The α Macroglobulins of Rat Serum

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A three-stage method for isolation of α_1 macroglobulin and α_2 macroglobulin from the serum of normal and injured rats is described. The methods successively used, namely gel filtration, ultracentrifugation and chromatography on DEAE-cellulose, were chosen to minimize loss of tryptic esterase-protecting activity. The two proteins differed slightly with respect to the following properties: mol.wt., α_1 macroglobulin 7.46×10^5 , α_2 macroglobulin 7.16×10^5 ; isoelectric focusing, α_1 macroglobulin pI 4.4, α_2 macroglobulin pI 4.5. Amino acid analyses were identical, except with respect to tyrosine: α_1 macroglobulin 3.96 ± 0.24 , α_2 macroglobulin 3.16 ± 0.32 mol/100 mol of total amino acids. When isolated from the serum of uninjured rats, α_1 macroglobulin retained the capacity to bind 1.05 mol of trypsin/mol. However, if isolated from serum 2 days after injury only 0.78 mol of trypsin/mol of α_1 macroglobulin was bound. α_2 macroglobulin isolated from this latter serum bound on average 0.97 mol of trypsin/mol. When reduced with *N*-acetylcysteine, both molecules formed subunits of size corresponding to that expected for quarter molecules. When α_2 macroglobulin was reduced with dithiothreitol, quarter molecules were again produced. α_1 macroglobulin, however, when thus treated gave a more complex mixture, containing a component having a mol.wt. of less than 6×10^4 . Antisera raised against the two proteins permitted estimation of the concentration of each protein in the plasmas or sera of normal and injured rats. Plasma from normal male rats contained 3.76 ± 0.56 mg of α_1 macroglobulin/ml ($n = 33$) and 0.016 ± 0.001 mg of α_2 macroglobulin/ml ($n = 33$). After injury by injection of turpentine and cortisone, the concentrations in plasma were at 3 days 5.19 ± 0.81 mg of α_1 macroglobulin/ml ($n = 12$) and at 2 days 1.38 ± 0.35 mg of α_2 macroglobulin/ml ($n = 12$). Antisera to the two proteins did not cross-react with one another. The quarter molecules formed by reduction of both proteins showed increased antigenicity.

Previous studies of the macroglobulins of rat plasma have demonstrated the existence of two proteins of similar physicochemical properties. These proteins, α_1 macroglobulin (Boffa *et al.*, 1964; Fisher & Canning, 1966; Ganrot, 1968) and α_2 macroglobulin (Darcy, 1955, 1957; Weimer & Benjamin, 1965; Bogden & Gray, 1968; Heim, 1968; Menninger *et al.*, 1969; Ganrot, 1973), differ in that the plasma concentration of α_2 macroglobulin only, increases greatly as a result of injury or during inflammation. In such circumstances only relatively minor changes in the concentration of α_1 macroglobulin have been observed.

Bogden & Gray (1968) and many others have shown that the plasma concentration of α_2 macroglobulin provides a convenient index of the degree of injury that has been suffered by a rat. Thus many investigations dealing with acute-phase plasma proteins have made use of immunological estimations of the concentration of this protein. However, because α_2 macroglobulin has not been isolated in a pure form, the results of these estimations have been only relative.

As first demonstrated by Haverbach *et al.* (1962), α_2 macroglobulin of human serum (analogous to α_1 macroglobulin of rat serum) combines with proteinases in a special way (Harpel, 1973; Barrett & Starkey, 1973). Advantage was taken of this property of α_2 macroglobulin by Ganrot (1966a), who described a method for its estimation in human serum.

Experimental

Isolation procedures

Stages of purification. These were gel filtration, zonal ultracentrifugation and finally chromatography on DEAE-cellulose or alternatively electrophoresis in agarose gel. The latter method had the advantage that α_1 macroglobulin free from α_2 macroglobulin could be obtained from serum containing both proteins.

Chromatography on DEAE-cellulose was most often used to avoid the necessity of recovering the separated proteins from the agarose. The initial gel-filtration stage was omitted when small-scale

preparations, starting from 10–15 ml of serum, were undertaken.

Pretreatment and bleeding of rats. For preparation of α_1 macroglobulin, serum was obtained from 200–250 g male or female rats. For α_2 macroglobulin, larger male rats were used 2 days after subcutaneous injection of 0.4 ml of turpentine. At the same time 5 mg of cortisone (cortisone acetate Injection BP; Boots Ltd., Nottingham, U.K.) was injected intramuscularly.

The rats were bled by cardiac puncture under light ether anaesthesia. To increase the yield of serum they were immediately injected intraperitoneally with 10 ml of 0.9% NaCl; 5 h later they were again bled by heart puncture.

Serum intended for preparation of α_1 macroglobulin obtained from individual rats was assayed for the presence of α_2 macroglobulin and rejected if found to contain more than 0.03 mg of this protein/ml. All concentrations of α_1 macroglobulin and α_2 macroglobulin in the text are expressed as means $\pm$ s.d.

Gel filtration. Serum (50 ml volumes) was filtered on a column (180 cm $\times$ 5.5 cm) of Sephadex G-150, exactly as described by Gordon & Louis (1969), except that the preliminary dialysis was omitted. The material in the first peak that emerged was concentrated by ultrafiltration by using an Amicon PM 10 membrane. With the cell cooled in ice, filtration was continued until the protein content of the solution had risen to approx. 25 mg/ml.

Zonal centrifugation. A portion (10 ml) of the above solution was dialysed overnight against 0.3 M-NaCl containing 10 mM-sodium phosphate, pH 7.7. Centrifugation was carried out for 5 h at 43 000 rev./min in an MSE 65 BXIV zonal rotor containing a linear gradient of sucrose (7½–22½%, w/v). The time-integrated g value at an average radius of 6.5 cm was 1.53×10^6 g-s. The sucrose solutions also contained the same phosphate buffer. The E_{280} of 15 ml fractions thus obtained indicated the presence of a well-separated macroglobulin peak. The appropriate fractions were combined and dialysed at 4°C against 0.08 M-NaCl. Next day the solution thus obtained was concentrated by ultrafiltration as described above to approx. 50 ml.

Chromatography on DEAE-cellulose. Amounts up to 100 mg were dialysed against 0.05 M-NaCl/0.05 M-Tris/HCl, pH 8.0, and applied to a column (65 cm $\times$ 2.0 cm diam.) of DEAE-cellulose DE-52 (Whatman Ltd., Maidstone, Kent, U.K.).

The column was eluted by application of a linear gradient from 0.1 to 0.25 M-NaCl in Tris buffer. All these procedures were carried out at room temperature (20°C).

The conductivities at which α_2 macroglobulin and α_1 macroglobulin emerged were respectively 1.40×10^{-2} and 1.16×10^{-2} mhos.

Electrophoresis in agarose gel. This was carried out exactly as described by Johansson (1972) and Johansson & Hjerten (1974). Not more than 50 mg of the macroglobulin mixture was applied to a gel slab 14 cm $\times$ 20 cm $\times$ 0.3 cm. The final position of the protein bands was located by printing with Whatman no. 1 filter paper. The damp paper was transferred directly to 0.05% solution of Amido Black in 7% (v/v) acetic acid and then de-stained in 7% acetic acid.

Analytical methods

Isoelectric focusing. This was carried out as described by Awdeh *et al.* (1968) in a 3.5% acrylamide/5% bisacrylamide gel containing pH 4–6 Ampholine. To allow sufficient time for the macroglobulins to diffuse into the gel, focusing was started 6 h after application of the samples. To ensure completion of focusing, current was passed for 22 h. The positions of the macroglobulin components were then apparent as regions of opalescence or light precipitate in the gel. This behaviour suggested that the macroglobulins might, as a result of insolubility, fail to reach the positions corresponding exactly to their isoelectric points. Thus, as a check, the samples were placed on the gel on either side of the positions that they were expected to reach.

Reduction and alkylation of α_1 macroglobulin and α_2 macroglobulin. Reduction was carried out either with *N*-acetylcysteine or alternatively, when stronger reducing conditions were required, with dithiothreitol (Harpel, 1973), i.e. to the macroglobulin dissolved in 0.015 M-Tris, pH 8.7, was added an equal volume of a solution containing 10 M-urea, 1% sodium dodecyl sulphate and 14 mM-dithiothreitol. The mixture was then kept at 37°C for 45 min.

When *N*-acetylcysteine was used, an excess of the reagent was added to a solution of α_2 macroglobulin at room temperature in 0.4 M-Tris/HCl buffer, pH 8.0. Thus 10 mg of *N*-acetylcysteine was added to 3 mg of α_2 macroglobulin dissolved in 1.2 ml of the buffer. Immediately 60 μ l of 1 M-NaOH was also added. The mixture was left for 40 min, and then 20 mg of iodoacetamide dissolved in 1 ml of water was added, and finally 60 μ l of 1 M-NaOH.

Disc electrophoresis. This was carried out in either 4% polyacrylamide gels containing 0.05 M-glycine and 0.015 M-Tris/HCl, pH 8.7, with the same buffer solution in the electrode compartments or 5.5% polyacrylamide gels, containing 0.1% sodium dodecyl sulphate, by the method of Weber & Osborn (1969).

Measurement of protein concentration. This was carried out spectrophotometrically by the method of Waddell (1956), and by radial immunodiffusion (Mancini *et al.*, 1965).

Measurement of tryptic esterase-protecting activity of α_1 macroglobulin and α_2 macroglobulin. The capacity to bind trypsin with retention of enzymic activity

EXPLANATION OF PLATE I

Electrophoresis in polyacrylamide gels

(a) Isolated α_1 macroglobulin and α_2 macroglobulin after electrophoresis in 4% polyacrylamide gel as described under 'Disc electrophoresis'. (1) α_2 macroglobulin; (2) α_1 macroglobulin; (3) rat albumin. (b) (1) α_2 macroglobulin; (2) α_2 macroglobulin reduced by *N*-acetylcysteine; (3) α_2 macroglobulin reduced by dithiothreitol; (4) α_1 macroglobulin; (5) α_1 macroglobulin reduced with *N*-acetylcysteine; (6) α_1 macroglobulin reduced with dithiothreitol; (7) rat albumin and albumin polymers. Electrophoresis of (2), (3), (5), (6) and (7) was for 3.5 h, whereas that for (1) and (4) was for 5 h. Electrophoresis was in 5.5% polyacrylamide gels containing 0.1% sodium dodecyl sulphate (see under 'Disc electrophoresis').

EXPLANATION OF PLATE 2

Two-dimensional immunoelectrophoresis

(a) Isolated α_1 macroglobulin after two-dimensional immunoelectrophoresis (for details see under 'Two-dimensional immunoelectrophoresis'); (b) isolated α_2 macroglobulin similarly treated; (c) serum from a rat 2 days after injection of turpentine and cortisone, similarly treated. The same antiserum raised in rabbits against serum from a rat which had received turpentine and cortisone was used for all three plates. For further details see under 'Two-dimensional immunoelectrophoresis'.

against BzArgNPhNO₂* was measured as described by Ganrot (1966a), except that the solution was left for 1h after the addition of the trypsin. This modification was introduced because binding of trypsin by the macroglobulin was incomplete after 5–10s. When calculating tryptic esterase-protecting activity bound trypsin was assumed to be 80% active against BzArgNPhNO₂ (Barrett & Starkey, 1973). Because, as described below, 50% of the sample of trypsin used titrated with *p*-nitrophenyl *p*'-guanidinobenzoate, this factor was also allowed for (Table 2). Trypsin standards were included in each assay. These were made up just before addition of the BzArgNPhNO₂ because trypsin is unstable at pH 8.2. The results thus obtained with α_1 macroglobulin and α_2 macroglobulin are referred to below as tryptic esterase-protecting activity.

Amino acid analysis. The protein was hydrolysed in 6M-HCl in an atmosphere of N₂ at 110°C for 22h. The analyser was a Beckman 120C.

Tryptophan contents were calculated from molar tyrosine/tryptophan ratios measured spectrophotometrically in 0.1M-NaOH, at the wavelengths (280, 294.4nm) proposed by Goodwin & Morton (1946), as recommended by Beaven & Holiday (1952).

Carbohydrate analysis. Carbohydrate analysis was carried out by g.l.c. (Clamp, 1974).

Specific extinction coefficients. The u.v.-absorption characteristics of aqueous solutions of α_1 macroglobulin and α_2 macroglobulin were measured by using a Cary 118 spectrophotometer. The measured absorbances at 280nm were corrected for cell zeros and non-specific absorption (based on a linear extrapolation of the absorption in the 330–350nm region; these corrections decreased the measured absorption at 280nm by 4.4% and 2.9% for α_1 macroglobulin and α_2 macroglobulin respectively). The protein concentrations of these solutions were measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard; a specific extinction coefficient ($E_{1\text{cm}}^{1\%}$) of 6.6 was used to determine the protein concentration of the standard (Foster & Sterman, 1956).

Results

Purity of α_1 macroglobulin and α_2 macroglobulin

All analyses were done on material that had been through the three stages of purification, of which chromatography on DEAE-cellulose was the last.

Disc electrophoresis. As shown in Plate 1(a) electrophoresis of α_1 macroglobulin in 4% polyacrylamide gel showed a single band and a trace of a slower-moving contaminant. Under the same conditions α_2 macroglobulin showed only faint traces of contaminants. The position of the slower-moving

band obtained from α_1 macroglobulin is consistent with that expected for the dimer of α_1 macroglobulin. The absence of a slower-moving component when α_1 macroglobulin was examined by two-dimensional immunoelectrophoresis is also consistent with this hypothesis.

Electrophoresis of both α_1 macroglobulin and α_2 macroglobulin in gels containing 0.1% sodium dodecyl sulphate gave only single, rather broad, bands. These were either too close to the surface of the gel to permit any estimation of molecular weight or just separated from the surface suggesting mobilities corresponding to those of half-molecules of α_1 macroglobulin or α_2 macroglobulin.

Two-dimensional immunoelectrophoresis. The method of Laurell (1965) was used with antisera raised against the serum of an injured rat. As shown in Plate 2, α_1 macroglobulin showed a minor component which moved ahead of albumin in the first dimension, whereas α_2 macroglobulin gave a single peak.

Ultracentrifugation. Velocity sedimentation at 25°C and 59780rev./min of α_1 macroglobulin and α_2 macroglobulin both at 1.5mg/ml gave respectively $s_{20,w}$ 18.7 and 19.8S. A minor component with $s_{20,w}$ 27.8S was present in the α_1 macroglobulin (see under 'Disc electrophoresis' of this section).

The molecular weights of the two macroglobulins were determined by the high-speed equilibrium method of Yphantis (1964). A partial specific volume of 0.73 calculated from the amino acid composition was used. The plot of $\ln c$ versus r^2 for α_1 macroglobulin was slightly curved. On the assumption that the departure from linearity was due to the presence of a small proportion of a heavier component (considered to be a dimer), the best fit obtained was for a mixture of 8.5% of this with a monomer of mol.wt. 7.46×10^5 . The plot for α_2 macroglobulin showed very little curvature and gave a mol.wt. of 7.16×10^5 .

Isoelectric focusing. Focusing of α_1 macroglobulin and α_2 macroglobulin in polyacrylamide gel under the conditions described under 'Analytical methods' led to the formation of easily visible opalescent areas. Subsequent staining of the gel with Bromophenol Blue (Gordon, 1971) revealed that each of these areas contained two separate bands.

Measurements of the gel pH with a surface electrode indicated that the position of the pair of bands obtained from α_1 macroglobulin corresponded approximately to pH 4.4 and that for α_2 macroglobulin to pH 4.5. Perhaps because of insolubility of the macroglobulins at pH values near to their isoelectric points, the band positions were slightly variable. It was notable, however, that the difference of pH between the band pair derived from α_1 macroglobulin and that from α_2 macroglobulin remained constant.

* Abbreviation: BzArgNPhNO₂, benzoylarginine *p*-nitroanilide.

Radial immunodiffusion. α_2 macroglobulin, prepared as described above from the serum of rats 2 days after injection of turpentine and cortisone, the mixtures of α_1 macroglobulin and α_2 macroglobulin that were eluted from the DEAE-cellulose column after the peak of α_2 macroglobulin, and α_1 macroglobulin, prepared from the serum of unstimulated rats, were all subjected to radial immunodiffusion against specific antisera to the two proteins. The α_2 macroglobulin preparation contained over 99% of α_2 macroglobulin, whereas the following peak eluted from the DEAE-cellulose column contained 16% of α_2 macroglobulin and 84% of α_1 macroglobulin (averages from seven preparations). These values are percentages of α_1 macroglobulin plus α_2 macroglobulin as determined by radial immunodiffusion. Evidence for the absence of other proteins was obtained by two-dimensional immunoelectrophoresis. The amounts of α_2 macroglobulin and α_2 macroglobulin plus α_1 macroglobulin, constituting the first and second peaks eluted from the DEAE-cellulose column as estimated by radial immunodiffusion, corresponded to 113 and 120% of the amounts of protein in the same solution estimated spectrophotometrically (Waddell, 1956). Serum from unstimulated rats gave a single main peak from the DEAE-cellulose column, which consisted of 98% of

α_1 macroglobulin and less than 2% of α_2 macroglobulin.

Tryptic esterase-protecting activity. The amounts of trypsin bound by both sera from large and small rats and isolated α_1 macroglobulin and α_2 macroglobulin were estimated by using BzArgNPhNO₂ as described in the Experimental section. The results, corrected for the amount of active trypsin present (see below), expressed on a molar basis appear in Table 1. In the sera from the large and small uninjured rats respectively 1.19 and 1.30 mol of trypsin were bound per mol of α_1 macroglobulin. In serum from injured rats the amounts of trypsin bound varied greatly but were lower. To obtain the ratios given in Table 1, concentrations of α_1 macroglobulin and α_2 macroglobulin in the sera were estimated by radial immunodiffusion. Similar measurements were also made for the isolated macroglobulins. However, because these concentrations could also be estimated spectrophotometrically, the ratios from the isolated α_1 macroglobulin and α_2 macroglobulin have been calculated on this basis.

To ascertain whether the slightly lower average tryptic esterase-protecting activity found for isolated α_1 macroglobulin compared with that for α_2 macroglobulin, as given in Table 1, represented a real difference, the tryptic esterase-protecting acti-

Table 1. *Molecules of trypsin bound per molecule of α macroglobulin*

Numbers of estimations carried out are shown in parentheses. Each was on a pool of serum from six to ten rats. To calculate the concentrations of trypsin bound to macroglobulin, the values of bound trypsin estimated with BzArgNPhNO₂ after addition of soya-bean trypsin inhibitor (see under 'Measurement of tryptic esterase-protecting activity of α_1 macroglobulin and α_2 macroglobulin') were used. These values were converted into 'active trypsin' concentrations by using the factor ($\times 0.50$) obtained by titration of free trypsin with *p*-nitrophenyl *p*'-guanidinobenzoate. Additional correction ($\times 1.25$) was made for the lower activity of trypsin against BzArgNPhNO₂ when it is bound to macroglobulin (see under 'Tryptic esterase-protecting activity'). For the results of this calculation to be valid, it is necessary to assume that active trypsin molecules are active in relation to both BzArgNPhNO₂ and *p*-nitrophenyl *p*'-guanidinobenzoate and that only those trypsin molecules active in this sense are bound by α_1 and α_2 macroglobulins. Then because BzArgNPhNO₂ was standardized against trypsin, only part of which is active, the amount of trypsin measured with this standard must be corrected by application of the appropriate factor, which in this case is $\times 0.50$. The concentrations of α_1 macroglobulin and α_2 macroglobulin in the sera were measured by radial immunodiffusion (see under 'Radial immunodiffusion'), whereas those of the isolated macroglobulins are based on absorption (see under 'Specific extinction coefficients'). Molecular weights of 7.46×10^5 for α_1 macroglobulin, 7.16×10^5 for α_2 macroglobulin and 2.33×10^4 for trypsin are assumed.

		Mol. of trypsin bound/mol of α macroglobulin			
Rat weights (g) ...		300-500		80-85	
Pretreatment of rats		α_1 macroglobulin	α_2 macroglobulin	α_1 macroglobulin	α_2 macroglobulin
In sera	None	1.19 (4)	—	1.30 (2)	—
	Turpentine injected	—	—	0.74* (2)	0.72* (2)
	Turpentine+cortisone injected	0.94* (5)	0.91* (5)	0.61* (2)	0.58* (2)
Macroglobulins	None	1.05 (4)	—	—	—
	isolated from Turpentine+cortisone injected serum after	0.78 (8)	0.97 (8)	—	—

* Estimates of the ratios for α_1 macroglobulin and α_2 macroglobulin in sera were obtained by dividing the total concentration of bound trypsin in proportion to the relative concentrations of α_1 macroglobulin and α_2 macroglobulin as estimated by radial immunodiffusion.

vities of samples of α_1 macroglobulin and α_2 macroglobulin which had been separated by electrophoresis in agarose were estimated. Initially 14 ml of serum from injured rats was subjected to zonal centrifugation as described in the Experimental section. The solution of macroglobulins thus obtained provided the starting material for three electrophoretic separations. After each, the regions containing α_1 macroglobulin and α_2 macroglobulin were removed, extracted and assayed separately for tryptic esterase-protecting activity and also by radial immunodiffusion. The averages were 0.63 and 0.99 mol of trypsin/mol of α_1 macroglobulin and α_2 macroglobulin respectively.

Active-site titrations. Because of the probability that the trypsin used in this work was not fully active, it was titrated with *p*-nitrophenyl *p'*-guanidinobenzoate as described by Chase & Shaw (1967). The burst of nitrophenol thus produced was measured spectrophotometrically at 410 nm. The resultant $E_{410} \times 6.025 \times 10^{-5}$ then gave the molarity of the nitrophenol, which is known to equal that of the active trypsin present. This was 50% of the weight of the trypsin (uncorrected for moisture or traces of salt).

As suggested by Barrett & Starkey (1973), the same procedure was used to estimate trypsin bound to isolated α_1 macroglobulin and α_2 macroglobulin. In this instance, excess of trypsin was inactivated by adding soya-bean trypsin inhibitor (Koch-Light Laboratories, Colnbrook, Bucks., U.K.). The molar concentrations of bound trypsin thus found, expressed as a percentage of the molarity of the α_1 macroglobulin or α_2 macroglobulin, measured with the Lowry *et al.* (1951) reagent, were respectively 72 and 62.

Recoveries of α_2 and α_1 macroglobulin from the sera of normal rats and from those after injection with turpentine

The concentration of α_1 macroglobulin in the serum of uninjured rats, estimated by radial immunodiffusion (Mancini *et al.*, 1965), was 3.96 ± 0.56 mg/ml ($n=33$). The concentration of α_2 macroglobulin in serum of male rats 2 days after injection of turpentine and cortisone was 1.38 ± 0.35 mg/ml ($n=12$). Values for concentration of both α_1 macroglobulin and α_2 macroglobulin in sera of male and female rats of the same strain, at times up to 12 days after injection of turpentine, have been reported by Bosanquet *et al.* (1976). The concentrations of α_1 macroglobulin and α_2 macroglobulin as estimated by radial immunodiffusion, were obtained by using α_1 macroglobulin and α_2 macroglobulin isolated by the methods given above as standards. For this purpose the two macroglobulins are assumed to be pure.

The yields of purified α_1 macroglobulin and α_2 macroglobulin obtained by the procedures described above were similar. Further, recoveries estimated by the trypsin-binding assay and by radial immunodiffusion did not differ greatly. The most considerable losses occurred during the final chromatographic stage. Overall recoveries ranged between 15 and 25%.

Properties of isolated α_1 macroglobulin and α_2 macroglobulin

Extinction measurements. The specific extinction coefficients, $E_{1\text{cm}}^{1\%}$, at 280 nm found for α_1 macroglobulin and α_2 macroglobulin were 9.8 and 9.2 respectively for 1% (w/v) concentration in a 1 cm cell. The absorption maxima were at 277 nm and 278 nm for α_1 macroglobulin and α_2 macroglobulin respectively, and the corresponding maximal $E_{1\text{cm}}^{1\%}$ values were 9.95 and 9.3. These values are in fair agreement with the specific extinction coefficients calculated from the measured tyrosine contents and the spectrophotometric tyrosine/tryptophan ratios.

Amino acid analyses. The molar proportions of the amino acids obtained by hydrolysis of α_1 macroglobulin and α_2 macroglobulin are shown in Table 2. Except for those for tyrosine, the average values for the proportions of most of the amino acids obtained for three preparations of α_1 macroglobulin differed very little compared with those of α_2 macroglobulin.

Table 2. *Amino acid composition of α_1 and α_2 macroglobulins*

Results are expressed as mol/100 mol of amino acids present in hydrolysate, $\pm$ s.d. Values for α_1 macroglobulin and α_2 macroglobulin are averages from respectively three and five analyses of separate preparations. The values for tryptophan were obtained spectrophotometrically and were based on the average tyrosine values.

	α_1 macroglobulin	α_2 macroglobulin
Lysine	6.47	5.83
Histidine	2.25	2.11
Arginine	3.13	3.56
Aspartic acid	9.29	9.34
Threonine	6.52	7.41
Serine	7.53	6.88
Glutamic acid	11.59	11.90
Proline	5.47	5.05
Glycine	5.96	6.08
Alanine	6.78	7.07
$\frac{1}{2}$ Cystine	1.37	1.23
Valine	8.37	8.90
Methionine	1.69	1.70
Isoleucine	4.97	4.11
Leucine	9.46	10.41
Tyrosine	3.96 ± 0.24	3.16 ± 0.32
Phenylalanine	4.26	4.47
Tryptophan	0.90	0.81

The tyrosine/tryptophan ratios for α_1 macroglobulin and α_2 macroglobulin were 4.38 and 3.96 respectively. These values, together with the corresponding mol % of tyrosine values, were used to derive the mol % of tryptophan values given in Table 2.

Carbohydrate analyses. α_1 macroglobulin and α_2 macroglobulin contained very similar amounts of mannose plus galactose. The contents of glucosamine were also close. Means for four preparations expressed as percentages of the amount of protein present were: mannose plus galactose, α_1 macroglobulin 5.44 and α_2 macroglobulin 6.26 respectively; glucosamine 2.60 and 2.61. Fucose could not be detected in either macroglobulin.

Changes brought about by reduction. As shown in Plate 1(b), electrophoresis, in gels containing sodium dodecyl sulphate, of α_1 macroglobulin and α_2 macroglobulin after similar treatment of the two proteins with *N*-acetylcysteine followed by iodoacetamide yielded almost identical patterns. Each single band thus formed had a position near to that expected for molecules one-quarter the size of the original macroglobulin. Treatment with dithiothreitol, on the other hand, revealed a difference between α_1 macroglobulin and α_2 macroglobulin. Thus whereas the products from α_1 macroglobulin separated into several bands on a sodium dodecyl sulphate-containing gel, α_2 macroglobulin gave quarter-molecules, just as after treatment with *N*-acetylcysteine. The strongest band obtained from α_1 macroglobulin after dithiothreitol treatment had the mobility expected for quarter-molecules. It is notable that this band had moved slightly faster than that believed to correspond to quarter-molecules of α_2 macroglobulin.

Radial immunodiffusion of the products obtained by reduction of both α_1 macroglobulin and α_2 macroglobulin with *N*-acetylcysteine, in gels containing specific antisera against whole α_1 macroglobulin or α_2 macroglobulin, gave rings of greatly increased diameter. Expressed per unit weight of protein this varied between two and four times that of each of the original macroglobulins.

Discussion

As yet, physicochemical data for rat plasma α_1 macroglobulin and α_2 macroglobulin (Boffa *et al.*, 1964; Jamieson *et al.*, 1972a) have been insufficient to indicate whether the small separation obtained by electrophoresis in polyacrylamide gel results only from the difference in charge of these proteins or whether differences in size and shape also play a part. The present finding that the mol.wt. of α_1 macroglobulin (7.46×10^5) is slightly larger than that of α_2 macroglobulin may be taken to indicate that if the shapes are equivalent, α_1

macroglobulin will be relatively retarded in a sieving gel, thus tending to affect the separation due to charge (Plates 1a and 2). However, caution is necessary, because the smaller $s_{20,w}$ value for α_1 macroglobulin may mean that shape differences are important. The mol.wt. now obtained by the method of Yphantis (1964) for α_1 macroglobulin agrees well with the value of 7.39×10^5 found for the same protein by Ho *et al.* (1974). A rather higher value (7.68×10^5) was reported by Fisher & Canning (1966).

In view of the more complex structure of α_1 macroglobulin suggested by the pattern of bands obtained when its reduction products are separated by electrophoresis in sodium dodecyl sulphate on a disc gel, it is perhaps surprising that the results of amino acid analysis of α_1 macroglobulin and α_2 macroglobulin are so similar. Probably both α_1 macroglobulin and α_2 macroglobulin will be found to consist of sets of similar chains held together only in part by S-S bridges, as has been found for human α_2 macroglobulin by Harpel (1973).

Both the slightly higher molecular weight and the extra proportion of tyrosine in α_1 macroglobulin may be due to the subunit, which after reduction appears as a band corresponding to a mol.wt. of less than 6×10^4 .

The value for the isoelectric point of α_2 macroglobulin now obtained by focusing in polyacrylamide gel agrees with that found by Gauthier & Mouray (1976), when the experiment was carried out in a sucrose density gradient. Because of the greater resolving power of the gel system, both α_1 macroglobulin and α_2 macroglobulin have now been found to consist of at least two components.

The use in this study of serum rather than plasma as the source of the α_1 macroglobulin and α_2 macroglobulin involves the possibility that part of the tryptic esterase-protecting activity of the two macroglobulins is satisfied before the start of the isolation. However, the observation (Table 1) that in the serum from large uninjured rats 1.19 mol of trypsin was bound/mol of α_1 macroglobulin suggests the existence of a binding ratio of 1:1 for trypsin and α_1 macroglobulin. A similar ratio was found by Barrett & Starkey (1973), who measured binding of ^3H -labelled trypsin to human α_2 macroglobulin.

The molar ratios for the binding of trypsin to α_1 macroglobulin in rat serum given above and in Table 1 were calculated by assuming 50% active-site trypsin and 80% activity against BzArgNPhNO₂ of trypsin bound to α_1 macroglobulin or α_2 macroglobulin. As indicated under 'Active-site titrations', titration of the sample of trypsin used in these experiments with *p*-nitrophenyl *p*'-guanidinobenzoate led to this proportion of active site in free trypsin. Active-site titration of trypsin when bound to

human α_2 macroglobulin was shown by Barrett & Starkey (1973) to give a binding ratio of 1:1. As mentioned under 'Active-site titrations', active-site titration of α_1 macroglobulin and α_2 macroglobulin as isolated by the present method from rat serum gave similar binding ratios, both close to 1:1. The present finding of substantially the same binding ratios by active-site titration with *p*-nitrophenyl *p*'-guanidinobenzoate and by hydrolysis of BzArgNPhNO₂ (a rate estimation) is further evidence for a 1:1 trypsin/macroglobulin binding ratio rather than that of 2:1 as reported by Ganrot (1966b) for human α_2 macroglobulin. Evidence in favour of 2 mol of proteinase/mol of macroglobulin has, however, been obtained by Gauthier & Mouray (1976); thus by taking advantage of the greatly decreased esterolytic activity of chymotrypsin when bound to rat α_1 macroglobulin or α_2 macroglobulin, they were able to estimate the binding ratio of this enzyme. Further investigations of proteinase-macroglobulin interactions are needed to establish the binding ratios that may exist for particular proteinases.

As shown in Table 1, α_1 macroglobulin as a constituent of the serum obtained from injured rats, and also after isolation from such serum, bound trypsin at a lower ratio than when in the serum of uninjured rats. The release of proteinases brought about by the injury may explain the observed lower ratios. However, for this to be true it is also necessary to assume that such proteinases, after binding by α_1 macroglobulin or α_2 macroglobulin, lose any ability that they may have had to hydrolyse BzArgNPhNO₂. The rather lower tryptic esterase-protecting activity found for α_1 macroglobulin as compared with α_2 macroglobulin may be explained similarly. If so, isolated α_1 macroglobulin, which has been observed to bind less trypsin than does α_2 macroglobulin, must be assumed to have shown, when still in the serum, a relatively stronger tryptic esterase-protecting activity.

During isolation, estimates of the degree of purity attained can be based on any available property of the substances in question which is not shared with other substances in the mixture. However, meaningful estimates of purity necessarily require that the chosen property remains stable. In the present work both tryptic esterase-protecting activity and antigenicity have been used as bases for indices of purity. The use of both these properties of α_1 macroglobulin and α_2 macroglobulin was necessary because each property proved to have its own limitation. Thus in polyacrylamide gels the tryptic esterase-protecting activity was found to be labile.

The present work confirmed the findings of Ganrot (1966b) that gel filtration of rat serum does not lead to inactivation of tryptic esterase-protecting

activity. In addition, little inactivation was brought about by zonal centrifugation or chromatography on DEAE-cellulose.

Although the work of Ganrot (1973), who likewise used chromatography on DEAE-cellulose, indicated that the α_2 macroglobulin thus obtained from rat serum retained some degree of tryptic esterase-protecting activity, no claim for full retention of activity was made. Consequently it seemed worth while to compare in Table 1 the ratios of tryptic esterase-protecting activity to protein concentration given by isolated α_1 macroglobulin and α_2 macroglobulin with the corresponding ratios for the two proteins in the original sera. These data are presented in support of the claim that the isolated macroglobulins are substantially pure and retain almost all their tryptic esterase-protecting activity.

It is notable that, by using an immunochemical method similar to that used by Ganrot (1973) and α_1 macroglobulin and α_2 macroglobulin isolated by the present method as standards, much lower concentrations of the macroglobulin were found in the sera of normal and injured rats (see under 'Recoveries of α_2 and α_1 macroglobulin from the sera of normal rats and from those after injection with turpentine'). Evidently the values obtained by Ganrot (1973) and the even higher concentration of α_2 macroglobulin found by Jamieson *et al.* (1972b) in the serum of injured rats must depend on the purity of the α_1 macroglobulin and α_2 macroglobulin used as standards. The present method was used by Bosanquet *et al.* (1976) to follow the changes in plasma concentration of α_1 macroglobulin and α_2 macroglobulin after injury to male and female rats. It is of some interest that in males but not in females the plasma concentration of α_1 macroglobulin increases, reaching 5.19 ± 0.81 mg/ml ($n = 12$) 3 days after injury.

The present investigation has demonstrated certain chemical differences between α_1 macroglobulin and α_2 macroglobulin of the rat. In particular the much greater lability of α_1 macroglobulin when reduced with dithiothreitol is of special interest. Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate of the products thus obtained showed clearly that the subunit structures of the two molecules are different.

Electron micrographs of macroglobulin obtained from rabbits (Morelis *et al.*, 1969), humans, pigs (Barrett *et al.*, 1974) and rats (Gauthier & Mouray, 1975) show complex structures. As originally reported by Morelis *et al.* (1969), addition of trypsin to the macroglobulin leads to a degree of conformational change. Electron micrographs of rat α_1 macroglobulin and α_2 macroglobulin as now prepared showed more compact and regular shapes as a result of trypsin treatment (E. B. Brown & N. G.

Wrigley, unpublished work). However, no difference between α_1 macroglobulin and α_2 macroglobulin either before or after addition of trypsin could be detected.

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