Biochemical Pharmacology, Vol. 26, pp. 283–288. Pergamon Press, 1977. Printed in Great Britain.

CIRCULAR DICHROIC INVESTIGATIONS OF THE BINDING OF SALICYLATE AND RELATED COMPOUNDS TO HUMAN SERUM ALBUMIN

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(*Received* 10 *June* 1976; *accepted* 7 *September* 1976)

Abstract—The binding of salicylate and some related compounds has been investigated by circular dichroism. The binding constants, where possible, were determined by direct titration, otherwise by their ability to displace salicylate. The binding constants for the first two sites for salicylate were found to be 1.05×10^5 l. mole^{-1} and 5.10×10^3 l. mole^{-1}. The induced circular dichroism of salicylate was diminished by addition of acetate ions. Attempts to correlate binding constants with partition coefficients and Hammett σ values suggested hydrophobic and electrostatic forces to be involved in the binding of these benzoic acid derivatives. Aspirin, indomethacin and phenylbutazone appeared to share the primary site with salicylate.

The binding of salicylic acid and its analogues has been quantitatively investigated by several authors [1–5]. Using equilibrium dialysis, Davison and Smith [1] studied the binding of salicylate and related molecules to bovine serum albumin (BSA).

Moriguchi [2] investigated the relationship between structural features and binding constants for some benzoic acid derivatives including salicylate following the interaction with BSA. From a clinical point of view, the possible displacement of a range of drugs by salicylate has been reported [5–10]. Perrin and co-workers have previously reported that circular dichroism (CD) is a useful technique to determine binding parameters as well as to investigate competition at low drug to albumin ratios [11–13] and have shown that salicylic acid gives a small induced circular dichroic signal on binding to HSA [14]. This signal was too small to be quantitatively investigated but the more sensitive circular dichroism equipment now commercially available makes such investigations possible.

Perrin and Nelson [11] have shown, using the CD technique that salicylate displaces sulfaethidole from its primary binding site on BSA to a greater extent than does the more hydrophobic aspirin. This is in agreement with literature values for their binding constants. In the work reported here CD has been used to investigate the binding of salicylate and various analogues to human serum albumin (HSA) in the hope of elucidating the nature of the binding.

MATERIALS AND METHODS

HSA fraction V (lot No A 2386) was obtained from Sigma Chemical Co, St. Louis, Mo. and was used as supplied. Butyl-*p*-hydroxy benzoate was obtained from Eastman Kodak Co, Rochester, N.Y., sodium salicylate and its analogues were obtained from Aldrich, Europe, Beerse Belgium. For the displacement investigations, tolbutamide (Hoechst, Frankfurt, Germany), isopropamide iodide (Smith Kline and French, Philadelphia PA.), indomethacin (Merck Sharp and Dohme, West Point PA.), sodium 2-(*p*-chlor phenoxy) 2-methyl propionate (I.C.I. Ltd., Macclesfield, Cheshire), Phenylbutazone, sulfadiazine, pentobarbital and phenobarbital, all from Nogepha Alkmaar Holland, were used as supplied. All other materials were reagent grade and solutions were prepared in deionised and distilled water.

All solutions were prepared in 0.1 M phosphate buffer of pH 7.4 at 22° HSA concentrations of $1.45–5.80 \times 10^{-5}$ M (mol. wt 69,000) were used. CD measurements were made on a Dichrographe III. (Jobin Yvon. Long Jumeau, France) using 10, 20 and 50-mm cells. All solutions were scanned from wavelengths at which no induced optical activity was observed. The induced ellipticity is defined as the ellipticity of the drug albumin mixture minus the ellipticity of the albumin alone at the same wavelength and is expressed in degrees. For the displacement experiments, fixed concentrations of salicylate and HSA were used, and the concentration of competing drug was varied. Only drugs, having little or no measurable induced CD at the relevant wavelengths could be used as competitors. None of the compounds modified the CD spectrum of albumin at wavelengths between 200 and 250 nm.

RESULTS AND DISCUSSION

The induced CD spectra of the binding of the hydroxy benzoates and aspirin to HSA are shown in Fig. 1. The *o*-hydroxy benzoate (salicylate) gives the largest induced CD signal. The more sensitive Dichrograph III detects a small induced CD signal following the binding of aspirin to HSA, this had not been observed in previous investigations. Table 1 summarizes the induced ellipticities for the salicylate analogues investigated; the absence of data indicates that no measurable peaks were obtained above 270 nm. In all cases the induced ellipticities observed were positive in sign. The hydroxy group, whether ortho, meta, or para seems important for the generation of induced optical activity because the benzoic acid derivatives possessing amino, methyl, methoxy, and chloro groups showed significantly lower induced

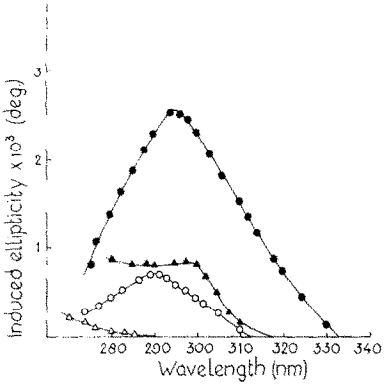

Fig. 1. Induced optical activity of salicylate analogues binding to 1.45×10^{-5} M HSA. ●——● *o*-hydroxy benzoic acid (salicylate); ○——○ *m*-hydroxy benzoic acid; △——△ *p*-hydroxy benzoic acid; ▲——▲ acethyl salicylate (aspirin).

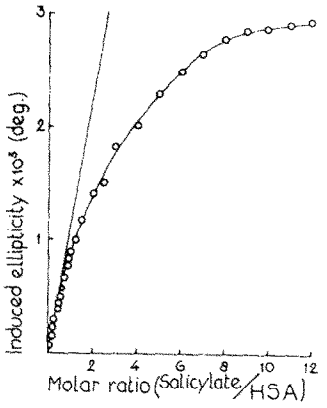

Fig. 2. Estimation of free and bound drug by the method of Rosen [16]. Measurements were made in 10-mm cells and with a constant HSA concentration of 2.90×10^{-5} M. Measurements were made at 296 nm.

optical activities. It has been previously reported that the phenolic group of the salicylate is important in the binding process [14], and all of the above observations support this hypothesis. The lack of an induced CD signal in some cases may be due to the lack of rigidity of the complex [15].

Figure 2 shows the induced ellipticity at 296 nm for various ratios of salicylate to HSA. All points are the average of ten determinations. It is assumed that the subtraction of the CD curves obtained by subtraction of the spectrum of HSA alone from that of the drug–HSA mixtures correspond to the bound drug and contain no small contribution from the disturbance of the aromatic residues or from the N → B transition of the protein [16, 17]. When the shape of this curve is compared to that previously obtained for sulfaethidole–BSA complexes [18], when only one binding site was capable of inducing optical activity, then it is obvious that more than one site is contributing to the induced optical activity. To interpret this curve in terms of amount bound and free drug concentration, it is assumed that the induced ellipticity is due entirely to the bound drug and making CD measurements at very low drug to HSA ratios enables the appropriate intensive factor to be calculated for the first binding site. This is usually done by drawing

Table 1. Induced CD characteristics of benzoate analogues bound to 1.45×10^{-5} M HSA

Compound	Concentration of drug (M × 10^4)	Peak characteristics	
		Wavelength (nm)	Observed ellipticity × 10^3 (degrees)
(1) *o*-Hydroxybenzoate (Salicylate)	2.0	295	2.55
(2) *m*-Hydroxybenzoate	2.0	289	0.70
(3) *p*-Hydroxybenzoate	2.0	—	—
(4) *o*-Methylbenzoate	4.0	—	—
(5) *m*-Methylbenzoate	4.0	—	—
(6) *p*-Methylbenzoate	4.0	—	—
(7) *o*-Methoxybenzoate	4.0	—	—
(8) *m*-Methoxybenzoate	4.0	286	139
(9) *p*-Methoxybenzoate	4.0	278 (shoulder)	0.50
(10) *o*-Aminobenzoate	2.0	312	0.53
(11) *m*-Aminobenzoate	4.0	—	—
(12) *p*-Aminobenzoate	0.8	—	—
(13) *o*-Chlorobenzoate	4.0	—	—
(14) *m*-Chlorobenzoate	4.0	285	0.64
(15) *p*-Chlorobenzoate	4.0	—	—
(16) 2,3-Dihydroxybenzoate	2.5	306	1.65
(17) 2,4-Dihydroxybenzoate	1.3	—	—
(18) 2,5-Dihydroxybenzoate	2.5	320	1.20
(19) 2,6-Dihydroxybenzoate	1.4	306	3.47
(20) 3,5-Dihydroxybenzoate	3.0	292	0.65
(21) 3-Hydroxy-3-methylbenzoate	1.2	298	4.16
(22) Aspirin	10.0	297	0.83
(23) Methyl paraben	1.8	298 (shoulder)	1.02
(24) Ethyl paraben	1.8	298 (shoulder)	1.09
(25) Propyl paraben	1.8	298 (shoulder)	1.16
(26) Butyl paraben	1.8	298 (shoulder)	1.68

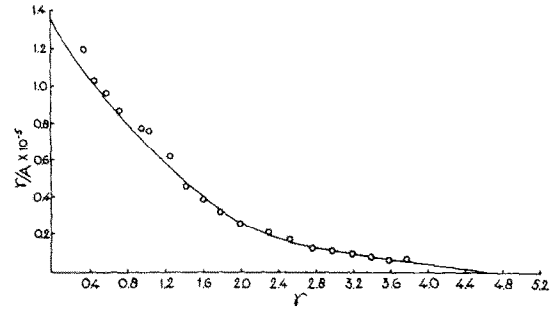

Fig. 3. Scatchard plot of salicylate–HSA binding.

the tangent to the curve of Fig. 2 at zero drug concentration [19]. This method of deriving concentrations of free and bound drug, only allows precise interpretation in terms of binding constants when a single site is involved. When more than one site contributes to the ellipticity, the method is less precise. The observed induced ellipticity is a function of the amounts bound at the individual sites and the associated molar ellipticities. If the binding constants of the primary and secondary sites are very different from one another and the ellipticities of the secondary sites are small then reasonable estimates of the primary binding constant can be obtained, the values for the secondary sites being less reliable. It is the first site that is of major clinical importance and is the one of interest in the current work. These problems are not peculiar to the CD technique, they are shared by other spectroscopic techniques where the intensive factor for the second and higher binding sites may be impossible to obtain. Figure 3 shows the Scatchard plot for salicylate, using data derived in the manner described above, and Table 2 gives the binding parameters of the analogues which were determined by direct titration. It is the primary binding constant that is of interest, and the value for salicylate is in agreement with those reported by Hultmark *et al.* (1.20×10^5 l. mole^{-1}) [3], Karesztes-Nagy *et al.* (0.7×10^5) [4] and Meyer *et al.* (2.0×10^5) [5], but higher than that reported by Davison and Smith (3.0×10^4) [1]. Differences can be due to buffer composition, pH, albumin source, temperature effects as well as techniques.

The binding constants for the methyl, ethyl and propyl parabens are of the same order as those reported by Jun *et al.* [20] for the binding to BSA, but significantly higher than those for binding to BSA reported by Patel *et al.* [21]. These low values seem to be due to the lack of data at low drug to albumin ratios. The binding constants of Jun *et al.* are relatively independent of the side chain, contrary to the present work which shows a significant increase in binding constant on going from propyl to butyl.

Table 3. Log primary binding constants for benzoic acid derivatives

Compound	This study	Moriguchi*
Benzoic acid		4.7
o-Hydroxybenzoic acid (salicylic acid)	5.1	5.0
m-Hydroxybenzoic acid	4.3	4.7
p-Hydroxybenzoic acid	3.9	3.9
o-methylbenzoic acid	4.1	4.4
m-Methylbenzoic acid	4.4	5.4
p-Methylbenzoic acid	4.3	4.9
o-Methoxybenzoic acid	4.1	
m-Methoxybenzoic acid	4.3	
p-Methoxybenzoic acid	4.2	4.8
o-Aminobenzoic acid		3.8
m-Aminobenzoic acid		3.5
p-Aminobenzoic acid		2.9
o-Chlorobenzoic acid	3.9	
m-Chlorobenzoic acid	4.4	
p-Chlorobenzoic acid	4.3	
Methylsalicylate	3.5	
Salicylamide	3.1	

* Using u.v. technique, BSA 0.15 M Tris buffer pH 7.4 37°.

It is not unexpected to find a significant role for the side chain in the binding process and the situation can be compared to the observations of Yalkowsky *et al.* [22] who found that the crystal structure of the lower alkyl *p*-aminobenzoate esters is probably determined by the aromatic ring, but that the aliphatic chains begin to exert a dominant effect at four carbon atoms. It was not possible to investigate higher homologues in the present study because of their low solubility.

The binding constants of the compounds in Table 3 could not be determined directly because of their small induced ellipticities, but were determined, as described previously, from their abilities to displace salicylic acid from HSA [11]. The values obtained are in good agreement with those of Moriguchi [2]. It should be noted that the ortho isomers of methyl, methoxy and chloro benzoic acids have lower binding constants than the meta- and para-isomers, contrary to the situation with hydroxy substituents. From Tables 2 and 3 it is apparent that acids with hydroxy groups ortho to the carboxylate group have significantly higher binding constants than the other molecules. Two explanations are that intramolecular hy-

Table 2. Binding parameters for salicylate analogues

Compound*	n_1	$K_1 \times 10^{-5}$ M^{-1}	n_2	$K_2 \times 10^{-3}$ M^{-1}
Salicylate	1.38	1.15	3.73	5.10
2,6-Dihydroxybenzoate	1.10	4.09	3.10	9.01
3-Methylsalicylate	1.01	8.30	2.39	9.92
Methylparaben	1.68	0.66	7.20	2.34
Ethylparaben	1.71	0.69	7.43	2.24
Propylparaben	1.60	0.70	8.19	2.03
Butylparaben	1.19	1.18	6.60	4.01

* To HSA at pH 7.4 in 0.1 M phosphate buffer.

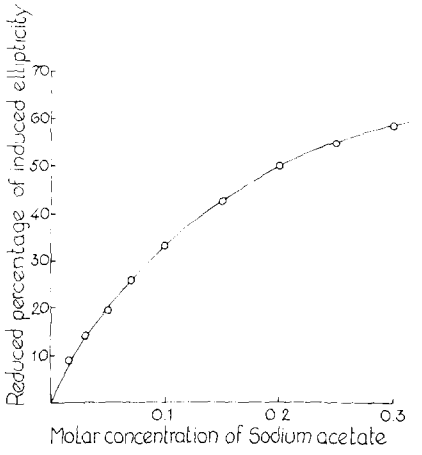

Fig. 4. Effect of acetate on the induced ellipticities of salicylate binding to HSA. Concentration: HSA 5.8×10^{-5} M salicylate 6×10^{-5} M. Measurements were made in 50 mm cells.

drogen bonding facilitates the binding to HSA, or that the binding site on albumin has closely spaced positive and negative centres. In either case a strong involvement of electrostatic forces is suggested for the binding process. The importance of the negative carboxyl group on the binding is emphasised by the lower binding constants found (Table 3) for salicylamide and methyl salicylate. Zaroslinski *et al.* [23] have recently reported a negative enthalpy and negative entropy for the primary binding of salicylate to HSA. In Fig. 4 the effect of large concentrations of sodium acetate on the induced ellipticity of salicylate–HSA complexes is shown. The acetate caused a small change in the pH of the solution, however in this pH region, pH has no effect on the ellipticity of the salicylate–HSA complexes [14]. If the effect of acetate is interpreted as a displacement rather than a conformational change then a binding constant of about 200 can be estimated. This value is of the same order of magnitude as has been reported for acetate–BSA complexes [24]. Davison and Smith [1] have also reported the displacement of salicylate by acetate from HSA. It is interesting to note the greatly increased binding of the more hydrophobic 3-methyl-2-hydroxy benzoate (Table 2).

Figure 5 shows the correlation of the binding constants of meta and para substituted benzoic acids with the Hammett sigma constants. The correlation coefficient of 0.670 (eight samples) is significant at a 90

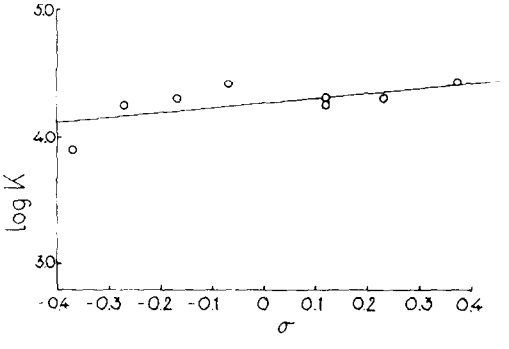

Fig. 5. Correlation between log K and Hammett sigma values for meta- and para-substituted benzoic acids.

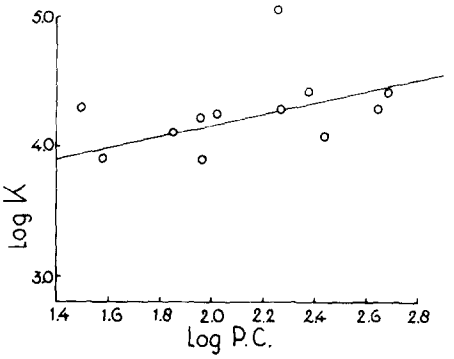

Fig. 6. Correlation between log K and log $P.C$ for benzoic acids. Salicylate has the highest binding constant.

per cent level, and suggests a significant influence of electrostatic forces on the binding process. Partition coefficients, as a measure of hydrophobicity, correlate with the binding constants to a significant level (95 per cent) with a correlation coefficient of 0.702 (ten samples) as shown in Fig. 6. However, when an attempt was made to combine these terms in a single equation no significant correlation could be obtained at 95 per cent confidence level as shown below.

$$\log K = 0.340 \log PC - 0.353\,\sigma + 3.52, \quad n = 11, r = 0.415, s = 0.343. \tag{1}$$

The narrow range of log K values made such correlations difficult. The abnormally high binding constant of salicylate is again emphasised by Fig. 6. The low values of the slopes in Figs. 5 and 6 show a very small influence of substituent on the binding process, the acids are fully ionised under these experimental conditions and except in the case of salicylate the binding does not deviate markedly from that of the benzoate ion.

Table 4 shows the binding constants of some drugs, whose complexes with HSA have little or no induced optical activity, obtained by the displacement method [11]. These binding constants are in reasonable agreement with the primary binding constants reported in the literature. Sulfadiazine has been shown to have a binding constant of less than 10^3 [25] and so is unlikely to displace salicylic acid under the experimental conditions of Table 4.

The values for pentobarbital and phenobarbital are in good agreement with those obtained by Bränstad *et al.* [26], as also are the values for tolbutamide [27, 28] and clofibric acid [29], indicating that a wide range of acidic drugs do share a primary binding on HSA. The low value for aspirin again stresses the importance of the hydroxy group in the binding of salicylate to HSA.

This value is of the same magnitude as that previously reported [11] for the binding to BSA, however it is much lower than that reported by Davison and Smith [1] for BSA. With aspirin, there is the danger of hydrolysis during a long dialysis experiment as well as possible acetylation of the HSA [30].

Indomethacin and phenylbutazone are frequently prescribed with salicylate or aspirin in the treatment of rheumatoid arthritis. Muiden *et al.* [10] have recently reported that indomethacin and phenylbutazone cause appreciable increases in unbound salicylate at therapeutic levels. Induced CD curves for com-

Table 4. Binding constants competing drugs found by displacement

Drugs	$K_{app}(M^{-1})$*	$K_{lit}(M^{-1})$	Method	Reference
Sulfadiazine	No significant competition	0.8×10^3	Fluorescene (HSA)	23
Phenobarbital	1.00×10^3	2.5×10^3	Dialysis (HSA)	24
Pentobarbital	2.34×10^3	6.2×10^3	Dialysis (HSA)	24
Isoproamide iodide	No significant competition			
Tolbutamide	3.98×10^4	4.05×10^4	Dialysis (HSA)	25
		9.04×10^4	Fluorescene (BSA)	26
Clofibric acid	3.70×10^4	2.47×10^4	Spectrophotometry (HSA)	27
Aspirin	2.77×10^3	3.5×10^4	Dialysis (BSA)	1
		4.52×10^3	CD (BSA)	11

* HSA (5.80×10^{-5} M) and salicylate (6×10^{-5} M) in 5-cm cells.

plexes of these drugs are larger than those of salicylate–HSA complexes and so quantitative investigations of their competition for binding sites cannot be attempted under the conditions of Table 4, but it is of interest to determine whether or not they do share a primary binding site on HSA in a qualitative manner. Induced CD curves for low concentrations of a mixture of indomethacin and salicylate were compared to the addition of the induced curves of both drugs when alone with HSA. The curves did not match, indicating a mutual displacement. Similar results were obtained with phenylbutazone and salicylate mixtures. Indomethacin–HSA complexes give measurable CD curves (Fig. 7) at higher wavelengths than do salicylate–HSA complexes, so allowing a binding constant for salicylate to be determined from its ability to displace indomethacin. The literature value for the primary binding constants of indomethacin is 3×10^5 l. mole^{-1} [6], allowing a value of 5.5×10^4 l. mole^{-1} for salicylate to be estimated (Fig. 7). This value is lower than that found by direct titration and may indicate that the literature value for indomethacin is too low, a frequent problem when insufficient data is collected at low drug to protein ratios. Unfortunately the induced CD spectra of phenylbutazone and salicylate complexes with HSA overlap and so no similar quantitation can be attempted with mixtures of these drugs.

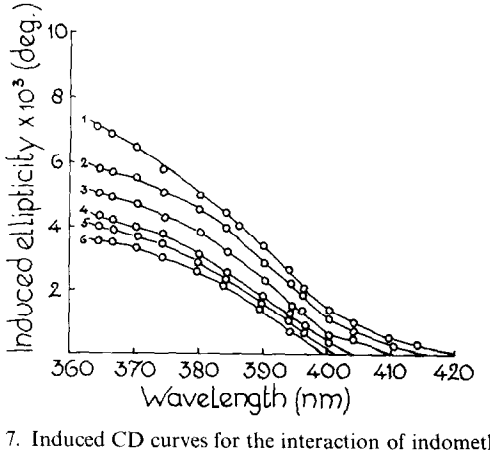

Fig. 7. Induced CD curves for the interaction of indomethacin and salicylate with HSA. Measurements were made in 50-mm cells with a HSA concentration of 5.8×10^{-5} M and indomethacin concentration of 6.0×10^{-5} M salicylate concentration (curve 1) 0; (curve 2) 6×10^{-5} M (curve 3) 12×10^{-5} M (curve 4) 18×10^{-5} M (curve 5) 24×10^{-5} M (curve 6).

This limited *in vitro* displacement data does stress the precautions necessary when prescribing salicylate or the easily hydrolysed aspirin with a range of strongly bound acidic drugs.

Acknowledgements—The authors would like to thank Dr. Eric Lien of the University of Southern California for helpful discussions over the structure activity correlations. Some of the preliminary experiments of this work were done by Dr. J. J. Vallner and J.H.P. whilst at the University of Wisconsin.

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