(1959).
(11) S. Safe, O. Hutzinger, and D. Ecobichon, *Experientia*, **30**, 720 (1974).
(12) S. Safe, O. Hutzinger, and D. Jones, *J. Agric. Food Chem.*, **23**, 851 (1975).
(13) O. Hutzinger, D. M. Nash, S. Safe, A. S. W. DeFreitas, R. J. Norstrom, D. J. Wildish, and V. Zitko, *Science*, **178**, 312 (1972).
(14) S. Safe, N. Platonow, and O. Hutzinger, *J. Agric. Food Chem.*, **23**, 259 (1975).
(15) P. J. Creaven, D. V. Parke, and R. T. Williams, *Biochem. J.*, **96**, 879 (1965).
(16) R. Ammon, H. Berninger, and I. Berninger, *Qual. Plant. Mater. Veg.*, **16**, 263 (1968).
(17) H. Berninger, R. Ammon, and I. Berninger, *Arzneim.-Forsch.*, **18**, 880 (1968).
(18) P. Raig and R. Ammon, *ibid.*, **20**, 1266 (1970).
(19) D. M. Jerina, *Lloydia*, **37**, 212 (1974).
(20) H. D. West, J. R. Lawson, I. H. Miller, and G. R. Mathura, *Arch. Biochem. Biophys.*, **60**, 14 (1956).
(21) G. C. Mills and J. L. Wood, *J. Biol. Chem.*, **219**, 1 (1956).
(22) M. M. Barnes, S. P. James, and P. B. Wood, *Biochem. J.*, **71**, 680 (1959).
(23) S. Safe, D. Jones, and O. Hutzinger, *J. Chem. Soc., Perkin Trans. I*, **4**, 357 (1976).

Optical Studies on Interaction of Biliary Contrast Agents with Native and Modified Human Serum Albumin

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Abstract □ The interaction of two homologous series of biliary contrast agents with native human and bovine serum albumin and with modified human serum albumin was investigated using circular dichroism and equilibrium dialysis. For most derivatives, extrinsic Cotton effects were observed for the interaction with both albumins. In some cases, these effects were strongly affected by only small changes in the chemical structure of the drugs. These large differences in extrinsic Cotton effects can be explained by definite effects of the chemical structures on the binding site selectivity of some drugs. For example, iopodate preferentially binds to the warfarin binding site of human serum albumin, while an ethyl group into the propionic acid side chain reduces the affinity for the warfarin site but strongly increases the affinity for the diazepam binding site of human serum albumin.

Keyphrases □ Biliary contrast agents—interaction with native and modified human serum albumin, optical studies on warfarin and diazepam binding sites of albumin □ Albumin, human serum—interaction of biliary contrast agents with native and modified albumin, optical studies of warfarin and diazepam binding sites □ Binding sites—warfarin and diazepam binding sites on human serum albumin molecule, interaction of biliary contrast agents with albumin □ Structure–activity relationships—interaction of biliary contrast agents with native and modified human serum albumin, optical studies of warfarin and diazepam binding sites

Contrast agents for the intravenous or oral visualization of the biliary tract are, in general, highly bound to albumin in the blood of humans and laboratory animals (1). Because of their strong binding to albumin, their low lipophilicity, and their low volumes of distribution, plasma protein binding of biliary contrast agents seems to be an important determinant of the pharmacokinetics of these drugs (2, 3). Furthermore, since the toxicity of the biliary contrast agents increases with increasing plasma protein binding, binding mechanisms leading to high albumin binding and high toxicity may have similarities (1, 4).

Therefore, detailed knowledge of the degree of albumin binding of the biliary contrast agents and of the binding mechanisms is of great interest. Thus, it must be determined which moieties of the contrast medium molecules are important for high binding and to which sites of the human serum albumin molecule the substances are bound. While some quantitative data on affinity constants and numbers of binding sites for two groups of homologous biliary contrast agents were published previously (5), this study describes qualitatively their interaction with human serum albumin, using optical methods and modified albumin derivatives.

EXPERIMENTAL

Materials[1]—The human serum albumin[2] had an electrophoretic purity of 100%. All other chemicals were obtained from commercial suppliers.

Preparation of Albumin Derivatives—The modification of human serum albumin with 2-hydroxy-5-nitrobenzyl bromide (XII·Br) was reported in detail elsewhere (6). Human serum albumin was dissolved in 10 *M* urea adjusted to pH 4.4 by acetic acid, and a 1100-fold molar excess of XII·Br was added (XII–albumin). Another albumin sample was treated similarly but without the reagent and was called urea–albumin (control). Albumin modified with *o*-nitrophenylsulfenyl chloride (XIII·Cl) was prepared with a 22-fold molar excess of the reagent in 20% acetic acid (7) to yield XIII–albumin. With both reagents, the lone tryptophan residue of human serum albumin was modified completely (7).

For the preparation of tetranitromethane- (XIV) modified human serum albumin derivatives (XIV–albumin), a 4-, 15-, or 64-fold molar excess of tetranitromethane was added to human serum albumin dissolved in 0.05 *M* tromethamine buffer and adjusted to pH 8.0, resulting in the modification of 2.2, 5.1, or 7.8 tyrosine residues per albumin molecule, respectively. The modification was described in detail elsewhere (7).

The degree of tryptophan and tyrosine residue modification of the albumin derivatives was determined spectrophotometrically and by amino acid analysis (6, 7).

Circular Dichroism Measurements—Circular dichroism measurements were performed with a spectropolarimeter[3] calibrated with *d*-camphorsulfonic acid. All solutions were prepared in 67 m*M* phosphate buffer (pH 7.4). Results are expressed as molar ellipticities $[\theta]$ (degree square centimeter per decimole), calculated with reference to the albumin concentration, using a molecular weight of 69,000. The optical path length was 10 mm, and the albumin concentration was 30 or 13.1 μM. Difference spectra were obtained by subtracting the effect of the corresponding albumin derivative as the blank.

Equilibrium Dialysis Experiments—The binding of the ^{125}I-labeled

[1] Biliary contrast agents were the gifts of Dr. U. Speck (Schering AG, Berlin), except iopanoic and iophenoxic acids, which were gifts of Dr. G. H. Mudge (Dartmouth Medical School).
[2] Behringwerke, Marburg, West Germany.
[3] Cary 61.

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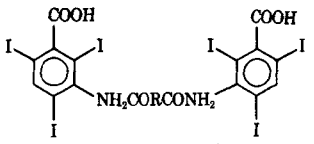

Table I—Structural Requirements for the Generation of Extrinsic Cotton Effects: Intravenous Biliary Contrast Agents [a]

Compound	R	Human Serum Albumin λ_{max}, nm	Human Serum Albumin $[\theta] \times 10^{-4}$ [b]	Bovine Serum Albumin λ_{max}, nm	Bovine Serum Albumin $[\theta] \times 10^{-4}$ [b]
I (iodipamide)	$(CH_2)_4$	310	+0.44 ± 0.02	—	—
		285	−0.70 ± 0.03		
		270	−0.97 ± 0.02		
II (ioglycamide)	CH_2OCH_2	270	−1.32 ± 0.06	—	—
III	$(CH_2OCH_2)_2$	295	−0.20 ± 0.03	260	+1.45 ± 0.08
IV (iotroxamide)	$(CH_2OCH_2)_3$	280	+0.37 ± 0.06	315	−0.45 ± 0.05
				280	+1.12 ± 0.07
V (iodoxamide)	$CH_2—(CH_2OCH_2)_4—CH_2$	280	−0.49 ± 0.05	290	−0.38 ± 0.08
				260	+2.48 ± 0.12

[a] Extrinsic Cotton effects are given as molar ellipticities ($[\theta]$), calculated with reference to the concentration of drug bound at the wavelength of the induced circular dichroism maximum (λ_{max}). All data are difference values, using the Cotton effects of human or bovine serum albumin at the same wavelength as the blank. Albumin concentrations of 30 μM and drug concentrations of 60 μM were used. [b] $\bar{x} \pm s_{\bar{x}}$ = mean ± *SEM*; $n = 6$.

biliary contrast agents, [^{14}C]warfarin, and [^{14}C]diazepam to the albumin derivatives was determined by equilibrium dialysis using an albumin concentration of 36 μM and varying concentrations of the contrast agents. All solutions were prepared with 67 mM phosphate buffer (pH 7.4). The albumin solution (0.9 ml) was dialyzed for 16 hr at 25° in the dark against the buffer (0.9 ml) containing the labeled drugs. Dialysis cells (1 ml) and cellophane dialysis membranes[4] were used. The radioactivity on both sides of the membrane was determined by liquid scintillation spectrometry.

RESULTS

Interaction with Native Human and Bovine Serum Albumin—The binding of most of the intravenous and oral biliary contrast agents to native human and bovine serum albumin significantly changed the circular dichroism spectra of the proteins between 250 and 350 nm (Fig. 1). Maxima and shoulders of the induced circular dichroism spectra correlated sufficiently with maxima and shoulders of the UV absorbance spectra of the ligands. Thus, the induced Cotton effects undoubtedly were extrinsic in origin and originated by binding-induced optical perturbations of electronic transitions of the contrast agent molecules.

The extrinsic Cotton effects of iodipamide (an example of the intravenous contrast agents) consisted of at least three Gaussian components (8), with a small positive band at ~310 nm, a larger negative one at 270 nm, and a shoulder at ~285 nm (Fig. 1). Increasing the length of the aliphatic bridge between both triiodobenzoic acid nuclei of the intravenous contrast agents significantly altered the induced Cotton effects (Table I). The weak band at 310 nm disappeared completely, while the strong band at ~270 nm was present in all intravenous contrast agents investigated, although great differences in the intensity and sign of this band were observed (Table I).

The intensity of the extrinsic Cotton effects depended on the concentration of the ligands, as shown for I (iodipamide) in Fig. 2, where the three extrinsic circular dichroism bands of I increased differently with increasing concentrations. The positive band at 310 nm seemed to be associated mainly with binding to the single high-affinity binding site (5) since this band increased only to about one molecule bound per molecule of human serum albumin (Fig. 2). The band at 270 nm also was generated at secondary binding sites. The induced Cotton effects at 285 nm seemed to be higher at the secondary sites than at the high-affinity binding site, as might be concluded from the shape of the curve in Fig. 2.

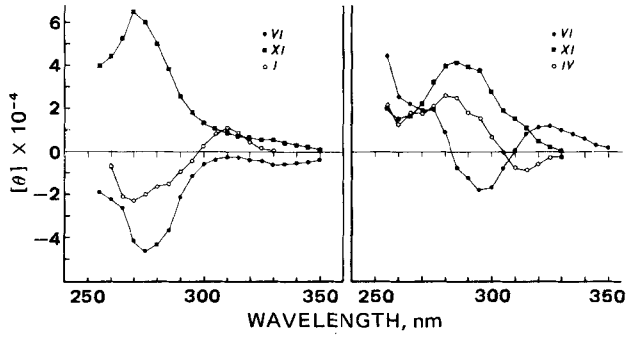

Figure 1—*Extrinsic Cotton effects of some biliary contrast agents (60 μM) in the presence of human (left) or bovine (right) serum albumin. The albumin concentration was 30 μM. The data are difference values, using the Cotton effects of the albumins at each wavelength as a blank. Each point represents the mean of three determinations.*

While I and II are bound to human serum albumin more strongly than III–V, I and II are bound less strongly to bovine serum albumin than the other three derivatives (5). Accordingly, only for the interaction of III–V with bovine serum albumin were induced Cotton effects found (Fig. 1 and Table I). The positions of the circular dichroism band were in agreement with those found in the interaction with human serum albumin (Table I).

As with the intravenous agents, the binding of the oral biliary contrast agents to human serum albumin induced extrinsic Cotton effects with a strong band at ~270 nm and a weak band above 300 nm (Fig. 1 and Table II). The band intensity at 270 nm differed little for the six compounds, but the sign of this band was highly dependent on the substitu-

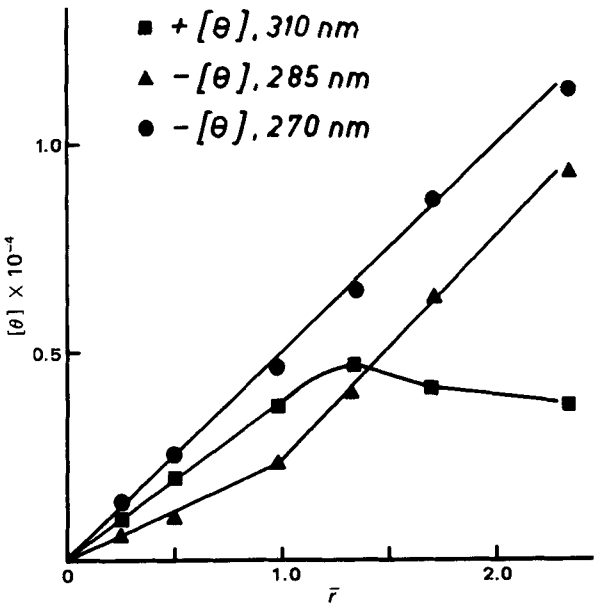

Figure 2—*Correlation between the concentration of I bound to human serum albumin (30 μM) and the intensity of the induced Cotton effects at three different maxima (given as molar ellipticity, [θ], calculated with reference to the albumin concentration). The concentration bound is given as r̄ (moles of I bound per mole of albumin). Each point represents the mean of six determinations.*

[4] Union Carbide.

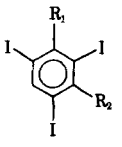

Table II—Structural Requirements for the Generation of Extrinsic Cotton Effects: Oral Biliary Contrast Agents [a]

Compound	R_1	R_2	Human Serum Albumin λ_{max}, nm	Human Serum Albumin $[\theta] \times 10^{-4}$ [b]	Bovine Serum Albumin λ_{max}, nm	Bovine Serum Albumin $[\theta] \times 10^{-4}$ [b]
VI	$CH_2CH(C_2H_5)COOH$	$N{=}CH{-}N(CH_3)_2$	330 275	−0.13 ± 0.01 −1.85 ± 0.03	325 295 270	+0.39 ± 0.01 −0.42 ± 0.06 +0.82 ± 0.08
VII	CH_2CH_2COOH	$N{=}C(CH_3){-}NHCH_3$	330 270	+0.30 ± 0.02 +1.52 ± 0.12	305	−0.07 ± 0.01
VIII	CH_2CH_2COOH	$N{=}C(CH_3){-}NH_2$	330 295 270*	+0.28 ± 0.01 −0.26 ± 0.02 +1.76 ± 0.16	300 260*	+0.33 ± 0.01 +1.19 ± 0.04
IX	CH_2CH_2COOH	$N{=}C(C_2H_5){-}NH_2$	295 270*	−0.60 ± 0.07 +1.36 ± 0.15	265*	+1.93 ± 0.04
X	$CH_2CH(C_2H_5)COOH$	$N{=}C(CH_3){-}NHC_2H_5$	270	+1.07 ± 0.04	330 300 270	+0.35 ± 0.01 −0.74 ± 0.00 +1.48 ± 0.00
XI (iopodate)	CH_2CH_2COOH	$N{=}CH{-}N(CH_3)_2$	270	+2.43 ± 0.04	280	+1.35 ± 0.03

[a] Extrinsic Cotton effects are given as molar ellipticities ($[\theta]$), calculated with reference to the concentration of drug bound at the wavelength of the induced circular dichroism maximum (λ_{max}) or shoulder (*). All data are difference values, using the Cotton effects of human or bovine serum albumin at the same wavelength as the blank. Albumin concentrations of 30 μM and drug concentrations of 60 μM were used. [b] $\bar{x} \pm s_{\bar{x}}$; $n = 6$.

ents of the triiodobenzene moiety (Table II). While this band was positive for VII and XI, the introduction of an ethyl group in the propionic acid side chain of XI made this band negative (VI in Table II). This dramatic effect was not found for the binding to bovine serum albumin since the band at 270 nm, if present, was positive for all compounds.

As found for the intravenous contrast agent I, the intensity of the extrinsic Cotton effects of the oral contrast agents depended on the ligand concentration. The induced optical activity of XI (iopodate) bound to human serum albumin increased only until the first molecule of XI was bound per albumin molecule but decreased at higher concentrations (Fig. 3). Again, the introduction of an ethyl group in XI was followed by a binding change to optically active binding sites, as shown by the increase of both bands of VI with increasing numbers of molecules bound per molecule of human serum albumin (Fig. 3).

Optical Studies with Tryptophan- and Tyrosine-Modified Human Serum Albumin—Previous studies labeled the specific warfarin binding site of human serum albumin by modification of the lone tryptophan residue with XII-Br or XIII-Cl (8). The highly reactive tyrosine residue is part of the indole and benzodiazepine binding site, which can be modified selectively with XIV (7). Accordingly, XII–albumin and XIV–albumin were used to investigate the significance of the warfarin and the diazepam binding sites for the interaction of the biliary contrast agents with human serum albumin. Since the tryptophan modification by XII-Br includes an unfolding of the protein in acidic urea solution, urea–albumin was used to measure the effect of the acidic unfolding on the binding of the compounds to human serum albumin.

The unfolding procedure only slightly changed the extrinsic Cotton effects of I, VI, and XI and those of iophenoxic acid and iopanoic acid, two oral biliary contrast agents that are structurally related to VI and XI (Table III). However, the tryptophan modification reduced the extrinsic Cotton effects of I and iophenoxic acid at both concentrations investigated by ~40–50%. The most striking observation was made for VI where the large negative band at 270 nm disappeared after the tryptophan modification and a small positive band appeared at ~260 nm (Fig. 4 and Table III). On the other hand, the extrinsic Cotton effects of XI and of iopanoic acid were not reduced by the tryptophan modification.

The modification of about two out of the 18 tyrosine residues of human serum albumin (XIV–albumin) had no influence on the extrinsic Cotton effects of I at 310 nm, while those at 270 nm were reduced to ~50% (Table IV). Furthermore, the extrinsic Cotton effects of iophenoxic acid were influenced only slightly by the modification of two tyrosine residues, but

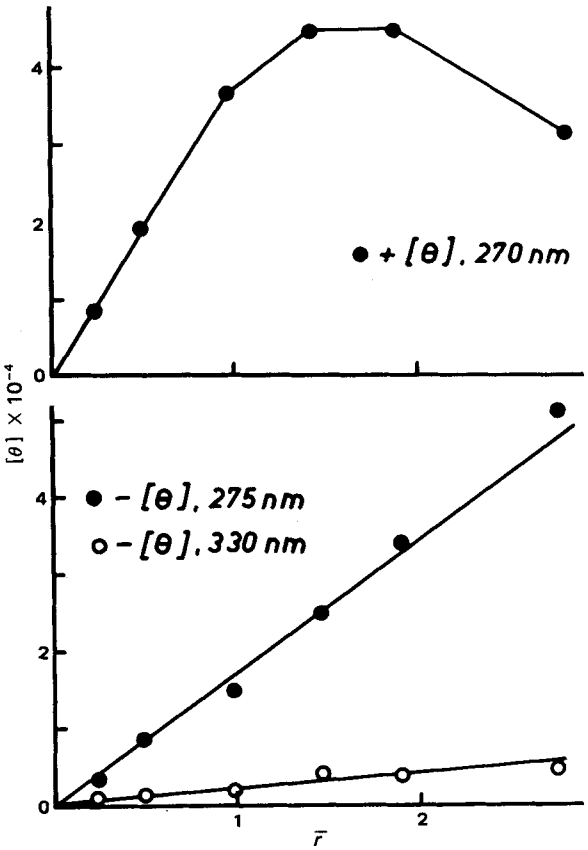

Figure 3—*Correlation between the concentration of XI (upper) and VI (lower) bound to human serum albumin (30 μM) and the intensity of the induced Cotton effects at different maxima (given as molar ellipticity, [θ], calculated with reference to the albumin concentration). The concentration bound is given as r̄ (moles of drug bound per mole of albumin). Each point represents the mean of six determinations.*

Table III—Tryptophan and Tyrosine Modification of Human Serum Albumin: Influence of the Extrinsic Cotton Effects [a]

Drug	λ, nm	Drug/Protein, [M/M]	$[\theta] \times 10^{-3}, \bar{x} \pm s_{\bar{x}}$ Control (n = 4)	XIV-Albumin (n = 4) (%)	Urea-Albumin (n = 3)	XII-Albumin (n = 6) (%)
I	310	1	+8.9 ± 0.5	+9.9 ± 0.2 (111)	+7.4 ± 0.3	+3.8 ± 0.3 (51)
	310	3	+10.5 ± 0.3	+12.0 ± 0.2 (114)	+11.3 ± 0.3	+5.4 ± 0.3 (48)
	270	1	−5.7 ± 0.5	−5.0 ± 0.6 (88)	−8.9 ± 0.8	−6.5 ± 0.4 (73)
	270	3	−16.8 ± 1.1	−8.9 ± 0.5 (53)	−17.2 ± 0.9	−10.8 ± 0.3 (63)
VI	275	1	−9.6 ± 0.7	−23.8 ± 0.4 (248)	−10.1 ± 0.4	+4.2 ± 0.4 (−42)
	275	3	−34.8 ± 2.0	−61.0 ± 0.8 (175)	−34.7 ± 1.0	−2.7 ± 0.9 (8)
XI	270	1	+35.9 ± 0.8	+1.1 ± 0.3 (3)	+35.8 ± 0.1	+35.2 ± 0.6 (98)
	270	3	+41.7 ± 0.3	−31.6 ± 0.8 (−76)	+25.3 ± 0.2	+45.4 ± 0.6 (179)
Iophenoxate	320	1	−41.5 ± 0.4	−27.6 ± 0.3 (67)	−41.8 ± 0.7	−25.6 ± 0.3 (61)
	320	3	−34.9 ± 0.4	−39.3 ± 0.6 (113)	−38.1 ± 0.8	−27.3 ± 0.3 (72)
Iopanoate	303	1	−13.8 ± 0.3	−4.1 ± 0.3 (30)	−12.5 ± 0.2	−12.7 ± 0.3 (102)
	303	3	−5.6 ± 0.3	−1.1 ± 0.3 (20)	−4.5 ± 0.4	−9.8 ± 0.3 (218)

[a] The molar ellipticities ([θ]), calculated with reference to the albumin concentration, of various contrast agents bound to native (control) and tryptophan- (XII-albumin) and tyrosine- (XIV-albumin) modified human serum albumin, at the wavelength of their induced circular dichroism maxima (λ). Albumin concentrations of 13.1 μM were used with two different molar drug to albumin concentration ratios (D/P).

Table IV—Binding of I (Iodipamide) and XI (Iopodate) to Tryptophan- and Tyrosine-Modified Human Serum Albumin [a]

	I				XI			
	n_1	$K_1 \times 10^{-4}$, M^{-1}	n_2	$K_2 \times 10^{-4}$, M^{-1}	n_1	$K_1 \times 10^{-4}$, M^{-1}	n_2	$K_2 \times 10^{-4}$, M^{-1}
Control	1.1	307	2.0	9	1.1	254	4.1	12
Urea-albumin	1.0	314	1.8	8	1.1	198	3.7	12
XII-albumin	0.8	71	1.8	6	1.0	167	4.0	6
XIII-albumin	0.8	61	2.1	6	1.2	313	3.8	11
XIV-albumin	0.8	228	1.9	6	0.9	60	3.9	13

[a] The binding of the two contrast agents to the human serum albumin derivatives (36 μM) was investigated by equilibrium dialysis. Curved Scatchard plots were resolved to obtain n (number of binding sites) and K (association constant) by a graphical method according to Pennock (9). For each individual Scatchard plot, 30–40 experiments at eight to 10 different concentrations of the drugs were performed.

those of iopanoic acid were strongly reduced at both ligand concentrations. However, the modification of only two tyrosine residues (XIV-albumin) had an interesting effect on the extrinsic Cotton effects of the structurally related compounds, VI and XI. While the negative Cotton effects of VI were increased by nearly 100% at both ligand concentrations (Fig. 4 and Table III), the positive band of XI disappeared at a molar drug to albumin ratio of one and became negative at higher drug to albumin ratios (Fig. 5 and Table III). Increasing the degree of tyrosine modification to 5.1 was followed by a reduction of the Cotton effects of VI and XI, and the Cotton effects of both contrast agents became positive again when 7.8 tyrosine residues were modified per molecule of albumin (Figs. 4 and 5).

Equilibrium Dialysis Studies on Binding of I and XI to Native and Modified Human Serum Albumin—As calculated from the curved Scatchard plot (Fig. 6) by the method of Pennock (9), I binds to native human serum albumin *via* one high-affinity and two low-affinity binding sites (Table IV). While the unfolding in acidic urea (urea-albumin) was nearly without effect on the binding of I, the modification of the lone tryptophan residue with XII-Cl as well as with XIII-Br strongly reduced the association constant of the high-affinity binding site for I (Fig. 6 and Table IV). The secondary binding sites remained nearly unaffected by the tryptophan modifications. The modification of about two tyrosine residues with XIV, on the other hand, had a significantly smaller effect on the high-affinity binding site of I than did the modification of the tryptophan residue. In all three modified albumin derivatives, the number of high-affinity binding sites was reduced from ~1.0 to ~0.7–0.82, possibly not an effect of the modification of a specific amino acid residue.

In contrast to I, neither tryptophan modification affected the high-affinity binding site of XI. The slightly reduced affinity found for XII-albumin also was found for urea-albumin and, therefore, may be an effect of unfolding during modification. Also in contrast to I, the tyrosine modification reduced the affinity of the high-affinity binding site up to ~25% of the value found for native albumin. Neither tryptophan nor the tyrosine modifications significantly changed the number of high-affinity

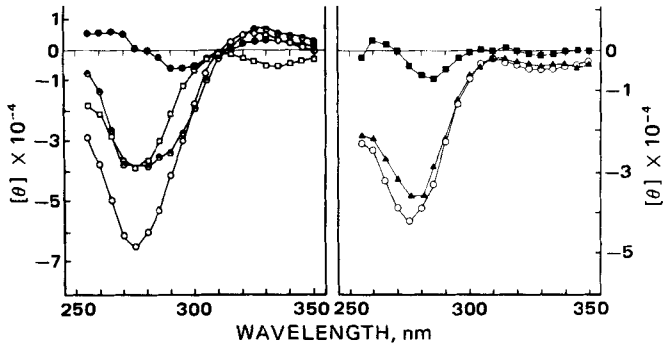

Figure 4—*Induced circular dichroism spectra of VI bound to human serum albumin and modified albumin derivatives (all were 13.1 μM). Intensity is given as molar ellipticity, [θ], calculated with respect to the albumin concentration. Compound VI was used at a molar drug to albumin ratio of 3. Data are difference values, using the Cotton effects of the albumin alone as a blank. Each point is the mean of three determinations. Key (left): □, native human serum albumin; ○, XIV-albumin (degree of modification 2.2); ◒, XIV-albumin (5.1); and ●, XIV-albumin (7.8). Key (right): ○, albumin; ▲, urea-albumin; and ■, XII-albumin.*

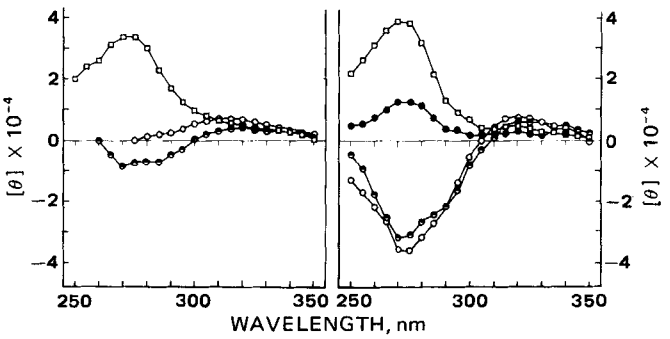

Figure 5—*Induced circular dichroism spectra of XI bound to human serum albumin and modified albumin derivatives (all were 13.1 μM). Intensity is given as molar ellipticity, [θ], calculated with respect to the albumin concentration. Compound XI was used at molar drug to albumin ratios of 1 (left) and 3 (right). Data are difference values, using the Cotton effects of the albumins alone as a blank. Each point is the mean of three determinations. Key: □, native human serum albumin; ○, XIV-albumin (degree of modification 2.2); ◒, XIV-albumin (5.1); and ●, XIV-albumin (7.8).*

Table V—Inhibition (Percent Displacement, $\bar{x} \pm s_{\bar{x}}$, $n = 3$) of Diazepam and Warfarin Binding to Human Serum Albumin by Biliary Contrast Agents [a]

Displacer	Diazepam		Warfarin	
	D/P 1.5	D/P 4.5	D/P 1.5	D/P 4.5
Iopanoic acid	32.2 ± 1.0	38.2 ± 0.7	10.3 ± 0.6	46.6 ± 1.6
Iophenoxic acid	6.1 ± 0.9	26.9 ± 1.0	37.8 ± 0.6	78.7 ± 1.1
I (iodipamide)	1.8 ± 0.9	3.4 ± 1.0	23.1 ± 0.8	43.4 ± 1.5
VI	16.0 ± 1.0	36.2 ± 1.1	8.0 ± 1.4	29.0 ± 1.7
XI (iopodate)	7.1 ± 0.4	14.0 ± 0.2	7.0 ± 1.4	23.2 ± 1.0

[a] The inhibition of the binding of diazepam (40 μM) and of warfarin (72 μM) to human serum albumin was investigated by equilibrium dialysis, using the displacing drugs at two different molar drug to albumin concentration ratios (D/P). The data are given as percent displacement of the fraction bound under control conditions (diazepam = 84.9%; warfarin = 79.7%).

binding sites nor the properties of the secondary binding sites of XI (Table IV).

Effect of Biliary Contrast Agents on Binding of Diazepam and Warfarin to Native Human Serum Albumin—Only a few specific binding sites exist on the human serum albumin molecule, which mediate the high-affinity binding of most strongly albumin-bound drugs (10). Two of the most important are the warfarin and diazepam binding sites (10). To determine to which site the contrast agents are bound, displacement of the two specific ligands, warfarin and diazepam, was studied by equilibrium dialysis (Table V).

At low concentrations (drug to protein ratio of ~1), iopanoic acid preferentially bound to the diazepam site and iophenoxic acid bound to the warfarin site. At higher concentrations, both drugs seemed to bind to both binding sites.

By contrast, even at higher concentrations, I did not interfere with the diazepam binding site, as was demonstrated by its failure to displace diazepam. The high potency in displacing warfarin, however, suggests that the high-affinity binding site of I might be identical with the warfarin binding site.

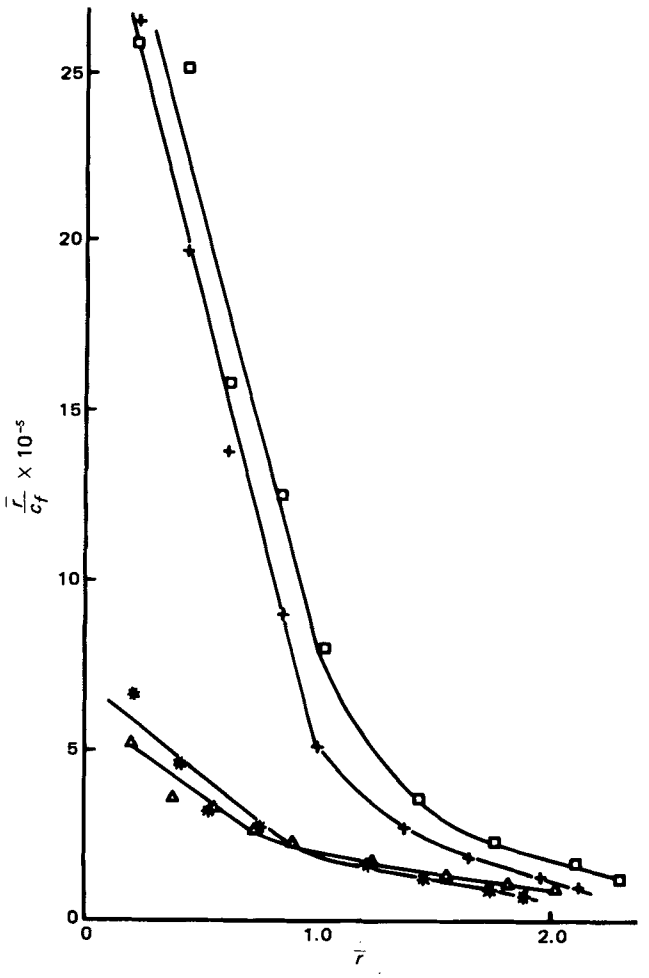

Figure 6—*Scatchard plot of the binding of I to human serum albumin and modified albumin derivatives. The albumin concentration was 36 μM. Each point is the mean of three or four determinations. Key: □, albumin; △, XIII-albumin; +, urea-albumin; and *, XII-albumin.*

Compounds VI and XI, which differ largely regarding their extrinsic Cotton effects in the presence of the modified albumin derivatives, seemed to bind to both sites. However, VI preferentially interacted with the diazepam site while XI had a higher affinity for the warfarin site, as suggested by their potencies in inhibiting the binding of diazepam and warfarin, respectively (Table V).

DISCUSSION

The binding of optically inactive small organic molecules to optically active macromolecules can induce optical activity into the ligand molecule so that, within the range of the UV or visible absorption spectra of the ligands, induced Cotton effects can be observed (11, 12). Such induced circular dichroism spectra have proved to be extremely useful in gaining insight into the molecular mechanism of the interactions of drugs with serum albumins (7, 8, 11–13).

The induced circular dichroism spectra of the investigated intravenous biliary contrast agents, as far as induced optical activity was observed, had one component in common: an induced band around 270 nm whose sign and intensity varied for the compounds. This band might be due to optical perturbations of electronic transitions in the triiodobenzoic acid moieties of the compounds, the only common structure of the investigated intravenous biliary contrast agents. However, the structural parameters leading to high induced optical activity of the intravenous contrast agents are not obvious since neither the degree of albumin binding (5) nor the length or lipophilicity of the aliphatic bridge between both triiodobenzoic acid moieties seemed to be the major determinant for high induced optical activity.

In general, considering their extremely high binding to human serum albumin, the intravenous biliary contrast agents gave only weak extrinsic Cotton effects when bound to human serum albumin. This result and the failure to find any clear correlation between intensity of the extrinsic Cotton effects and their chemical structure may indicate that the complex between these compounds and human serum albumin lacks the structural specificity found for other drugs when bound to albumin (14–17).

As was found for the intravenous biliary contrast agents, all oral biliary contrast agents had a common induced circular dichroism band around 270 nm when bound to human or bovine serum albumin, possibly due to the triiodobenzene moiety of the drugs. With human serum albumin, the band intensity varied little for the oral compounds, suggesting a similar spatial relationship of their complexes with human serum albumin. For the binding of VI and XI to human serum albumin, their Cotton effects at 270 nm had opposite signs, even though the only difference in their chemical structure was the introduction of an ethyl group in position 2 of the propionic side chain (VI). This observation supports earlier findings on iophenoxic acid, indicating that the optical properties of the complexes of the oral biliary contrast agents are extremely sensitive to even small changes of the ligand chemical structure (18).

As already mentioned, the positive Cotton effects of XI seemed to be associated mainly with its high-affinity binding site, while the negative Cotton effects were induced at all binding sites of XI. Accordingly, only negative Cotton effects were observed after tyrosine modification, presumably induced at the secondary binding sites.

Previously it was shown that the lone tryptophan residue of human serum albumin is part of the warfarin binding site and can be selectively modified by XII-Br and XIII-Cl (8), while a highly reactive tyrosine residue, which can be nitrated selectively by low concentrations of XIV, is part of the indole and benzodiazepine binding site (7). Thus, the interaction of drugs with both specific binding sites of human serum albumin can be studied indirectly by displacement of the marker ligands, diazepam and warfarin, and directly by the binding of the drugs to the

modified albumin derivatives.

The results obtained with I give some evidence that the single high-affinity binding site of the intravenous biliary contrast agents (5) might be identical with the warfarin binding site of human serum albumin. This conclusion can be made from the strong displacement of bound warfarin, the large reduction of the extrinsic Cotton effects of I bound to XII–albumin at 310 nm that are associated only with binding to the high-affinity binding site, and the large reduction of the association constant of the high-affinity binding site for XII–albumin or XIII–albumin. By contrast, binding of I to the diazepam binding site may be negligible, as shown by the lack of any displacement of human serum albumin-bound diazepam and by the small effects of the tyrosine modification on the binding parameters of I.

With the oral biliary contrast agents, the specificity for one of the two sites of human serum albumin is not so pronounced. However, as revealed by their displacing potencies for diazepam and warfarin and their binding behavior for tyrosine- and tryptophan-modified albumin, VI is bound preferentially to the warfarin binding site but XI is bound to the diazepam binding site. This observation is interesting considering the small difference in the chemical structures of these contrast agents. A similar observation was made for the structurally related oral biliary contrast agents, iopanoic and iophenoxic acids, which differ only by the substitution of a phenolic hydroxyl group in iophenoxic acid and by an amino group in iopanoic acid (18). The data support earlier observations that the high-affinity binding sites of both drugs are different (18, 19); it seems that iophenoxic acid is bound preferentially to the warfarin site while iopanoic acid is bound preferentially to the diazepam binding site.

The latter results indicate that it will be difficult to find common characteristics for drugs bound specifically to the warfarin binding site and for drugs bound specifically to the indole and benzodiazepine binding site since only small changes in the chemical structure of the ligands can have pronounced effects on the binding site selectivity of the drugs.

In summary, the data clearly showed that the serum albumin binding of biliary contrast agents is quite unusual in respect to the structural parameters leading to strong binding to human and bovine serum albumin. Furthermore, small variations of the chemical structure of the contrast agents can effect large changes in the binding site selectivity. The diazepam and the warfarin binding sites of human serum albumin (two important drug binding sites of the protein) are involved in the binding of the biliary contrast agents. Since many other drugs also are bound to these sites, the biliary contrast agents must be considered as potent displacers of many drugs *in vivo*, especially with regard to their high plasma concentration *in vivo* (20, 21). However, the short plasma half-life of the intravenous biliary contrast agents or the short use of the oral biliary contrast agents makes it unlikely that such displacements are clinically important. However, evidence is accumulating that the plasma protein binding of the biliary contrast agents is extremely important for their distribution, hepatic uptake, and biliary and renal elimination (20–23). Therefore, exact knowledge about the mechanisms involved in the plasma protein binding of these drugs could be helpful for understanding the pharmacokinetics of the biliary contrast agents.

REFERENCES

(1) P. K. Knoefel, in "Radiocontrast Agents," vol. 1, P. K. Knoefel, Ed., Pergamon, New York, N.Y., 1971, pp. 133–145.

(2) S. K. Lin, A. A. Moss, and S. Riegelman, *J. Pharm. Sci.*, **66,** 1670 (1977).

(3) S. K. Lin, A. A. Moss, R. Motson, and S. Riegelman, *ibid.*, **67,** 930 (1978).

(4) E. C. Lasser, R. S. Farr, T. Iujimagari, and W. N. Tripp, *Am. J. Roentgenol.*, **87,** 338 (1962).

(5) W. E. Müller, *Naunyn-Schmiedebergs Arch. Pharmacol.*, **302,** 227 (1978).

(6) K. J. Fehske, W. E. Müller, and U. Wollert, *Hoppe-Seylers Z. Physiol. Chem.*, **359,** 709 (1978).

(7) K. J. Fehske, W. E. Müller, and U. Wollert, *Biochim. Biophys. Acta*, **577,** 346 (1979).

(8) K. J. Fehske, W. E. Müller, U. Wollert, and L. Velden, *Mol. Pharmacol.*, **16,** 778 (1979).

(9) B. E. Pennock, *Anal. Biochem.*, **56,** 306 (1973).

(10) W. E. Müller and U. Wollert, *Pharmacology*, **19,** 59 (1979).

(11) C. F. Chignell, *Adv. Drug Res.*, **5,** 55 (1970).

(12) J. H. Perrin and P. A. Hart, *J. Pharm. Sci.*, **59,** 431 (1970).

(13) J. J. Vallner, *ibid.*, **66,** 447 (1977).

(14) W. E. Müller, *Klin. Wochenschr.*, **55,** 105 (1977).

(15) J. H. Perrin, *J. Pharm. Pharmacol.*, **25,** 208 (1973).

(16) C. F. Chignell, *Mol. Pharmacol.*, **5,** 455 (1969).

(17) T. Sjödin, N. Roosdorp, and I. Sjöholm, *Biochem. Pharmacol.*, **25,** 2131 (1976).

(18) K. J. Fehske and W. E. Müller, *Res. Commun. Chem. Pathol. Pharmacol.*, **19,** 119 (1978).

(19) G. H. Mudge, N. Desbienz, and G. R. Stibitz, *Drug. Metab. Disp.*, **6,** 432 (1978).

(20) R. N. Berk, P. M. Loeb, and B. A. Ellzey, in "Radiocontrast Agents," R. E. Miller and J. Skucas, Eds., University Park Press, Baltimore, Md., 1977, pp. 223–250.

(21) R. N. Berk and P. M. Loeb, in *ibid.*, pp. 195–221.

(22) W. E. Müller and A. Stillbauer, *Arch. Int. Pharmacodyn. Ther.*, **246,** 187 (1980).

(23) W. E. Müller and A. Stillbauer, *Pharmacology*, in press.

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Interaction of Xanthan Gum with Suspended Solids

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Abstract □ Xanthan gum was adsorbed significantly by magnesium carbonate, aluminum hydroxide, zinc oxide, and calcium carbonate, giving Langmuir-type isotherms. Saturation adsorption was higher from 0.9% NaCl than from water due to reduced mutual repulsion of polymer segments in the presence of the salt. Adsorption resulted from electrostatic attraction between positively charged particles and the negatively charged polymer. ζ-Potential measurements correlated with the adsorption data but were not predictive of the flocculation state. The results indicate that flocculation of magnesium carbonate and aluminum hydroxide by xanthan gum is consistent with a bridging mechanism.

Keyphrases □ Xanthan gum—interaction with magnesium carbonate, aluminum hydroxide, zinc oxide, and calcium carbonate □ Adsorption—xanthan gum by suspended solids □ Flocculation—bridging mechanism, magnesium carbonate and aluminum hydroxide with xanthan gum

Xanthan gum produces flocculation of pharmaceutical suspensions (1, 2). Studies utilizing sedimentation volume, microscopy, and particle-size measurement showed that changes in interparticulate structure could be induced by the polymer (2). Flocculation was attributed to the joining of several particles by adsorbed polymer molecules, which

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