Study of Drug–Protein Binding by Affinity Chromatography: Interaction of Bovine Serum Albumin and Salicylic Acid

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Abstract □ The affinity chromatographic technique was used to study the interaction of bovine serum albumin and salicylic acid at 3.3 ± 1.1°. Beaded agarose gel, on which the albumin was immobilized by covalent linkage, was packed in a column as an affinity adsorbent. Frontal analysis was performed on this column to evaluate the binding parameters for the interaction. The effect of albumin immobilization on drug binding was investigated by comparing the binding parameters of two affinity adsorbents, directly coupled albumin and albumin coupled through a spacer arm. The latter mode of attachment gave binding characteristics comparable to those of the soluble albumin. The method is simple and precise. The affinity adsorbent can be used repeatedly for many months for various drugs, including those that do not diffuse through dialysis membranes.

Keyphrases □ Salicylic acid—binding to bovine serum albumin, affinity chromatographic study □ Binding, protein—salicylic acid to bovine serum albumin, affinity chromatographic study □ Chromatography, affinity—study of binding of salicylic acid to bovine serum albumin □ Keratolytic agents—salicylic acid, binding to bovine serum albumin, affinity chromatographic study

The interaction of drugs with serum albumin is well known, and its consequences are manifested in various aspects of drug disposition. Recently, it was observed that the displacement of a bound drug from albumin by another drug can have profound pharmacological and toxicological effects in multiple drug therapy (1, 2). The methods of quantitating serum protein binding are numerous (3, 4), but a simple and direct procedure particularly suitable for systems containing more than one drug is not available. The development of such a method through the affinity chromatographic technique was initiated by studying serum albumin–single drug interactions. This report describes the application of this technique for studying interactions of bovine serum albumin and salicylic acid and the evaluation of affinity adsorbents.

The present method differs from gel filtration (5, 6) or other methods (7) employing gel beads in that the protein is immobilized on a gel matrix through covalent linkage and packed in a chromatographic column as an adsorbent; a drug in an appropriate buffer is passed through the column. In gel filtration, the gel is employed to exclude the protein molecule from the gel matrix. Similar use of affinity columns was described previously (8, 9). The interaction between an enzyme and its inhibitor was studied by immobilization of the small inhibitor molecules on a gel matrix. Bovine serum albumin immobilized on agarose was employed in a batchwise procedure in competition with free protein to evaluate the binding parameters for the interactions of hydrocortisone, bilirubin, fatty acids, and tryptophan with several proteins or protein fragments (10).

Although affinity chromatography by means of immobilized albumin has not been employed in quantitative investigations of drug–protein interactions, these interactions were utilized to separate or isolate substances. DL-Tryptophan was resolved successfully on an albumin–agarose column on the basis of differences in the binding of the two optical isomers to albumin (11). The isolation of albumin from serum also was attempted by passing serum samples through a column of affinity adsorbents prepared by coupling to agarose substances known to interact strongly with albumin, such as a dye (12), fatty acids (13), and bilirubin (14).

EXPERIMENTAL

Materials—Agarose[1], bovine serum albumin[2], salicylic acid[3] (recrystallized from hot water), 6-aminohexanoic acid[3], cyanogen bromide[3], sodium azide[3], and *N*-ethyl-*N*-(3-dimethylaminopropyl)carbodiimide hydrochloride[4] were used. Chemicals in the buffer solutions were reagent grade. Water was deionized and double distilled, with the second distillation performed in an all-glass apparatus. Cellulose tubing[5] was used for the equilibrium dialysis study.

Immobilization of Albumin to Agarose—Bovine serum albumin directly coupled to cyanogen bromide-activated agarose (Affinity Adsorbent I) was prepared according to the general method of Axén and Ernback (15). The activation of agarose was carried out at pH 11 for 6 min, and approximately 50 ml of the activated gel was coupled with 2 g of bovine serum albumin at about 3° overnight under constant shaking. The reaction mixture was filtered and extensively washed with 0.1 *M* $NaHCO_3$.

The gel was suspended in 100 ml of 1 *M* ethanolamine hydrochloride (pH 9.4) for 2 hr at room temperature to block the unreacted active groups and packed into a glass column. It was then washed thoroughly to remove the adsorbed albumin with 0.025 *M* $Na_2B_4O_7$–acetic acid containing 1 *M* NaCl (pH 8.5), followed by 0.1 *M* acetate buffer containing 1 *M* NaCl (pH 4.1) and, finally, 0.1 *M* CH_3COONa at the maximum flow rate of the column under gravity.

Bovine serum albumin coupled to cyanogen bromide-activated agarose through a six-carbon-atom spacer (Affinity Adsorbent II) was prepared according to the Kaufman method (16) for the preparation of methotrexate–aminohexylagarose; 50 ml of the cyanogen bromide-activated agarose was reacted with 100 ml of a solution of 0.5 *M* 6-aminohexanoic acid and 0.5 *M* NaCl (pH adjusted to 10 with 2 *N* NaOH) at about 3° for 16 hr. The spacer arm coupled agarose (carboxypentylagarose[6]) thus obtained was extensively washed, and the unreacted groups were blocked as described previously.

After exhaustive washing with 1 *M* HCl, water, 1 *M* NaOH, and then water, the gel was reacted with 1 g of albumin at pH 4.5 in the presence of 0.5 g of the carbodiimide hydrochloride for 16 hr at room temperature. Affinity Adsorbent II thus prepared was subjected to the same treatment as described for Affinity Adsorbent I.

Assay of Coupled Albumin—The determination of the albumin immobilized per milliliter of gravity-packed Affinity Adsorbents I and II was carried out by direct spectrophotometry (17) at 278 nm. An aliquot of adsorbent gel suspension was gravity packed into a previously weighed and graduated column equipped with a sintered-glass disk (G3) to determine the volume of the packed gel under normal column packing. This volume of adsorbent was either dried directly in the column with water–acetone mixtures, as described by Axén and Ernback (15), or freeze

[1] Sepharose 4B, Pharmacia Fine Chemicals, Uppsala, Sweden.
[2] Fraction V, powder lot 243, Miles Laboratories, Elkhart, Ind.
[3] Wako Pure Chemical Industries, Osaka, Japan.
[4] Fluka AG, Buchs SG, Switzerland.
[5] Visking cellulose tubing, size 18/32, Union Carbide Corp., Chicago, Ill.
[6] Carboxypentylagarose is also available under the name CH-Sepharose from Pharmacia Fine Chemicals, Uppsala, Sweden.

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Table I—Column Characteristics

Column	Affinity Adsorbent	Column Dimensions, i.d. × height, cm	Gel Volume, ml	Bovine Serum Albumin, mg/ml of gel	Flow Rate, ml/hr
1	I	0.98 × 29.2	22.0	4.2	4–12
2	I	1.60 × 10.8	21.7	4.1	5.0 or 11.0
3	II	0.79 × 20.1	9.8	8.6	6.6
4	II	0.96 × 27.0	19.6	8.6	6.0
5	II	1.60 × 11.5	23.1	8.6	5.6 or 11.4

dried to determine the corresponding dry weight. Approximately 10 mg of the dried adsorbent was weighed directly into a 1-cm cell and suspended in 3 ml of 50% glycerin.

The turbidity due to the gel beads was balanced at 350 nm against agarose or carboxypentylagarose beads in the reference cell compartment of the spectrophotometer[7] (cells were positioned directly in front of the photomultiplier). Then the difference spectrum was recorded and showed no difference from the spectrum of an albumin solution. A molecular weight of 66,210 (18) was used for bovine serum albumin.

Affinity Chromatography by Frontal Analysis—The column characteristics are listed in Table I. The columns were preequilibrated in a cold room (3.3 ± 1.1°), with buffer flowing for at least 24 hr before the experiment. The buffer employed throughout the binding study was 0.05 *M* tromethamine [tris(hydroxymethyl)aminomethane] containing 0.1 *M* NaCl at pH 7.40 (ionic strength of $\mu = 0.142$). A solution of salicylic acid in the buffer containing 0.004% NaN_3 was applied to a column continuously either by gravity flow or a peristaltic pump[8], and the eluate was collected in 1–6-ml fractions[9], depending on the concentration of salicylic acid, until the concentration in the eluate fractions reached that of the applied solution.

The amount of the drug adsorbed, D_b, by the total amount of albumin immobilized in the column was determined by the equation $D_b = [D_0](V - V_0)$, where $[D_0]$ is the concentration of the drug applied to the column and V_0 and V are eluted volumes of 0.004% NaN_3 and the drug, respectively. The latter values were obtained from the frontal chromatogram as the volumes of eluate corresponding to the concentrations that reached 50% of those of the applied solution. The concentrations of the drug and sodium azide were determined spectrophotometrically[7] at 295 and 247 nm, respectively.

Equilibrium Dialysis—The dialysis tubing was pretreated for 30 min in hot 8% Na_2CO_3 (19) and washed thoroughly with water. Dialysis bags were made from the tubing and soaked in water for about 48 hr at refrigerative temperature. Water was replaced several times during this period until no significant UV absorbance (λ 360–250 nm) was detected. Finally, the bags were soaked and rinsed with the buffer containing 0.004% NaN_3.

Dialysis studies were carried out under the same conditions as the affinity chromatographic studies, using the soluble albumin instead of the immobilized one. Thus, 0.004% NaN_3 was incorporated in the buffer, and experiments were performed at two albumin concentrations (0.45 and 0.8%), corresponding approximately to those of Affinity Adsorbents I and II, respectively. The solution containing the albumin and the drug (5 ml) was placed inside the bag and dialyzed against 50 ml of the buffer system in a 100-ml erlenmeyer flask.

Dialysis was allowed to proceed at 3.3 ± 1.1° for about 16–24 hr under agitation of both the inside and outside solutions by magnetic bars. A small magnetic bar (0.3 × 1.0 cm) was placed inside each bag. Care was taken to arrange the flask on a magnetic stirrer so that neither magnetic bar excessively touched the bag during movement.

The concentration of salicylic acid outside the bag was determined spectrophotometrically at 295 nm. Blank studies with the buffer system alone showed that the absorbance at 295 nm outside of the bag increased by at most 0.003 over that of the buffer system during the equilibration period of over 20 hr, and correction was made for this factor. When the albumin was placed alone inside the bag, no substance that interfered with the salicylic acid assay was dialyzed out of the bag.

Data Analysis—Binding data of a small molecule to a macromolecule are often analyzed according to (20):

$$r = \sum_{i=1}^{m} \frac{n_i K_i [D_0]}{1 + K_i [D_0]} \qquad \text{(Eq. 1)}$$

where r is the molar ratio of bound drug, D_b, to total protein, P_t; n_i is the number of binding sites in the ith class; K_i is the intrinsic binding constant for the sites in the ith class; and $[D_0]$ is the concentration of unbound (free) drug.

For only one class of n binding sites, Eq. 1 reduces to:

$$r = \frac{nK[D_0]}{1 + K[D_0]} \qquad \text{(Eq. 2)}$$

and Eq. 2 is often transformed to the following familiar Scatchard equation (21):

$$\frac{r}{[D_0]} = Kn - Kr \qquad \text{(Eq. 3)}$$

Substituting $(V - V_0)[D_0]/P_t$ for r in the reciprocal of Eq. 2 yields:

$$\frac{1}{(V - V_0)} = \frac{1}{nKP_t} + \frac{1}{nP_t}[D_0] \qquad \text{(Eq. 4)}$$

This form for the value of D_b relates directly to the affinity chromatography frontal analysis data. Thus, a plot of $1/(V - V_o)$ *versus* $[D_0]$ should be linear. The binding constant, K, can be determined from the slope and ordinate intercept of the straight line, and n can be determined from the slope if P_t is known.

When this plot or a plot according to Eq. 3 does not give a straight line, the existence of multiple classes of sites on a protein molecule is assumed and the extrapolation of a Scatchard plot curve to the abscissa and ordinate allows estimation of $\Sigma_{i=1}^{m} n_i$ and $\Sigma_{i=1}^{m} K_i n_i$, respectively (22).

Resolution of K_i and n_i is usually attempted on the assumption that there exist only two classes of sites; K_1, K_2, n_1, and n_2 are calculated by a nonlinear least-squares fit by computer (23). Since this assumption is made only for convenience, in this report the graphically obtainable quantity, $\Sigma_{i=1}^{m} K_i n_i$, the total binding capacity (10), is reported either from the extrapolation of the plots of $r/[D_0]$ *versus* r to the ordinate or from the intercept of the plots of $1/(V - V_0)$ *versus* $[D_0]$ and P_t. The apparent K_1 (app K_1) and n_1 (app n_1) values also were obtained from the limiting linear portions of the plots according to Eqs. 3 and 4 for comparison only.

RESULTS AND DISCUSSION

Concentration-Dependent Elution Profiles—Figure 1 shows typical elution profiles of sodium azide and salicylic acid when salicylic acid solutions of varying concentrations, each containing 0.004% NaN_3, were applied to a column of Affinity Adsorbent II. These elution profiles indicated that the elution volume of 0.004% NaN_3 was independent of salicylic acid concentration but that the elution volume of salicylic acid depended on its own concentration. That the concentration-dependent

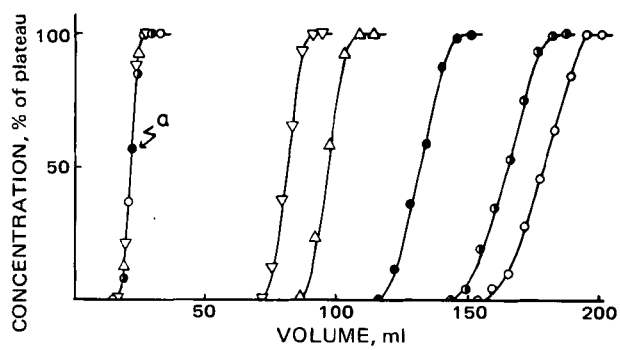

Figure 1—*Concentration-dependent elution profiles of salicylic acid from Column 4 (see Table I for the column characteristics). Key (concentrations of salicylic acid): ○, 1.07 × 10⁻⁵ M; ◑, 1.40 × 10⁻⁵ M; ●, 2.49 × 10⁻⁵ M; △, 5.04 × 10⁻⁵ M; and ▽, 7.10 × 10⁻⁵ M. Curve a is the elution profile of 0.004% NaN_3 incorporated in these salicylic acid solutions.*

[7] Double-beam spectrophotometer, model UV-300, Shimadzu Manufacturing Co., Kyoto, Japan.

[8] P-3, Pharmacia Fine Chemicals, Uppsala, Sweden, or MultiPerpex 2115, LKB, Bromma, Sweden.

[9] Fraction collector model LKB 7000A UltroRac, LKB, Bromma, Sweden, or model SF-160K, Toyo Scientific Co., Tokyo, Japan.

Table II—Binding Parameters for the Interaction of Bovine Serum Albumin with Salicylic Acid

State of Albumin	Buffer	pH	Temperature	$\Sigma K_i n_i \times 10^{-4}, M^{-1}$	app $K_1 \times 10^{-4}, M^{-1}$	app n_1	Method[a]	Reference
Immobilized (Affinity Adsorbent I)	$\mu = 0.143$[b], tromethamine	7.40	3.3 ± 1.1°	7.09	6.03	1.18	AF	This work
Immobilized (Affinity Adsorbent II)	$\mu = 0.143$[b], tromethamine	7.40	3.3 ± 1.1°	9.33	4.73	1.97	AF	This work
Soluble	$\mu = 0.143$[b], tromethamine	7.40	3.3 ± 1.1°	9.83	5.02	1.96	ED	This work
	$\mu = 0.05, 0.04$ *M* phosphate	7.4	4°	1.88[c]	2.44[c]	0.78[c]	ED	29
		7.0	25°	20.9[c]	19.1[c]	1.09[c]	DD	30
	0.016 *M* phosphate + 0.122 *M* NaCl	7.4	25°	15[d]	10[d]	1.5[d]	ED	31
	0.128 *M* phosphate	7.38	37°	5.4[c]	4.82[c]	1.12[c]	DD	32

[a] AF = affinity chromatography, ED = equilibrium dialysis, and DD = dynamic dialysis. [b] μ = ionic strength, including 0.1 *M* NaCl and 0.004% NaN_3. [c] Two classes were assumed; thus, $\Sigma K_i n_i = n_1K_1 + n_2K_2$, app $K_1 = (n_1K_1^2 + n_2K_2^2)/(n_1K_1 + n_2K_2)$, and app $n_1 = [(n_1K_1 + n_2K_2)^2]/(n_1K_1^2 + n_2K_2^2)$ (Ref. 23). [d] Estimated from Scatchard plots.

elution patterns of the drug were primarily the consequence of the specific interaction between the immobilized albumin and salicylic acid was apparent when similar drug solutions were applied to an agarose column. From the column void of albumin, salicylic acid was eluted within experimental error at the elution volume of sodium azide. A similar observation was made with a carboxypentylagarose column, indicating that the six-carbon-atom spacer also contributed little to the observed effect.

When 0.004% NaN_3 alone was applied to a column of albumin-coupled agarose, its elution volume was the same as that of 0.004% NaN_3 added to salicylate solutions of varying concentrations (Fig. 1). Even at a salicylate concentration as high as 1.0×10^{-3} *M*, the elution volume of 0.004% NaN_3 was not significantly influenced. In the absence of salicylic acid, the elution volume of sodium azide from the albumin-coupled column was independent of its concentration (0.004–0.1%). Furthermore, the elution volume of salicylic acid at a relatively low concentration of 2.17×10^{-5} *M* was not altered significantly by 0.004% NaN_3.

These observations suggest that 0.004% NaN_3 does not significantly influence the specific interaction of salicylic acid with albumin immobilized on agarose[10]. Thus, 0.004% NaN_3 was employed as an internal void volume indicator throughout these studies, because the column heights sometimes decreased suddenly for unknown reasons and settled to a constant level during continuous use for several months. The void volume here means the void volume of the column plus the volume of tubing.

Effect of Flow Rate—Investigations concerning the effect of flow rate on the elution profile on Column 5 (Table I) indicated that the elution volumes were unaffected within a range of 5–12 ml/hr, although the sigmoidal elution curve became less steep as the flow rate increased. At a flow rate of 17.6 ml/hr, elution volumes decreased only by about 1.5%, but the gradual saturation of the column was noted. With Column 1, the flow rate of which was not controlled by a peristaltic pump, elution volumes were little affected by the variation in flow rates shown in Table I.

Data Treatment—Figure 2 shows plots of $1/(V - V_0)$ *versus* $[D_0]$ according to Eq. 4 for Columns 1, 2, and 4 (Table I). Points are shown for only low $[D_0]$ data, and the straight lines correspond to the least-squares fit. It is possible to estimate app K_1 and app n_1 from such plots if P_t is known. Equation 4 is thus useful for obtaining these parameters directly from chromatographic data, *i.e.*, the elution volume and concentration of the drug applied to an affinity adsorbent column. If P_t immobilized in each column is known, data obtained from all columns can be presented in the familiar Scatchard form (Eq. 3).

The results of the determination of albumin coupled per milliliter of packed gel (Table I) reveal that nearly twice as much albumin was coupled to agarose through the spacer. Although the direct coupling was undertaken at refrigerative temperature and the carbodiimide coupling was carried out at room temperature, separate carbodiimide coupling reactions showed that the amount of albumin coupled was the same whether carried out overnight at room or refrigerative temperature. Thus, these results may indicate that the direct coupling of the albumin is considerably sterically unfavorable.

Since the total number of moles of albumin, P_t, immobilized on the column and the moles of drug bound, D_b, were known from elution data, the ratio $r = D_b/P_t$ was calculated for each free concentration of drug for all columns and plotted against $r[D_0]$ (Fig. 3). The points all converged into two separate curves, one for Affinity Adsorbent I and the other for II. Even the two different batches of Affinity Adsorbent I (Columns 1 and 2) showed the trend satisfactorily. These results indicated that the albumin coupled through the spacer bound more salicylic acid per mole of albumin than the directly coupled albumin.

Comparison with Equilibrium Dialysis Data—The equilibrium dialysis data obtained under the same conditions as for the affinity chromatographic study for two different soluble albumin concentrations, *i.e.*, one corresponding closely to the concentration of coupled albumin in the packed gel bed of Affinity Adsorbent II (0.8%) and the other corresponding to that of I (0.45%), fell close to the curve for Affinity Adsorbent II (Fig. 3).

Table II summarizes the binding parameters for the interaction of salicylic acid with bovine serum albumin. The parameters from the present studies were obtained from the straight lines drawn by the least-squares method for the linear portion ($r < 1$–1.4) of the Scatchard plots (Fig. 3). The correlation coefficients for the straight lines were greater than 0.98 for the affinity chromatographic studies and 0.96 for the equilibrium dialysis data. Apparent K_1 for Affinity Adsorbent I was greater than that for the Affinity Adsorbent II, although the total binding capacity of I was reduced by about 24% over that of II. Employing a batchwise method with bovine serum albumin directly coupled to agarose, Reed *et al.* (10) similarly noted that the tryptophan binding capacity of immobilized albumin was approximately one-tenth of that of soluble albumin but that the app K_1 was slightly higher than that for soluble albumin.

The role played by the spacer groups in affinity chromatography is largely to relieve the steric hindrance for specific interactions, thereby increasing the capacity of affinity adsorbents (24–27); six-carbon atom chains are generally considered long enough. Good agreement between the data obtained on Affinity Adsorbent II and those of equilibrium dialysis, under controlled experimental conditions, may indicate that the spacer largely eliminates the steric hindrance imposed on the directly coupled albumin and that immobilization of albumin through the spacer

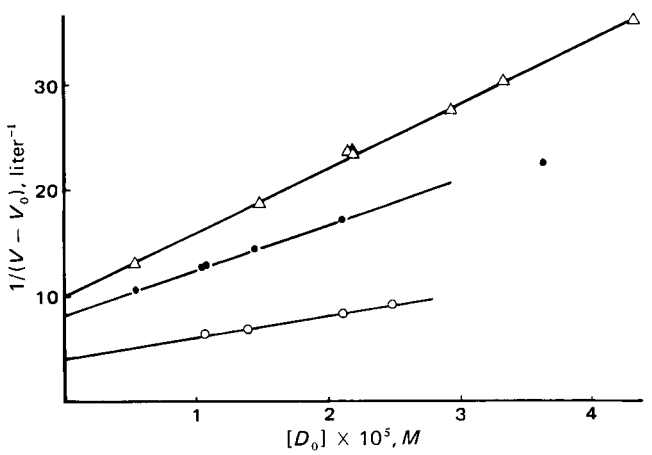

Figure 2—*Plots of* $1/(V - V_0)$ versus $[D_0]$ *according to Eq. 4. Key:* △, *Column 1;* ●, *Column 3; and* ○, *Column 4 (see Table I for the column characteristics).*

[10] This does not mean that sodium azide does not interact with the albumin. In fact, when 0.1% NaN_3 was incorporated in 2.17×10^{-5} *M* salicylic acid, the elution volume of salicylic acid decreased in comparison with that in the absence of sodium azide, indicating that sodium azide may possibly influence salicylate binding at higher concentrations. At 0.004% NaN_3, however, this effect was negligible.

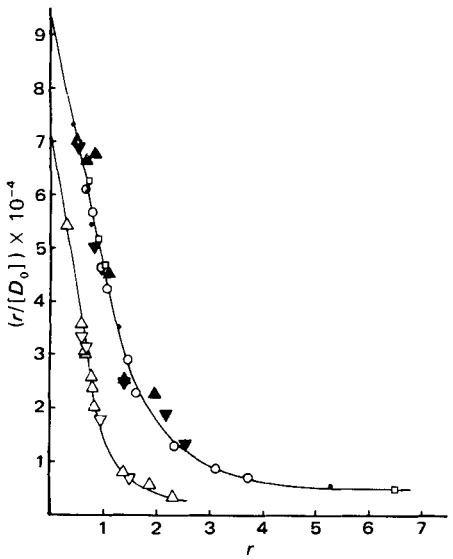

Figure 3—*Scatchard plots for the interaction of salicylic acid with bovine serum albumin at 3.3 ± 1.1°, pH 7.40, and μ = 0.143. Key for affinity chromatographic technique:* △, *Column 1;* ▽, *Column 2;* ●, *Column 3;* ○, *Column 4; and* □, *Column 5 (see Table I for the column characteristics). Key for equilibrium dialysis technique:* ▲, *0.8% bovine serum albumin; and* ▼, *0.45% bovine serum albumin.*

does not significantly impair the binding capacity of the albumin, at least toward salicylic acid.

Table II presents some previously published data for the interaction of bovine serum albumin with salicylate. In spite of the good agreement between the binding parameters obtained by this equilibrium dialysis and affinity chromatographic study employing Affinity Adsorbent II, these parameters are not in agreement with those of other workers. The present app K_1 values, for instance, are generally smaller than those of other workers when the differences in temperature at which the studies were carried out are considered. An exact comparison is not possible because binding parameters are affected not only by temperature but also by the preparation of serum albumin (28), the composition of buffer, the pH and ionic strength of the medium, *etc.* As for the preparation of albumin, the difference in the content of free fatty acids or other impurities tightly bound to albumin or other factors may be responsible for the disparity. Also, the azide incorporated in the present studies might have inhibited salicylate binding, particularly at low salicylate concentrations, although this was not obvious in the affinity chromatographic study.

CONCLUSIONS

Although the conformation on the soluble albumin may not be kept intact after immobilization on agarose, the present study demonstrated that the salicylate binding capacity was not altered significantly by immobilization, particularly through the spacer. It is not the maintenance of conformation upon immobilization that is important in this method but that the drug binding characteristics of immobilized albumin are similar to that of soluble albumin. Thus, Affinity Adsorbent II is better than I for drug binding studies by affinity chromatography. Even if the drug binding capacity is reduced upon immobilization, as with Affinity Adsorbent I, the technique would still be of value in *in vitro* comparison of affinities of drugs toward serum albumin if the degrees of reduction are the same for many drugs.

This method is simple and much more precise than the batchwise method of Reed *et al.* (10), since measurement of the volume of gel suspension, which is essential in the batchwise method, inherently introduces uncertainty about the amount of albumin present. The salicylate binding capacity of immobilized albumin was unaltered for at least 8 months under continuous use. When the columns were not in use for 1 week or more, the buffer was continuously applied at a low flow rate. During the 8 months, many drugs were applied to the columns, but subsequent elution with the buffer after each run apparently left the column unaltered. These observations support the conclusions that albumin itself and the covalent linkage between agarose and albumin are stable and that the conformation of albumin is likely to be restored each time upon elution with the buffer. Therefore, repeated use of the column renders this method economical.

Affinity chromatography facilitates the quantitation of salicylic acid binding by frontal analysis, and the salicylate binding capacity of immobilized bovine serum albumin depends on the mode of immobilization. Many other drugs were tested similarly on these columns, and the data will be published separately. Theoretically, any drug can be studied by this method, provided that the eluate can be assayed for the drug, including drugs that do not diffuse through the dialysis membrane. However, the effect of immobilization of albumin may have to be investigated for each drug. Such studies, in turn, may shed light on the sites of drug interaction on the albumin molecule.

REFERENCES

(1) R. A. O'Reilly, *Ann. N.Y. Acad. Sci.*, **226**, 293 (1973).

(2) H. M. Solomon, in "Handbook of Experimental Pharmacology," vol. 28/1, B. B. Brodie and J. R. Gillette, Eds., Springer-Verlag, Berlin, Germany, 1971, p. 234.

(3) J. Steinhardt and J. A. Reynolds, "Multiple Equilibria in Proteins," Academic, New York, N.Y., 1969, pp. 34–83.

(4) M. C. Meyer and D. E. Guttman, *J. Pharm. Sci.*, **57**, 895 (1968).

(5) J. P. Hummel and W. J. Dreyer, *Biochim. Biophys. Acta*, **63**, 530 (1962).

(6) P. F. Cooper and G. C. Wood, *J. Pharm. Pharmacol.*, **20**, 150S (1968).

(7) W. Scholtan, *Arzneim.-Forsch.*, **14**, 146 (1964).

(8) B. M. Dunn and I. M. Chaiken, *Proc. Natl. Acad. Sci. USA*, **71**, 2382 (1974).

(9) K. Kasai and S. Ishii, *J. Biochem.*, **77**, 261 (1975).

(10) R. G. Reed, T. Gates, and T. Peters, Jr., *Anal. Biochem.*, **69**, 361 (1975).

(11) K. K. Steward and R. F. Doherty, *Proc. Natl. Acad. Sci. USA*, **70**, 2850 (1973).

(12) J. Travis and R. Pannell, *Clin. Chim. Acta*, **49**, 49 (1973).

(13) T. Peters, Jr., H. Taniuchi, and C. B. Anfinsen, Jr., *J. Biol. Chem.*, **248**, 2447 (1973).

(14) M. Hierowski and R. Brodersen, *Biochim. Biophys. Acta*, **354**, 121 (1974).

(15) R. Axén and S. Ernback, *Eur. J. Biochem.*, **18**, 351 (1971).

(16) B. T. Kaufman, in "Methods in Enzymology," vol. 34, W. B. Jakoby and M. Wilchek, Eds., Academic, New York, N.Y., 1974, p. 272.

(17) A. Miyakawa and N. Okuyama, *Seikagaku*, **48**, 413 (1976).

(18) T. Peters, Jr., in "The Plasma Proteins," vol. 1, F. W. Putnum, Ed., Academic, New York, N.Y., 1975, p. 141.

(19) D. Hultmark, K. O. Borg, R. Elofsson, and L. Palmer, *Acta Pharm. Suec.*, **12**, 259 (1975).

(20) G. Scatchard, J. S. Coleman, and A. L. Shen, *J. Am. Chem. Soc.*, **79**, 12 (1957).

(21) G. Scatchard, *Ann. N.Y. Acad. Sci.*, **51**, 660 (1949).

(22) I. M. Klotz and D. L. Hunston, *Biochemistry*, **10**, 3065 (1971).

(23) J. J. Vallner, J. H. Perrin, and S. Wold, *J. Pharm. Sci.*, **65**, 1182 (1976).

(24) P. Cuatrecasas, *J. Biol. Chem.*, **245**, 3059 (1970).

(25) E. Steers, P. Cuatrecasas, and H. Polland, *ibid.*, **246**, 196 (1971).

(26) C. R. Lowe, M. J. Harvey, D. B. Craven, and P. D. G. Dean, *Biochem. J.*, **133**, 499 (1973).

(27) C. R. Lowe and P. D. G. Dean, "Affinity Chromatography," Wiley, New York, N.Y., 1974, p. 23.

(28) R. H. McMenamy, *J. Biol. Chem.*, **240**, 4235 (1965).

(29) C. Davison and P. K. Smith, *J. Pharmacol. Exp. Ther.*, **133**, 161, (1961).

(30) M. C. Meyer and D. E. Guttman, *J. Pharm. Sci.*, **59**, 39 (1970).

(31) T. E. Huntley, J. K. Neitzel, and M. K. Elson, *Biochim. Biophys. Acta*, **490**, 112 (1977).

(32) C. A. Cruze and M. C. Meyer, *J. Pharm. Sci.*, **65**, 33 (1976).