–6 and 5–8 Ampholine remained sharp.

The question of whether material which had been kept for some time in concentrated urea reverts completely to its native state after removal of the urea by dialysis was not examined in detail. In a single experiment treatment with 8M-urea at 4°C for 23 h, as described by Salaman & Williamson (1971) for albumin, led to the formation of a component of slightly higher isoelectric point. However, most of the material was unchanged by this treatment.

Isoelectric focusing in polyacrylamide gel in the presence of 6M-urea

As shown in Plate 2, material which appeared to be homogeneous by isoelectric focusing in the absence of urea, when examined by the same technique in gels containing 6M-urea, was found to subdivide into at least five bands. To check whether these bands represented individual molecular species or artifacts caused by interaction with the polyacrylamide, several experiments, including isoelectric focusing in a non-polyacrylamide system, were carried out (see below). These are briefly reported, not because they proved useful in relation to isolation of individual α_1 -globulin components, but because information concerning the behaviour of α_1 -globulin in polyacrylamide gels may be of value for interpretation of the behaviour of other proteins in similar conditions. When protein eluted from pieces of gel known to contain single bands and concentrated as described above was re-examined by isoelectric focusing in gels containing 6M-urea a number of bands were obtained. As indicated in Plate 3 these were mainly in regions of lower pH value.

To see if this apparent instability of the material from individual bands was due to the conditions used for extraction, electrophoresis in two dimensions

Table 1. *Isoelectric points of materials constituting single bands obtained by isoelectric focusing in polyacrylamide gel in the presence and the absence of 6M-urea*

The differences between the isoelectric points of bands in 6M-urea and the native state are also given. The acid-treated material was kept at 80°C for 1 h in 0.025M- H_2SO_4 . The neuraminidase treatment was at 37°C for 72 h at pH 4.4. See the Methods section for further details. All values of pH which were measured in urea have been corrected for the presence of urea. These corrections were 0.5 unit at pH 4.4 and 0.36 unit at pH 5.3.

	Untreated α_1 -globulin			Acid-treated α_1 -globulin			Neuraminidase-treated α_1 -globulin		
	Native	Urea	Urea-native	Native	Urea	Urea-native	Native	Urea	Urea-native
Strongest band	4.35*	4.65	0.30	4.80	5.35	0.55	4.75	5.27	0.52
Band nearest anode	4.35*	4.54	0.19	4.80	5.20	0.40	4.64	5.00	0.36
Band nearest cathode	4.45†	4.70	0.25	5.03	5.90	0.87	4.80	5.52	0.72

* Material same as band B in Plate 1.

† Material same as band C in Plate 1.

was done as described above. Although the two gels were as far as possible identical, material which constituted one band in the first dimension subdivided into positions corresponding to more than one value of pI in the second. As shown in Plate 4, however, the extent to which the bands were interconverted was much less than occurred after extraction from the gel, concentration and reinvestigation.

Effect of 6M-urea on isoelectric points of material forming individual bands

As shown in Table 1 the isoelectric points observed in the presence of 6M-urea were increased considerably. This effect was more pronounced for the bands with higher isoelectric points.

After treatment with neuraminidase and 0.025M- H_2SO_4 as described above the effect was more than doubled. The necessary measurements of pH were made by means of a surface electrode as described in the Methods section.

Isoelectric focusing in a bed of Sephadex in the presence of 6M-urea

Isoelectric focusing of 20 mg of protein, known to be homogenous by isoelectric focusing in polyacrylamide gel in the absence of urea, yielded a pattern of bands which was well resolved except for the fainter bands in the region of lower pH values. The degree of separation was such that by means of a print on two strips of filter paper the bed of Sephadex could be subdivided to yield material derived from four individual bands (2, 3, 4 and 5, Plate 2). To check the stability after separation from the Sephadex and concentration as described above, the isolated materials were subjected to isoelectric focusing in polyacrylamide gel in the presence of 6M-urea. As shown in Plate 5 each fraction consisted mainly of a single component.

Electrophoresis in polyacrylamide gel containing sodium dodecyl sulphate

Material obtained after stage 2, which was almost homogenous by isoelectric focusing in the absence of urea, was subjected to electrophoresis in a 7.5% polyacrylamide gel containing 0.1% sodium dodecyl sulphate (Maizel, 1969); only one band was formed. By comparison with the movement of a number of marker proteins, including rat albumin of molecular weight 63 500, the molecular weight of the α_1 -globulin was approx. 55 000, and there was no change after reduction and alkylation.

Neuraminidase

This enzyme released 6.4% sialic acid from a fraction containing bands B and C of Plate 1. Isoelectric focusing in the absence of urea showed that this

treated material was shifted to a position corresponding to slightly higher isoelectric points than that of band E material before treatment with neuraminidase. The isoelectric points of the material constituting these bands in the presence and the absence of urea are given in Table 1. The neuraminidase-treated materials, when subjected to isoelectric focusing in 6M-urea, showed patterns of regularly spaced bands with isoelectric points higher than obtained with the untreated materials (Plate 6). The number of bands obtained by isoelectric focusing in 6M-urea was not affected by the enzyme treatment.

Hydrolysis for 1 h at pH 1.5 and 80°C

After this treatment, which liberated 7.65% sialic acid, isoelectric focusing in the absence of urea led to patterns of sharp bands displaced considerably towards the region of higher pH. Thus material which in this system originally gave two broad bands yielded several irregularly spaced but sharper bands after hydrolysis (Plate 6).

In the presence of 6M-urea isoelectric focusing of the hydrolysed material led to a pattern of bands displaced considerably towards the region of higher pH, but the number of bands obtained by isoelectric focusing in 6M-urea was not affected by this treatment (Plate 6).

Borate buffer pH 9–11

After treatment for 18 h at 37°C in borate buffer at pH 11, isoelectric focusing in 6M-urea showed a pattern of bands with slightly lowered isoelectric points. Unfortunately, the bands were accompanied by much unresolved material.

Analytical results

Amino acids. Amino acid analysis of material after stage 2 and after stage 3 gave results (Table 2) which differed insignificantly from the material prepared by Gordon & Louis (1969) (stage 2).

To see if different proportions of basic and dicarboxylic amino acids might be responsible for the separation which occurred during isoelectric focusing both in the presence and the absence of urea, material from individual bands was analysed. Very similar ratios were obtained (Table 2) and it is especially noteworthy that no trend occurred. To see if progressively increased or decreased proportions of basic or dicarboxylic acids, sufficient to account for the differences of isoelectric points of the bands obtained by isoelectric focusing in 6M-urea, might have been missed because of analytical error the results were calculated as shown in Table 3. The change of percentage composition to be expected for addition of a single residue of any one of these amino acids

Table 2. *Basic/dicarboxylic amino acid ratios in preparations of α_1 -globulins after stage 2 (electrophoresis in polyacrylamide gel) and after stage 3 (isoelectric focusing in 6M-urea)*

The protein was hydrolysed in 6M-HCl in an atmosphere of N₂ at 110°C for 22h. Corrections for the breakdown of amino acids during hydrolysis were not applied. The analyser was supplied by Evans Electroselenium Ltd. (Halstead, Essex, U.K.). Fractions 1, 2 and 3 represent successive fractions obtained by preparative electrophoresis and are comparable with (e), (f) and (g) in Plate 1.

(Arg+His+Lys)/(Asp+Glu) ratio				
After stage 2		After stage 3		
Fraction	Ratio	Band	Ratio	
1	0.584	6	0.597	0.573*
2	0.589	3	0.645	
3	0.581	2	0.626	
Material after stage 2				
Gordon & Louis (1969)				
Ratio				
0.545		6+7	0.606	
		5	0.599	
		4	0.600	
		2	0.607	

* Repeat analysis.

Table 3. *Amino acid analysis of materials constituting individual bands separated by isoelectric focusing in Sephadex G-150 containing 6M-urea and pH3-6 Ampholine as shown in Plate 2(a)*

The analysis was carried out in the same way as in Table 2. The difference between values for material from bands 2 and 6+7 or vice versa is expressed as a percentage of the average value. The calculated difference for a change of 1 extra residue/molecule is also shown, expressed in the same way.

	Difference (%)	Calculated difference (%)
Lys	0.99	3.5
His	5.52	8.8
Arg	3.88	8.1
Asp	1.03	2.8
Glu	1.44	2.3

materials from which they were prepared. Calculated as residues of sialic acid, after stage 2 preparation C contained 9.7 residues/molecule of mol.wt. 45000. The bands nearer the cathode which were well separated contained amounts of sialic acid corresponding to slightly less than one extra residue per band (Fig. 1). To check that all the sialic acid in the α_1 -globulins had been removed by treatment with 0.025M-H₂SO₄ at 80°C for 1h the treatment was repeated. Less than 1% of sialic acid released by the first treatment then appeared.

Free thiol groups. Measurement by the method of Ellman (1959) gave approx. 0.3SH groups/mol of α_1 -globulin.

Amide groups. After preliminary precipitation in acidic ether-propanol to remove non-covalently bound ammonia an additional 1.5mol of ammonia/mol of α_1 -globulin was liberated at room temperature at pH11.5 as measured by the Conway (1947) method.

exceeded the difference actually observed between material from the bands with highest and lowest isoelectric points.

Sialic acid. The proportions of sialic acid in material obtained after stage 2 were determined by the method of Aminoff (1961), and are given in Table 4.

Analyses for sialic acid were also done on material from the individual bands obtained by isoelectric focusing in the presence of 6M-urea. The values given in Fig. 1 were considerably lower than those for the

Discussion

In the present work we have distinguished clearly between two types of microheterogeneity. The first of these causes differences in isoelectric points under physiologically normal conditions, whereas the second is evident only in the presence of a denaturing solvent. Many examples of the first type of heterogeneity are now known, e.g. D-amino acid oxidase (Hayes & Wellner, 1969), ovomucoid (Beeley, 1971)

Table 4. Analysis for sialic acid of α_1 -globulins after separation by electrophoresis in polyacrylamide gel (stage 2)

Determinations were done by the method of Aminoff (1961). The serum for preparation A was from Sprague-Dawley rats; the serum for preparations B and C was from rats at I.C.I. Pharmaceuticals Division, Alderley Park, Cheshire, U.K. Fractions obtained in preparation B were less-well separated than those in preparation A.

Material from fraction number	Preparation ...	Sialic acid (%)		
		A	Composition by isoelectric focusing shown in Plate	B C
1		7.65	1 (d)	6.27
2		6.41	1 (e)	
3		6.03	1 (f)	6.10
4		5.76	1 (g)	5.26 6.87*

* Isoelectric focusing showed this material to be almost the same as preparation A, fraction 4.

Fig. 1. Percentage of sialic acid found in materials constituting bands separated by isoelectric focusing in 6M-urea in a bed of Sephadex

For details of separation see the Methods section. For homogeneity of certain of these materials see Plate 5. Material from single bands was used except where intermediate positions between bands are shown. In these cases material from pairs of bands was used. Band numbers correspond to those in Plate 2. ● and ■ refer to separate experiments.

and α_1 -acid glycoprotein (Schmid *et al.*, 1967). However, clear demonstration of the second type of microheterogeneity has been more difficult but has been mentioned for ovalbumin (Salaman & Williamson, 1971). Investigations of fetuin and α_1 -acid glycoprotein clearly show that heterogeneity of the first type may be due to differences either in the protein moiety or to the number of attached carbohydrate residues. Thus Nimberg *et al.* (1971) have shown that

the two variants of α_1 -acid glycoprotein are the result of substitution of arginine for glutamine, and Oshiro & Eylar (1968) found that different amounts of sialic acid are present in the variants of fetuin.

The present work has led to the conclusion that for α_1 -globulin heterogeneity of the first type must result both from differences in sialic acid content and also because of certain other structural features. The effect of the latter are especially evident after isoelectric focusing of acid-treated material (Plate 6 and Table 1). Because different proportions of sialic acid were found in material from individual bands after isoelectric focusing in 6M-urea and because multi-band patterns in 6M-urea were also obtained with α_1 -globulin from which all sialic acid had been removed, it seems necessary to reach a similar conclusion about the microheterogeneity of the second type.

Table 1 shows the isoelectric points of material which constitutes bands B and C in Plate 1 both before and after treatment with acid and also with neuraminidase. In addition the changes in isoelectric point of these components when examined in presence of 6M-urea are also shown. It seems likely that the positions of successive bands in the 6M-urea system correspond to a difference of one unit charge. Comparison of the acid-treated material with neuraminidase-treated material in this system (Plates 6e and 6f) shows that the patterns are displaced by approximately the width of the separation of two bands. The difference in sialic acid between these two fractions is 1.2%, or from Fig. 1 approx. 2 residues of sialic acid. The difference is likely to be only in the number of sialic residues, the acid treatment being too mild to disrupt peptide or amide bonds, the usual conditions for the latter being 0.5M- or 0.1M- H_2SO_4 at 100°C for 2–7h (Light & Smith, 1963). Also in the case of the α_1 -acid glycoprotein Schmid *et al.* (1967) were able to find only small chemical and physico-

chemical differences between acid-desialylated and neuraminidase-desialylated protein.

As already mentioned it seems certain that structural features other than the presence or the absence of sialic residues must play an important part in determining the microheterogeneity. Apart from the indication, obtained by treatment at pH 11.5, that amide groups are involved, no further evidence is yet available.

To reach these conclusions concerning the types of heterogeneity of the α_1 -globulins it seemed essential to ascertain whether the observed bands might be artifacts caused by interaction of a single protein species with anything present during separation. In particular it was necessary to determine whether the formation of a number of bands when isoelectric focusing was done in 6M-urea, by material which had given only a single band in the absence of urea, might be due to reaction with traces of cyanate formed from the urea. Any such conclusion was rendered very unlikely because, as described above, no change in pattern could be detected which could be attributed to the presence of 6M-urea at room temperature. Further, only when 1M-sodium cyanate had been allowed to react at 50°C for 1 h were anything more than very minor changes in the pattern of bands observed after isoelectric focusing. The possibility of some interaction between the ampholytes and the protein also seems unlikely. Although some interaction has been shown to occur with bovine plasma albumin (Kaplan & Foster, 1971), this interaction disappears when the albumin is focused in 6M-urea (Williamson *et al.*, 1972). The further possibility that the bands, though not artifacts in the above sense, might be formed by materials of such limited stability that they would be found to have different properties after recovery from the system used for separation was also considered. In fact, material from individual bands recovered after separation in Sephadex was stable in the sense that when subjected to isoelectric focusing in polyacrylamide the reformed bands were almost completely homogenous and of unchanged isoelectric point (see Plate 5).

Because identical band-patterns were formed by isoelectric focusing in 6M-urea stabilized in Sephadex and in polyacrylamide gel the latter material cannot have been responsible for the formation of multiple components. However, interaction between a small part of the protein eluted from acrylamide and polyacrylamide solubilized during this procedure has been clearly demonstrated. Confirmation that artifacts can be formed in this way was obtained from

experiments employing two-dimensional isoelectric focusing in polyacrylamide containing 6M-urea (Plate 4). Evidently conclusions regarding homogeneity of proteins cannot safely be derived from experiments of this kind, since the extra bands which appear in the second dimension may well be due to interaction of protein molecules with soluble components of the polyacrylamide. Because of such considerations only materials recovered from Sephadex were further studied.

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