Isolation of Human β_{1A} -Globulin by Modern Techniques

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1. Human β_{1A} -globulin was isolated from serum by precipitation with ammonium sulphate, gel filtration and electrophoresis in polyacrylamide gel. 2. The product was found by ultracentrifugation, analytical electrophoresis in polyacrylamide gel and two-dimensional immunoelectrophoresis to be of satisfactory quality for further study. 3. The amino acid composition of β_{1A} -globulin was determined. 4. In ordinary dilute buffers near neutrality, β_{1A} -globulin had $S_{20,w}^0$ 6.42s and M 131 000, but some reversible aggregation occurred at lower pH. In neutral 6M-guanidine hydrochloride the molecular weight was not measurably different from that in dilute buffer.

Electrophoretic analysis of human serum (or plasma) that has been stored reveals a component (Müller-Eberhard, Nilsson & Aronsson, 1960), now known usually as β_{1A} -globulin, but originally called the 'p' protein by Poulik & Smithies (1958), that has developed at the expense of one of the components of fresh serum. The isolation of β_{1A} -globulin, as well as that of its presumed precursor (β_{1C} -globulin), was first achieved by Müller-Eberhard *et al.* (1960). As β_{1C} -globulin is a constituent of the complement complex, and is present in abnormally large amounts in some pathological conditions (Schultze, Heide & Haupt, 1962), most investigations of these related proteins have been concerned with their biological properties. Müller-Eberhard *et al.* (1960) suggested that β_{1A} -globulin arises from the splitting of the molecule of β_{1C} -globulin into two parts of approximately equal size, but no measurements of molecular weights or chemical studies have been made to substantiate this. The objects of the present work were to use modern techniques to isolate β_{1A} -globulin and then to study its properties as a first stage towards settling some of the questions about its derivation.

MATERIALS AND METHODS

Isolation of β_{1A} -globulin. Human plasma and 40% (w/v) CaCl_2 were mixed in a volume ratio of 100:1 and left overnight in a refrigerator. The serum, after separation by centrifugation, was sterilized by Seitz filtration and incubated at 37° for a week.

To 80 ml. of incubated serum 15 ml. of sodium phosphate buffer, pH 8.0 and 11.0, was added, followed by 55 ml. of water. The mixture was stirred as 150 ml. of 4M-(NH_4)₂SO₄ was added slowly. The suspension, which had pH 7.0, was left for 30 min. before being filtered through Whatman no. 1 paper. The solid from the paper was dissolved in 8 ml. of

0.15M-NaCl-25mM-tris-HCl buffer, pH 7.2, and dialysed against buffer of double this concentration.

The fraction obtained in this way was placed on a column (3.5 cm. $\times$ 150 cm.) of Sephadex G-150 (Pharmacia, Uppsala, Sweden) that had been prepared in the same solvent as used for dialysis. Elution with this solvent, at 25–30 ml./hr., was followed by recording of the extinction (at 253.7 nm.) of the effluent, fractions (5.5 ml.) of which were collected. Preliminary experiments showed that β_{1A} -globulin was in the latter part of the second zone, as confirmed in Fig. 1. Requisite fractions were pooled and concentrated, either by precipitation with (NH_4)₂SO₄, as in the first step, or by dialysis under pressure. Final dialysis in either case was in 50mM-glycine-17mM-tris buffer, pH 8.7.

Two batches of material from the Sephadex-column stage were pooled and fractionated by electrophoresis in polyacrylamide gel [cross-linkage (methylenebisacrylamide) content 5% of total acrylamide] in apparatus of the Brownstone (1969) type. The gel (4½%; 9.4 cm. diam. $\times$ 4.6 cm. long) was set in the above glycine-tris buffer and soaked in it overnight. The gel was then put in its holder and a current of 150 mA was passed for 4 hr. to remove mobile u.v.-absorbing material. The sample, diluted to 40 ml. and having 2 g. of sucrose dissolved in it, was layered on the gel surface and the same current passed. For the first 2 hr. buffer circulation was not carried out, but by the end of this time most of the protein had entered the gel. Electrophoresis was continued overnight (16 hr.) with buffer circulation between the electrode compartments. The pump delivering buffer into the collecting chamber was set to provide 1.8 ml./cycle, but fractions were somewhat bigger than this because of electro-endosmosis. For the first 3 hr., fractions were removed every 7 min., but the interval was increased gradually to 70 min. during the night. Extinctions of fractions were recorded automatically at 253.7 nm. The middle absorption peak (Fig. 2) represented β_{1A} -globulin. Appropriate fractions were pooled and concentrated by dialysis under pressure. Usually two such batches were combined for a second electrophoretic stage, which was done similarly, except that the gel (6.5 cm. diam. $\times$ 7.5 cm. long) was 8%, current (75 mA) and input volume (0.85 ml.) were both

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Fig. 1. Extinction at 253.7nm. (—) and radioactivity (c.p.m.) (----) of fractions obtained from a column (3.5cm. $\times$ 150cm.) of Sephadex G-150. The sample consisted of serum protein precipitated in 2M-(NH₄)₂SO₄ (final concn.), to which was added a small amount of ¹²⁵I-labelled β_{1A} -globulin. The solvent was 0.3M-NaCl-50mM-tris-HCl buffer, pH7.2. Fractions (5.5ml.) were collected. The flow rate was 25–30ml./hr.

Fig. 2. Extinction at 253.7nm. of fractions obtained by electrophoresis, in 4½% polyacrylamide gel, of 500mg. of protein prepared by Sephadex chromatography. The gel was 9.4cm. diam. $\times$ 4.6cm. long. The buffer was 50mM-glycine-17mM-tris, pH8.7, the temperature 8° and the current 150–200mA. The mean fraction volume was 2.2ml.; the total time was 18hr.

lower, and the time for running-in was reduced from 2hr. to 30min.

The product from the last stage, concentrated by dialysis under pressure, was placed on a Sephadex column (1.5cm. $\times$ 90cm.) under the same conditions as originally and eluted so that the most concentrated fraction could be used for molecular-weight determinations without further treatment except simple dialysis. The rest of the protein in the zone was dialysed under pressure.

Electrophoretic analysis. Fractions from various stages were examined by disc electrophoresis (Davis, 1964) in 8mm.-diam. gel columns which consisted of a top layer of 5% polyacrylamide [cross-linkage (methylenebisacrylamide) content 2% of total acrylamide] (5cm. high) above a layer of 9.5% polyacrylamide [cross-linkage (methylene-

bisacrylamide) content 1% of total acrylamide] (7.5cm. high). Sucrose (20mg./ml.) was dissolved in the protein samples to facilitate layering for running-in. The buffer was 30mM-glycine-5mM-tris, pH8.4. After a current of 0.75mA/tube had been passed for 18hr. the gels were fixed. Staining was done with 0.05% Amido Black-7% (w/v) acetic acid, excess of dye being removed by diffusion into 7% acetic acid.

Two-dimensional immunoelectrophoresis. Final products were tested by the method of Clarke & Freeman (1968), both to check for the absence of contaminants and to confirm the authenticity of the β_{1A} -globulin. The antiserum used was prepared in goats by immunization with whole human serum.

Sedimentation-velocity measurements. These were done at 59780rev./min. in a Spinco model E ultracentrifuge equipped with temperature control. Records were made through schlieren optics, which included a Wratten 77A filter to isolate light of λ 546nm. and a phase plate. Measurements and calculations were done in the usual way (Charlwood, 1961), all sedimentation coefficients being corrected to water at 20° and expressed in Svedberg units (s).

Diffusion measurements. These were made at 25° in the double synthetic-boundary cell of the ultracentrifuge at 6995rev./min. (J rotor), by layering solvent over 0.15ml. of protein solution. In each experiment the height (h) of the schlieren peak was measured at 15 different times, and the regression of h^{-2} on time as well as the intercept on the time axis, the zero-time correction, was calculated. The mean area under the peak was derived by trapezoidal integration from measurements made on three of the earlier photographs.

Molecular-weight measurements. These were done in 2mm. columns at 25°, mainly by newer equilibrium techniques developed with interference optics (Charlwood, 1965, 1967, 1968), but in a few cases by the meniscus-depletion method of Yphantis (1964). For the latter, 1% of sucrose was included as recommended by Kanarek, Marler, Bradshaw, Fellows & Hill (1964). Experiments at lower pH or in guanidine hydrochloride were done in a short-column multichannel cell (Yphantis, 1960) in conjunction with schlieren optics. In all cases refractive increment was assumed to be proportional to protein concentration. The final equilibrium patterns recorded with interference optics gave concentration distributions directly, and molecular weight M was calculated from the expression:

$$M = \frac{2RT}{\omega^2(1-\bar{v}\rho)} \frac{d(\ln c)}{d(r^2)}$$

where $d(\ln c)/d(r^2)$ was the statistical regression coefficient, the partial specific volume ($\bar{v}$) was taken as 0.74 (see below) and the other symbols have their usual significance. The results of multichannel experiments were calculated in the manner described by Charlwood & Utsumi (1969).

Protein concentrations. Those in guanidine hydrochloride solution were determined as follows. When the globulin had been dialysed to equilibrium with buffer (0.1M-NaCl-0.05M sodium phosphate, pH7.0) its concentration was measured in a differential refractometer (Cecil & Ogston, 1951). Solution, buffer of ten times the above concentration, solid guanidine hydrochloride and water were mixed in weighed amounts to give a final solution whose density was calculated from the data of Kawahara & Tanford (1966). The guanidine hydrochloride was 6M, and the final sodium salt

concentrations were as given above. A similar solution, not containing protein, was prepared to use in the comparison channels of the cell, and also to dilute the stock protein solution so that the apparent molecular weight could be found as a function of concentration. These dilutions were done with accurate microsyringes (Scientific Products Inc., Flushing, N.Y., U.S.A.). Where concentrations are quoted on a weight basis the factor 0.00180 has been used for the specific refractive increment.

Amino acid analysis. Samples containing 2 mg. of protein in 1.5 ml. of 6M-HCl were put in hydrolysis tubes and degassed either by repeated freezing in ethanol-solid CO_2 mixtures and thawing under vacuum, or by gently bubbling argon through them. The tubes were sealed and heated at 110° for 22 hr., after which they were opened and the contents dried either in a rotary evaporator or in a desiccator containing NaOH pellets. The resulting solid was dissolved in buffer and portions corresponding to 0.4 mg. of protein were analysed as described by Jacobs (1969).

Iodinated proteins. These were prepared with carrier-free ^{131}I or ^{125}I mixed with buffered ICl, either by the procedure of McFarlane (1958) or in borate buffer (Helmkamp, Goodland, Bale, Spar & Mutschler, 1960). Radioactivity of fractions was measured in an Auto-gamma counter.

RESULTS

The pattern given by the effluent from the large Sephadex columns (Fig. 1) showed the great decrease in the concentration of smaller proteins (mainly albumin) that had resulted from the ammonium sulphate stage. The distribution of radioactivity relative to u.v. absorption showed that a useful elimination of IgG* was effected when fractions corresponding to the peak of radioactivity were pooled, but that a substantial amount of IgG would still be included.

The pattern resulting from preparative-electrophoresis experiments (Fig. 2) showed three main peaks, corresponding to caeruloplasmin, β_{1A} -globulin and IgG in descending order of mobility. The caeruloplasmin was located by its blue colour, but relative positions after disc electrophoresis and behaviour on two-dimensional immunoelectrophoresis were the main characteristics used to identify the components.

The ultracentrifuge pattern of β_{1A} -globulin immediately before its final passage through Sephadex (Fig. 3a) indicates the reason why this stage was added. In many preparations there was detectable a small amount of slowly sedimenting substance that derived from the polyacrylamide gel and was most easily eliminated by means of Sephadex.

An indication of the composition of fractions at various stages in the isolation is given by the disc-electrophoresis patterns shown in Fig. 4, and the two-dimensional immunoelectrophoresis pattern and amino acid analysis of the final product are given in Fig. 5 and Table 1 respectively.

* Abbreviation: IgG, immunoglobulin G.

Fig. 3. Ultracentrifuge patterns of a β_{1A} -globulin preparation (a) before and (b) after final passage through Sephadex G-150. Sedimentation (right to left) was for (a) 62 min. in 3 mm. cell and (b) 90 min. in 12 mm. cell, both at 59780 rev./min. The protein concentrations were 12 and 15 mg./ml. respectively. The solvent was 0.1M-NaCl-0.05M-sodium phosphate buffer, pH 7.0, and the temperature 20.5° .

Fig. 4. Disc-electrophoresis patterns at pH 8.4 of material at various stages in the isolation: (a) original serum; (b) fraction precipitated at pH 7.0 by 2M-(NH_4) $_2\text{SO}_4$; (c) concentrate of latter part of middle zone from Sephadex column (see Fig. 1); (d) and (e) products of first and second step respectively of polyacrylamide-gel electrophoresis.

As judged by radioactively labelled material, the precipitation and gel-filtration stages gave recoveries of at least 90–95% of β_{1A} -globulin. Electrophoresis was undoubtedly less efficient but, although the yields from these stages were not assessed on a tracer basis, the overall yield of β_{1A} -globulin must have been at least 60%, judging by the value given by Schultze & Heremans (1966) for the content of β_{1C} -globulin in serum.

Fig. 5. Two-dimensional immunoelectrophoresis of purified β_{1A} -globulin. First stage; migration right to left. The anti-serum used was prepared by injection of whole human serum into goats.

Table 1. *Amino acid composition of human β_{1A} -globulin*

The values are means of four determinations. See the text for hydrolysis conditions. Cystine was detected, but is not included in the table as it was difficult to measure; it corresponded to no more than 5–10 half-cystine residues/mol. Tryptophan was not measured.

Residue	Amino acid composition (moles/100 moles of total residues) (mean $\pm$ s.e.m.)	Residues/mol of mol.wt.131 000
Lys	7.43 $\pm$ 0.042	86.9
His	1.73 $\pm$ 0.033	20.2
Arg	4.55 $\pm$ 0.087	53.2
Asp	9.78 $\pm$ 0.099	114.3
Thr	6.72 $\pm$ 0.153	78.6
Ser	6.93 $\pm$ 0.166	81.1
Glu	12.99 $\pm$ 0.065	151.9
Pro	5.12 $\pm$ 0.147	59.8
Gly	6.01 $\pm$ 0.066	70.2
Ala	5.26 $\pm$ 0.072	61.5
Val	9.39 $\pm$ 0.182	109.8
Met	1.99 $\pm$ 0.059	23.2
Ile	5.30 $\pm$ 0.090	61.9
Leu	9.05 $\pm$ 0.130	105.9
Tyr	3.84 $\pm$ 0.042	44.8
Phe	3.91 $\pm$ 0.063	45.7
Hexosamine	Trace	

The results of sedimentation-velocity measurements (Fig. 6) gave $S_{20,w}^0$ as 6.42 ± 0.03 s (95% fiducial limits) in neutral solution, whereas at pH 5 values were higher but not reproducible. However, material that had been exposed to this lower pH and then dialysed back to neutrality had normal sedimentation characteristics. Some of the filled circles in Fig. 6 refer to such samples.

The standard errors of slope of the regressions from which the diffusion coefficients (Table 2) were calculated ranged from about 0.5 to 1.0% of the respective slopes. When these errors, and those

Fig. 6. Sedimentation coefficients of human β_{1A} -globulin. The solvents were: ●, 0.1M-NaCl-0.05I-sodium phosphate buffer, pH 7.0; ○, 0.1M-NaCl-0.05I-sodium acetate buffer, pH 4.9.

Table 2. *Diffusion coefficients of β_{1A} -globulin*

The solvent was 0.1M-NaCl-0.05I-sodium phosphate buffer, pH 7.0. For other details see the text.

Approx. concn. (mg./ml.)	$10^7 \times D_{20,w}^0$ (cm. ² sec. ⁻¹)	Zero time (min.)
3	4.64	1.2
6	4.47	2.0
9	4.54	1.2
12	4.60	3.4

involved in arriving at the areas, are taken into account, the diffusion coefficients show no significant concentration dependence. The best estimate of $D_{20,w}^0$ is then the simple mean, 4.56×10^{-7} cm.² sec.⁻¹.

The results of the main equilibrium molecular-weight measurements (Table 3) indicate that M is 131 000 for β_{1A} -globulin in ordinary neutral solution, a value in excellent agreement with that obtained by combining sedimentation-velocity and diffusion values, and further supported, within their limits of error, by the meniscus-depletion results. At lower pH there was some aggregation of the protein, as previously suspected from the velocity behaviour. The concentration-dependence of the apparent molecular weight in 6M-guanidine hydrochloride was fairly large, and extrapolation rather uncertain, but it is clear that the molecular weight in this solvent is sufficiently close to that in ordinary buffer at pH 7 to exclude the possibility of any appreciable dissociation.

DISCUSSION

Earlier methods for the isolation of β_{1A} -globulin (Müller-Eberhard *et al.* 1960; Schultze *et al.* 1962; Steinbuch, Quentin & Pejaudier, 1963) also in-

Table 3. *Molecular weight of human β_{1A} -globulin under various conditions*

Solvent	Method	Concn. (mg./ml.)	Speed (rev./min.)	M
0.1M-NaCl-0.05 <i>I</i> -sodium phosphate buffer, pH 7.0	Interference equilibrium	1.5-3.5	6995	131100±770*
		1.5-2.5	6995	131200†
	Meniscus depletion	0.3	20410	135800‡
		0.6	20410	130800‡
		0.9	20410	134000‡
	Combined $S_{20,w}^0$ and $D_{20,w}^0$			131300
0.1M-NaCl-0.05 <i>I</i> -sodium acetate buffer, pH 4.9	Short-column schlieren	2.5	6166	226000
		5.0	6166	277000
0.1M-NaCl-0.05 <i>I</i> -sodium phosphate buffer, pH 7.0+6M-guanidine hydrochloride	Short-column schlieren	2.0	14290	78900
		3.0	14290	71700
		4.0	14290	65100
		5.0	14290	61800

* Mean and S.E.M. of 15 determinations on various batches.

† After dialysis back from pH 4.9 buffer.

‡ Sucrose (1%) was included in these solutions (see the text).

volved precipitation. It seems difficult to avoid this, because the usefulness of chromatographic or electrophoretic methods applied directly to serum is greatly restricted by the preponderance of albumin. Gel filtration is very valuable in the elimination of macroglobulins, residual albumin etc., but electrophoresis seems the most convenient way of separating β_{1A} -globulin from IgG and caeruloplasmin because in most other respects their properties are rather similar. A gel of lower concentration and raised buffer concentration in the sample are used in the first electrophoretic stage because otherwise IgG tends to block the top surface of the gel. Since IgG is virtually eliminated during this stage it is possible to use a higher polyacrylamide concentration to improve resolution in the next step.

The earlier methods of isolation used a lower pH for some stages. Since it has been shown (Table 3) that β_{1A} -globulin aggregates to some extent even at pH 5, material exposed to this pH and then dialysed back to neutrality was examined. The evidence of Table 3 and sedimentation-velocity coefficients show that it reverted to its normal behaviour.

The partial specific volume of β_{1A} -globulin was calculated from the amino acid composition (Table 1) by using factors from Cohn & Edsall (1943). As the value came to 0.739 it was rounded off to 0.74 for use in ultracentrifuge calculations, although it is likely that the value in guanidine hydrochloride differs a little from that in more ordinary solvents.

Although it contains many tyrosine residues, and was iodinated without difficulty, β_{1A} -globulin gradually lost some of the iodine. Thus, although labelled material was useful in following the course of fractionation, care had to be taken in drawing quantitative conclusions.

The value of $S_{20,w}^0$ obtained here is rather lower

than that of Müller-Eberhard *et al.* (1960) or Schultze *et al.* (1962). The reason for this is not obvious, but it could arise from specific ion effects.

The molecular weight obtained here (131 000) taken in conjunction with the sedimentation coefficient (6.42s) gives a frictional ratio of 1.38, which is typical of a globular protein. If the molecule contains subunits they must be linked either by covalent bonds, such as disulphide bridges, or by some other powerful forces, possibly of a hydrophobic character (cf. stFc fragment of rabbit IgG; Charlwood & Utsumi, 1969).

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