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Ultracentrifugal Studies of Rat, Rabbit and Guinea-Pig Serum Albumins

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Rat, rabbit and guinea-pig serum albumins have been used in many investigations, but no systematic study of their behaviour in the ultracentrifuge has been reported, except for a short preliminary account of part of the present work (Charlwood, 1959) and a graph showing the dependence on concentration of the sedimentation coefficient of rat albumin at pH 7.5 (Anderson, Canning, Anderson & Shellhamer, 1959). Some measurements have now been completed with the intention of defining the molecular weights of the albumins as accurately as possible, of discovering whether different methods of preparation give essentially the same product and whether the sedimentation coefficients are dependent on pH in the same manner as those of bovine, human and horse albumins (Charlwood & Ens, 1957). In assessing possible differences between preparations, it was necessary to obtain an estimate of the reproducibility of measurements and of the probable magnitudes of errors entering into the various factors concerned. A stock preparation of human albumin, designated A (Charlwood, 1954), was used for many of these experiments.

METHODS

Protein isolation. Some samples of albumin were obtained by electrophoresis of diluted serum in veronal buffer (pH 8.6, *I* 0.1) in the Tiselius apparatus, with automatic control of the compensator, as described by Charlwood (1954) and Warren & Charlwood (1953). Some sera were subjected to preliminary salt fractionation, globulins being mostly removed by saturation with magnesium sulphate followed by filtration (Popják & McCarthy, 1946).

Two organic solvent systems were used for serum

fractionation. In the first, 10 vol. of aq. ethanol (96%, v/v, of the alcohol) containing 1% (w/v) of trichloroacetic acid were slowly mixed with 1 vol. of serum at room temperature (Delaville, Delaville & Delaville, 1954). After 30 min., either at room temperature or in the cold (5°), the mixture was centrifuged. The supernatant was dialysed under pressure in the cold against several changes of water. After removal of any precipitate by centrifuging, the albumin solution was dialysed against a suitable buffer. This was veronal (pH 8.6, *I* 0.1) if electrophoresis was to follow, or sodium acetate (pH 4.5, *I* 0.1) if the next stage was to be ultracentrifuging. Solutions not clear at this stage were treated as described below. In some initial experiments the preliminary dialysis against water was omitted, but this proved inadvisable (see Results section). The second method of isolation involving an organic solvent was the same as the previous one, except that the trichloroacetic acid-ethanol system was replaced by 0.4M-tartaric acid in aq. methanol (90%, v/v, of the alcohol) (Michael, 1958).

When a preparation, made in an organic solvent system, yielded an opalescent aqueous solution which could not be clarified in the ordinary centrifuge, it was often possible to remove the unwanted impurity by centrifuging for 15-30 min. at 39 460 rev./min. in the Spinco preparative rotor SW39. Some electrophoretically separated rat-albumin solutions that contained soluble materials of higher sedimentation coefficients were treated similarly for a period of several hours; this was calculated to bring down the larger molecules but to leave a high proportion of the albumin in the middle of the tubes, whence it could be removed by syringe. This method of fractionation was also applied to one freeze-dried sample of rat albumin that had originally been prepared by the trichloroacetic acid-ethanol method. Many preparations were made on a scale sufficiently large for a comparison to be possible between freeze-dried material and a sample of the same batch which had remained in solution throughout.

Protein quality control. All albumin preparations were examined in the Spinco ultracentrifuge (model E) at high speed (59 780 or 52 640 rev./min.) at a protein concentration of 0.7–1.2%, usually in 0.15M-potassium chloride containing sodium phosphate buffer (pH 6.4, I 0.05). Samples prepared by electrophoresis were checked by examination in the microcell of the Tiselius apparatus at a concentration of 0.8–1.5% in the same buffer as was used for the separation. Many of the other preparations were also analysed in veronal buffer (pH 8.6, I 0.1) in the Tiselius apparatus. When the quantity was more limited, electrophoresis on paper was sometimes preferred. This was done in the manner of Jencks, Jetton & Durrum (1955). The three rat and four rabbit albumins indicated in Table 1 and rat albumin EL/S (see Fig. 1*b*), were examined by immunoelectrophoresis with a combination of the techniques of Grabar & Williams (1953) and Wieme (1959).

Molecular-weight measurements. These were done in the Spinco ultracentrifuge (model E) at low speed (8210 or 12 590 rev./min.), with albumin solutions of concentration 0.6–0.8% previously dialysed against buffer; the buffer was usually sodium phosphate (pH 6.4, I 0.05) containing 0.15M-potassium chloride. Five photographic exposures were recorded at 8 min. intervals, commencing about 30 min. after the ultracentrifuge had attained operating speed. Schlieren bar angles were in the range 45–65°.

The refractive index increment between buffer and protein solution was measured in a differential refractometer of the type described by Cecil & Ogston (1951) at the wavelength used for recording the sedimentation (λ 546 m μ). The refractometer was carefully calibrated with potassium chloride solutions, by using the results of Gosting (1950). From the known constants of the optical system, refractive increments were used to calculate equivalent peak areas. Although confirmatory synthetic-boundary-cell experiments were made occasionally, refractometer measurements were the preferred method for determining the initial concentration.

Enlarged tracings of the curves obtained with the ultracentrifuge were analysed according to the procedures of Charlwood (1957*b*), and molecular weights were calculated by the method of Archibald (1947), from the relation

$$M = RT (\partial c / \partial x)_{x_0} / c_{x_0} \omega^2 (1 - \bar{v} \rho), \quad (1)$$

where c_{x_0} is the protein concentration at the meniscus, x_0 the distance of the meniscus from the axis of rotation, and the other symbols have their usual significance. Partial specific volumes, $\bar{v}$, were taken as 0.736, the value for human albumin near the isoelectric point (Charlwood, 1957*a*). The molecular weight of a sample was taken as the mean value of M derived from the five exposures.

Sedimentation velocity measurements. These were made at 59 780 or 52 640 rev./min., considerable economy of time being effected by the use of wedge window cells. Below pH 5, cells with Kel-F (polychlorotrifluoroethylene) plastic centre-pieces were used for all experiments. The protein concentration was normally about 0.35% and the total ionic strength of solutions was 0.2, of which 0.15 was contributed by potassium chloride. This system was adopted to decrease as much as possible the specific ion effects which have been studied by Kronman & Foster (1957) and Pedersen (1958). Other details were as given by Charlwood & Ens (1957). In addition to the buffers previously used, several sodium citrate buffers were made up covering the

range pH 3–4.5. In a few instances the effect of increasing the buffer strength, or using a different protein concentration, was determined.

The ideal of a series of measurements at different protein concentrations at each pH value and extrapolation would have involved a prohibitive amount of work. The measurements made at higher and lower protein concentrations confirmed that concentration dependence of $S_{20,w}$ was in no case so great that conclusions would be materially affected by use of the standard concentration of about 0.35% of protein.

Results were all corrected to water at 20° in the usual way, and expressed in Svedberg units. Correction factors were calculated from viscosity and density information given by Svedberg & Pedersen (1940), Gosting (1950), Lyons & Thomas (1950) and Levien (1955). Independent measurements were made of the viscosity and density (at 25.00 $\pm$ 0.02°) of the acetate buffers at pH 3.4 and of the citrate buffers. Viscosity measurements were done in a no. 50 Cannon-Fenske type viscometer (British Standard 188: 1957), and densities in a specially designed flask of about 18 ml. capacity. Except for one acetate buffer (0.15M-potassium chloride + 0.05 ionic strength acetate, pH 3.4), the computed densities and viscosities agreed so closely with the experimental determinations that alterations were unnecessary. As the measured viscosity of this acetate buffer was about 1% higher than the computed value, an appropriate correction was made. In calculating $S_{20,w}$ it was assumed that $\bar{v} = 0.736$ because, although it varies slightly with pH (Charlwood, 1957*a*) even for a buffer density increment of 0.03, 1% error in $\bar{v}$ affects $S_{20,w}$ by less than 0.3%.

RESULTS

Quality of preparations. Rat albumin separated by electrophoresis of serum, although monodisperse electrophoretically, gave rise in the ultracentrifuge to three components (Fig. 1*a*), the fastest of which could be eliminated by preparative ultracentrifuging. However, the main component was still contaminated by the constituent of intermediate sedimentation velocity (Fig. 1*b*). Prior fractionation with magnesium sulphate eliminated these heavier components (Fig. 1*c*). Rabbit and guinea-pig albumins, separated electrophoretically from serum, appeared to be free from these complications.

In those experiments with the organic solvent systems in which the solution was dialysed in the early stages against acetate buffer rather than water, the albumin produced was often contaminated with material of higher sedimentation coefficient (Fig. 2*a*). On the other hand, this did not apply when the alcoholic solution was first dialysed thoroughly against water (Fig. 2*b*), although the preparations when examined by electrophoresis usually showed a small amount of material with the mobility of α -globulin and sometimes β -globulin as well (Fig. 3*a-d*).

The albumins examined by immunoelectrophoresis all gave a strong arc in the usual position,

but, except with rat albumin EL/S, no other definite components were detected, although there was a slight suggestion of traces of α -globulin in the albumins isolated by means of alcohols. Rat albumin EL/S gave a second, but much weaker, arc beneath the main one.

Fig. 1. Ultracentrifuge patterns obtained with rat albumins separated by (a) electrophoresis of serum, (b) electrophoresis of serum, followed by preparative ultracentrifuging (EL/S) and (c) electrophoresis of the fraction of serum not precipitated by saturation with magnesium sulphate. Protein concentration approx. 0.8%. Temperature 20.5°. Sedimentation (right to left) for (a) 39 min. at 59 780 rev./min. in veronal buffer, pH 8.6, I 0.1, (b) 46 min. at 59 780 rev./min. in 0.15M-KCl + phosphate buffer, pH 6.4, I 0.05 and (c) 72 min. at 52 640 rev./min. in 0.15M-KCl + phosphate buffer, pH 6.4, I 0.05.

Fig. 2. Ultracentrifuge patterns obtained after precipitation of (a) rat serum with ethanol-trichloroacetic acid, followed by dialysis against acetate buffer, pH 4.5, I 0.1, (b) rabbit serum with methanol-tartaric acid, followed by dialysis against water. Sedimentation (right to left) at 59 780 rev./min. for 82 min. at (a) 25.2° in acetate buffer, pH 4.5, I 0.1, (b) 20.0° in 0.15M-KCl + phosphate buffer, pH 6.4, I 0.05. Protein concentrations (a) 1.2%, (b) 0.75%.

Molecular weights. The molecular weights of those preparations which appeared by all criteria to be of best quality are given in Table 1. All samples which showed a definite shoulder or faster peak during ultracentrifuging (except at lower pH) have been excluded. The mean values for rat, rabbit and guinea-pig albumin are respectively 64 300, 63 900 and 66 500. The results obtained with human albumin (Table 2) fall within a range of $\pm 2\%$ of the mean (65 200), as expected (see Discussion), whereas in Table 1 the scatter is about $\pm 5\%$.

The results given in Tables 3 and 4 demonstrate that there is no significant difference between molecular weights measured at two ultracentrifuge speeds, nor (with one possible exception) any difference after freeze-drying.

The effect of lower pH on molecular weights is shown in Table 5. The figures, which are weight averages, show that in many cases alterations do occur, but, except for guinea-pig albumin, changes are generally not very large and are reversed when the solution is dialysed back to pH 6.4. Clearly, too, there are often quite marked specific ion effects, citrate especially promoting association.

Sedimentation coefficients. In the present work, values of $S_{20,w}$ for a given solution were usually reproducible within $\pm 1\%$, often within $\pm 0.5\%$. The results of measurements on rat and rabbit albumins in glycine, acetate, phosphate and borate buffers are shown in Figs. 4 and 5. Where points coincide, or are too close to be shown distinctly, some have been omitted. With a few possible

Fig. 3. Ascending electrophoretic patterns at 2.0° in veronal buffer, pH 8.6, I 0.1 of (a) rat albumin 2 TAR, (b) rat albumin 1 TCA, (c) rabbit albumin 1 TAR, (d) rabbit albumin 1 TCA. Protein concentration approx. 1%. Migration from right to left at 3.1 v/cm. for (a) 192 min., (b) 168 min., (c) 150 min., (d) 159 min. For abbreviations see Table 1.

Table 1. *Molecular weights of serum albumins*Measurements in 0.15M-KCl + phosphate buffer, pH 6.4, *I* 0.05.

Sample	Isolation method	Electrophoretic analysis		Molecular weight
		Paper	Tiselius	
Rat				
1 EL	Salt fractionation + electrophoresis	No globulin	No globulin*	62 800†
2 EL	Salt fractionation + electrophoresis	No globulin	No globulin	61 500†
1 TAR	Methanol-tartaric acid	No globulin	—	65 700†
2 TAR	Methanol-tartaric acid	Trace of α -globulin	Some α -globulin, β -globulin*	66 500
1 TCA	Ethanol-trichloroacetic acid	—	No globulin	67 700
2 TCA	Ethanol-trichloroacetic acid	—	Trace of α -globulin*	61 700†
Rabbit				
1 EL	Electrophoresis	—	Trace of α -globulin*	62 800
2 EL	Salt fractionation + electrophoresis	No globulin	No globulin*	60 100†
1 TAR	Methanol-tartaric acid	Some α -globulin, β -globulin	Some α -globulin, β -globulin*	67 900
2 TAR	Methanol-tartaric acid	No globulin	No globulin	63 900
1 TCA	Ethanol-trichloroacetic acid	Trace of α -globulin	Some α -globulin	65 400
2 TCA	Ethanol-trichloroacetic acid	—	Trace of α -globulin, β -globulin*	63 100†
Guinea pig				
1 EL	Electrophoresis	—	No globulin	65 600
2 EL	Electrophoresis	—	No globulin	68 100
1 TAR	Methanol-tartaric acid	—	—	64 900
2 TAR	Methanol-tartaric acid	—	Some α -globulin, β -globulin	67 700
1 TCA	Ethanol-trichloroacetic acid	—	—	69 500
2 TCA	Ethanol-trichloroacetic acid	—	Some α -globulin	64 900†

* Also examined by immunoelectrophoresis (see text).

† Mean of two separate sets of determinations.

Table 2. *Molecular-weight determinations on human albumin*

Buffer	Molecular weight
Acetate, pH 4.6, <i>I</i> 0.2	65 100
	66 000
Acetate, pH 4.5, <i>I</i> 0.05, +0.15M-KCl	66 400
Phosphate, pH 6.4, <i>I</i> 0.05, +0.15M-KCl	65 000
	65 800*
	63 800†
	64 800†
	64 800‡

* Mean of two separate sets of determinations.

† After exposure to ethanol-trichloroacetic acid.

‡ After exposure to methanol-tartaric acid.

exceptions, points lie fairly close to the curves shown, although not within quite such fine limits as if they referred to a single preparation. Any differences between preparations (whether fresh or freeze-dried) are, therefore, negligible so far as these velocity measurements show. The curves are very similar to those obtained under corresponding conditions with bovine, human and horse albumins (Charlwood & Ens, 1957). In the neutral range (pH 6–8) $S_{20,w}$ is close to 4.4.

Below pH 5, doubling the buffer concentration resulted in partial suppression of the decrease of

Fig. 4. Sedimentation coefficients of rat albumins as a function of pH. Protein concentration approx. 0.35%. *I* 0.2, except for rat 3 TCA measurements at *I* 0.15. $\blacktriangle$, 3 EL; $\circ$, 1 TAR; $\bullet$, 1 TAR (freeze-dried); $\triangle$, 1 TCA (freeze-dried); $\square$, 3 TCA. For abbreviations see Table 1.

$S_{20,w}$, the general character of the pH-dependence curves remaining unaltered (Figs. 6, 7). Citrate buffers (*I* 0.2) also decreased the fall in $S_{20,w}$ (Figs. 6, 7). Excellent reproducibility was obtained when duplicate determinations were made immediately after mixing protein and buffer solutions, and after a lapse of some days (Fig. 7).

Guinea-pig albumin preparations, more especially below pH 4, showed measurable differences in behaviour (Fig. 8), but displayed a common characteristic. The general fall in $S_{20,w}$ as the pH decreased was interrupted at about pH 3.6–3.2 by a sharp rise. This rather abrupt transition was accentuated if the protein concentration was decreased or the buffer strength increased (Fig. 9). Although the increase in $S_{20,w}$ coincided with the change from acetate to glycine buffer, similar behaviour was obtained in citrate buffers (Fig. 10) in which the change of environment was much more gradual as the pH altered. In the neutral range the mean value of $S_{20,w}$ is slightly lower (4.3) than for rat and rabbit albumins, but extrapolation of values over a range of concentration gave $S_{20,w}^0$ 4.46 within experimental error for all albumins.

Table 3. Comparison of measurements at different ultracentrifuge speeds

Measurement in 0.15M-KCl + phosphate buffer, pH 6.4, I 0.05. For abbreviations see Table 1.

Albumin	Molecular weight	
	At 8210 rev./min.	At 12 590 rev./min.
Guinea pig		
2 TCA	65 600	64 200
Human*	67 900	63 600
Rabbit		
1 TAR*	67 400	68 600
1 TCA*	66 300	67 200
2 TCA	62 500	63 700
Rat		
2 TCA	60 000	63 300

* Freeze-dried material.

Table 4. Molecular weights of albumins before and after freeze-drying

Measurements in 0.15M-KCl + phosphate buffer, pH 6.4, I 0.05. For abbreviations see Table 1.

Albumin	Molecular weight	
	Before	After
Guinea pig		
1 EL	65 600	72 800
1 TAR	64 900	68 300
2 TAR	67 700	66 000
1 TCA	69 500	71 100
Rabbit		
1 TAR	67 900	68 000*
1 TCA	65 400	66 800*
Rat		
1 TAR	65 700*	62 500
1 TCA	67 700	67 200*

* Mean of two separate sets of determinations.

DISCUSSION

Nature of contaminants

The attempts to prepare rat albumin by electrophoresis of serum showed that there are other proteins present with a similar mobility but higher sedimentation coefficients; this makes the separation only partly effective. These proteins were assumed to be the two globulins which Grabar & Courcon (1958) detected by immunoelectrophoresis.

Table 5. Effect of pH on molecular weight

For abbreviations see Table 1.

Albumin	Buffer†	pH	Molecular weight	Molecular weight after redialysis‡
Guinea pig				
2 TAR	Glycine	3.00	95 300	—
2 TAR	Acetate	3.40	75 400	—
2 TCA	Citrate	3.37	117 000	63 100
2 TCA	Citrate	3.92	82 400	—
Human*				
	Glycine	1.95	67 400	—
	Glycine	2.93	67 800	65 700
	Glycine	2.99	73 100	—
	Acetate	3.34	72 900	—
	Acetate	4.02	64 500	—
Rabbit				
1 TCA*	Glycine	1.92	70 600	71 900
1 TCA*	Glycine	2.92	73 600	71 600
1 TCA*	Acetate	3.37	70 100	73 000
2 TCA	Citrate	3.37	90 300	65 300
Rat				
1 TAR*	Glycine	1.92	57 500	63 200
1 TAR*	Glycine	2.92	63 500	64 000
1 TAR*	Acetate	3.38	61 600	63 900
2 TCA	Citrate	3.39	73 600	65 000

* Freeze-dried material.

† I 0.05, 0.15M-KCl.

‡ In 0.15M-KCl + phosphate buffer, pH 6.4, I 0.05.

Fig. 5. Sedimentation coefficients of rabbit albumins as a function of pH. Protein concentration approx. 0.35%. I 0.2. $\circ$, 1 EL; $\bullet$, 3 EL (freeze-dried); $\triangle$, 1 TAR (freeze-dried); $\blacksquare$, 3 TAR; $\blacktriangle$, 1 TCA (freeze-dried); $\square$, 3 TCA. For abbreviations see Table 1.

This was confirmed for the component of intermediate sedimentation coefficient by the result of immunoelectrophoresis of rat albumin EL/S. No doubt one also corresponds to the component described recently by Jacox (1959). Although rabbit and guinea-pig albumins isolated by electrophoresis of serum were monodisperse in the ultracentrifuge, they might still have contained material of different molecular weight, but similar sedimentation coefficient. However, with rabbit albumin the failure to detect any other component by immunoelectrophoresis suggested that this possibility could probably be excluded.

The work of Cann, Brown, Singer, Shumaker & Kirkwood (1951) gave an indication of the danger

of artifacts arising when serum proteins come in contact with organic solvents, but Schwert (1957) and Ram & Maurer (1958) showed that bovine serum albumin appeared unchanged in its sedimentation velocity, electrophoretic, immunochemical and other properties after exposure to trichloroacetic acid-ethanol mixtures. Table 2 shows that the molecular weight of human albumin is unaltered after similar treatment. Differences in the metabolic behaviour of human albumin isolated with trichloroacetic acid-ethanol (Freeman, 1959) are most probably due to changes brought about during an extra stage of ether precipitation. Immunologically the rat and rabbit albumins tested appeared quite normal. It would thus seem most

Fig. 6. Sedimentation coefficients of rat albumins as a function of pH. Protein concentration approx. 0.35%. $\square$, 1 TAR (freeze-dried) in buffers, *I* 0.4. Other points refer to citrate buffers, *I* 0.2, $\bullet$, 1 TAR (freeze-dried); $\circ$, 2 TCA (freeze-dried). Dotted line corresponds with curve of Fig. 4. For abbreviations see Table 1.

Fig. 7. Sedimentation coefficients of rabbit albumins as a function of pH. Protein concentration approx. 0.35%. $\square$ and $\blacktriangle$ represent respectively 1 TCA (freeze-dried) in buffers, *I* 0.4, immediately after mixing and about 14 days later. Other points refer to citrate buffers, *I* 0.2, $\circ$, 1 TAR (freeze-dried); $\square$, 1 TCA (freeze-dried); $\bullet$, 2 TCA. Dotted line corresponds with curve of Fig. 5. For abbreviations see Table 1.

Fig. 8. Sedimentation coefficients of guinea-pig albumins as a function of pH. Protein concentration approx. 0.35%, *I* 0.2. $\circ$, 2 EL; $\bullet$, 1 TAR; $\square$, 2 TAR (freeze-dried); $\triangle$, 1 TCA (freeze-dried); $\blacktriangle$, 3 TCA. For abbreviations see Table 1.

Fig. 9. Sedimentation coefficients of guinea-pig albumins as a function of pH. Protein concentration approx. 0.18%. $\blacktriangle$, 1 TAR (freeze-dried) in buffer, *I* 0.4; $\circ$, 1 TCA (freeze-dried) in buffers, *I* 0.4; $\triangle$, 1 TCA (freeze-dried) in buffers, *I* 0.2. For abbreviations see Table 1.

likely that protein impurities in the albumins which have been in contact with an alcohol (Table 1) consist, not of altered albumin, but of those globulin fractions which are also soluble. It is known that protein solubility in acid-ethanol mixtures is not a property exclusive to albumins, since, for instance, Chibnall, Mangan & Rees (1958) have shown that insulin and β -lactoglobulin are soluble in ethanol containing hydrogen chloride. The contaminants of the animal albumins behaved on electrophoresis as α - or β -globulins, their proportions varying with the individual sera fractionated.

Accuracy of results

The main assumptions underlying the derivation of equation (1) are that the solution contains only two components, and that it is sufficiently dilute for the ratio of sedimentation and diffusion coefficients of the protein to be nearly the same as at infinite dilution. However, the equation applies under less stringent conditions to a good degree of approximation. In buffer systems, strictly speaking, various other interaction factors ought to be taken into account (Williams, van Holde, Baldwin & Fujita, 1958). Assuming that these are small enough to be neglected under the present conditions, the accuracy of determination of M can be assessed from a consideration of the factors of equation (1).

The factors which are most susceptible to error are c_{x_0} , $(\partial c/\partial x)_{x=x_0}$ and $\bar{v}$. It is unnecessary to

know the specific refractive increment of the protein, as this enters into both c_{x_0} and $(\partial c/\partial x)_{x=x_0}$. The bar angle of the schlieren system must be carefully adjusted, as otherwise it leads to errors in c_{x_0} and $(\partial c/\partial x)_{x=x_0}$, which are additive. The value of c_{x_0} depends on the original concentration, c_0 , measured in the differential refractometer (estimated error not more than 0.5%) and on the area below the schlieren curve between the meniscus and the plateau region. This area is measured with an accuracy of about 2%, but the term proportional to this area is generally only about 30% of c_0 . Thus c_{x_0} is known to an accuracy of about 1.5% (see Charlwood, 1957*b*). The factor $(\partial c/\partial x)_{x=x_0}$ is proportional to the height above the base-line at which the curve intercepts the meniscus. By using slightly higher speeds, Ehrenberg (1957) was able to reduce the error involved in measuring this intercept, but only at the expense of increased uncertainty in c_{x_0} which is then the relatively small difference of two large numbers. Owing to diffraction effects it is necessary to extrapolate the schlieren curve in the immediate neighbourhood of the meniscus (Ginsburg, Appel & Schachman, 1956). This is mainly responsible for the fluctuations in M (sometimes as great as $\pm 5\%$) within a series of five photographs. The error in the mean from this cause should not, however, be more than $\pm 3.5\%$. Taking into account errors in c_{x_0} , therefore, differences of M much greater than 5% between batches ought to be regarded as significant.

The value of $\bar{v}$ used does not affect the relative values of M for different preparations of albumin, although a small error in the assumed value of $\bar{v}$ is approximately trebled in the calculation of the absolute value of M . As $\bar{v}$ is determined mainly by the amino acid composition of a protein, and is nearly the same for bovine, human and horse serum albumins (Charlwood, 1957*a*), errors arising on this account are probably not very great.

It has been assumed above that the protein is monodisperse in the ultracentrifuge. Polydisperse aggregates, or contaminants of moderate size sometimes not easy to detect in velocity ultracentrifuging, will give rise to a spurious molecular weight. As the method yields a weight average, aggregates have a noticeable effect, giving initially too high a value of M , which decreases with time as the solution near the meniscus becomes differentially depleted of larger components (Smith, Wood & Charlwood, 1956). It is theoretically possible for very large aggregates to sediment so fast during the preliminary stages that assumptions about the concentration in the plateau region are no longer correct, and an erroneously low value of M is obtained. However, it is improbable that this would occur undetected. As a check on this

Fig. 10. Sedimentation coefficients of guinea-pig albumins as a function of pH in citrate buffers, I 0.2. Protein concentration approx. 0.18% for 1 TCA, 0.35% for the rest. $\circ$, 2 TAR (freeze-dried); $\triangle$, 1 TCA (freeze-dried); $\square$, 2 TCA; $\bullet$, 3 TCA. Dotted lines correspond with curves of Fig. 8. For abbreviations see Table 1.

a comparison was made of the starting material and the centre layer from the rat-albumin solution centrifuged in the preparative rotor SW 39. The molecular weights were indistinguishable. Although, in experiments quoted in this paper, there was never a significant drift of M with time, the fluctuation of $\pm 5\%$ within a series of five exposures could obviously have obscured any small trend.

The agreement between results at different speeds indicates that there is insufficient vibration to cause significant anomalies.

Ultracentrifugal findings

Independent measurements of $S_{20,w}$ and diffusion coefficients are available for the human albumin (Charlwood, 1954). Strict comparison with the present work requires a correction of 2.5% to the sedimentation coefficients (Charlwood, 1954), $\bar{v}$ to be taken as 0.736, and values of $S_{20,w}$ and $D_{20,w}$ to refer to an albumin concentration of about 0.6%, corresponding to the mean value of c_{x_0} . The good agreement between M 64 300 thus obtained, and M 65 200 from Table 2 substantiates the reliability of the figures.

The molecular weights of different preparations of the animal albumins varied more than was to be expected on the basis of errors of measurement alone. It must be concluded that this was due to the small amounts of contaminants demonstrated in some cases, and possibly present but not detected in others. The variations seemed sufficiently small for the best estimates of M to be taken as the mean values in Table 1. These differ slightly from the figures of Charlwood (1959), but are probably more nearly correct, being based on a larger number of preparations and more rigorous quality control. The apparently significant small difference between the molecular weight of guinea-pig and the other albumins no longer exists.

The reasonable consistency of M among the preparations obtained by different methods implies that no changes in the state of aggregation of the albumin are involved, in contrast with what probably happens when γ -globulin is exposed to some systems containing ethanol (Cann *et al.* 1951).

There have been reports of aggregation taking place when albumin is stored in the freeze-dried state (Halwer, Nutting & Brice, 1951; Christiansen, Jensen & Marcker, 1957). No aggregates were observed in high-velocity ultracentrifuging of freeze-dried samples when dialysed in phosphate buffer (I 0.05, pH 6.4) containing 0.15 M-potassium chloride, and there was little, if any, evidence of molecular-weight changes. Bovine albumin, dissolved in acetate buffer (I 0.2, pH 4.6) and examined immediately in the ultracentrifuge, although it revealed the usual small proportion of a

faster component, showed no sign of the aggregation which would have been expected from the measurements of Christiansen *et al.* (1957). This work, therefore, gives no indication that freeze-drying is responsible for any aggregation of albumins. Nevertheless, traces of contaminants may aggregate in these circumstances and might thus give rise to misleading conclusions.

The general decrease of sedimentation coefficients below about pH 5 is presumably due to the same factors as for bovine and human albumin, i.e. to a general expansion of the molecule due in part to electrostatic repulsion. This is borne out by the observation that increase of ionic strength partly suppresses the fall of $S_{20,w}$, much in the same way as with bovine serum albumin (Reichmann & Charlwood, 1954). The smaller decrease in $S_{20,w}$ in citrate buffers is most likely due to a decrease in the net charge brought about by binding of citrate ions to the protein, since Kronman & Foster (1957) showed that $S_{20,w}$ for bovine albumin decreased as the net charge on the molecule increased. The reproducibility of measurements after a time interval (Fig. 7) shows that, in this instance at least, the changes in protein configuration at lower pH are complete within an hour or two at most.

It was thought that differences between preparations might be revealed by measurements of sedimentation coefficient at lower pH if the ion-binding characteristics differed, a more sensitive test than comparison of molecular weight or sedimentation near neutrality. There were no clear indications of such differences among the rat and rabbit albumins, but guinea-pig albumins were by no means identical in sedimentation coefficients at low pH. However, these differences may be partly a reflexion of the critical dependence of the state of molecular association on pH for guinea-pig albumin.

The interruption in the fall of $S_{20,w}$ below pH 5, which takes place at pH 3.6–3.2, seemed much more likely to be due to the formation of a dimer (or higher aggregate) than to a sudden reversion of the guinea-pig albumin molecule to a more compact shape. This supposition was confirmed by molecular-weight measurements (Table 5), which also indicated that association had already begun above pH 3.6. The failure to observe more than one peak at 0.35% protein concentration may be due to various causes. Thus, if equilibrium between monomer and dimer is rapid, resolution cannot occur (Gilbert, 1959). Moreover, the lower the protein concentration, the more difficult it becomes to distinguish a given proportion of a second component, although careful re-examination of some of the records did reveal that there was sometimes asymmetry of peaks. If monomer and dimer exist together in comparable amounts only over a very

narrow range of pH, this may not include the limited number of buffers used. The behaviour of guinea-pig albumin is similar to that of bovine albumin, which tends to associate near pH 3 (Charlwood & Ens, 1957), but the effect is more marked here. Human albumin displays a similar tendency (Table 5), but rat albumin did not associate except in citrate buffer (Table 5). The low value of M for rat albumin at pH 1.92 is most probably due to the influence, under these conditions, of the factors which have been generally assumed to be negligible (see comment on Williams *et al.* 1958 above).

The apparent slight increase in $S_{20,w}$ between pH 3.4 and pH 3.2 for rat and rabbit albumins may be a reflexion of a small amount of association, but the change coincides with an alteration from acetate to glycine buffer, the acetate buffer necessitating very large viscosity and density corrections which may not be perfectly valid, especially at I 0.4. However, there does seem to be some suggestion of a similar effect in citrate buffer where the corrections are considerably smaller.

The association of guinea-pig albumin occurs mainly over a narrow range of pH (which is affected slightly by the buffer used), and goes nearer to completion than does the corresponding reaction for other albumins under comparable conditions. If a necessary prerequisite for association is the exposure of a particular site on the molecule, it might be supposed that this would occur at a particular degree of expansion (or extension), and that $S_{20,w}$ would therefore drop to a definite value at this point. However, comparison of results in citrate and other buffers (Fig. 10) is not consistent with this simple interpretation. Further work will therefore be required to elucidate the nature of the mechanism by which the association occurs.

SUMMARY

1. Albumins have been prepared from rat, rabbit and guinea-pig sera by trichloroacetic acid-ethanol and tartaric acid-methanol systems as well as by free electrophoresis. Salt fractionation with magnesium sulphate was sometimes used in conjunction with these methods.

2. The molecular weights of all these albumins, measured near neutrality in the ultracentrifuge, were close to 65 000. Differences were due to a combination of random error and the residual small amounts of globulins. There was no demonstrable effect of freeze-drying on the molecular weights.

3. The pH-dependence of $S_{20,w}$ for rat and rabbit albumins was closely similar to that for bovine, human and horse albumins, $S_{20,w}$ falling steadily from about pH 5 to pH 2. The effect of variations in

the environment indicated that electrostatic factors were implicated.

4. The pH-dependence of $S_{20,w}$ for guinea-pig albumin involved a steady fall below pH 5, interrupted by a large increase between approximately pH 3.6 and 3.2. This was shown to be due to dimer formation, but there was not sufficient information to reveal the details of this process.

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A Colorimetric Method for the Estimation of Monoamine Oxidase

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The reaction catalysed by monoamine oxidase is generally represented (Zeller, 1951; Blaschko, 1952) by the equation

Quantitative assay methods have been described based on measurement of the disappearance of substrate, the uptake of oxygen, or the liberation of ammonia, but none of these is entirely satisfactory for studies of the kinetics of the reaction. The most widely used method, measurement of the oxygen consumption, is relatively insensitive and is complicated by secondary reactions which occur, owing to the presence of other oxidizing enzymes, in normal tissue or mitochondrial-monoamine-oxidase preparations. The hydrogen peroxide is decomposed by catalase, which is found in all the tissue preparations, thus reducing the oxygen uptake from 2 atoms/mole of substrate as indicated in equation (1) to 1 atom/mole. The aldehyde may be further oxidized to the corresponding carboxylic acid or to resinous polymeric products; this results in a time-dependent and uncontrolled absorption of further oxygen (Cotzias & Greenough, 1959). This decomposition of the aldehyde may be prevented if aldehyde trapping agents, such as cyanide or semicarbazide, are added to the assay mixture, and under these conditions the oxygen absorption can be limited to 1 atom/mole of substrate (Creasey, 1956) but only at the expense of a further loss in sensitivity. Further, the presence of cyanide ions in the assay mixture may produce a dramatic change in the susceptibility of the enzyme to certain inhibitors (Davison, 1957). Measurement of the ammonia liberated provides a reliable and

sensitive assay method but the procedure is rather laborious (Cotzias & Dole, 1951). Measurement of substrate disappearance means there must be an

appreciable change in substrate concentration during the assay; this is undesirable for studies of kinetics.

The aldehyde semicarbazone, which is formed quantitatively when the enzymic oxidation is carried out in the presence of an excess of semicarbazide, can be readily converted into the corresponding 2:4-dinitrophenylhydrazone (Richter, 1937). Dinitrophenylhydrazones have an intense colour in alkaline solution (Friedemann & Haugen, 1943), and this offers a convenient means of measuring the enzymic activity. The present paper describes an assay procedure based on this dinitrophenylhydrazone formation. The method is sensitive and can be used over a wide range of substrate concentrations without significant depletion of the substrate during the reaction.

EXPERIMENTAL AND RESULTS

Materials. Guinea-pig-liver mitochondria, prepared by differential centrifuging of guinea-pig liver homogenized in 0.25M-sucrose (Hawkins, 1952), were used as the enzyme source. The sedimented mitochondria were suspended in 0.1M-phosphate buffer, pH 7.4 (83 ml. of 0.2M- Na_2HPO_4 + 17 ml. of 0.2M- NaH_2PO_4 + 100 ml. of water); 1 ml. of suspension was equivalent to 1 g. wet wt. of liver. This preparation was stored at -10° , and showed little loss of activity even after storage for several months.

Tyramine hydrochloride was obtained from L. Light and Co. Ltd., Colnbrook, Bucks.

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