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Effect of Chloride Ions on the Interaction between Salicylic Acid and Human Serum Albumin Studied by Frontal Affinity Chromatography[1)]

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The interaction of salicylic acid with human serum albumin (HSA) was studied at 4 °C by a frontal affinity chromatographic technique employing HSA monomer immobilized on agarose beads in two buffer systems, *i.e.* Tris-HCl and phosphate buffers. A large difference in the apparent binding constants in these buffer systems was almost entirely attributed to the presence of chloride ions in Tris-HCl buffers. The inhibitory effect of chloride ions was directly demonstrated to be due to the binding of chloride ions to HSA, and other components of the buffers had little effect. The data can be interpreted on the basis of competitive binding at sites other than the primary chloride ion binding sites.

Keywords——human serum albumin; immobilized albumin; frontal affinity chromatography; interaction; protein binding; chloride binding; salicylic acid binding; displacement

In *in vitro* drug binding studies, various experimental conditions are known to influence the drug binding parameters. In particular, physiologically important Cl^- ions, which exist in the plasma at a concentration of about 0.1 M, are often added to the binding system as a buffer component (as in Tris-HCl buffers). Sodium chloride is also often added to the medium in order to adjust the ionic strength or osmotic pressure, to suppress the Donnan's effect in equilibrium dialysis studies, or just to simulate the physiological condition of 0.1 M Cl^-. However, there are several reports that Cl^- binds to serum albumins[2)] and also inhibits the binding of many drugs such as tolbutamide,[3)] sulfaethisole,[4)] a benzodiazepin,[5)] warfarin,[6)] *etc.* to serum albumins. Further, the inhibitory effect of Cl^- has been attributed to competitive binding.[3,6b,c,7)] Others,[8)] however, considered Cl^- to alter either the charge on the albumin molecule or its conformation.

In this communication, we report a study of the effect of Cl^- on the binding of salicylic acid to human serum albumin (HSA) monomer immobilized on agarose beads in phosphate and Tris-HCl buffers by frontal affinity chromatographic techniques.[9)] A pronounced reduction in the salicylate binding parameters in Tris-HCl buffers over those in phosphate buffers was almost entirely attributed to the presence of Cl^- in the Tris-HCl buffer, while other components of the buffers had little effect. The inhibitory effect of Cl^- was demonstrated to be due to the binding of Cl^- to HSA. An analysis based on a competitive binding model and a direct demonstration of the release of a bound ligand by another ligand suggest that the secondary Cl^- binding sites are the same as the primary salicylate binding sites on HSA.

Experimental

Materials——Human serum albumin (HSA) monomer obtained as described previously[10] by gel filtration of fraction V, essentially fatty acid-free (Sigma Chemical Co., St. Louis, Mo., U.S.A., lot No. 76C-7480) on a Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) column, was reacted as described previously with activated CH-Sepharose 4B (Pharmacia Fine Chemicals) to immobilize it on the insoluble carrier. Salicylic acid (Wako Pure Chemical Industries, Osaka, Japan) was recrystallized from hot water. Disodium ethylenediaminetetraacetate dihydrate (EDTA) was purchased from Wako Pure Chemical Industries. Radioactive chloride $^{36}Cl^-$ (10.4 μCi/mg Cl^-) was purchased as a sodium chloride solution from The Radiochemical Centre, Amersham, England. Tritium-labeled water supplied by Packard Instrument Co., Inc., Dower Grove, Ill., U.S.A. and a scintillation fluid, Aquasol-2 (New England Nuclear, Boston, Mass., U.S.A.), were employed. All other chemicals used were of reagent grade and water was deionized and double-distilled with the second distillation performed in an all-glass apparatus.

Binding Studies——Determination of the amounts of ligand bound or displaced was carried out at 4 °C as described previously by continuous frontal chromatography[11] and by the affinity chromatographic method reported for analyzing the interactions of two drugs with HSA.[12] Two buffer systems were employed, (a) Tris-HCl buffers of various concentrations and pH values (pH values reported are at 4 °C) in the presence and absence of added NaCl, (b) phosphate buffers of various concentrations and pH values containing 0.01% EDTA in the presence and absence of added NaCl. The concentration of salicylic acid was determined spectrophotometrically at 295 nm on a Shimadzu model UV-300 spectrophotometer or by fluorescence measurement at 410 nm (excited at 310 nm) on a Shimadzu model RF-503 spectrofluorophotometer. Chloride ions were determined either by titration with $AgNO_3$ for higher concentrations or by radioactivity measurements with a Packard model 3385 Tri-Carb liquid scintillation spectrometer. For radioactivity measurements, column eluates were collected in preweighed counting vials in about 0.5 ml fractions by means of an LKB model 2112 Redirack fraction collector. To each of the vials, 10 ml of Aquasol-2 was added and the radioactivity was counted. The vial-to-vial variation in the amount of eluate collected was corrected by converting the count of the vial to the count per gram of eluate collected.

Data Analysis——Binding data were treated on the basis of competition[13] of salicylic acid (SA) and Cl^- for the same n binding sites in the concentration range of free salicylic acid of 1.43—3.52 × 10^{-5} M where the Scatchard plot appeared linear. The number of mol of SA bound per mol of HSA (r_{SA}) is then given by eq. (1)

$$r_{SA}=\frac{nK'_{SA}(D_f)_{SA}}{1+K'_{SA}(D_f)_{SA}} \tag{1}$$

$$K'_{SA}=\frac{K_{SA}}{1+K_{Cl^-}(D_f)_{Cl^-}} \tag{2}$$

where K'_{SA} is the apparent binding constant of SA in the presence of Cl^-, K_{SA} and K_{Cl^-} are the binding constants of SA and Cl^-, respectively, and $(D_f)_{SA}$ and $(D_f)_{Cl^-}$ are the concentrations of free SA and Cl^-, respectively. Rearrangement of eqs. (1) and (2) gives eqs. (3) and (4), respectively.

$$\frac{1}{r_{SA}}=\frac{1}{n}+\left(\frac{1}{nK'_{SA}}\right)\frac{1}{(D_f)_{SA}} \tag{3}$$

$$K_{Cl^-}=\frac{1}{(D_f)_{Cl^-}}\left(\frac{K_{SA}}{K'_{SA}}-1\right) \tag{4}$$

From eqs. (3) and (4), K'_{SA} and K_{Cl^-}, were calculated respectively. When a correction for the electrostatic effects of bound anions on the subsequent binding of the anions is included in this model, according to the method of McMenamy,[14] eq. (2) becomes

$$K'_{SA}=\frac{K^0_{SA}\exp(-2\omega\bar{Z}_pZ_{SA})}{1+a_{Cl^-}K^0_{Cl^-}\exp(-2\omega\bar{Z}_pZ_{Cl^-})} \tag{5}$$

In eq. (5), K^0_{SA} and $K^0_{Cl^-}$ are the intrinsic association constants of SA and Cl^-, respectively, at the reference state of the 1/15 M phosphate buffer; ω is the electrostatic interaction parameter, calculated from the relationship[2d] $\omega=0.115-(1.12\sqrt{\mu}/(1+10.53\sqrt{\mu}))$ where μ is the ionic strength of the medium; $\bar{Z}_p$ is the net charge on HSA including the charges from the groups on HSA and Cl^- binding (the binding constants of Cl^- for HSA at 0 °C employed for the calculation of $\bar{Z}_p$ were $K_1=1132$, $n_1=1$; $K_2=74$, $n_2=4$; and $K_3=10$, $n_3=22$[2d]); Z_{SA} and Z_{Cl^-} are the charges on SA and Cl^-, respectively (these are -1 at the pH of the present study); and a_{Cl^-} is the activity of Cl^-. Rearrangement of eq. (5) gives eq. (6).

$$K^0_{Cl^-} = \frac{1}{a_{Cl^-}\exp(2\omega\bar{Z}_p)}\left[\frac{K^0_{SA}}{K'_{SA}}\exp(2\omega\bar{Z}_p)-1\right] \quad (6)$$

Next, eq. (6) was used to calculate the $K^0_{Cl^-}$ values for the interaction in the 1/15 M phosphate buffer systems.

Results and Discussion

Figure 1 shows the binding of salicylic acid to the immobilized HSA in the two buffers in the form of Scatchard plots. The Tris-HCl buffer has been used in our affinity chromatographic studies, whereas 1/15 M phosphate buffer is often employed for binding studies involving albumin. In the phosphate buffer, salicylic acid eluted from the column was partly decomposed, but this was found to be preventable by the addition of 0.01% EDTA. The fact that the added EDTA does not influence the binding of salicylic acid was confirmed by incorporating EDTA into the Tris-HCl buffer (Fig. 1). McMenamy[15] also reported that the binding of EDTA to albumin was negligible.

Brown and Crooks[3] have shown that the binding of tolbutamide in 1/15 M phosphate buffer is 20 times greater than that in 1/15 M Tris-HCl buffer. The binding of ibuprofen[16] was also found to be greater in phosphate buffers than in Tris-HCl buffers. In both cases the reduced binding in Tris-HCl buffers was attributed to the presence of Cl^-.

The contribution of Cl^- to the marked difference in the binding characteristics of salicylic acid in the two buffers was evaluated by adding NaCl to both buffers.

Figure 2 shows the effect of added NaCl on the salicylate binding in the Tris-HCl buffer.

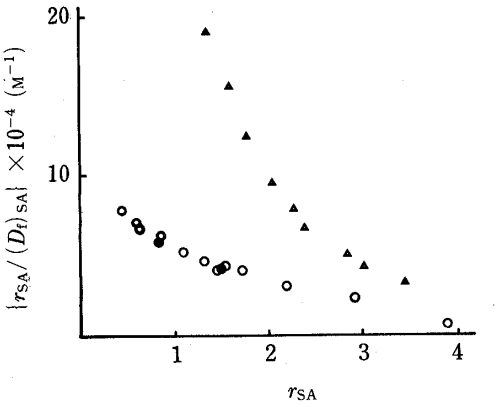

Fig. 1. Scatchard Plots for Salicylate Binding to HSA at 4 °C

▲, 1/15 M phosphate buffer+0.01% EDTA; ○, 0.05 M Tris-HCl buffer+0.1 M NaCl; ●, 0.05 M Tris-HCl buffer+0.1 M NaCl+0.01% EDTA.

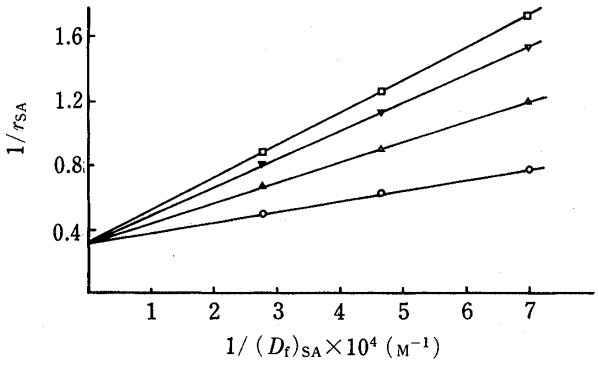

Fig. 2. Double-Reciprocal Plots Showing the Effect of Added NaCl on the Binding of Salicylate to HSA in 0.05 M Tris-HCl Buffer, pH 7.87, at 4 °C

○, in the absence of added NaCl; ▲, 0.1 M NaCl; ▼, 0.2 M NaCl; □, 0.3 M NaCl.

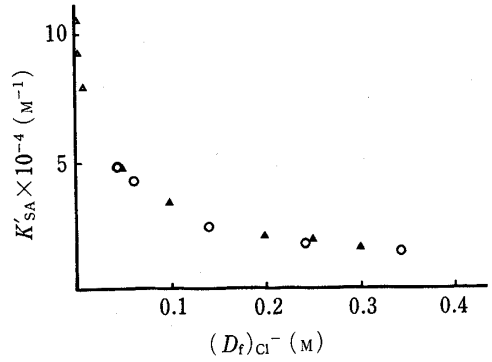

Fig. 3. Effect of Chloride Concentration on the Apparent Binding Constant of the Interaction between Salicylate and HSA at 4 °C

○, in 0.05 M Tris-HCl buffer, pH 7.87; ▲, 1/15 M phosphate buffer, pH 7.45, containing 0.01% EDTA.

TABLE I. Effects of Buffer Concentration and pH on the Binding of Salicylate to HSA[a]

Buffer concn. M	pH	$K'_{SA} \times 10^{-4}$ M^{-1}	n
0.030	7.45	12.1	2.9
0.067	7.45	10.1±0.9	3.0±0.1
0.067	7.98	9.40	2.9
0.100	7.45	9.84	3.0

a) Binding parameters K'_{SA} and n (±S.D.) were determined at 4 °C by continuous frontal analysis affinity chromatography.

TABLE II. Binding Parameters for Chloride Ions Calculated on the Basis of Competitive Binding with Salicylate Ions for the Same Binding Sites on Human Serum Albumin[a]

1/15 M Phosphate buffer, pH 7.45				0.05 M Tris-HCl buffer, pH 7.87			
$(D_f)_{Cl^-}$ M	K'_{SA} $M^{-1} \times 10^{-4}$	n	K_{Cl^-} M^{-1}	$(D_f)_{Cl^-}$ M	K'_{SA} $M^{-1} \times 10^{-4}$	n	K_{Cl^-} M^{-1}
—	10.1±0.9	3.03±0.09	—				
0.001	9.22	3.10	91.6				
0.010	7.52	3.15	34.3				
0.050	4.83	3.17	21.6	0.047	4.76	3.21	23.9
0.100	3.43	3.04	19.4	0.062	4.28	3.09	21.8
0.200	2.12	3.22	18.7	0.142	2.52±0.03	3.16±0.03	21.1±0.3
0.250	1.92	3.16	17.0	0.244	1.85	3.10	18.2
0.300	1.67	3.23	16.8	0.344	1.55	3.16	16.0

a) Determined at 4 °C by continuous frontal affinity chromatography (±S.D.).

Since the straight lines all pass through a common intercept on the y-axis, Cl^- appears to reduce the binding of salicylic acid by competing for the same sites (Scatchard *et al.*[2a] showed that Na^+ ions do not bind to HSA). Similar results were obtained in the phosphate buffer. The apparent binding constants K'_{SA} of salicylic acid in the presence of added salt were calculated from eq. (3) and are plotted against $(D_f)_{Cl^-}$ in Fig. 3. The data in both buffer systems fell on a common curve, indicating that the difference in the binding parameters in the two buffers is essentially due to the presence of Cl^-. We have, nevertheless, investigated the effects of pH and buffer concentration for the two buffer systems. The results in the phosphate buffer system (presented in Table I) show that buffer concentration and pH have little effect on the parameters of salicylic acid and the effect of Cl^- far outweighs these. Although the results are not shown, the same trend was observed in the Tris-HCl buffer system. The pH value was varied only slightly as albumins are known to undergo conformational transition.[7]

Thus, we attempted to explain the variation in K'_{SA} with $(D_f)_{Cl^-}$ on the basis of a competitive inhibition model, and the binding constant of Cl^-, K_{Cl^-}, was calculated for each $(D_f)_{Cl^-}$ using eq. (4). The results are presented in Table II. In the concentration range of 0.05—0.3 M, the K_{Cl^-} values were about 20 M^{-1}, although the values appear to decrease with increasing concentration of Cl^-. Wilting *et al.* reported[6c] a value of 20 M^{-1} at 0.1 M Cl^- as the binding constant of Cl^- for the primary warfarin binding site on the basis of a competitive model.

The decrease in the K_{Cl^-} value with increasing concentration of Cl^- may be due to the

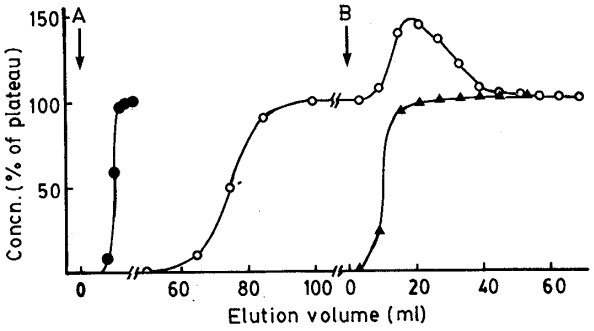

Fig. 4. Displacement of Bound Salicylate by Chloride

●, elution of ^3H_2O; ○, elution of salicylate; ▲, elution of chloride.

The following solutions were applied to the column at 4 °C. At arrow A, either a solution containing ^3H_2O or a solution of 0.01 M Cl^{-1} and 3.57×10^{-5} M salicylate, in 1/15 M phosphate buffer, pH 7.45, containing 0.01% EDTA. At arrow B, after equilibration of the column with the salicylate solution, a similar salicylate solution but containing 0.05 M Cl^{-1}.

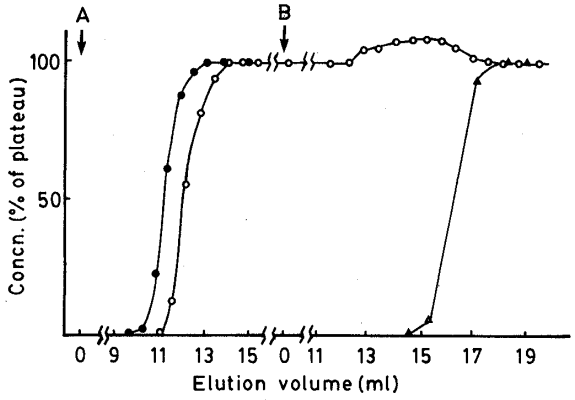

Fig. 5. Displacement of Bound Chloride by Salicylate

●, elution of ^3H_2O; ○, elution of Cl^{-1}; ▲, elution of salicylate.

The following solutions were applied to the column at 4 °C. At arrow A, either a solution containing ^3H_2O or a solution of 2.8×10^{-5} M Cl^{-1}, in 1/15 M phosphate buffer, pH 7.45, containing 0.01% EDTA. At arrow B, after equilibration of the column with the chloride solution, a similar chloride solution but containing 3.2×10^{-3} M salicylate.

electrostatic effect of already-bound Cl^- on the binding of subsequent Cl^-. A correction for this effect was made according to the method employed by McMenamy[14] for the data obtained in the phosphate buffer. Intrinsic binding constants of salicylic acid (K^0_{SA}) and Cl^- ($K^0_{Cl^-}$) at the reference state of the medium were calculated to be 3.2×10^5 and 59 ± 9, respectively, at 4 °C with no apparent tendency of $K^0_{Cl^-}$ to increase with decreasing $(D_f)_{Cl^-}$ at 0.01—0.3 M Cl^-. However, at 0.001 M Cl^-, $K^0_{Cl^-}$ was calculated to be 200. The $K^0_{Cl^-}$ value roughly corresponds to the value of 74 determined by Scatchard and Yap[2d] at 0 °C for the second class ($n=4$) of chloride binding sites on HSA. Therefore, we attempted to observe the mutual displacement phenomena by monitoring both salicylic acid and Cl^-, the latter particularly at low concentration, in order to check whether the primary chloride binding sites are involved or not.

Figure 4 shows a direct demonstration of the release of bound salicylic acid when the concentration of Cl^- was increased from 0.01 to 0.05 M. Figure 5 shows the reverse case, *i.e.* the release of bound Cl^- by salicylic acid. At arrow A, a solution of ^3H_2O or 2.8×10^{-5} M Cl^- in the phosphate buffer was applied to a column of HSA immobilized agarose beads. Since no such delay in the elution of Cl^- over that of ^3H_2O was observed in the blank column (in the absence of immobilized HSA) of similar dimensions, the delay shown in Fig. 5 indicates the binding of Cl^- to the HSA in the column. Part of the bound Cl^- was shown to be released by the application of a solution containing 3.2×10^{-3} M salicylic acid, which is over 100 times the concentration of Cl^-. The dependence of the amount of Cl^- released on the concentration of salicylic acid is summarized in Table III. In spite of the large excess of salicylic acid over Cl^- and the high ratio of the calculated binding constants ($K_{SA}/K_{Cl^-}\doteqdot5000$), the percentage of Cl^- released was not so great. This observation supports the view that the primary Cl^- binding sites are different from the primary binding sites of salicylic acid. Kragh-Hansen[17] has assigned different high-affinity binding sites for Cl^- and salicylate. The observed mutual displacement is not likely to be a result of competitive binding to common primary binding sites for these ligands, but rather is likely to be due to a competition at the secondary Cl^- binding sites. A similar interpretation was suggested for the effect of Cl^- on the binding of warfarin[18] and benoxaprofen[7] to HSA on the basis of circular dichroic and dialysis studies.

TABLE III. Displacement of Bound Cl^- by Salicylate Ions[a)]

Column	$(D_f)_{SA}$ M × 10^4	$(D_b)_{Cl^-}$ mol × 10^8	% of Cl^- released
1	—	1.67	—
1	1.07	1.68	*ca.* 0
1	14.5	1.32	21
2	—	2.91	—
2	32.0	1.93	34

a) In 0.067 M phosphate buffer, pH 7.45, when $(D_f)_{Cl^-} = 2.8 \times 10^{-5}$ M at 4 °C.

Although our present observations suggest that the primary salicylate binding sites and the secondary Cl^- binding sites of HSA are common, and competitive binding takes place at these sites when these ligands are present simultaneously, some other mechanism such as modulatory effects among bound ligands at different regions on HSA,[17)] which may be a result of conformational changes in HSA brought about by binding of ligands, cannot be ruled out at present as the cause of the observed mutual displacement of these ligands.

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