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EVIDENCE FOR A CONCENTRATION-DEPENDENT POLYMERIZATION OF A COMMERCIAL HUMAN SERUM ALBUMIN

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SUMMARY

Polymerization of a commercial human serum albumin (Sigma A-1887) was investigated by two different techniques, high-performance liquid chromatography and gel electrophoresis. The chromatographic technique was based on the frontal analysis principle using a column which excludes polymers but retains monomers. The results allowed the determination of the monomer–polymer affinity constant, $X = 526 \pm 100$. The electrophoresis technique was performed with a polyacrylamide gel containing sodium dodecyl sulphate in order to separate the different polymer species according to their molecular weights. The two techniques gave results in good accordance and showed a concentration-dependent aggregation. The higher the human serum albumin concentration, the more the monomer proportion decreases.

INTRODUCTION

Many workers[1–9] have found that drug–protein binding parameters depend upon the human serum albumin (HSA) concentrations used. Some of these authors[4–9] have postulated that this phenomenon might be related to HSA aggregation upon increasing the concentration. In this paper, an attempt has been made to demonstrate a concentration-dependent molecular aggregation using two different methods: sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), and high-performance liquid chromatography (HPLC) based on frontal analysis, *i.e.*, a saturation method.

The elution of a HSA sample from a column capable of separating molecular species according to their size leads to a complete separation of those species. When these species can undergo a self association, the dilution which occurs along the column can lead to a dissociation of the species initially present in the HSA sample. In order to investigate the increase in self association with increasing HSA concentra-

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tion, and HPLC method in which each protein concentration remains constant during the elution must be employed; the only one is the frontal analysis based on saturation of the column by the solution. It is also the reason why this method was previously employed to assess the HSA retention volume at different concentrations[10].

EXPERIMENTAL

Crystalline HSA, fraction V (Sigma A-1887, essentially free fatty acid), was dissolved in a 0.067 *M* phosphate buffer, pH 7.4 ($I = 0.173$).

HPLC

A Waters Assoc. 6000 A pump and 450 UV detector were used for all experiments. The injection of a large amount of HSA (30 ml), in the case of frontal analysis, was done directly by use of the 6000 A pump. A Waters Assoc. U6K injector was included for injections of 25-μl samples.

The size exclusion column (600 × 3.9 mm I.D.) for frontal analysis was packed with LiChrosorb-Diol (pore diameter 100 Å, particle diameter 5–10 μm) (E. Merck, Darmstadt, G.F.R.). This support was chosen because previous work[11] had shown that only HSA monomer can enter the pores and polymers are excluded. Determinations of polymers were performed with 30 ml of different HSA solutions in phosphate buffer, pH 7.4. The HSA concentrations were 7.25, 14.5, 29, 58, 145, 217.5, 435 and 580 μ*M*. HSA solutions were placed on the column which was equilibrated with degassed phosphate buffer. The column eluate was monitored at 363 nm in order to avoid detector saturation.

To demonstrate the variation of HSA retention volume, the frontal analysis method was used. The elution profiles obtained were normalized to take into account any variation in the shapes of such profiles; the median bisector was used to define the weight average elution volume, V_w. V_w was estimated by using the equilibrium saturation chromatographic method[10]; HSA elution volumes were determined by injecting 25 μl phosphate buffer solution into an HSA eluting solution at different concentrations. Under these conditions, the chromatogram shows a negative peak which allows the determination of V_w without perturbing equilibria due to the different associations.

Polyacrylamide gel electrophoresis

The presence of polymers was also determined by SDS–PAGE. These 5.5% (v/v) acrylamide gels (70 × 5 mm) were prepared as described by Baruch[12]. Proteins were incubated at room temperature overnight in 0.1 *M* sodium phosphate buffer, pH 7.1, containing SDS (1%, w/v) and β-mercaptoethanol (0.5%). 100-μl HSA samples containing 7.25, 14.5, 29 or 58 μ*M* HSA and 10-μl HSA samples containing 145, 217.5, 435 or 580 μ*M* were run in order not to exceed an amount of 58 μmoles. After staining with Amidoschwarz, bands were quantitated with an Acta C III Beckman spectrophotometer.

THEORETICAL

Calculation of monomer concentrations

Nichol *et al.*[13] have worked out a method which allows the determination of a monomer concentration. The monomer concentration is assessed from the retention volume given by a sample (i) containing several associated species. Considering the similarity of the chemical potentials of the different species in both the mobile (γ) and the stationary (β) phases, we can write

$$C_i^{\beta} Y_i^{\beta} / C_i^{\gamma} Y_i^{\gamma} = P \tag{1}$$

where C_i is total concentration of solute, Y_i is the activity coefficient and P a constant independent of the total concentration of all species. On the other hand, the partition coefficient can be defined as:

$$\sigma_i = \frac{C_i^{\beta}}{C_i^{\gamma}} \tag{2}$$

When the column has a pore diameter chosen so as to exclude polymers and retain monomers, the weight average partition coefficient, σ_w, depends on the total solute concentration, $\bar{C}^{\gamma}$, according to

$$\sigma_w = \frac{\sigma_1 C_1^{\gamma}}{\bar{C}^{\gamma}} \tag{3}$$

where σ_1 and C_1^{γ} denote the monomer partition coefficient and the monomer concentration respectively. From an experimental viewpoint it should be noted that σ_w is identical with the commonly used distribution coefficient parameter, K_D, and hence

$$\sigma_w \approx K_D = \frac{(V_w - V_0)}{(V_T - V_0)} \tag{4}$$

where V_w denotes the weight average elution volume of a species w from a column with void volume V_0 and total accessible volume V_T.

Nichol *et al.*[13] calculated the activity coefficient (Y_1^{γ}) from the equation given by Ogston and Winzor[14]

$$\ln Y_1^{\gamma} = \frac{\alpha_{11} C_1^{\gamma}}{M_1} + \sum \frac{\alpha_{1j} C_j^{\gamma}}{M_j} \tag{5}$$

where α_{11} and α_{1j} are constant coefficients expressing interactions between the species 1 and the species $1 - j^n$. According to Adams and Fujita[15], let us assume that

$$\frac{\alpha_{1j}}{M_j} = BM_1 \tag{6}$$

where M_1 and B are the monomer molecular weight (66,000) and the virial coefficient

respectively. BM_1 was calculated from the theoretical equation according to Tanford[16]

$$BM_1 = 1.5 + \frac{1000\, z^2 V_1}{4\, m_3 M_1} \tag{7}$$

where V_1 is the specific volume of solvent, z the charge of the monomer and m_3 the molality of the medium.

At pH 7.0, HSA has 18 negative charges[17]. Knowing that the calculated change in pH for addition of a single anionic charge is 0.07 pH units[18], HSA will have 24 negative charges (z) at pH 7.4. Then eqn. 5 becomes:

$$\ln Y_1^{\gamma} = BM_1 \cdot \bar{C}^{\gamma} \tag{8}$$

Another expression is required to estimate σ_1 at each $\bar{C}^{\gamma}$ according to Nichol *et al.*[13]

$$\sigma_1 = \sigma_1^{\circ} \cdot \exp\left[BM_1 \cdot \bar{C}^{\gamma}\,(1 - \sigma_w)\right] \tag{9}$$

where σ_1° is the limiting value obtained by extrapolating a plot of σ_w *versus* C^{γ} to infinite dilution.

Under these conditions it is possible to determine from eqn. 3 the monomer concentration in the mobile phase, C_1^{γ}, by using the experimental values σ_w (eqn. 5). The determination of C_1^{γ} is of value in establishing the polymerization pattern of the self-associating system. For such an indefinitely associating system governed by a single equilibrium constant, X, it has been established[19] that the total weight concentration is related to the monomer weight concentration by:

$$X = \frac{(M_1 \bar{C}^{\gamma} - M_1 \sqrt{C_1^{\gamma} \bar{C}^{\gamma}})}{\bar{C}^{\gamma} C_1^{\gamma}} \tag{10}$$

RESULTS

HPLC

The HSA elution volume was measured by injecting different HSA concentrations into a column eluted with a phosphate buffer. For the different concentrations, a single HSA peak was observed with a constant retention volume.

Fig. 1 shows normalized elution profiles obtained in frontal chromatography of 7.25–580 μM HSA solution. The profiles clearly demonstrate pronounced concentration-dependence of the elution volume. However, at half-height of the plateau, saturation was not achieved. In order to determine the HSA retention volume, the chromatographic saturation method was used[10]. The retention volume, V_w, allows one to calculate σ_w via eqn. 4 by using $V_0 = 3.0$ ml and $V_T = 5.7$ ml; the last two parameters were obtained from elution chromatography of ferritin and lysozyme respectively. The monomer concentration present in each HSA concentration can then be determined via eqn. 3, and there the association constant, X, by means of eqn.

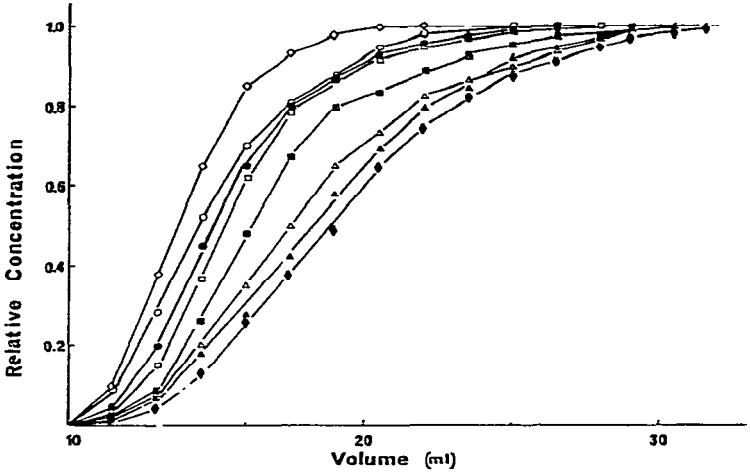

Fig. 1. Normalized elution profiles obtained in frontal chromatography of HSA in 0.066 *M* phosphate buffer, pH 7.4, at 21°C. Monomer plateau concentrations, $\bar{C}^{\circ}$: 7.25 (◇); 14.5 (○); 29 (●); 58 (□); 145 (■); 217.5 (△); 435 (▲) and 580 (◆) μM HSA.

TABLE I

HSA MONOMER PROPORTIONS AND ASSOCIATION CONSTANTS OF MONOMER–POLYMER SPECIES

The monomer quantitation was performed by frontal chromatography. The weight average partition coefficient, σ_w, and the concentration of monomeric HSA species were used to calculate the HSA monomer percentages and the association constant, X, as described in Experimental.

HSA, $\bar{C}^{\circ}$ *(μM)*	V_w *(ml)*	σ_w	C_1 *(μM)*	*Monomer (%)*	X (M^{-1})
7.25	3.38	0.141	7.20	99	667
14.5	3.38	0.141	14.40	99	334
29	3.39	0.144	27.99	97	514
58	3.40	0.148	54.33	94	534
145	3.45	0.166	128	88	464
217.5	3.48	0.177	179	82	507
435	3.58	0.215	296	68	567
580	3.65	0.241	350	60	618
					526 ± 100
					Mean ± S.D.

10. The results are listed in Table I. Since X remains approximately constant, it can be assumed that there is an isodesmic self association system[19].

SDS–PAGE

As shown in Fig. 2 and in Table II, the appearance of the protein bands in the gels indicates an HSA heterogeneity. As the HSA concentration increased this heterogeneity was enhanced. These bands represented different polymers up to the hep-

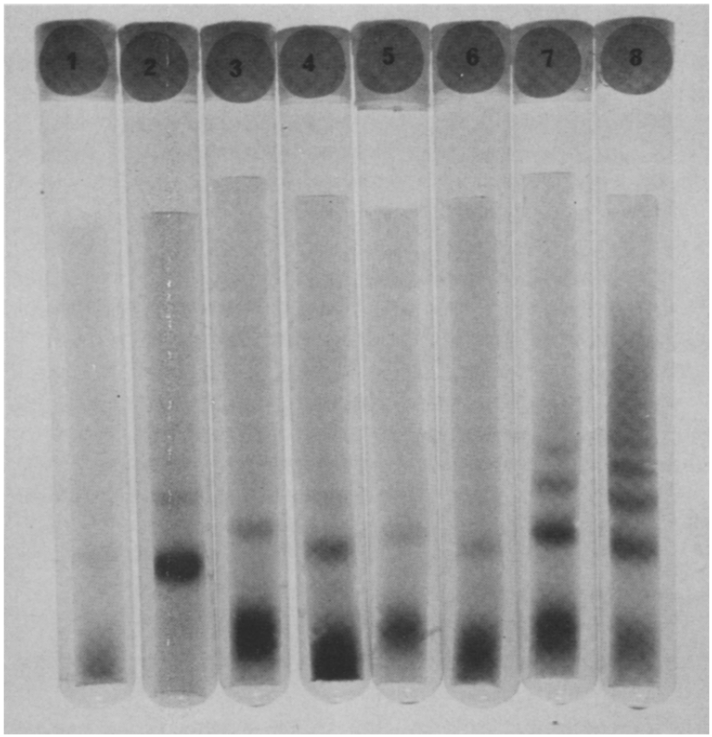

Fig. 2. SDS polyacrylamide gel electrophoresis showing the influence of protein concentration on the degree of polymerization of HSA. A 580 μM HSA solution in 0.066 M phosphate buffer, pH 7.4, was diluted in the same buffer to give samples 1–8 containing 7.25, 14.5, 29, 58, 145, 217.5, 435 and 580 μM HSA respectively. The reaction procedure is described in Experimental.

tamer. Monomer concentration increased while the monomers/polymers ratio decreased with increasing HSA concentration. The data show that initially the percentages of monomers found at each HSA concentration are approximately the same as those revealed by HPLC (Table II).

TABLE II

PROPORTIONS OF HSA MONOMERS AND POLYMERS

The quantitation of the degree of HSA polymerization was by gel electrophoresis and HPLC (only monomers).

HSA (μM)	*Monomer (%)*		*SDS–PAGE*				
	HPLC	*SDS–PAGE*	*Dimer (%)*	*Trimer (%)*	*Tetramer (%)*	*Pentamer (%)*	*Hexamer (%)*
7.25	99	95	5	–	–	–	–
14.5	99	95	5	–	–	–	–
29	97	92	8	–	–	–	–
58	94	77	22	1	–	–	–
145	88	70	25	5	–	–	–
217.5	82	65	25	8	2	–	–
435	68	62	24	10	3	1	–
580	60	33	46	12	6	2	1

DISCUSSION

The content of aggregates in some commercial HSA preparations have previously been estimated[17,20–22]. Our sample of HSA (Sigma A-1887) contained aggregates ranging from monomer to high-molecular-weight polymers. This phenomenon has been demonstrated by two different and independent methods: SDS–PAGE and HPLC. It should be emphasized that our HPLC data are obtained from a steady dynamic equilibrium system. This HPLC method based on saturation is much more adequate than standard elution chromatography since in the latter a progressive dilution of the injected sample occurs leading to total dissociation of the species present in HSA solution. Our data clearly showed a variation of the elution volumes according to HSA concentration, while data obtained by elution chromatography would give a constant elution volume.

Our HPLC results are dependent on the properties of the packing whose characteristics were analysed by Schmidt *et al.*[11]. With this packing all proteins of mol. wt. > 90,000 are excluded; consequently the HSA monomer alone can easily penetrate into pores of the packing. Moreover, this situation is identical to that described by Nichol *et al.*[23]. So, the chromatographic behaviour of HSA can be explained according to this scheme. The wide range of HSA concentrations (7.25–580 μM total HSA and 7.20–350 μM monomer HSA) (Table I) can be assumed to lead to saturation of the packing pores. The increase of the elution volume of HSA with its concentration cannot be due to a shift of the reversible binding isotherm caused by saturation of the packing. In this situation, saturation of the packing would lead to a decreased binding of HSA. Consequently the elution volume should decrease with the HSA concentration. Our HPLC results allow us to discard the above possibility.

Freeze-drying leading to protein crystallization was shown by some workers[22,24] to induce oxidation of sulphydryl groups. Such groups in different HSA molecules could promote the formation of intermolecular disulphide bridges, thus resulting in polymerization. However, this mechanism alone cannot account for aggregation since the proportion of polymer markedly increases with HSA concentration. Increasing monomer fraction upon increasing dilution clearly indicates that a reversible equilibrium between monomer and polymer species is involved. This could not occur with S–S bonding.

The proportions of HSA monomers quantitated by both HPLC and SDS–PAGE are in a good qualitative accordance. Eqns. 5 and 6 allowed us to calculate the percentage of monomers from the chromatographic results. However, a discrepancy between chromatographic and electrophoretic data was observed. This is not surprising because the percentages of monomers are calculated for HPLC, whereas they are measured directly in SDS–PAGE. It can be concluded that HSA (Sigma A-1887) exhibits reversible aggregation with increasing concentration.

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