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FRACTIONATION OF A HUMAN SERUM ALBUMIN PREPARATION AND THE SALICYLATE BINDING CHARACTERISTICS

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SUMMARY

A defatted human serum albumin preparation was fractionated on Sephadex G-200 columns equilibrated with 0.1 M NaCl at 4°C. The salicylate binding parameters, determined by equilibrium dialysis, of the unfractionated preparation was compared with those of various fractions. The monomeric fraction showed the highest binding capacity. The dimeric fraction and the unfractionated preparation showed a similar binding capacity, approximately 80% of the monomeric fraction. The highest molecular weight species appeared to reduce the salicylate binding capacity of the albumin preparation. The absorbance values, $A^{1\%}_{1cm}$ at 278 nm, in 0.05 M Tris buffer containing 0.1 M NaCl, pH 7.40 at 20°C for the monomeric and dimeric fractions were determined to be 5.30 ± 0.02 and 5.47 ± 0.04, respectively.

INTRODUCTION

Although commercial albumin preparations are known to contain usually 5–10% polymers of albumin and sometimes as much as 50% in some crystalline albumin preparations (Foster, 1977), in in vitro drug binding studies very little attention has been paid to this matter. A literature survey revealed the following two relevant reports – McMenamy (1965) briefly stated in his paper on the binding of indole analogues that human serum albumin (HSA) dimer has binding characteristics similar to those of two monomers. Binding of progesterone to defatted monomer and dimer of HSA has been characterized by the same binding parameters per monomeric unit, but the binding capacity of the higher polymers was reported to be 30% lower (Westphal and Harding, 1973).

Too often, albumin samples obtained from commercial sources are used without further purification in drug binding studies. Investigators tend to select, in view of

expected purity, a crystallized albumin preparation or a defatted albumin preparation rather than Fraction V. Hardee et al. (1978) have raised doubts about the merit of the defatting process as far as the homogeneity of albumin and contamination with inorganic ions are concerned.

The dimer and polymers of HSA are likely to arise as artifacts during the isolation procedures and storage, and would not be present in the blood (Andersson, 1966; Janatova, 1974; Kistler, 1974; Foster, 1977), although in patients with nephrotic syndrome they may be present (Boesken and Noller, 1975). It is therefore worthwhile to know what effects these polymeric species have on in vitro drug binding parameters. Since successful fractionation of albumin preparations has been demonstrated previously by gel filtration on Sephadex G-150 or G-200 (Pedersen, 1962; Friedli and Kistler, 1970; Finlayson et al., 1974), we have chosen columns of Sephadex G-200 to fractionate a defatted HSA preparation and studied the salicylate binding characteristics of mainly the monomeric and dimeric fractions. We have also investigated the effects of other fractions on the binding capacity of the HSA preparation.

MATERIALS AND METHODS

Materials

Human serum albumin preparations, Fraction V (Sigma, A-2386, lot no. 18C-0519) and essentially fatty acid-free (Sigma A-1887, lot no. 76C-7480) which was prepared from the Fraction V, were employed. Sephadex G-25 (superfine) and G-200 were purchased from Pharmacia Pure Chemicals. A molecular weight marker (mol. wt. 53,000–265,000) was purchased from BDH Chemicals, Poole, England. Salicylic acid was a product of Koso Chemicals, Tokyo, Japan and recrystallized from water. All other chemicals were reagent grade and water was double-distilled with the second distillation in an all glass apparatus. To concentrate eluate fractions by ultrafiltration, Diaflo membranes (UM-10, Amicon, Lexington, Mass.) were used.

Fractionation of HSA preparations

A HSA preparation (0.1 g) was dissolved in 5 ml of 0.1 M NaCl and the solution was applied to a Sephadex G-200 column (2.6 cm (i.d.) × 92.5 cm) equilibrated with 0.1 M NaCl at 4°C. The column was eluted at 4.5–10 ml/h with 0.1 M NaCl and about 6–10 ml fractions were collected. The absorbance of each fraction at 280 nm was determined to construct the elution profile (Fig. 1). The eluate fractions of the defatted HSA preparation were pooled to form Fractions A–H as indicated in Fig. 1a. Fractions A–D were subjected to polyacrylamide electrophoresis. For preparative purposes, 1 g of the defatted preparation was dissolved in 5 ml 0.1 M NaCl and applied to the same column. Appropriate fractions were collected and pooled for binding studies.

Polyacrylamide electrophoresis

Polyacrylamide electrophoresis was carried out according to the method described by Zwaan (1967) in the vertical electrophoresis apparatus developed by Raymond (1962; E.C. Apparatus Corp., Philadelphia, Pa.). A 7.5% gel (7.12% acrylamide and 0.38% N,N′-methylbisacrylamide) and 0.0825 M Tris–EDTA–boric acid buffer of pH 9.2 were

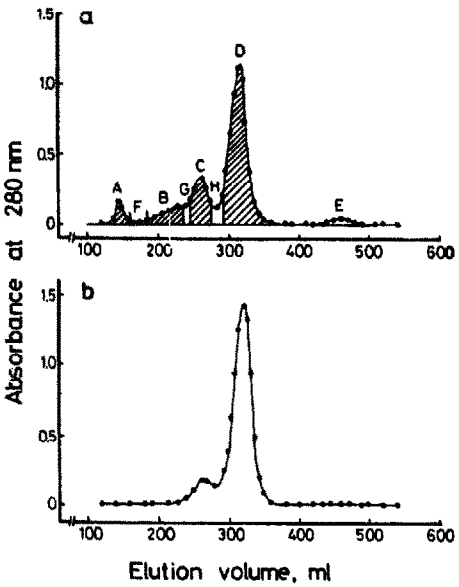

Fig. 1. Elution profiles of (a) 0.1 g of a defatted HSA preparation and (b) 0.1 g HSA Fraction V through a Sephadex G-200 column (2.6 × 87.7 cm) equilibrated at 4°C with 0.1 M NaCl flowing at a rate of 4.5 ml/h. A–H indicate various fractions into which the eluate fractions were pooled.

employed. Samples (50–75 μl) were applied to the sample slots and electrophoresis was carried out for 2.5 h at 250 V and 140 mA. After the run, the gel was stained with amido black and destained according to the standard procedure.

Sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis was carried out using glass columns (0.5 × 7.0 cm) following the instructions supplied by BDH Chemicals with the molecular weight standard. The procedure was based upon the method of Weber and Osborn (1969). Samples (20 μl) containing about 30 μg of HSA and bromophenol blue as tracking dye were applied to 3.3% gel and electrophoresis was carried out at 2 mA per column for the first 30 min and at 5 mA per column for a further 4 h. The gels were stained with 0.25% Coomassie brilliant blue. The relative mobility of each band was calculated with respect to the mobility of the reference dye allowing for the change in the length of the gel before and after staining.

Equilibrium dialysis (binding studies)

Equilibrium dialysis was carried out for 16 h as described previously (Nakano et al., 1978) at 4°C in 0.05 M Tris · HCl buffer containing 0.1 M NaCl without sodium azide. Albumin concentration was adjusted to 0.7–0.8%. The number of moles of total salicylate placed in the dialysis bag ranged from 5.5×10^{-7} to 6.1×10^{-6}.

Fraction D was shown to contain over 95% monomer by applying 5 ml of this fraction to the same Sephadex G-200 column and used directly for binding studies. Fraction C of 6–7 fractionations were pooled and the mixture was concentrated by ultrafiltration or freeze–drying and reapplied to the Sephadex G-200 column to eliminate the monomeric and trimeric fractions as much as possible. The fraction thus obtained contained about

90% dimer and suitably concentrated by ultrafiltration for binding studies. Prior to binding studies this concentrated dimer fraction was dialyzed extensively against 0.1 M NaCl. The macromolecular fractions (Fractions A–D and F–H) of a single gel filtration of 1 g of the defatted HSA preparation were pooled and the mixture was concentrated and dialyzed as described above for binding studies. Similarly, Fractions B–D, G and H were pooled for binding studies.

Binding data were analyzed as described previously (Nakano et al., 1978) assuming that only one class of n-binding sites were present per monomeric unit of albumin. A molecular weight of 66,250 (Peter, 1975) was employed for calculations.

The monomeric and dimeric fractions employed in the binding studies were desalted through a column of Sephadex G-25, superfine, freeze–dried and further dried over P_2O_5 to a constant weight prior to determination of their absorbance values, $A^{1\%}_{1cm}$ at 278 nm in the Tris buffer. The values were determined to be 5.30 ± 0.02 and 5.47 ± 0.04, for the monomer and the dimer, respectively, on a Shimadzu model UV-300 spectrophotometer in the second sample compartment where cells were placed directly in front of the photomultiplier. These values were comparable to the previously reported values of 5.17 and 5.38 for HSA monomer and dimer, respectively (Westphal and Harding, 1972), and 5.30 for human mercaptalbumin (Clark et al., 1962). A value of 5.30 was used as $A^{1\%}_{1cm}$ at 278 nm for the binding studies of fractions other than those of the dimer for which a value of 5.47 was used.

RESULTS AND DISCUSSION

Fractionation of HSA preparations and characterization of the main component of the fractions

Fig. 1 shows elution profiles of two HSA preparations, i.e. defatted HSA (Fig. 1a) and Fraction V (Fig. 1b). The defatted preparation is far less homogeneous than Fraction V, not only with respect to the higher molecular weight species, but also to small molecular weight impurities (Fraction E).

The electrophoretic analyses of the original or unfractionated defatted HSA and Fractions A–D are shown in Fig. 2. Since no band is visible in Fig. 2 for Fraction A, Fractions B–D and the unfractionated HSA, after concentrating Fractions B and C to approximately the same concentration as that of Fraction D and the unfractionated HSA were subjected to SDS gel electrophoresis together with the molecular weight marker and the results are presented in Fig. 3. Treatment of Fraction C with 1% 2-mercaptoethanol for 2–3 min at 90°C influenced not only the relative migration of bands but also the relative intensity of bands (see Fig. 3, C-1 and C-2). Such fast migration of unreduced bovine serum albumin (BSA) over reduced and alkylated BSA was also reported by Lane (1978) in SDS–polyacrylamide gel electrophoresis. Westphal and Harding (1973) did not observe the reduction of HSA dimer with 2-mercaptoethanol to the monomeric species, but noted a slightly higher mobility of the unreduced HSA components. Shrivastava et al. (1972) also observed no conversion of the dimer to monomer in SDS-polyacrylamide gel electrophoresis under exhaustive reducing conditions. Other investigators (Hartley, 1962; Andersson, 1966; Janatova, 1968) consider that the dimeric fractions of BSA are heterogeneous with regard to reduction with thiol reagents, and thus not all components of the

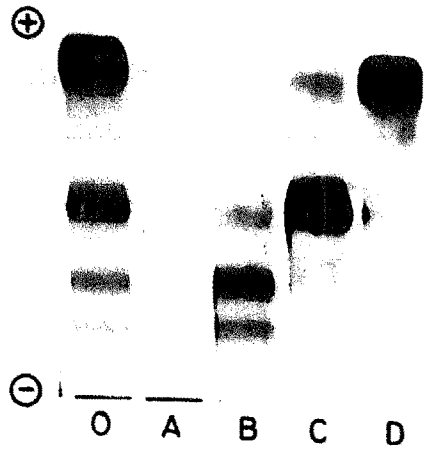

Fig. 2. Polyacrylamide electrophoresis of various fractions of a defatted HSA preparation. O, original (or unfractionated) and A–D correspond to Fractions A–D in Fig. 1.

dimer fractions are converted to monomer under reducing conditions.

Since partial conversion of the dimer to the monomer was observed in the presence of 2-mercaptoethanol, the unfractionated HSA, Fraction D and the standard only were treated with 2-mercaptoethanol and the molecular weight of each band was determined as shown in Table 1. For Fractions B and C (Fig. 3), 2-mercaptoethanol was excluded to characterize the main bands in these fractions. The results in Table 1 show that Fractions D, C and B are rich in the monomer, dimer and trimer, respectively, and none of these fractions is completely homogeneous. Fraction B, particularly, contains a large number of species besides trimer. The defatted HSA preparation, thus, contained about 62% monomer, 23% dimer and 15% polymer, whereas the Fraction V contained 85% monomer and 15% dimer only. Since the former was prepared from the latter, polymer-

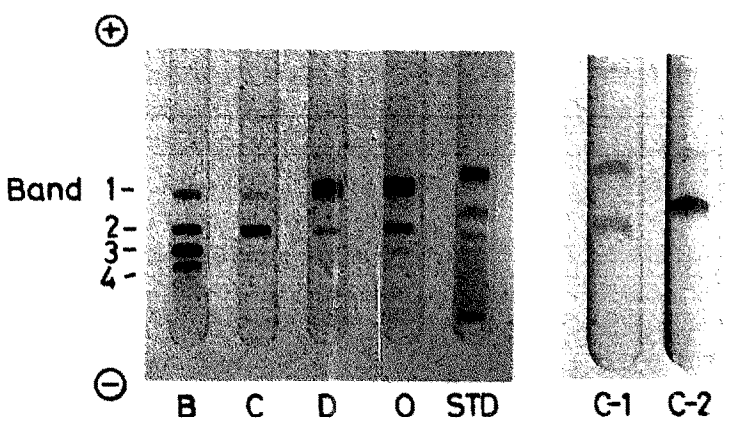

Fig. 3. Sodium dodecyl sulfate polyacrylamide electrophoresis of various fractions of a defatted HSA preparation. O, B–D: as for Fig. 2. STD: a molecular weight standard mixture. STD, O, D and C-1 were treated with 1% 2-mercaptoethanol, but B, C and C-2 were not treated.

TABLE 1

MOLECULAR WEIGHT ESTIMATION OF COMPONENTS OF A DEFATTED HSA PREPARATION BY SDS–GEL ELECTROPHORESIS IN 3.3% GEL

Band number	Mol. wt. X 10^{-4} [a]	Theoretical mol. wt. X 10^{-4} [b]
1	6.83 ± 0.27 (6)	Monomer 6.63
2	14.3 ± 0.64 (6)	Dimer 13.3
3	21.3 ± 0.66 (4)	Trimer 19.9
4	28.3 ± 0.35 (2)	Tetramer 26.5

[a] Determined value ± S.D. (number of determinations).
[b] From Peter, 1975.

ization of albumin molecules is likely to accompany the delipidation process or defatted albumins tend to polymerize easily.

Binding studies

Fig. 4 shows the salicylate binding characteristics of the monomeric, dimeric and unfractionated defatted HSA in the form of Scatchard plots, i.e. r/D_f is plotted against r, where r is the number of moles of salicylate bound per mole of a monomeric unit of HSA and D_f is the concentration of free (unbound) salicylate. Since, for $r < 2$, the Scatchard plots showed a fair linearity, the data were treated on the basis of the presence of only one class of n-binding sites per monomeric unit, which possess an equal intrinsic binding constant K. The binding parameters, K and n, together with the total binding capacity, nK, are summarized in Table 2 for various fractions together with the limits of error for a

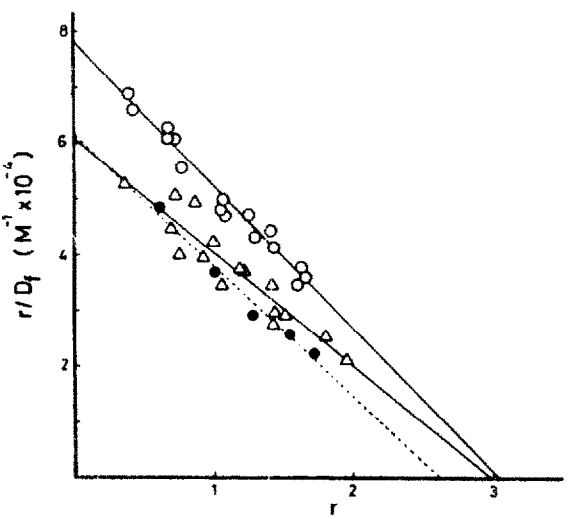

Fig. 4. Scatchard plots for the interactions of monomer (○———○), dimer (△———△) and unfractionated defatted HSA (●- - - - -●) at 4°C in 0.05 M Tris buffer containing 0.1 M NaCl, pH 7.87 (at 4°C).

TABLE 2

SALICYLATE BINDING PARAMETERS [a] OF VARIOUS FRACTIONS OF A DEFATTED HSA PREPARATION

HSA fraction [b]	K [c] × 10^{-4} (M^{-1})	n [c]	nK [c] × 10^{-4} (M^{-1})	No. [d]
Monomer (D)	2.58 ± 0.24	3.03 ± 0.28	7.82 ± 0.28	16
Dimer (from C)	2.05 ± 0.43	2.96 ± 0.54	6.07 ± 0.54	16
A–F (unfractionated)	2.35 ± 0.69	2.62 ± 0.41	6.15 ± 0.89	5
A–D and F–G	2.55 ± 0.32	2.57 ± 0.21	6.56 ± 0.32	5
B–D, G and H	2.29 ± 0.20	3.02 ± 0.16	6.90 ± 0.25	8

[a] Determined at 4°C in 0.05 M Tris buffer containing 0.1 M NaCl, pH 7.87 (at 4°C).
[b] Letters A–H are as indicated in Fig. 1.
[c] These values (limits of error for $P = 0.95$) were calculated, by the least-squares method, from the Scatchard plots for $r < 2$, assuming only one class of binding site is present on a HSA molecule. For the last 3 fractions a $A^{1\%}_{1cm}$ at 278 nm value of 5.30 was used in the calculations.
[d] Number of data used for calculation of the binding parameters.

probability level of 0.95. The binding parameters of fractions other than the monomer and dimer in the table cannot be interpreted in the same strict sense as for those of the monomer and dimer. Firstly, the amount of data of these fractions are less than that of the monomer and dimer. Secondly, the $A^{1\%}_{1cm}$ at 278 nm values are unknown for polymers higher than the trimer. However, the results show that the salicylate binding characteristics vary among the different fractions examined. The most homogeneous monomeric fraction showed the highest total binding capacity, followed by the last fraction, B–D, G and H, which contains monomer, dimer, trimer, tetramer and possibly higher polymers. The dimeric fraction obtained from Fraction C which contained about 90% dimer showed a total binding capacity about 20% lower than that of the monomer. This difference appears to be related to the difference in their K values rather than their n values. Thus, it would be reasonable to expect that the dimer and higher polymers of HSA have somewhat reduced total salicylate binding capacities in comparison with the monomer.

Further comparison of nK values among the last 3 fractions in Table 2 indicates that the removal of the highest molecular weight species (Fraction A mainly) improves the total binding capacity of the preparation, but that the effect of low molecular weight impurities (Fraction E) is not so significant.

CONCLUSIONS

Two commercial HSA preparations, Fraction V and defatted HSA showed considerable difference in their molecular weight heterogeneity. The defatted preparation was far less homogeneous and contained only about 62% monomer, whereas Fraction V contained about 85% monomer.

In contrast to the binding of progesterone (Westphal and Harding, 1973) and indole analogues (McMenamy, 1965), for which both HSA monomer and dimer were reported to have the same binding parameters, the salicylate binding capacity of the dimer per

monomeric unit, was about 80% of the monomer. Although not to a great extent, other fractions also influenced the salicylate binding capacity indicating the need for purification of HSA preparations to obtain the monomer for in vitro binding study. Gel filtration through a column such as Sephadex G-200 is simple and would be suitable to obtain the monomer in high yields.

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