α_1 Acute-Phase Globulins of Rats

ISOLATION AND SOME PROPERTIES

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1. A method is described for the isolation of certain of the α_1 -globulins of rat plasma that are known to increase in concentration after tissue damage (acute-phase globulins). 2. Although apparently homogeneous when examined by disc electrophoresis at pH 9, these proteins could be subdivided further by isoelectric fractionation. 3. Treatment with neuraminidase removed approx. 60% of the sialic acid originally present in these proteins and gave almost completely homogeneous material of decreased mobility when examined by disc electrophoresis in polyacrylamide gel. When subjected to immunoelectrophoresis this material gave a single arc. 4. The homogeneity of the isolated materials was examined by ultracentrifugation. The single peak thus found is consistent with molecular weights of 45 000–46 000. 5. The isolated materials were shown to be glycoproteins containing approx. 15% of carbohydrate, and to have isoelectric points in the range pH 4.4–4.8.

As a result of tissue damage, and when tumours are present, greatly increased concentrations of certain α -globulins occur in the plasma. The behaviour of an α_1 glycoprotein or group of glycoproteins of this kind, present in rat plasma, has been investigated in some detail by Darcy (1957, 1960*a,b*, 1964, 1965, 1966, 1967) and Gordon & Darcy (1967). Other investigators searching in animal plasmas for an equivalent to orosomucoid have isolated a number of acid glycoproteins both from plasma and from Yoshida ascites-tumour fluid. The properties of the glycoprotein isolated by Kawasaki, Koyama & Yamashina (1966) from normal rat plasma were very similar to those of human orosomucoid. On the other hand the amino acid composition of the glycoproteins of normal rat plasma (Zito, Marcaute & Caputo, 1964) was very different from that of orosomucoid (Heimbürger, Heide, Haupt & Schultze, 1964). The properties of the glycoproteins investigated in the present work differ considerably from those of almost all other rat plasma proteins for which information is available, except for the proteins described by Darcy (1967). In particular, the isoelectric points, which are in the range pH 4.4–4.8, are considerably higher than those of orosomucoid and of the similar acid glycoproteins of rat plasma isolated by Weimer & Winzler (1955), Zito *et al.* (1964) and Kawasaki *et al.* (1966). The existence of a component of rat plasma with properties rather similar to those of the glycoproteins described here was, however, described by Sarcione (1963).

METHODS

Pretreatment of rats

Serum was obtained either at 2 days after injection of turpentine (0.5 ml. subcutaneously at each of two sites) or from rats carrying Walker tumours. The former treatment was carried out on hooded rats of the strain maintained at the National Institute for Medical Research. Serum from the tumour-bearing rats was kindly supplied by Dr D. A. Darcy.

Isolation procedure

Initially the serum was subjected to chromatography on Sephadex G-150. Further purification was then carried out first by electrophoresis in a polyacrylamide gel and then by the isoelectric-focussing procedure originally introduced by Svensson (see Vesterberg & Svensson, 1966). The material thus obtained consisted of two or sometimes three components. Because it seemed likely that these differed only in their content of sialic acid, the material at this stage of purification was subjected to treatment with neuraminidase. After repetition of the isoelectric fractionation only a single component was apparent. Details of a typical preparation are as follows.

Chromatography on Sephadex G-150. A 50 ml. volume of serum from rats that had been pretreated as described above, after dialysis overnight against 25 mM-tris-citric acid buffer, pH 8.6, containing 0.5 M-NaCl and 5 mg. of NaN_3 /100 ml., was applied to a column (3 cm. $\times$ 200 cm.) of Sephadex G-150 that had previously been equilibrated against the same buffer. Elution with the same buffer at a rate of 50 ml./hr. took approx. 36 hr. On the basis of E_{253} measurements obtained with a u.v. monitor, a fraction consisting of the leading half of the last band to emerge was collected (Figs. 1*a* and 1*c*).

Fig. 1. Ultraviolet-absorption patterns obtained after stage 1 (chromatography on Sephadex G-150; see Table 1). (a) and (b) were obtained with serum from rats at 2 days after turpentine injection (see the Methods section). Pattern (b) was obtained by chromatography of the material obtained from the stippled area of pattern (a). (c) and (d) were obtained in a similar way with serum from tumour-bearing rats.

After concentration by pressure dialysis against the same buffer to approx. 25 ml., this material was reappplied to the same column and the chromatography was repeated. As shown in Figs. 1(b) and 1(d), the main peak thus obtained was subdivided and the fractions were concentrated by pressure dialysis against water. Both the stippled fractions shown in Figs. 1(b) and 1(d) were found to be suitable for the next stage.

Electrophoresis in polyacrylamide gel. This was carried out as described by Gordon & Louis (1967) by using an 8% $\times$ 3% gel containing 0.37 M-tris-HCl buffer, pH 8.8. However, to obtain gels with a flat surface it was found to be an advantage to decrease the setting time by adding twice as much NNN'-N'-tetramethylethylenediamine and ammonium persulphate as was originally used. In the present work the elution of the proteins was followed with a u.v. monitor. In this way fractions were selected for further treatment (cf. Fig. 4a).

Isoelectric fractionation. The LKB 8101 apparatus was filled with a sucrose gradient containing 0.42% of the pH 3-6 ampholine buffer. To obtain a suitable gradient, 60 ml. of 0.21% ampholine was placed in a beaker equipped with a magnetic stirrer. Then 60 ml. of 0.63% ampholine also containing 47% (w/v) of sucrose was slowly introduced into this solution by a triangle pump (Bainbridge & Wright, 1964). At the same time the mixture thus formed was pumped into the bottom of the column at twice the rate of entry of the 0.21% ampholine solution. This was achieved by using two further channels connected in parallel on the same pump. After 40 ml. of the gradient had entered the

column, the sample, in a volume of approx. 4 ml. and not containing any sucrose, was pumped at a rate of 15 ml./hr. into the beaker in which the stirrer was operating. Addition of the sample into the lower part of the gradient and thus some distance from the negative electrode was found to be important, because if this was not done precipitation in the upper part of the gradient always occurred. The pumping in of the gradient and sample required about 1 hr., after which dilute H_2SO_4 plus sucrose and dilute ethanolamine were added at the bottom (+) and top (-) respectively of the apparatus. Next the valve was opened and, with refrigerant at 1° circulating through the cooling system, 500 v was applied. After a few hours the potential was increased to 750 v. Soon after commencement the current fell to approx. 1 mA and remained at this value for 75 hr. Finally the valve was closed and water was pumped into the top of the column at a rate of 2 ml./min., thus causing the gradient to flow via the lower exit into a fraction collector.

After measurements of E_{280} and pH values had been made, appropriate tubes were combined and sucrose was removed by pressure dialysis against water.

Treatment with neuraminidase. Neuraminidase from *Vibrio cholerae* was employed. After addition of the enzyme each sample was adjusted to pH 5.6 in the presence of 1 mM-CaCl₂ and kept at 37° for 18 hr. (Ada, French & Lind, 1961).

Repeat of isoelectric focussing. This was carried out exactly as described above except that the neuraminidase-treated material was employed. The Ca²⁺ buffer was not removed.

Analytical methods

Determination of protein. The methods of Gornall, Bardawill & David (1949) and Waddell (1956) were used for the determination of protein.

Disc electrophoresis. Tubes (12 cm. $\times$ 0.8 cm. diam.) containing a 5% polyacrylamide gel in the upper half and a 10% gel in the lower half were used. Both these gels were at pH 8.9 and contained 0.37 M-tris, *NNN'*-tetramethylethylenediamine and ammonium persulphate. No spacer gel was used. Other conditions, except for the use of 50 mM-tris-30 mM-glycine buffer, pH 8.4, in the electrode vessels, were as described by Gordon (1969).

Amino acid analysis. The protein was hydrolysed in 6 M-HCl in an atmosphere of N_2 at 110° for 22 hr. Corrections for the breakdown of amino acids during hydrolysis were not applied. The analyser was supplied by Evans Electrochemical Ltd. (Halstead, Essex).

Determination of carbohydrates. Total hexose was determined by the orcinol method as described by Weimer & Moshin (1953), hexosamine by that of Rimington (1940), sialic acid by that of Aminoff (1961) and methylpentose by that of Dische & Shettles (1948). All these methods were scaled down so that amounts of approx. 1 mg. of protein could be analysed.

Immunoelectrophoresis. This was carried out as described by Jones (1969). The antiserum used was raised in rabbits against a preparation of albumin and α - and β -globulins obtained from rat serum by zone electrophoresis in a block of Pevikon (Bocci, 1962).

RESULTS

Yields obtained at each stage. The recoveries of protein at each stage from serum of rats at 2 days after an injection of turpentine are shown in Table 1.

The properties of the materials obtained at each stage were investigated by the following methods.

Ultracentrifugation. Stages 1 and 2 (Table 1) yielded material that on velocity centrifugation was sufficiently homogeneous to produce a single peak. As shown in Table 2, the molecular weights of the proteins isolated in this way were almost the same (45 000–46 000) irrespective of their mobilities in the polyacrylamide gel.

Electrophoretic properties. The materials obtained

after stages 1 and 2, when investigated by the method of disc electrophoresis described above, were found to consist only of either one of two bands (Fig. 2) or of mixtures of these two bands. The mobility of the faster of these corresponded to the mobility of a substance in the original serum that moved slightly slower than albumin (Fig. 3, band A).

Fig. 3 also shows disc-electrophoresis patterns of material obtained after stage 1 only. The patterns in Figs. 3(b) and 3(c) were obtained from

Table 2. *Sedimentation results for α_1 -globulins*

The values of molecular weight (*M*) were measured by the Archibald method in phosphate buffer, pH 6.8 and *I* 0.1, assuming $\bar{v}$ 0.69. The mobilities in polyacrylamide gel of samples A and B were similar to those of bands A and B in Fig. 2.

Sample	Concn. (mg./ml.)	<i>S</i> _{20,w} (s)	<i>M</i>
A	8.0	3.3	45 200
	2.7	3.6	
B	8.0	3.3	46 000
	2.7	3.6

Fig. 2. Disc-electrophoresis patterns at pH 8.8 of materials obtained after stage 2 (Table 1). (a) The material of higher mobility thus obtained. (b) The same material as in (a) but after treatment with neuraminidase. (c) (a) + (b). (d) The material of lower mobility from stage 2, after treatment with neuraminidase. (e) (a) + (d). O, Origin.

Table 1. *Stages of the isolation procedure used for α_1 -globulins*

Stage no.		Proteins present (% of amount present in original serum)
1	Chromatography on Sephadex G-150	10.0
2	Electrophoresis in polyacrylamide gel	3.0
3	Isoelectric focussing	1.5
4	Treatment with neuraminidase and further isoelectric focussing	0.7

Fig. 3. Disc-electrophoresis patterns at pH 8.8 of serum from a tumour-bearing rat and α_1 -globulins at different stages of purification. (a) Whole serum. (b) and (c) Material after stage 1 (Table 1) only. Equal amounts of the same pool of serum were applied to two Sephadex G-150 chromatograms. After elution, concentration and rechromatography on the same columns similar fractions were collected. (d) Material containing albumin, eluted immediately after that shown in (c). (e) Material at the final stage (including treatment with neuraminidase) with mobility much lower than that of band A, from which it was obtained. See Fig. 1(d) for the elution diagram of the Sephadex G-150 chromatography that yielded the materials analysed in (b), (c) and (d). O, Origin.

separate chromatograms each eluted with the same volume of buffer solution. No difference is apparent but the complexity of the pattern behind band A is evident. The pattern in Fig. 3(d) represents material eluted from the Sephadex G-150 column immediately after that shown in Fig. 3(c). In Fig. 3(d) only two bands appear in this region, and the separation of these is less distinct than that of bands A and B in the original serum.

Because material that gave both the patterns shown in Figs. 3(b) and 3(d) was used for stage 2, some degree of heterogeneity in the products obtained from the polyacrylamide gel was to be expected. In fact as shown in Figs. 2(b), 2(c), 2(d) and 2(e), the slower band obtained by disc electrophoresis, although rather diffuse, did not suggest the existence of resolvable subcomponents.

As shown in Figs. 4(b) and 4(c), isoelectric

focussing (stage 3) permitted further resolution of the material responsible for both band A and band B in Fig. 2. By this method components with isoelectric points at pH values 4.4, 4.6 and 4.8 were obtained. These could not be distinguished by disc electrophoresis because, although single bands were sometimes obtained, the results depended greatly on the conditions used during the preliminary concentration and removal of salt. When this was done by pressure dialysis against water, prolonged treatment led to material showing several bands (Fig. 5b). Traces of albumin were also revealed.

Treatment with neuraminidase. The products obtained on treatment of different preparations of these proteins with neuraminidase differed somewhat, according to the degree of purity already achieved. Thus after the electrophoresis in polyacrylamide gel (stage 2) the result of the enzyme action was to decrease the mobility of the substance responsible for the faster of the two bands obtained by disc electrophoresis to that of the slower (Fig. 2). This treatment left the mobility of the slower band unchanged. In contrast with these results, the action of neuraminidase on the materials obtained after isoelectric focussing (stage 3) led to homogeneous material of still lower mobility by disc electrophoresis (Figs. 3e and 5c). This was true even when the neuraminidase was allowed to act on partially polymerized material that itself showed a complex pattern on disc electrophoresis (e.g. that shown in Fig. 5b). Hence the isoelectric-focussing procedure, as well as separating individual components from the apparently homogeneous fractions led to alterations in at least some of the protein molecules. As shown in Figs. 5(b), 5(c) and 5(d), the complex pattern obtained with the materials after stage 3 was greatly simplified after neuraminidase action. However, at least with some of the material (Fig. 5d) having pI 4.6 before treatment with neuraminidase, disc electrophoresis led to a partly resolved band.

Immuno-electrophoresis. When examined against the antiserum with broad specificity mentioned above, all the isolated materials except that prepared by the whole procedure including the final treatment with neuraminidase gave complex patterns. On the other hand, as shown in Fig. 6, the material obtained after all four stages gave a single arc together with a very faint diffuse area suggesting the probable presence of traces of a minor component.

Analytical results

Amino acid analyses were carried out both on material after stage 2 and on material after additional purification by isoelectric fractionation.

Fig. 4. Preparative electrophoresis in polyacrylamide gel at pH 8.8. (a) Ultraviolet absorption of eluates obtained after stage 2 (Table 1). Approx. 100 mg. of protein was applied to the gel (see the Methods section). (b) Ultraviolet-absorption diagram obtained after isoelectric focussing as described in the Methods section. Approx. 20 mg. of the material of higher mobility obtained by stage 2 (Table 1) was used. (c) Same as (b) except that material of lower mobility from stage 2 was used. (d) Material from the stippled part of (b) after concentration, treatment with neuraminidase and repeated isoelectric focussing (stage 4, Table 1). (e) Same as (d) except that material from (c) was used. Numbers on the graphs are the pI values of the peaks.

Analysis of four preparations after stage 2 led to the mean values ($\pm$ s.d.) for the molar proportions of each amino acid shown in Table 3, column (a). In addition, in Table 3, column (b), are shown similar values for a single preparation that had been subjected to isoelectric focussing (stage 3). This material formed a single band with pI 4.7. Analysis of this material revealed (Table 3) less of the dicarboxylic amino acids and more of the basic amino acids than were found on average in the material that had not been subjected to this procedure.

Results obtained by analysis for sialic acid, hexosamine, hexose and methylpentose are shown in Table 4. Materials after stage 2 and after stage 3 contained almost the same proportion of sialic acid. Also, no difference in sialic acid content could be found between material of higher and lower mobility after stage 2 (bands A and B in Fig. 2). On the other hand, treatment of materials after stage 2 and also after stage 3 with the enzyme decreased the content of sialic acid to approx. 35% of the previous value. The individual preparations were obtained from the serum of either normal or tumour-bearing rats or from normal rats after turpentine injection. No difference with respect

to the various carbohydrates in the preparation made from the different sources was found.

DISCUSSION

Among the acute-phase proteins of the rat the group of α_1 -globulins investigated by Darcy (1957, 1960a,b, 1964, 1965, 1966, 1967) and Gordon & Darcy (1967) are of special interest because of the very slight stimulus required to elicit increased synthesis and the greatly increased concentrations occurring in the plasma after tissue damage. In addition Gordon & Koj (1968), using the perfused rat liver, demonstrated that the time-course of the synthesis of this group of proteins differs from that of fibrinogen and of serumucoid. Thus it seemed worth while to attempt to isolate a protein belonging to this group and to investigate some of its properties.

During the work it became apparent that certain of the properties common to the group of plasma proteins under investigation made for difficulties in isolation. These were of two kinds, both of which manifested themselves in the formation of components with altered mobility on disc electrophoresis. Some of these changes probably occurred as a result of minor increases in ionization of

Fig. 5. Disc-electrophoresis patterns at pH 8.8 of material after stages 3 and 4 (Table 1). (a) After stage 1 only, as a control. (b) After stage 3, pI 4.4. (c) Same as in (b) but after stage 4. (d) Same as in (c) except that it originated from material with pI 4.6 at stage 3. (e) Mixture of (b) and (c). The mobilities of bands A and B are very similar to those of bands A and B in Figs. 2 and 3. O, Origin.

Fig. 6. Immunoelectrophoresis of material obtained after stage 4 (see the Methods section and Table 1). The antiserum used had broad specificity against rat α - and β -globulins and albumin. The material before treatment with neuraminidase had given complex patterns against this antiserum. (c) and (d) are the same as (a) and (b) respectively except that three times as much protein was added to each.

carboxyl groups during isolation. Thus the material responsible for the slower of the two bands in the original serum after isolation by chromatography and then by electrophoresis in polyacrylamide gel then had slightly increased mobility towards the

Table 3. *Amino acid analysis of isolated α_1 -globulin*

For hydrolysis conditions see the Methods section. Methionine sulfoxide, when found, is expressed as methionine. The *P* values indicate the probability (by a *t* test) that the values for material after stage 3 (column *b*) differ from the mean values for material after stage 2 (column *a*). The stages are defined in Table 1.

	Amino acid composition (moles/100 moles of total amino acids)		<i>P</i>
	Material after stage 2 (means $\pm$ s.d. of four analyses) (a)	Material after stage 3 (single analysis) (b)	
Lys	8.10 $\pm$ 0.142	8.87	0.01
His	3.32 $\pm$ 0.450	4.18	—
Arg	3.02 $\pm$ 0.170	3.67	0.02
Asp	11.98 $\pm$ 0.684	10.74	—
Thr	7.57 $\pm$ 0.656	6.48	—
Ser	6.05 $\pm$ 0.640	5.54	—
Glu	14.50 $\pm$ 0.632	12.96	—
Pro	4.70 $\pm$ 1.600	3.50	—
Gly	6.65 $\pm$ 1.062	4.77	—
Ala	7.75 $\pm$ 1.506	5.46	—
Cys	6.00*	6.39	—
Val	6.30 $\pm$ 0.496	6.56	—
Met	1.08 $\pm$ 0.708	1.28	0.02
Ile	3.75 $\pm$ 0.208	3.07	0.05
Leu	7.92 $\pm$ 0.754	7.59	—
Tyr	3.02 $\pm$ 0.288	2.73	—
Phe	4.42 $\pm$ 0.428	6.14	0.02

* This value was assumed to be the same as in the material after stage 3. Actual recoveries were low owing to losses during hydrolysis.

Table 4. *Analysis for carbohydrates of isolated α_1 -globulins*

Results are given as means with the numbers of preparations analysed in parenthesis. The stages are defined in Table 1.

	Composition (% of dry wt.)	
	After stage 2	After stage 3
Sialic acid		
Before treatment with neuraminidase	3.7 (6)	3.3 (5)
After treatment with neuraminidase	1.3 (3)	1.3 (3)
Hexosamine	5.9 (10)	—
Hexose	4.05 (13)	—
Methylpentose	Trace (9)	—

anode. Larger changes of mobility leading to greatly decreased anodic migration of part of the material present occurred after isoelectric focussing when the solution thus obtained had been con-

centrated by pressure dialysis against water. As mentioned above, these changes may well have resulted at least in part from polymerization. Since, however, regularly spaced patterns with bands of decreasing intensity such as are formed by haptoglobins were not obtained, changes of other kinds must also have occurred. The formation of polymers and other altered molecules all derived from material that had previously been electrophoretically homogeneous effectively prevented the further use of disc electrophoresis for characterization of the most highly purified materials. Fortunately, however, removal of part of the sialic acid from material at this stage of purity by treatment with neuraminidase led to a product that remained stable during concentration by the same method. The homogeneity of material thus obtained and then subjected to disc electrophoresis is shown in Fig. 3(e). Evidently the tendency to polymerize that seems to be characteristic of highly purified preparations of these proteins is greatly diminished after treatment with neuraminidase.

Further information on the heterogeneity of the mixture of proteins obtained by the electrophoresis in polyacrylamide gel was obtained by means of the isoelectric-focussing method. Thus, as shown in Figs. 4(b) and (c), three components were originally present, two of which disappeared after treatment with the enzyme. From the isoelectric points of these components it appears that loss of sialic acid from those with pI values 4.2 and 4.4 led to conversion into the least acid component. Although such loss must indeed be presumed it also seems certain that further changes must have taken place as a result of the enzyme treatment, at least in the component with pI 4.7. Thus not only did it change its mobility by disc electrophoresis, but like the other two components it lost its tendency to polymerize. In addition to these changes brought about by the enzyme, the slightly different proportions of amino acids found after treatment by isoelectric focussing indicate that the final material had been separated by this means from small amounts of other proteins. The finding that treatment with neuraminidase had less effect on the mobility of material after stage 2 than on the mobility of products that had also been subjected to isoelectric focussing suggests that this latter procedure must result in sufficient intramolecular rearrangement for previously buried sialic acid groups to become available. That only a few residues of sialic acid are concerned in these changes in mobility is indicated, however, since on analysis no difference in content of sialic acid could be detected.

Although the original aim of the present work was the isolation of a single α -globulin in the same state as in the plasma, it became apparent that a more realizable objective would be the isolation of a

modified glycoprotein from which part of the sialic acid had been removed. A method for the preparation of such a material is described above, but the properties of the modified protein have not been investigated in detail. However, it seemed worth while to describe the method of isolation and the properties of the mixture of proteins used for the final separation by isoelectric fractionation. This information is included because it is believed to be sufficient to indicate the relationship of this group of α -globulins to other plasma proteins that have already been described. As mentioned above, the isoelectric points of the present proteins are sufficient to distinguish them from the acid glycoprotein of rat serum isolated by Kawasaki *et al.* (1966). In addition, their content of carbohydrate (15% before treatment with neuraminidase) is much less than that of Kawasaki's glycoprotein, which contained 35% of carbohydrate. Amino acid analysis of the present glycoprotein indicates a considerable similarity to the proportions of amino acids found by Zito *et al.* (1964) in the glycoprotein that they obtained from normal rat serum. However, less than 3% of tyrosine was found in the present material compared with 10% found by Zito *et al.* (1964). Ultracentrifugation of the present material gave values of S_{20} (Table 2) that when interpolated to 0.5% concentration are slightly higher than the corresponding value obtained by Zito *et al.* (1964). Further, Kawasaki *et al.* (1966) reported molecular weights of only 30 000 whereas at the same concentration (0.8%) the values found for the present material were 45 200 and 46 000.

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