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Macroglobulins

1. STUDIES ON THE ISOLATION AND PHYSICAL PROPERTIES OF PATHOLOGICAL MACROGLOBULINS

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On examination in the ultracentrifuge, human sera are shown to possess small concentrations of protein which sediment some two or three times faster (i.e. sedimentation coefficients in excess of 15s) than the more usual globulin components. These macroglobulin components normally account for up to 5% of the total protein, but Waldenström (1944) pointed out that certain pathological sera contain much larger quantities of a protein (or group of proteins) whose sedimentation behaviour is very similar to that of the normally occurring macroglobulin. More recent work (Wallenius, Trautman, Kunkel & Franklin, 1957; Deutsch & Morton, 1958; Kunkel, 1960) has confirmed that a group of differently sedimenting components rather than a single species is involved, and that electrophoretically both α - and γ -globulin types occur, though not necessarily in a single case. The comparison of the properties, both physicochemical and immunological, of normal and pathological macroglobulins presents a problem of some complexity, but additional difficulty is introduced when only imperfect separations of the macroglobulins from normal serum components are available.

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At the time the present work began, the great resolution obtainable through the use of chromatographic columns of ion-exchange cellulose materials had become apparent (Sober, Gutter, Wyckoff & Peterson, 1956; Sober & Peterson, 1958; A. Feinstein, P. Johnson & C. B. Richards, 1958, unpublished work). Such columns have been used to isolate from a macroglobulinaemic serum the macroglobulins and other globulins for detailed physicochemical characterization and comparison with other serum proteins. The immunological properties of the various protein fractions have been compared and are described in the next paper.

EXPERIMENTAL

Blood was collected by venepuncture and allowed to clot at room temperature. After 3½ hr. the serum was separated and had a protein content of 10.4 g./100 ml. No precipitation occurred on cooling it to 2°, but dilution with water (Sia test: see Martin, 1960) caused precipitation.

Isolation of macroglobulins

Fig. 1 shows the sedimentation patterns of the serum from the case of macroglobulinaemia (b) alongside normal serum sedimentation patterns (a). In the pathological serum it is clear that a group of macroglobulins (approx. 32% of total protein) rather than a single component is

involved. Serum from this case was used exclusively as the source of the proteins whose isolation and properties are described in this and the next paper. The serum was dialysed against aq. 1.64 M-(NH₄)₂SO₄ at 2-4°; the supernatant liquid, containing most of the albumin and traces of S₇ globulin (a particular sedimenting species is denoted by the symbol *S* with, as subscript, the rounded value of its sedimentation coefficient) as well as macroglobulin (Fig. 1*d*),

was not further used for preparative purposes. The precipitate containing most of the S₇ globulins and macroglobulins (Fig. 1*c*) was redissolved in NaCl soln. up to the initial volume of the serum and was found to remain in solution at 2°. After dialysis against 5 mM-phosphate buffer (Na₂HPO₄, 5 mM) at pH 8.2, the solution was applied to a column of diethylaminoethylcellulose at 2-4°, prepared as described by Sober *et al.* (1956) and previously equilibrated against the same buffer solvent. Elution was performed by establishing a gradient of decreasing pH and increasing concentration of KH₂PO₄. The final concentration of KH₂PO₄ was 0.1 M and the pH 4.7. Further protein was removed by elution with a solution containing 0.1 M-KH₂PO₄ and 0.5 M-NaCl, giving a total yield in excess of 90%.

Two such fractionations (I and II), employing columns 23 and 55 cm. long, with internal diameters of 3 and 3.5 cm. (retardation volumes of about 80 and 300 ml. respectively), were carried out on the proteins from 12 and 45 ml. of the original sera respectively. Sample volumes were 5 and 15 ml. respectively. *E*_{280mμ} of each sample, measured on a Unicam SP. 500 spectrophotometer, was plotted against sample (or tube) number to give the elution diagrams, which were then utilized to suggest pooling procedure. The 10 or 11 fractions thus obtained were concentrated by pervaporation or freeze-drying before further examination. Fractions 1-5 were stored as solutions at -20° for some 6 months before the ultracentrifugal studies were completed. In some such cases, changes in the state of aggregation became apparent. The main fractions, particularly 6.I and 9.I, were freeze-dried to the solid before storage at -20°. Fig. 2 shows the elution diagram for fractionation I, which differed from that of fractionation II only in superficial details.

Ultracentrifugal examination was carried out in a Spinco model E instrument equipped with schlieren phase plate. Unless otherwise stated the solvent employed was phosphate-NaCl buffer, pH 7.8, *I* 0.1, and solutions were exhaustively dialysed against repeated changes before examination. In correcting observed sedimentation coefficients to water at 20° (*S*₂₀⁰), the partial specific volume assumed was 0.725 (Koenig, 1950; Dayhoff, Perlmann & MacInnes, 1952). Values of *S*₂₀⁰ extrapolated to zero protein concentration are denoted by [*S*₂₀⁰]. Electrophoretic measurements were performed in a Perkin-Elmer apparatus equipped with a diagonal-schlieren optical system and the usual sectional U-tube of rectangular cross-section. Both ultracentrifuge and electrophoretic records were measured on a two-dimensional Pye travelling microscope; sedimentation coefficients (*S*) were then computed from plots of ln *x* against time at constant speed (*x* being distance from centre of rotation) and electrophoretic mobilities (*u*) from plots of boundary position (*x*) against time at constant current: $u = [(dx/dt) AK]/i$, where *A* is area of cross-section of tube and *K* is the specific conductivity of the protein solution}.

Protein concentrations were determined from measurements of refractive index (*n*) in a Zeiss interferometer, assuming $dn/dc = 0.187$ ml./g. (Doty & Edsall, 1951) as for human γ-globulins. Since the actual value of dn/dc for macroglobulins was not determined (owing to the small quantities of purified material available) concentrations are quoted in terms of refractive-index differences. Occasionally, measurements of extinction were also used, for which

Fig. 1. Sedimentation patterns at comparable stages of sedimentation in phosphate-NaCl buffer, pH 7.8, *I* 0.1, for: (a) normal serum; (b) serum from a case of macroglobulinaemia; (c) precipitate from (b) with 1.64 M-(NH₄)₂SO₄; (d) supernatant from (b) with 1.64 M-(NH₄)₂SO₄. Rotational speed: 59 780 rev./min. Temperature: 16 ± 1°.

Fig. 2. Elution diagram for fractionation I of globulins from macroglobulinaemic serum on diethylaminoethyl (DEAE)-cellulose at 2-4°C. Tube volume: 5 ml.

an empirical relation, $\Delta n = 1.50 \times 10^{-4} E_{280}$, was found to apply at protein concentrations of up to 0.2 g./100 ml. In assessing the proportions of differently sedimenting components, peak areas at constant magnification and schlieren angle, corrected to the position of the meniscus, were utilized, equal refractive-index increments being assumed.

Materials

Buffer salts were of analytical or equivalent grade. 2-Mercaptoethanol was obtained from L. Light and Co. Ltd. and iodoacetamide from Hopkin and Williams Ltd.

The diethylaminoethylcellulose was obtained from L. Light and Co. Ltd. (type 40). It possessed approx. 1 m-equiv. of basic groups/g.

Bovine plasma albumin, used in the preparation of albumin solutions of varying concentration (≤ 5 g./100 ml.), was an Armour crystallized fraction (batch BF1670).

The carbohydrate content of various protein fractions was determined semi-quantitatively by the reaction with periodic acid-Schiff stain (Köiw & Grönwall, 1952). Equal weights of the proteins in solution were placed on Whatman 3MM paper and the red colours produced by the stain were compared visually. Total macroglobulinaemic serum and the isolated macroglobulin fractions gave definite positive tests for carbohydrate, but the slowly sedimenting fraction, 8.II, and the prominent S_7 fraction, 6.I, gave only weak and very weak reactions respectively.

RESULTS

The elution diagrams show that several minor components (1-5) and one major component (6) pass rapidly through the column with little or no

retardation. After this group of components, a considerable volume of eluent passes through the column before the next broad peak (8) emerges, followed by a major complex of peaks. The fine structure of the complex was not reproducible between the two fractionations and it has therefore been divided, somewhat arbitrarily, into two components, 9 and 10 in fractionation I and an additional one (11) in II. Fig. 3 contains sedimentation patterns of the components from the two fractionations; ultracentrifuge examination was performed immediately for fractions 6.I, 9.I and 9.II, or in other cases at only a few days (at most) after elution from the column. Fractions 1-4 sediment very largely as single well-defined boundaries, with sedimentation coefficients of approximately 7s. Fractions 5 and 6 also contain similar components but, in addition, significant proportions of very slowly sedimenting material (1.25s) occur. In fractionation II, similar slowly sedimenting material appeared in a later fraction (8.II).

Fraction 8.I consisted largely of material sedimenting at a rate similar to that of albumin with traces of macroglobulins. Since the globulin fraction applied to the column was not reprecipitated, the presence of some albumin was not unexpected and, with an isoelectric point lower than the globulins, its position in the elution pattern was as expected. However, in fractionation II, little or no albumin was observed and fraction 8.II con-

sisted largely of material with $S_{20}^0 = 1.25$ s. It would thus appear that albumin tends to displace the slowly sedimenting material.

The remaining fractions (9.I and 10.I, and 9.II, 10.II and 11.II) contained macroglobulins, and no difference between these fractions in their physical properties was apparent. Fractions 9.I and 9.II

were the most abundant and were chiefly used in the work. Three main sedimenting components with S_{20}^0 values (see below) of 17.0, 19.2 and 28.9s occurred in the proportions 5, 85 and 10%. Traces of slower (8, 11.5, 14s) and faster sedimenting components also occurred, for which accurate measurements are not available.

Fig. 3. Sedimentation patterns and data for representative fractions from two fractionations (I and II) of total globulins on DEAE-cellulose (cf. Fig. 2). Solvent: phosphate-NaCl buffer, pH 7.8, I 0.1. Speed: 59 780 rev./min.

Properties of the isolated macroglobulins

Since the differently sedimenting macroglobulin components did not separate on the chromatograms, the possibility arose that they formed an equilibrium system. In such a case the proportions of the components and measured properties should vary with the experimental conditions. Fig. 4 contains schlieren diagrams for experiments carried out at $17 \pm 2^\circ$ at different total protein concentrations and pH values; no change in relative proportions was detected. Similarly, dilution of the original whole serum and of the total globulin fraction was without effect on the macroglobulin sedimentation pattern. Ultracentrifugal examination at 37° also gave the same sedimentation pattern. Further, except for species of 17.0 and 19.2s, the components resolve completely from one another, giving a true base-line between them which indicates lack of interaction. The difference, 17.0 to 19.2s, is not sufficient to allow complete resolution of the species involved, but the separation observed appears to progress normally during sedimentation. Further, the sedimentation data were extrapolated linearly in the usual way to zero protein concentration for the different components (Fig. 6); this would not be expected if the various peaks contained components of a rapidly reacting equilibrium system.

Since previous work (particularly of Wallenius *et al.* 1957) had indicated that macroglobulins could show differing electrophoretic properties, electrophoretic examination over a range of pH was carried out on fraction 9.II. Except for the experiment at pH 4.15, in which 0.2M-acetate buffer was used, phosphate-NaCl buffers, I 0.2, were used. Electrophoretic patterns for pH 8 (Fig. 5a) demonstrate the essentially homogeneous electrophoretic nature of the material and particularly the absence of more rapidly moving (α or β) material. The pH-mobility curve of Fig. 5b gives an isoelectric point of pH 5.9, and comparison with published data (e.g. Tiselius, 1937; Porter, 1960) shows the present macroglobulins to be typical γ -globulins. The electrophoretic homogeneity demonstrates that the differently sedimenting macroglobulin species possess the same electrophoretic character and suggests that the macroglobulins may be a family of chemically similar or even identical members as discussed below.

Since the macroglobulin fractions were stable mixtures of differently and independently sedimenting systems, they were examined in the ultracentrifuge at different protein concentrations. The sedimentation coefficients of the different species, corrected to water at 20° , were plotted as a function of the total initial protein concentration. Fig. 6 summarizes the results obtained for the

fractions from both fractionations. Within experimental error, each sedimenting species gives its own linear plot for S_{20}^0 against c (where c is total initial protein concentration in g./100 ml.). By the method of least squares, the parameters $[S_{20}^0]_{c=0}$, K and k of equations (1) and (2),

$$S_{20}^0 = [S_{20}^0]_{c=0} - Kc \quad (1)$$

and

$$S_{20}^0 = \frac{[S_{20}^0]_{c=0}}{1 + kc} \quad (2)$$

Fig. 4. Sedimentation patterns for macroglobulins (9.II) under various conditions at $17 \pm 2^\circ$:

	Concn. of protein (g./100 ml.)	pH	Buffer
(a)	1.0	7.8	Phosphate, I 0.1
(b)	1.0	4.2	0.2M-Acetate
(c)	0.5	7.8	Phosphate, I 0.1
(d)	1.0	7.8	Phosphate, I 0.1 + 1% of mercaptoethanol

Fig. 5. Electrophoresis of macroglobulin fraction 9.II. (a) Ascending electrophoresis patterns in phosphate-NaCl buffer, pH 8.0, I 0.2. Time between exposures: 40 min. Potential gradient: 5.1 v/cm. (b) pH-mobility curve.

were computed (Table 1). The data of Fig. 6, and those derived from it, refer to a solution containing three main sedimenting species in different proportions. The S against c curve for the intermediate fraction, occurring to the extent of approx. 85%, has considerable significance. For the other two fractions only the intercept on the ordinate axis is important, since the slope in each of these cases is determined by the concentration of components other than itself.

For comparison, similar data for the earlier S_7 fractions and for bovine serum albumin (Harrington, Johnson & Ottewill, 1956; Johnson, 1957) are also provided with the corresponding S against c plots (Fig. 7). In all cases, the data fit linear plots within experimental error, but owing to the small quantities of some fractions available the concentration range was restricted.

The sedimentation behaviour of the dissociation products of macroglobulin fraction 9.II is also illustrated in Figs. 4 and 8. Dissociation was accomplished by dialysing for 50 hr. a solution of the macroglobulins containing 0.65 g./100 ml.

Fig. 6. Plots of S_{20}^0 against total initial protein concentration (or Δn) for macroglobulin components from the different chromatographic fractions. $\circ$, Fraction I, II, no. 9; $\square$, fraction I, II, no. 10; $\triangle$, fraction II, no. 11.

Table 1. Sedimentation parameters

Probable errors are given in parentheses.

Fraction	$[S_{20}^0]_{c=0}$	K (eqn. 1)	k (eqn. 2)	Concn. range (g./100 ml.)
Bovine serum albumin	4.45	0.32	0.072	0-1
6.I, 6.II, 7.II	6.82 (± 0.01)	0.36 (± 0.02)	0.052	0-1
1.I, 2.I	7.10 (± 0.03)	0.89 (± 0.1)	0.125	0-0.5
3.I	7.28 (± 0.05)	1.97 (± 0.3)	0.27	0-0.3
4.I, 5.I	7.14 (± 0.01)	1.80 (± 0.08)	0.25	0-0.3
9-11	(17.0)	1.94	0.12	0-1
I and II	(19.2)	2.50	0.13	0-1
	(28.9)	3.88	0.13 ₄	0-1
9.II Dissociated	7.40	0.44	0.060	0-1

against 1% (by vol.) of 2-mercaptoethanol in phosphate buffer, I 0.1, pH 7.8 (Fig. 8a). Removal of the mercaptoethanol by further dialysis for 24 and 72 hr. against the buffer alone was followed immediately by ultracentrifugal examination (Fig. 8b and 8c). Dialysis against 0.01M-iodoacetamide in phosphate buffer was used to stabilize the dissociation products (Fig. 8d) whose S against c behaviour was then investigated (Fig. 7). As shown above, fraction 9.II contains the three main, differently sedimenting, macroglobulin components as well as traces of minor ones; the contrast with the well-defined, single, much more slowly moving peak of the dissociated material (Figs. 4d and 8) is striking. Thus apart from the small proportion (approx. 20%) of additional material that consistently appears on the trailing edge of the peak of the dissociated macroglobulin, the latter appears comparable in respect of homogeneity with the earlier S_7 fractions. The S against c extrapolation was linear and the sedimentation coefficients are consistently and significantly higher than for the naturally occurring S_7 fractions. The dissociated material (unstabilized) reaggregated very slowly after removal of mercaptoethanol by dialysis (see Fig. 8b and 8c); though polymeric forms certainly appeared, the higher, naturally occurring polymers (particularly those with $S_{20}^{00} = 19.2, 28.9$ s) either did not appear or were in low concentration. The occurrence of the lower polymers confirmed that the minor components of the macroglobulin fractions were a part of the macroglobulin family.

Sedimentation of protein mixtures

Since macroglobulins are frequently observed sedimenting in serum, i.e. in the presence of considerable concentrations of albumin and globulin, a knowledge of the retarding effect of other proteins is important. Known mixtures of the macro-

globulins with the well-defined S_7 fraction, 6.I, and with human serum albumin, were therefore prepared and the sedimentation behaviour of the various boundaries was investigated quantitatively (Table 2). In mathematical consideration of the sedimentation coefficients, the term kc in the denominator of equation (2) is replaced by $\sum k_i c_i$ (by analogy with Wallenius *et al.* 1957), where c_i is the concentration of the i th component at the boundary in question and k_i is the constant derived for it from equation (2) which expresses the variation in its sedimentation coefficient with its own concentration. Thus for the corrected sedimentation

Fig. 7. Plots of S_{20}^{00} against concentration for different chromatographic fractions and for the iodoacetamide-stabilized product of macroglobulin dissociation (O). □, Fraction I, II, no. 6; +, x, Δ, ● and ○, fraction I, nos. 1-5 respectively.

Fig. 8. Sedimentation patterns illustrating dissociation and reaggregation of macroglobulin fraction 9.II. Protein concentration: 0.6 g./100 ml. Buffer: phosphate-NaCl, I 0.1, pH 7.8. (a) After dialysis against buffer containing 1% of mercaptoethanol; (b) after further dialysis against buffer for 24 hr.; (c) after further dialysis against buffer for a total of 72 hr.; (d) after (b), dialysed against 0.01M-acetamide for 24 hr.

coefficient (S_{20}^0) of a component at finite concentrations of itself and other components, equation (2) becomes

$$S_{20}^0 = \frac{[S_{20}^0]_{c=0}}{1 + \sum k_i c_i} \quad (3)$$

Values of k_i for fraction 6.I and bovine serum albumin have been given in Table 1.

Table 2 contains summarized results for the various mixtures. Column 3, headed S_{20}^0 (obs.), contains values of the sedimentation coefficient corrected to water at 20° by the usual expression

$$S_{20}^0 \text{ (obs.)} = S \times \frac{\eta_t}{\eta_{20}^0} \times \frac{1 - \bar{v}\rho_{20}^0}{1 - \bar{v}\rho},$$

where S is the experimentally observed coefficient at temperature t in buffer solvent of viscosity η_t and density ρ , and η_{20}^0 and ρ_{20}^0 are the viscosity and density of water at 20°. Thus the contribution of the slower sedimenting protein to the density and viscosity of the medium through which the faster sedimenting protein moves is not considered at this stage. Column 4 contains values of $[S_{20}^0]_{c=0}$ calculated from equation (3), and column (5) those determined by extrapolation of S against c curves for the pure proteins. Just as the calculation of $[S_{20}^0]_{c=0}$ from equation (2) for a single component at finite concentration includes the effect of in-

creased viscosity and density of the medium, so does that from equation (3), and no correction for change in properties of the medium arising from the presence of slower sedimenting components is required. Such a correction is involved in the $\sum k_i c_i$ term.

It is of interest, however, to calculate separately the retardation of the faster sedimenting material caused by the increase in density and viscosity of the medium due to slower components. If such a correction procedure were valid, the corrected sedimentation coefficient of the faster sedimenting material must correspond to a finite concentration of itself rather than $[S_{20}^0]_{c=0}$. Viscosity and density measurements over the necessary concentration range are not available for the various fractions, but were performed for bovine serum albumin, which in its physicochemical properties strongly resembles human serum albumin. Graphs of relative density and viscosity were constructed for concentrations approaching 5 g./100 ml., and were used to compute the appropriate correction factors. The corrected S_{20}^0 values are contained in column (6).

Changes in chromatographic fractions on storage

Whilst fractions 1-6, shortly after elution from the chromatographic column, consisted largely of

Table 2. *Sedimentation of protein mixtures*

Components	Concn. (g./100 ml.)	S_{20}^0 (obs.)	$[S_{20}^0]_{c=0}$		S_{20}^0 (corr.)*
			Calc. (eqn. 3)	Obs.	
9.II	0.56	17.23	19.0	19.2	—
		27.15	30.4	28.9	—
6.I	0.61	6.8	7.0	6.8 ₂	—
		15.52	16.9	17.0	—
9.II	0.56	17.52	19.0	19.2	—
		26.78	29.2	28.9	—
6.I	0.31 ₅	6.83	7.0	6.8 ₂	—
Macroglobulin (II)	Traces	14.77	19.15	19.2	18.1
Albumin	4.2	—	—	—	—
Macroglobulin (I)	Traces	16.35	19.4	19.2	18.9
Albumin	2.7	—	—	—	—
Macroglobulin (II)	0.26	14.87	18.0	19.2	17.0
		21.67	26.1	28.9	24.8
Albumin	2.52	—	—	—	—
Macroglobulin (I)	0.43 ₇	17.63	19.0	19.2	—
	0.06	27.28	29.4	28.9	—
Globulins	0.31	6.85	6.95	(7.0)	—
Macroglobulin (I)	2.19	12.59	17.1	17.0	—
	0.30	18.20	25.7	28.9	—
Globulins	1.45	6.08	6.50	(7.0)	—
Albumin	0.12 ₃	4.30	4.34	4.46	—

* Calculated by using increased density and viscosity of solvent due to presence of slower sedimenting components.

material with $S_{20}^0 \approx 7s$ with no significant proportion of faster material, after storage at -20° , both in solution and in the dry solid state, appreciable proportions of faster material were completely resolved (Fig. 9) from the main component. The new components must have been formed from the S_7 components, but if the change in pattern represented the approach to equilibrium, then, during any single sedimentation experiment, equilibration was too slow to affect sedimentation patterns. The S values computed could thus be taken to be quantitatively related to molecular weights. To obtain an approximate estimate of the sedimentation coefficient of the new products at zero concentration, the observed corrected S_{20}^0 values were plotted (Fig. 9b) against total initial protein concentration for the different fractions. When the differences between the various S_7 fractions are considered the plot is surprisingly good, and indicates a $[S]_{20}^0$ value of approx. $10s$. This may indicate dimerization of the S_7 molecules, with relatively minor changes in frictional ratio.

DISCUSSION

The macroglobulins described in this paper behave electrophoretically as typical γ -globulins. This supports the suggestion of Peterson & Sober (1960) that the slow elution (under the experimental conditions) of the macroglobulin relative to γ -globulins is due to the increased adsorption arising from high molecular weight. Elution of the macroglobulin was, in fact, very similar to that of albumin, so that for chromatographic purposes the increased adsorption due to higher-molecular-weight of the macroglobulins was more than equivalent to a lowering of the isoelectric point by one pH unit.

The well-defined character of the earlier S_7 fractions from the chromatogram probably arises also from the fact that higher states of aggregation, which form on storage, are held back on the column so that the 'monomer' only is eluted in the early stages. The aggregated forms would correspondingly elute at a later stage, possibly with the macroglobulins, and could thus give rise to contamination. Since the aggregated forms may be in equilibrium with the 'monomer' under normal conditions, during elution they would at least partially revert to monomer. The misleading impression would thus be obtained that the monomeric form was being partially retained after the first group of fractions containing it had been completely eluted.

From the nature of the processes employed (precipitation, chromatography and freeze-drying), it is clear that minor serum components which are normally undetected may be detected and concentrated. This probably explains the slowly sedimenting component (fraction 8.II, Fig. 3), occurring in both fractionations, which is invisible in the sedimentation patterns of the original serum or separated globulin fraction. This material is almost certainly of low molecular weight and its elution at the later stages probably indicates a low isoelectric point (< 4.5). It did not specifically affect the sedimentation of other protein fractions and in its chromatographic behaviour, as mentioned above, it appeared to be displaced by albumin.

Although the existence of a range of electrophoretic and associated properties within the γ -globulins has long been accepted (e.g. Sharp, Cooper & Neurath, 1942; Alberty, 1948), it is important to establish whether or not the fine structure of the elution diagram is meaningful. Previous work (Fahey & Horbett, 1959) tended to show definite electrophoretic differences between the different fractions; the present work has shown the occurrence of significant differences in sedimentation behaviour. Not only are differences in the extrapolated sedimentation coefficients, $[S_{20}^0]_{c=0}$, apparent, but the slopes of the linear S against c

Fig. 9. (a) Sedimentation pattern for fraction 5.I after storage at -20° (cf. Fig. 3d), showing occurrence of aggregation products. (b) Plot of sedimentation constants for aggregation products from various S_7 fractions against total initial protein concentration. Fraction 1, no. 3 (Δ), no. 4 (●), no. 5 (●) and no. 6 (□).

plots appear to vary with the latter. In view of the high precision of sedimentation-velocity measurements, it seems that real separations of the different S_7 species are performed by the chromatographic column. However, the different S_7 fractions have the common property of aggregating, probably to the dimer, on storage at -20° as the dry solid or in solution in the frozen condition. This property has not been attributed to S_7 material previously, and it may indicate that the S_7 fractions of the particular serum used have abnormal properties.

The macroglobulins discussed in this work are associated with a pathological condition, and for this reason are not necessarily comparable with normal macroglobulins. Except when reducing agents were present, the macroglobulins sedimented as several distinct species both in the original serum and in the purified form (cf. Martin, 1960). On the other hand, electrophoretically, the absence of α -globulin-type material is noteworthy as well as the typical γ -globulin, single-peak electrophoretic behaviour. The group of differently sedimenting macroglobulins with common electrophoretic properties provides a further example of the general statement that electrophoretic properties are largely independent of state of aggregation (e.g. Johnson & Naismith, 1953, 1954).

The subdivision of the macroglobulins by the column into two main and various subsidiary peaks was not reflected in any differences in sedimentation behaviour. However, a systematic and careful electrophoretic comparison was not performed, and electrophoretic differences, which would explain the slightly different chromatographic behaviour, may well exist.

As indicated by previous work (see, for example, Kunkel, 1960), the macroglobulin components show no sign of dissociation or aggregation with changing pH, protein concentration or temperature. Kunkel (1960) has shown that the distribution among the differently sedimenting members is similar in different cases of macroglobulinaemia; the present case, with the bulk of the material sedimenting with $[S_{20}^0] = 19.2s$, superficially fits into this scheme. Thus in general some 70–90% of the macroglobulin occurs as the component with $[S_{20}^0] \approx 19s$, with 10–20% of $[S_{20}^0] \approx 25$ –29s and smaller quantities of other components. The occurrence of carbohydrate in the macroglobulins is also common to many other cases (e.g. Kunkel, 1960; Martin, 1960). However, on careful examination differences are apparent. Thus even allowing for inaccuracy in the sedimentation coefficients, it is apparent that there is considerable variation in the sedimentation coefficients for the main species, e.g. 17.8–19.9s and 25.0–31.0s for prominent components (Deutsch & Morton, 1958; Kunkel, 1960; Martin 1960), and certain components occur in

some cases which are absent in others. Thus the component with $[S_{20}^0] = 17.0s$ occurring in the present work is absent in other published work (e.g. Deutsch & Morton, 1958; Kunkel, 1960; Metais, Kovacs-Jung & Daune, 1960), and components sedimenting faster than that of $[S_{20}^0] = 28.9s$ are largely absent from the present work but have been consistently reported elsewhere. Thus the different macroglobulin systems seem to resemble one another only superficially.

In response to sulphhydryl compounds (cf. Deutsch & Morton, 1958), however, the differently sedimenting species of macroglobulins all dissociate completely, to give mainly the same type of dissociation unit in view of the well-defined sedimenting boundary (Fig. 8a). There is a small proportion (about 20%) of more slowly sedimenting material, visible on the trailing edge of the peak, and this persists after stabilization of the product by iodoacetamide. No accurate sedimentation coefficient for this material could be obtained, but after stabilization with iodoacetate its sedimentation constant was approximately 5s. On removal of mercaptoethanol by dialysis (Fig. 8b), the slower-sedimenting dissociation products rapidly disappear, by contrast with the bulk of the dissociated material, which reaggregates only very slowly and incompletely. A small proportion of the macroglobulins appears to be broken down into units which differ from the main dissociation products not only in their slower sedimentation but also in their more rapid rate of recombination. The similarity of the recombination products with the minor components, seen in the isolated macroglobulin, suggests that these minor components are not impurities but, rather, lower states of aggregation of the macroglobulin system. The electrophoretic identity of the differently sedimenting components supports this view.

The main bulk of the dissociation product gave a good linear S against c plot that was significantly displaced above the corresponding lines for all of the earlier S_7 materials. That this displacement was not due to the uptake by the protein of iodoacetamide was shown by the fact that the sedimentation coefficient of unstabilized dissociated material, immediately after removal of mercaptoethanol by a short dialysis, fell close to the same line. Thus the dissociation products of the macroglobulins are not normal S_7 materials. Not only do they possess chemically active groups responsible for the polymerization into macroglobulins, but the molecules must be different in size or in shape (or both) to account for the differences in sedimentation.

A linear dependence of sedimentation coefficient on protein concentration satisfactorily explains experimental results. It was to be expected that

$K ([S_{20}^0] k)$ values would increase with increasing sedimentation coefficient and molecular weight, but no trend in k is apparent.

In considering the sedimentation of mixtures, perhaps the most obvious procedure in correcting to standard conditions the observed sedimentation of a fast component in the presence of slower-sedimenting materials would be based upon the viscosity and density changes arising from the presence of the slower. However, such a procedure, though not rigorously applied here, appears to 'undercorrect' considerably, even when it is realized that the corrected sedimentation coefficients (Table 2, column 6) refer to a finite concentration of the fast component. On the other hand, equation (3), particularly for the lower protein concentrations (< 2 g./100 ml.), yields values of $[S_{20}^0]_{c=0}$ from measurements in protein mixtures which are in good agreement with those obtained from single-protein systems. At the higher protein concentrations (> 2 g./100 ml.) the procedure also tends to undercorrect, but more data are required to substantiate this point. Equation (3) implies that the faster component is retarded by the slower to the same extent as the latter retards itself at the same concentration, i.e. specific interactions between the components are absent.

SUMMARY

1. The fractionation, by chromatography on diethylaminoethylcellulose, of the total serum globulins from a case of macroglobulinaemia has been described. By the use of a gradient of increasing salt concentration and decreasing pH, a series of globulins, distinguishable but all sedimenting at about 7s, was first eluted; macroglobulins emerged much later as a complex of peaks. The different peaks of the complex did not differ in their sedimentation behaviour.

2. The macroglobulin fractions all contained three main stable sedimenting species (85% with S_{20}^0 of 19.2s, 10% of 28.9s and 5% of 17.0s) and traces of minor components, the sedimentation pattern being independent of total concentration, pH and temperature over considerable ranges. The differently sedimenting species were typical γ -globulins electrophoretically, with an isoelectric point at pH 5.9, and contained carbohydrate.

3. Plots of corrected sedimentation coefficient (S_{20}^0) against total initial protein concentration were linear and the extrapolated values at zero concentration differed from those reported for other macroglobulins.

4. The different macroglobulin species were converted by 2-mercaptoethanol into a much slower and more homogeneously sedimenting material. On stabilization with iodoacetamide, the detailed

sedimentation behaviour was shown to be significantly different from that of the S_7 globulins from the same serum. Reversal of the dissociation was slow and incomplete, slower-sedimenting species comparable with the originally occurring minor components now occurring more prominently. This evidence, and the electrophoretic identity of the differently sedimenting macroglobulin species, suggest that the macroglobulins in this pathological case consist mainly of a number of different states of aggregation of the same monomeric unit.

5. From sedimentation-velocity experiments on mixtures, it was found that the faster sedimenting components were retarded by slower material to the same extent as the slower retarded itself from its infinite dilution value. On the other hand, the observed retardation of the faster components was greater than would be calculated from the increments in viscosity and density arising from the presence of the slower.

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Macroglobulins

2. IMMUNOCHEMICAL STUDIES ON ISOLATED PATHOLOGICAL MACROGLOBULINS

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Albert & Johnson (1961) described the isolation by chromatography of the macroglobulins and other protein fractions from the serum of a single case of macroglobulinaemia. The same macroglobulin and accompanying protein fractions have been further characterized and compared by immunochemical studies, which are now reported. Previous work along these lines has given conflicting results. Thus Morton & Deutsch (1958), on the basis of cross-reactivity and absorption experiments, conclude that several pathological macroglobulins 'do not contain any immunological determinants lacking in normal human serum' and that the iodoacetamide-stabilized dissociation products as well as the reaggregated molecules possess the same antigenic groupings. However, from similar experiments, Franklin & Kunkel (1957) consider that though 7s and 19s components of γ -globulin cross-react, they are not immunologically identical. Similarly Korngold & Van Leeuwen (1957), regarding pathological macroglobulins as immunologically related to normal (γ_1 -)macroglobulins and 7s γ -globulins, report the preparation of antisera to macroglobulins, which by absorption with normal γ -globulins can be made specific for homologous macroglobulin. In substantial agreement with this view, Fahey & Horbett (1959) show that 18s and 7s γ -globulins give rise to distinct and separate precipitation lines when allowed to diffuse against anti-(whole γ) serum.

The different views held might conceivably arise from the different states of purity achieved by different workers for both macroglobulins and 7s

components. Results on chromatographically isolated fractions thus become of considerable interest.

MATERIALS AND METHODS

Serum from a single case (P) of macroglobulinaemia was fractionated on diethylaminoethylcellulose as described by Albert & Johnson (1961) and the various macroglobulin and 7s globulin fractions were utilized in both gel-diffusion (Ouchterlony, 1949) and immunoelectrophoresis (Grabar, 1958) studies.

The macroglobulins used, in both native and dissociated form, were mainly fractions 9 from both fractionations (I and II). Fractions 10.I and 11.II were very similar in composition, but since much smaller quantities were available and they had been less thoroughly characterized physicochemically, they were not much utilized in this section of the work. Of the 7s γ -globulin fractions (all from fractionation I), fraction 6 (γ -6) was mainly used but γ -2, γ -3 and γ -4 were also studied. The different proteins were used in phosphate-NaCl buffer, 1.0.1, pH 7.8, at the following concentrations (g./100 ml.): macroglobulins (native and dissociated) 0.65–0.70; 7s globulin fractions (except γ -6), 0.2–0.4; γ -6, 1.0. In addition to the P serum and derived fractions, a small sample of a further serum (S) containing macroglobulins was made available by Dr R. Arden Jones of Newmarket General Hospital. Some months earlier, the patient (S) had shown a much higher content of macroglobulin ($\approx 47\%$), but, after treatment, the macroglobulin fraction now accounted for some 11% of the total protein. The following antisera were kindly provided by Professor J. R. Marrack and Dr A. Kelus. Rabbit antiserum against macroglobulin 9.II (AM) was prepared by a single multiple subcutaneous injection together with Freund's adjuvant. Anti- γ -globulin serum (A_γ) was prepared in a rabbit by injecting some of the animal's own red cells to which human γ -globulin (contaminated with other serum proteins) had been coupled by bisdiazotized benzidine. The rabbit received ten such injections intravenously.

A further anti- γ -globulin serum ($A_{\gamma T}$) was prepared in a

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