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Studies of Drug-Protein Binding by Affinity Chromatography. II.[1] Interactions of Bovine Serum Albumin with Various Ligands[2]

Naomi I. Nakano, Takayuki Oshio, Noriko Sato, Yoshimitsu Shimamori, and Shigenori Yamaguchi

Hokkaido Institute of Pharmaceutical Sciences[3]

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The use of albumin immobilized on agarose gel for the characterization of drug-protein binding by means of frontal analysis affinity chromatography is described for the interaction of bovine serum albumin with a variety of ligands including hydroxybenzoic acids, warfarin, phenylbutazone, D- and L-tryptophan, sulfonamides, *etc.* In all the systems, the binding capacity of the immobilized albumin was increased by the presence of a six-carbon-atom spacer between the agarose gel matrix and albumin, to a level comparable to that of soluble albumin as determined by equilibrium dialysis. The effect of residual fatty acids on the binding properties of albumin was similarly examined by coupling defatted bovine serum albumin with the gel. The results indicated that there is no marked difference in binding properties between the immobilized defatted and undefatted albumin preparations. The advantages and disadvantages of the present affinity chromatographic method are discussed.

Keywords——protein binding; immobilized bovine serum albumin; affinity chromatography; frontal analysis; equilibrium dialysis; hydroxybenzoic acids; phenylbutazone; warfarin; D- and L-tryptophan; sulfonamides

Utilizing bovine serum albumin (BSA) covalently attached to agarose beads, we have previously demonstrated the usefulness of an affinity chromatographic technique[1] for *in vitro* evaluation of drug-protein binding parameters in the BSA-salicylate interaction. We have shown that when BSA is coupled to agarose through a six-carbon-atom spacer, the binding characteristics of salicylate ions to the immobilized albumin are comparable to those in the case of soluble albumin.

The present paper demonstrates the applicability of this technique to interactions of BSA with a variety of ligands, including hydroxybenzoates, phenylbutazone, warfarin, L- and D-tryptophan, sulfonamides, *etc.* In all systems investigated, the binding parameters of the immobilized albumin were increased by the presence of the spacer to levels comparable to those obtained by equilibrium dialysis.

The use of immobilized albumin in the study of drug-protein binding has also been evaluated by Kober *et al.*,[4] who immobilized human serum albumin (HSA) by embedding it in microparticles of polyacrylamide. By comparison with equilibrium dialysis data they demonstrated that HSA retained its drug-binding properties when the HSA-immobilized gel was employed in a batchwise procedure. Bovine serum albumin covalently coupled to agarose gel has been utilized in a batchwise manner in competition with free proteins to evaluate the binding parameters of some ligand-protein interactions.[5]

The major advantage of the use of immobilized protein in a batchwise procedure is that the separation of free from bound drug can be accomplished very simply and quickly.

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This is because, in conventional equilibrium dialysis and other subtractive methods,[6] the time required to separate the free drug from the complex is far greater than that required for the establishment of binding equilibria. Even ultrafiltration or ultracentrifugation is more laborious and time-consuming than the batchwise method. However, the batchwise procedure suffers from the same disadvantages as subtractive methods, *i.e.*, the free drug concentration is determined and the bound drug concentration is estimated by subtracting the free drug from the total added. In the present affinity chromatographic technique, although it is more time consuming than batchwise procedures, the amount of the bound drug is directly and simply determined and the concentration of free drug is set at a predetermined value as in the gel filtration method of Hummel and Dreyer.[7] Therefore, precise data can be obtained even for low affinity drugs.

Experimental

Materials——Bovine serum albumin (BSA, Fraction V powder, lot 243) and essentially fatty acid-free BSA (BSA-FAF, prepared from Fraction V, lot 20, reported to contain 0.04 mol of fatty acids per mole of albumin by the manufacturer) were products of Miles Labs. Inc., Elkhart, Indiana, U.S.A. 3-Hydroxybenzoic acid, 4-hydroxybenzoic acid, and *n*-hexylamine were purchased from Wako Pure Chemical Inds. L-Tryptophan (Nakarai Chemicals Ltd.) and D-tryptophan (Tokyo Kasei Kogyo Co.) were heated to about 150° for one hr and cooled before use. Phenylbutazone, warfarin sodium, sulfamethizole, and sulfisoxazole, of either JP IX or USP XIX grade, were used without further purification. Bucolome was a product of Takeda Pharmaceutical Ind. All other chemicals used were as described previously.[1]

Preparation of Sepharose 4B Derivatives——1) *n*-Hexyl-Sepharose 4B was prepared according to the procedure described for carboxypentylagarose (CH-Sepharose 4B),[1] by reacting cyanogen bromide-activated Sepharose 4B with *n*-hexylamine.

2) Sepharose 4B-BSA (S4B-BSA, BSA coupled to Sepharose 4B through a six-carbon-atom spacer) was the same preparation described previously for salicylate binding.[1]

3) CH-Sepharose 4B-BSA-FAF (CHS4B-BSA-FAF) was prepared by coupling BSA-FAF to CH-Sepharose 4B as described previously.[1]

Affinity Chromatography——Frontal analyses were performed as described previously[1] employing 0.004 % NaN_3 as an internal void volume indicator. Briefly, either a blank gel or albumin-coupled gel was packed in a glass column and preequilibrated at $3.3 \pm 1.1°$ with buffer flowing for at least 24 hr before the experiment. The buffer employed throughout the present binding study was 0.05 M Tris [tris(hydroxymethyl)aminomethane]containing 0.1 M NaCl, the pH being adjusted to 7.40 with 2 N HCl at room temperature, ionic strength 0.142. A solution of ligand containing 0.004% NaN_3 in the buffer was applied to the column continuously at a flow rate of 10 ml/hr and the eluate was collected in 2—6 ml fractions, depending upon the concentration of ligand, until the concentration in the eluate fractions reached that of the applied solution. The elution of each ligand was followed spectrophotometrically at the following wavelengths: salicylic acid, 295 nm; 3-hydroxybenzoic acid, 286 nm; 4-hydroxybenzoic acid, 245 nm; phenylbutazone, 264 nm; warfarin, 308 nm; D- and L-tryptophan, 278 nm; sulfamethizole, 260 nm; sulfisoxazole, 253 nm; bucolome, 271 nm. The amount of ligand bound was determined as described previously.[1]

After adsorption of phenylbutazone, which binds strongly, its complete elution was facilitated by passing a salicylic acid solution (approximately 1×10^{-4} M) instead of the buffer normally used for the elution of bound ligands. The displacement of bound phenylbutazone by salicylic acid was followed spectrophotometrically by monitoring the UV spectrum of the eluate fractions until it coincided with that of the salicylic acid solution applied to the column. The adsorbed salicylic acid was then eluted with the buffer.

Equilibrium Dialysis——Dialysis data were also obtained according to the procedure described previously[1] in the buffer containing 0.004% NaN_3. The concentration of BSA was fixed at 0.6%. The concentration of free ligand was usually determined at the wavelength of maximum absorbance given in "Affinity Chromatography" above. However, phenylbutazone, sulfisoxazole, and sulfamethizole were determined at either 280 or 290 nm, because corrections for impurities arising from both the cellophane membrane and BSA preparations became appreciable at wavelengths shorter than about 260 nm.

Data Analysis——The binding data obtained with various ligands were analyzed according to Scatchard[8] as described previously.[1] The following equation for a single class of *n* equivalent binding sites was used:

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TABLE I. Summary of Binding Parameters for the Interaction of BSA with Various Ligands, and Comparison with Literature Values

	Our Data						Reported Data						
		Binding parameters[b]								Binding parameters[f]			
Ligand	State of BSA[a]	$\sum n_i K_i \times 10^{-4}$, M^{-1}	app $K_1 \times 10^{-4}$, M^{-1}	app n_1	N[c]	$-\rho$[d]	Albumin	Temp.	Method[e]	$\sum n_i K_i \times 10^{-4}$, M^{-1}	app $K_1 \times 10^{-4}$, M^{-1}	app n_1	Ref.
Salicylic Acid	S4B–BSA[g]	7.1	6.0	1.2	10	0.989	BSA	4°	ED	1.88	2.44	0.78	10
	CHS4B–BSA[g]	9.3	4.7	2.0	13	0.981	BSA	25°	ED	15[h]	10[h]	1.5[h]	11
	CHS4B–BSA–FAF	9.8	4.8	2.0	4	0.999	HSA	23°	IM	6.0	3.0	2.0	4
	BSA[g]	9.8	5.0	2.0	8	0.957							
3-Hydroxy-benzoic Acid	S4B–BSA	—	—	—	—	—	BSA	37°	SP		5.0		12
	CHS4B–BSA	3.3	2.7	1.2	6	0.965	HSA	22°	CD		2.0		13
4-Hydroxy-benzoic Acid	S4B–BSA	0.9	2.1	0.43	3	0.997	BSA	37°	SP		0.79		12
	CHS4B–BSA	1.4	2.1	0.68	3	0.999	HSA	22°	CD		0.79		13
Phenylbutazone	S4B–BSA	30	33	0.91	6	0.957	BSA	25°	F	37	12.2	3	14
	CHS4B–BSA	66	53	1.2	4	0.999	BSA–FAF	25°	ED	8.4	7.0	1.2	15
	CHS4B–BSA–FAF	69	52	1.3	3	0.978	HSA	37°	ED	35	22	1.6	16
	BSA[j]	—	—	—	—	—							
Warfarin	S4B–BSA	17	11	1.5	3	0.994	BSA	28°	F	15	4.9	3.0	14
	CHS4B–BSA	29	12	2.4	3	0.999	BSA–FAF	25°	UF	20	4.6	4.3	17
	BSA	38	16	2.4	11	0.973	HSA	23°	IM	24	24	1.0	4
	BSA–FAF	28	11	2.5	4	0.997	HSA	6°	GF	46	31	1.5	18
L-Tryptophan	CHS4B–BSA	4.0	4.5	0.89	6	0.986	BSA–FAF	20°	UF	2.1	2.3	0.90	19
	CHS4B–BSA–FAF	4.0	3.9	1.0	4	0.993	BSA	2°	ED	1.5	1.6	0.94	20
	BSA	4.5	4.9	0.92	9	0.773	BSA	25°	GF	16[m]	19[m]	0.87[m]	21
D-Tryptophan	CHS4B–BSA	0.27	0.28	0.96	3	0.967	BSA	25°	GF	0.84[m]	0.93[m]	0.9[m]	21
	CHS4B–BSA–FAF	0.27	0.31	0.87	3	0.981							
Bucolome	CHS4B–BSA	6.0	2.5	2.4	5	0.992	HSA	5°	ED	12	10	1.2	22
	CHS4B–BSA–FAF	6.9	3.1	2.2	5	0.985							
Sulfamethizole	CHS4B–BSA	2.4	1.1	2.2	3	0.983	BSA	27°	F		0.52	2.9	23
	BSA	2.7	1.3	2.0	6	0.995	BSA	37°	SP		2.0		24
Sulfisoxazole	CHS4B–BSA	6.3	4.6	1.4	12	0.962	BSA	27°	F		0.96	2.9	23
	BSA	7.6	4.2	1.8	10	0.934	BSA	37°	SP		10		24
	BSA–FAF	5.9	3.7	1.6	5	0.965							

a) S4B–BSA, CHS4B–BSA, *etc.* are forms of immobilized BSA; see the text for details. BSA and BSA–FAF indicate soluble undefatted and defatted preparations, respectively; binding data were obtained by equilibrium dialysis.

b) Conditions of experiments: in 0.05 M Tris–HCl buffer containing 0.1 M NaCl, pH 7.40 (measured at room temperature) containing 0.004% NaN_3 at 3.3 ± 1.1°

c) The number of experimental data used for determination of the binding parameters. *d*) Correlation coefficient.

e) ED, equilibrium dialysis; IM, immobilized albumin (batchwise); SP, spectrophotometric; CD, circular dichroism; F, fluorescence; UF, ultrafiltration; GF, gel filtration.

f) At pH 7.4. These were calculated from reported values of K_1, K_2, n_1 and n_2, *i.e.*

$$\sum n_i K_i = n_1 K_1 + n_2 K_2$$

$$\text{app } K_1 = \frac{n_1 K_1^2 + n_2 K_2^2}{n_1 K_1 + n_2 K_2}, \text{ and app } n_1 = \frac{(n_1 K_1 + n_2 K_2)^2}{n_1 K_1^2 + n_2 K_2^2}$$ (Ref. 25).

g) The salicylate binding parameters were reported in Ref. 1. *h*) Calculated from reported Scatchard plots.

j) Binding parameters not calculated, see Fig. 2. *m*) At pH 9.0.

$$\frac{r}{[D_0]} = nK - rK$$

where r is the number of moles of bound drug per mole of albumin, K is the association constant for drug-albumin complex, and $[D_0]$ is the concentration of free drug. When a Scatchard plot is curved, the existence of multiple classes of sites on the protein molecule is assumed, and extrapolation of a Scatchard plot to y axis gives $\sum n_i K_i$ (total binding capacity[9]), where the subscript i denotes the i-th class of binding sites. Thus, from limiting straight lines drawn by the least-squares methods for the linear portions (usually $r<1$—1.5 in the present study) of Scatchard plots, the values of $\sum n_i K_i$ (intercept on the y axis), apparent n_1 (app n_1, intercept on the x axis), and apparent K_1 (app K_1, slope of the straight line) were calculated.

Results and Discussion

The results of the binding studies are summarized in Table I together with some literature values. In all affinity chromatographic systems presented here, nonspecific interaction of these ligands with each of the blank gels (lacking albumin), Sepharose 4B, CH-Sepharose 4B (which has terminal carboxylate anionic groups at the pH of this study), and *n*-hexyl-Sepharose 4B (which has terminal methyl groups), was negligible in comparison with that of sodium azide. Thus, the delay in elution of these ligands from the affinity columns can be attributed entirely to the specific interaction of these ligands with the immobilized albumin. All ligands selected for the present study exist essentially as 100% anions in the buffer.

Mode of Attachment of BSA and Binding Characteristics——The binding characteristics of BSA directly coupled to Sepharose 4B (S4B-BSA) and those of BSA coupled to Sepharose 4B through the six-carbon-atom spacer (CHS4B-BSA) are displayed in Scatchard plots for several hydroxybenzoates, including salicylate, phenylbutazone, and warfarin, in Fig. 1. We have previously shown that the binding capacity of BSA is greater when it was coupled to Sepharose 4B through the spacer only for salicylate.[1] Figure 1 shows that this is also the case for these compounds as well. Similarity in the appearance of the curves in Fig. 1 (a) and (b) for these systems indicates that the spacer does not appear to influence the binding characteristics grossly. In the absence of the spacer, the general reduction in binding capacity is associated with the reduction in the number of binding sites. Comparison of app K_1 values for these systems with the literature values (Table I), even though the conditions and methods differ greatly, indicates a reasonable rank order correlation among the ligands studied.

Comparison with Equilibrium Dialysis Data

Previously it was shown that the salicylate binding capacity of CHS4B-BSA is not only greater than that of S4B-BSA, but is actually comparable to that of soluble BSA as deter-

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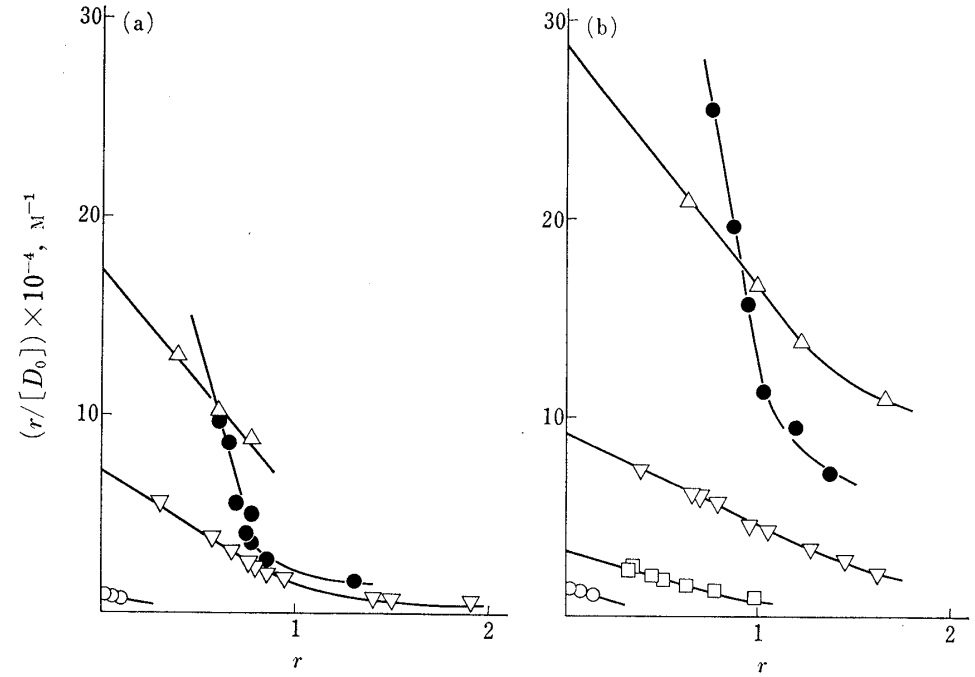

Fig. 1. Comparison of the Binding Characteristics of S4B–BSA (a) and CHS4B–BSA (b)

—●—, phenylbutazone; —△—, warfarin; —▽—, salicylic acid; —□—, 3-hydroxybenzoic acid; —○—, 4-hydroxybenzoic acid.

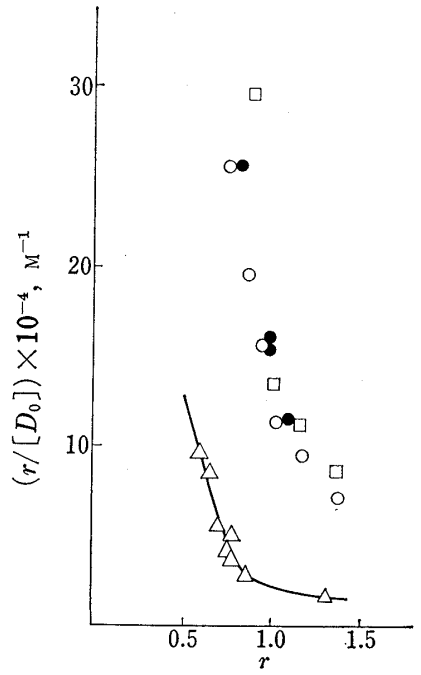

Fig. 2. Scatchard Plots for Phenylbutazone Binding to Immobilized BSA (by Affinity Chromatography) and to Soluble BSA (by Equilibrium Dialysis)

—△—, affinity chromatography, S4B–BSA;
○, affinity chromatography, CHS4B–BSA;
●, affinity chromatography, CHS4B–BSA–FAF;
□, equilibrium dialysis, undefatted BSA.

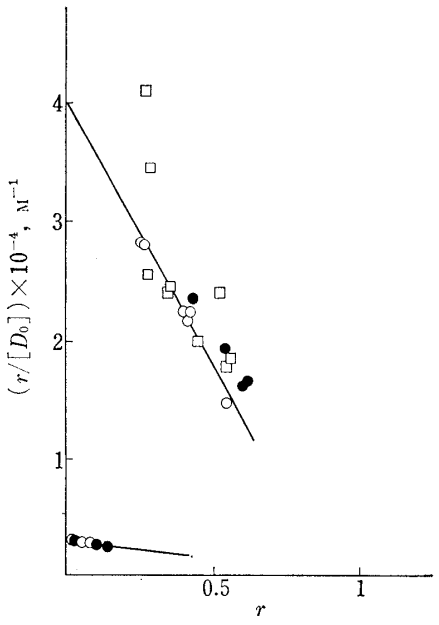

Fig. 3. Scatchard Plots for L- and D-Tryptophan Binding to Immobilized BSA (by Affinity Chromatography) and to Soluble BSA (by Equilibrium Dialysis)

○, affinity chromatography, CHS4B–BSA;
●, affinity chromatography, CHS4B–BSA–FAF;
□, equilibrium dialysis, undefatted BSA.
The upper line represents L-tryptophan binding to CHS4–BSA and the lower line D-tryptophan binding to CHS4B–BSA.

mined by equilibrium dialysis. The results of an extension of this study to phenylbutazone, presented as Scatchard plots in Fig. 2, show that the binding of phenylbutazone is analogous to that of salicylic acid. Similar results are presented for L- and D-tryptophan in Fig. 3. Since L-tryptophan is the only naturally occurring amino acid which is known to interact appreciably with serum albumin, and was reported[21] to have an affinity of approximately 20 times that of the D isomer, these compounds were selected as ligands for testing the general applicability of this technique. In the present study employing CHS4B-BSA, L-tryptophan bound to BSA with an affinity of about 15—16 times that of the D isomer. A fair agreement with equilibrium dialysis data (BSA) was also observed for L-tryptophan, although the experimental errors were greater in the latter method as indicated by the low correlation coefficient of -0.773 (see Table I and Fig. 3). With L-tryptophan, considerable experimental difficulty was encountered with the affinity chromatographic technique, particularly at low $[D_0]$. Binding equilibria were sometimes attained at lower concentrations than the experimentally fixed $[D_0]$, *i.e.*, the concentration of the eluate at equilibrium did not reach that of the solution applied to the column. Such data were excluded from the plots (Fig. 3) and the calculation of binding parameters, since the reasons for these effects could not be determined.

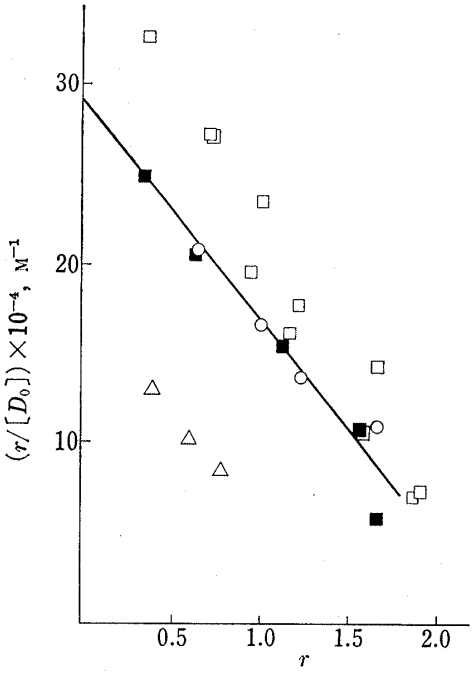

Fig. 4. Scatchard Plots for Warfarin Binding to Immobilized BSA (by Affinity Chromatography) and to Soluble BSA (by Equilibrium Dialysis)

△, affinity chromatography, S4B–BSA;
○, affinity chromatography, CHS4B–BSA;
□, equilibrium dialysis, undefatted BSA;
■, equilibrium dialysis, defatted BSA.
The line represents binding to CHS4B–BSA and defatted BSA.

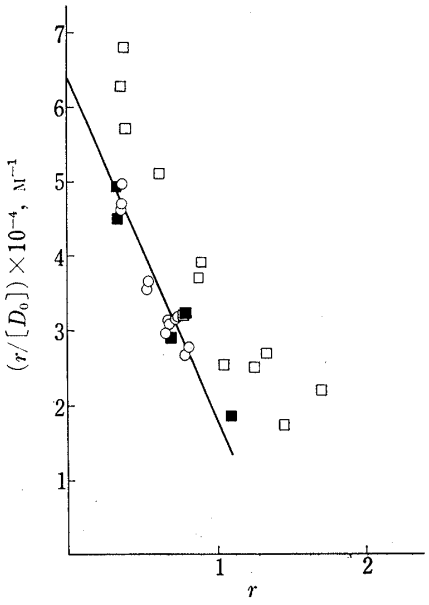

Fig. 5. Scatchard Plots for Sulfisoxazole Binding to Immobilized BSA (by Affinity Chromatography) and to Soluble BSA (by Equilibrium Dialysis)

○, affinity chromatography, CHS4B–BSA;
□, equilibrium dialysis, undefatted BSA;
■, equilibrium dialysis, defatted BSA.
The line represents the affinity chromatographyic data only.

The warfarin binding characteristics of CHS4B-BSA and soluble BSA are compared in Fig. 4. Although the soluble BSA preparation is closer to CHS4B-BSA than S4B-BSA in warfarin binding characteristics, some difference can be seen between soluble BSA and CHS4B-BSA, particularly at low molar ratios of warfarin to albumin. Figure 5 also indicates the soluble BSA preparation to have a somewhat higher binding capacity than CHS4B-BSA toward sulfisoxazole.

These studies thus suggest that, although the binding capacity of CHS4B-BSA approaches that of soluble albumin, depending upon the nature of the ligand, some difference may exist between the soluble albumin preparation and the immobilized BSA with the spacer (CHS4B-BSA). An attempt was made to elicit information concerning the reason for this difference by similarly examining another BSA preparation which is essentially fatty acid-free.

Effects of Residual Free Fatty Acids

Commercial preparations of serum albumin, unless described as defatted, are known to contain up to 2—3 mol of free fatty acids per mole of albumin.[26] A recent report[27] showed some HSA preparations to contain as much as 9 mol of fatty acids; the differences in binding properties among them were abolished upon defatting. Although physiologically important 16- and 18-carbon-atom fatty acids show affinities approximately 3—4 orders of magnitude greater than those of most drugs, these fatty acids are generally considered not to inhibit the binding of drugs and other physiological substances except at high concentrations.[28] However, recent studies indicate that depending upon the ligand even small amounts of fatty acids, at molar ratios to albumin of less than 1.5, may potentiate[17,29–32] or inhibit[33,34] the drug binding through an allosteric mechanism.

In the affinity chromatographic method, unless a defatted BSA preparation is employed in immobilization, the coupling conditions may favor the dissociation of albumin-residual free fatty acid complexes. Subsequent extensive washing with buffers at different pH values may also remove the residual fatty acids. Further, the repetitive use of a column for binding studies of many ligands over several months may be considered to gradually wash away the residual fatty acids, particularly if the ligand and fatty acid share the same binding sites on albumin. In view of these problems, a BSA preparation which was essentially fatty acid free (BSA-FAF) was similarly examined by immobilizing it on CH-Sepharose 4B (CHS4B-BSA-FAF). The bindings of warfarin and sulfisoxazole to BSA-FAF were also investigated by equilibrium dialysis.

The binding properties of CHS4B-BSA-FAF are compared with those of CHS4B-BSA for salicylate (Table I), phenylbutazone (Fig. 2), L- and D-tryprophan (Fig. 3), warfarin (Fig. 4), and bucolome (Table I). In no case was any significant difference observed between these two immobilized albumins. Thus, for salicylic acid, phenylbutazone, and L-tryptophan, these immobilized albumins appear to show binding properties similar to those of the undefatted BSA preparation. In the cases of warfarin and sulfisoxazole, however, the undefatted albumin preparation showed a slightly higher binding capacity than BSA-FAF or CHS4B-BSA. Moreover, the binding characteristics of the latter two were comparable. A number of recent reports[17,29,30] suggest that, at low molar ratios of fatty acid to albumin, fatty acids allosterically enhance warfarin binding. Our present results can be interpreted in this light as indicating that the bindings of warfarin and sulfisoxazole were enhanced by residual fatty acids in the undefatted BSA preparation and that the residual fatty acids were removed prior to binding experiments in the affinity chromatographic study. However, this remains unconfirmed, as we did not quantitate fatty acids in any way. Further studies in this direction are in progress in our laboratories.

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The results of the present study are consistent with the generally accepted view that the presence of small (residual) amounts of fatty acids does not grossly influence drug binding to albumin,[28] *i.e.*, the influence of fatty acids on drug binding is not competitive in nature. Fatty acid binding is likely to affect the conformational state of albumin[35] and to affect allosterically the interaction of albumin with other ligands.[36] Therefore, depending upon the nature of the ligand, the binding may be inhibited, enhanced, or unchanged by residual fatty acids.

General Discussion

Methods to study protein binding employing immobilized albumin, whether immobilization involves covalent linkage or embedding, may be classified into 2 categories, *i.e.*, (1) batchwise techniques and (2) affinity chromatographic techniques. The former technique offers rapid and simple procedures, while the latter, although more time-consuming, offers precise data with simple experimental procedures. The precision in the latter techique is due primarily to the fact that the free drug concentration is experimentally set and bound drug can be directly determined. The leakage of immobilized albumin is a major concern. However, the binding capacity of the albumin covalently linked to Sepharose 4B, employed in this study, remained essentially unaltered for several months. Even the less-stable defatted albumin showed adequate stability for 1—2 months after coupling. Thus, repeated use under the same experimental conditions would be of particular value when the availability of protein is limited. For strongly bound ligands, small columns are sufficient, while for weakly bound ligands, large columns are required to obtain precise data.

Disadvantages of the use of immobilized albumin lie in the nature of the gel matrix. Although Sepharose 4B is claimed to have low adsorption properties,[37] we have observed, for instance, that α-naphthol, 3-hydroxy-2-naphthoic acid, chlorpromazine, rose bengal, *etc.*, have some affinity for Sepharose 4B. Both Sepharose 4B derivatives (lacking albumin) employed in this study showed significant adsorption of some of these ligands. Thus, the present affinity chromatographic technique for such ligands is not as straightforward as presented here and may require correction for nonspecific interaction with the gel matrix. In such cases, a suitable choice of gel would be helpful.

The present study, however, demonstrated that Sepharose 4B is a suitable carrier for a wide variety of ligands known to interact with BSA and that BSA covalently coupled to this carrier through a spacer has binding parameters more comparable to those of soluble BSA than to those of directly coupled BSA.

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