Biological Significance of Serum Albumin Binding Parameters Determined *In Vitro* for Clofibrate-Related Hypolipemic Drugs: Use of 2-(4′-Hydroxybenzeneazo)benzoic Acid to Mirror L-Thyroxine Binding to and Displacement from Serum Albumins

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Abstract □ Clofibrate [ethyl 2-(*p*-chlorophenoxy)-2-methylpropionate] has been shown to be an effective lipid-lowering drug in man and to exert its action by multiple modes. To explore further the possibility that albumin binding parameters determined *in vitro* are predictive of hypolipemic activity determined *in vivo*, the binding of several cyclic and acyclic analogs of the corresponding carboxylic acid of clofibrate to bovine, rat, and human serum albumins was studied. This report compares the differential hypolipemic effects of the esters of these analogs with data derived from studies involving their ability to undergo hydrolysis by serum esterases *in vitro*, as well as with the pKa's, log *P* values, and albumin binding parameters of their respective free acids. The latter study involved the use of 2-(4′-hydroxybenzeneazo)benzoic acid as an optical probe which mirrors thyroxine binding *in vitro* as well as the use of the free acids alone and their ability to displace radiolabeled thyroxine. These investigations indicate that albumin binding parameters probably are important for drug transport *in vivo* but that hypolipemic activity is related to mechanisms other than thyroxine displacement.

Keyphrases □ Clofibrate-related hypolipemic drugs—*in vitro* serum albumin binding parameters determined using 2-(4′-hydroxybenzeneazo)benzoic acid, thyroxine displacement, relationship to hypolipemic activity □ Hypolipemic drugs related to clofibrate—*in vitro* serum albumin binding parameters, determined using 2-(4′-hydroxybenzeneazo)benzoic acid, thyroxine displacement, relationship to hypolipemic activity □ Serum albumin binding—clofibrate-related hypolipemic drugs, determined using 2-(4′-hydroxybenzeneazo)benzoic acid, thyroxine displacement □ 2-(4′-Hydroxybenzeneazo)benzoic acid—used to determine serum albumin binding of clofibrate-related hypolipemic drugs, thyroxine displacement

Clofibrate [ethyl 2-(*p*-chlorophenoxy)-2-methylpropionate, I] has been shown to be an effective lipid-lowering drug in man (1, 2). This drug rapidly undergoes hydrolysis *in vivo*, and the corresponding acid, II, is presumed to be the active drug (3). Studies *in vivo* with I and *in vitro* with II suggest that clofibrate may be exerting its effect by multiple modes of action (4). Aryloxyisobutyrates were reported (5) to cause a reduction of L-thyroxine binding to albumin in the range of $r < 1$ (r = moles drug bound per mole albumin). While clofibrate was reported to both alter (6) and not alter (7, 8) the distribution of labeled thyroxine *in vivo*, causing a reduction in plasma concentration with a concomitant increase in liver concentration, it was suggested that any alteration in distribution of thyroxine *in vivo* caused by clofibrate may have physiological significance and, in fact, correlate with *in vitro* binding studies. For example, the suggestion was made (6) that the displacement effects observed for hypolipemic drugs in the range r = 0–1 are likely of relevance to therapy whereas effects at $r > 1$ may be critical in the evaluation of toxicological studies. Studies by Thorp (9) involving interspecies differences in the binding of these drugs also led him to conclude that albumin binding studies *in vitro* could be used to predict the effects of a given compound on serum cholesterol levels *in vivo*.

To explore further the possibility that albumin binding parameters determined *in vitro* are predictive of hypolipemic activity determined *in vivo*, the binding of several cyclic and acyclic analogs of II to bovine, rat, and human serum albumins was studied. A comparative analysis was recently described (10) of the hypocholesterolemic and hypotriglyceridemic activity of the corresponding ethyl esters of these analogs in a hyperlipemic rat model in which hyperlipemia was induced by intraperitoneal injection of tyloxapol[1]. Under these conditions, clofibrate (I), ethyl 1,4-benzodioxane-2-carboxylate (V), ethyl 5-chloro-2,3-dihydro-2-benzofurancarboxylate (VII), and ethyl 6-chlorochroman-2-carboxylate (XI) significantly reduced serum cholesterol levels. The deschloro analogs IX and XIII were inactive. In these same hyperlipemic rats, esters I, V, and XI also significantly reduced plasma triglyceride levels. Deschloro analogs IX and XIII were inactive, and the apparent hypotriglyceridemic effect of chloro analog VII was not statistically significant. Acyclic analog III, administered as the DL-compound, also showed no significant hypocholesterolemic or hypotriglyceridemic activity in these hyperlipemic rats.

In this report, the differential hypolipemic effects of esters I, III, V, VII, IX, XI, and XIII are compared with data derived from studies involving their ability to undergo hydrolysis by serum esterases *in vitro*, as well as with the pKa's, log *P* (*P* = octanol–water partition coefficient) values, and albumin binding parameters of the respective free acids. Albumin binding studies also were carried out with optically pure L(*S*)-IV and D(*R*)-IV, alicyclic homolog XV, and 4-ketochroman (XVII) to determine whether stereochemistry and these minor structural modifications had a major influence on albumin binding parameters.

Since it was shown previously (11) that the optical probe 2-(4′-hydroxybenzeneazo)benzoic acid (XVIII) could be used as a model to mirror the binding of thyroxine to bovine and rat serum albumins, acid

[1] Triton WR-1339.

Table I—Molar Absorptivity and Wavelength Maximum Used to Study the Hydrolysis of Esters by Rat Plasma *In Vitro* at 37°

Ester	ϵ (Wavelength, nm)
I	11,600 (226)
III	13,047 (226)
VII	7,196 (231)
XI	8,965 (229)
XVI	19,290 (226)

XVIII also was utilized in these studies to gauge the displacement of thyroxine from serum albumins by the various analogs. Use of XVIII in place of thyroxine to estimate relative differences in competitive binding by the various analogs is simple and has the additional advantage of decreased expense over the usual assays for the hormone utilizing radiolabeled compound (12) or enzymatic reactions (13). To substantiate further the validity of results obtained in competitive binding studies of the various analogs using XVIII, the displacement of ^{125}I-thyroxine from rat serum albumin by II, XII, and XIV (12, 14) was investigated.

EXPERIMENTAL

Materials—Bovine serum albumin (Fraction V, fatty acid poor), human serum albumin (Fraction V), and 2-(4′-hydroxybenzeneazo)benzoic acid (XVIII) were used as purchased[2]. Rat serum albumin[3] (Fraction V), L-thyroxine[4], dimethyl sulfoxide[4], isooctane[5] (2,2,4-trimethylpentane, spectral grade), and ^{125}I-thyroxine[6] (specific activity 200 μCi/ml) were used without further purification. 1-Octanol[7] was purified as described under *Octanol–Water Partition Coefficient Determination.* All final solutions were made with 0.1 *M* sodium phosphate buffer, pH 7.4, using analytical grade chemicals. All carboxylic acids were synthesized as described by Witiak *et al.* (15–18). Dimethyl sulfoxide stock solutions of the carboxylic acids (1.0×10^{-2} *M*) were diluted with appropriate amounts of phosphate buffer to give the desired final concentrations.

Hydrolysis of Esters by Rat Plasma *In Vitro*—The hydrolysis of esters I, III, VII, XI, and XVI by rat plasma *in vitro* at 37° was determined according to the method of Thorp[8]. Blood collected from the abdominal aorta of anesthetized male Sprague–Dawley rats (180–200 g) was placed in tubes (Vacutainer) containing a small amount of ethylenediaminetetraacetic acid. The blood was immediately centrifuged for 15 min, and the supernatant plasma was separated and pooled. The esters were dissolved in spectral grade methanol to give 0.05 *M* solutions. The incubation system used was as follows: 0.1–0.3 ml of the ester solution was added to 8 ml of rat plasma, and the mixture was incubated at 37° on a metabolic shaker. Samples (1.0 ml) were removed at various times (0, 5, 10, and 60 min) for determination of the amounts of the parent esters present.

For the assay procedure, 1 ml of each sample was placed in a 10-ml glass-stoppered centrifuge tube. One-half milliliter of 3 *N* hydrochloric acid and 5 ml of an isooctane–absolute ethanol mixture (95:5 v/v) were added to each tube. The tubes were stoppered, shaken by hand, and allowed to stand. After separation of the layers, the UV absorption of the extracts was determined against a blank consisting of the solvent mixture. The wavelengths used for the esters as well as their ϵ values are given in Table I. This UV absorption of the extract is due to the presence of both ester and free acid. An aliquot of the isooctane–ethanol extract was extracted with an equal volume of 2% sodium bicarbonate to remove the free acid selectively. The amount of free acid was determined from the reduction in the UV absorption of the organic phase by comparison with suitable standards, blanks, and recoveries (at zero time) from rat plasma.

pKa and ϵ of Carboxylic Acids—The pKa's of the carboxylic acids were determined potentiometrically by titrating 0.02% solutions prepared by diluting 0.4% stock solutions in dimethyl sulfoxide with double-distilled water. Sodium hydroxide (0.01 *N*) was the titrant, and a pH meter[9] was used to measure the pH.

The molar absorptivity, ϵ, for the carboxylic acids was determined by measuring[10] the absorbance of solutions made by diluting 1.0×10^{-2} *M* dimethyl sulfoxide solutions with phosphate buffer. Reference solutions consisted of equivalent dilutions of dimethyl sulfoxide with buffer. The wavelength maxima at which ϵ was determined are listed in Table II. All studies were made at ambient temperature.

Binding of Carboxylic Acids to Serum Albumin—The binding of the carboxylic acids to bovine, human, and rat serum albumins was determined by the ultrafiltration technique (11) at ambient temperatures, using phosphate buffer solutions having a constant concentration of serum albumin (1.0×10^{-4} *M*, molecular weight 67,000) and a varying analog concentration (from 2.0×10^{-4} to 3.0×10^{-3} *M*). The ultrafiltrate was analyzed spectrophotometrically after suitable dilution. The ϵ value and wavelength of measurement are given in Table II.

Competitive Binding between XVIII and II, L(*S*)-IV, D(*R*)-IV, XIV, and XV on Bovine Serum Albumin—The binding of XVIII to bovine serum albumin in the presence of four analogs [L(*S*)-IV, D(*R*)-IV, XIV, and XV] was determined by varying the concentration of XVIII from 3.0×10^{-5} to 1.6×10^{-3} *M* at fixed albumin (1.0×10^{-4} *M*) and analog (1.0×10^{-4} *M*) concentrations. Three fixed concentration levels of II were used: 1.0×10^{-4}, 2.0×10^{-4}, and 5.0×10^{-4} *M*. Ultrafiltration was used to study binding where the amount of XVIII free was determined by spectrophotometric analysis at 350 nm. None of the analogs absorbs at this wavelength in phosphate buffer.

I: R = CH_2CH_3
II: R = H

III: R = (DL)-CH_2CH_3
IV: R = H

V: R = CH_2CH_3
VI: R = H

VII: R = CH_2CH_3, X = Cl
VIII: R = H, X = Cl
IX: R = CH_2CH_3, X = H
X: R = X = H

XI: R = CH_2CH_3, X = Cl
XII: R = H, X = Cl
XIII: R = CH_2CH_3, X = H
XIV: R = X = H

XV

XVI: R = CH_2CH_3
XVII: R = H

XVIII

[2] Nutritional Biochemical Corp., Cleveland, Ohio.
[3] Pentex, Inc., Kankakee, Ill.
[4] Eastman Kodak Co., Rochester, N.Y.
[5] Matheson, Coleman and Bell, Norwood, Ohio.
[6] Amersham-Searle Corp., Arlington Heights, Ill.
[7] Aldrich Chemical Co., Milwaukee, Wis.
[8] J. M. Thorp, Biological Research Department, Imperial Chemical Industries Ltd., Alderly Park, Macclesfield, Chesire, England, personal communication.

[9] Sargent, model DR.
[10] With a Cary model 16 spectrophotometer.

Table II—Molar Absorptivity and pKa for Various Analogs Used in Serum Albumin Binding Studies

Analog	ϵ (Wavelength, nm)	pKa
II	1018 (279)	4.46
L(*S*)-IV	1372 (279)	4.35
D(*R*)-IV	1400 (279)	4.35
VI	2300 (277)	4.23
VIII	2228 (279)	4.36
XII	1873 (283)	4.26
XIV	1613 (279)	4.14
XV	1048 (279)	3.90
XVII	3029 (337)	3.90

Table III—Octanol–Water Partition Coefficients of Analogs

Analog	ϵ (Wavelength, nm)	Log *P*
II	1038 (280)	2.57
D(*R*)-IV	1565 (281)	2.31
VI	2300 (277)	1.40
VIII	3010 (290)	2.11
XII	2120 (285)	2.40
XIV	1825 (282)	1.90
XV	1100 (281)	2.90
XVII	3240 (330)	1.77

Moriguchi Method—For comparative purposes, the binding of the carboxylic acids to bovine serum albumin also was studied by an indirect method (19, 20). The binding constants of the analogs were evaluated spectrophotometrically in terms of their ability to displace competitively 2-(4′-hydroxybenzeneazo)benzoic acid (XVIII). Bovine albumin and XVIII stock solutions were made in 0.05 *M* sodium phosphate buffer, pH 7.4. Stock analog solutions (1.0×10^{-4} *M*) were made in the phosphate buffer with 10% dimethyl sulfoxide. Solutions containing analogs (0, 1.0×10^{-4}, 1.5×10^{-4}, 2.0×10^{-4}, and 2.5×10^{-4} *M*), XVIII (1.0×10^{-4} *M*), and bovine serum albumin (5.0×10^{-5} *M*) were prepared, and the absorbances were measured at 482 nm 1 hr after preparation of the solutions. The results were analyzed according to Eq. 1:

$$\Delta E = E' - (E + E'') \qquad \text{(Eq. 1)}$$

where:

E' = absorbance of the solutions containing 1.0×10^{-4} *M* XVIII and 5.0×10^{-5} *M* bovine serum albumin with 0, 1.0×10^{-4}, 1.5×10^{-4}, 2.0×10^{-4}, and 2.6×10^{-4} *M* analogs at 482 nm

E = absorbance of the solution of 1.0×10^{-4} *M* XVIII at 482 nm

E'' = absorbance of the solution of 5.0×10^{-5} *M* bovine serum albumin at 482 nm

Log K_b, where K_b is the binding constant of the analog (presumably to the primary binding site), is determined from the ΔE value obtained for the various analogs through use of theoretical curves relating log K_b to ΔE as described by Moriguchi (20).

Competitive Binding between XVIII and II, XII, and XIV on Rat Serum Albumin—The binding of XVIII to rat serum albumin in the presence of two analogs (XII and XIV) was determined by varying the concentration of XVIII from 5.0×10^{-5} to 1.6×10^{-3} *M* at fixed albumin (1.0×10^{-4} *M*) and analog (2.0×10^{-4} *M*) concentrations. The effect of two fixed concentration levels (2.0×10^{-4} and 5.0×10^{-4} *M*) of II was studied under the same conditions. Ultrafiltration was used to study binding, and the amount of XVIII free was determined by spectrophotometric analysis at 350 nm.

Binding of Thyroxine to Rat Serum Albumin—Equilibrium dialysis with ^{125}I-thyroxine was used to study the binding of thyroxine to rat serum albumin (12, 14). Twenty-centimeter (8-in.) lengths of 2.1-cm (0.83-in.) dialysis tubing were soaked in 0.1 *M* nitric acid for 24 hr and then in 0.01 *M* nitric acid for 3 days to remove metal impurities. The tubing was thoroughly rinsed with distilled water and was finally stored in deionized water at 4°. Before use, the tubing was rinsed with glass-distilled water. The stock solution of thyroxine (1.0×10^{-3} *M*) was prepared in 0.04 *N* sodium hydroxide. The range of thyroxine concentrations used was from 2.6×10^{-6} to 2.6×10^{-5} *M*. These solutions were prepared by appropriate dilutions of the stock thyroxine solution using 0.1 *M* sodium phosphate buffer, pH 7.4, containing 3.0×10^{-4} *M* ethylenediaminetetraacetic acid. ^{125}I-Thyroxine (2.0 μCi) was added to the nonradioactive thyroxine solutions, and 5 ml of this solution was placed in 50-ml lusteroid centrifuge tubes (28 × 102 mm). Five milliliters of the rat serum albumin solution (0.05 g rat serum albumin/100 ml) was placed in the dialysis bag. The tubes were covered with aluminum foil and placed on a metabolic shaker at 37°. After 16 hr, the radioactivity in 2-ml samples from each compartment was counted[11]. The fraction bound was obtained from the relationship: (counts per minute inside − counts per minute outside)/total counts per minute.

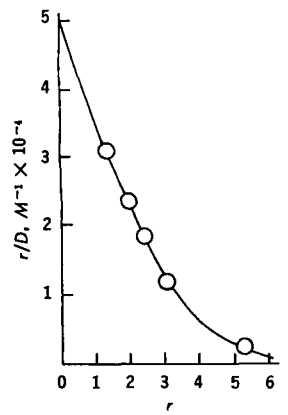

Figure 1—*Scatchard plot for the binding of XII to human serum albumin (1.0×10^{-4} M) at pH 7.4. Key: O, experimental values; and ——, computer fit assuming two independent classes of binding sites exist on serum albumin.*

Competitive Binding between Thyroxine and II, XII, and XIV on Rat Serum Albumin—The binding of thyroxine to rat serum albumin in the presence of two analogs (II and XIV) was determined by varying the concentration of thyroxine from 2.6×10^{-6} to 2.6×10^{-5} *M* at a fixed albumin concentration (7.5×10^{-6} *M*), employing an analog concentration of 1.0×10^{-4} *M*. Under similar experimental conditions, two fixed concentration levels of XII were used (1.0×10^{-4} and 2.0×10^{-4} *M*). Stock solutions of the analogs were made in 0.1 *M* sodium phosphate buffer, pH 7.4, containing 3.0×10^{-4} *M* ethylenediaminetetraacetic acid. Equilibrium dialysis, followed by analysis of ^{125}I-thyroxine as already described, was used to determine the fraction of thyroxine bound.

Octanol–Water Partition Coefficient Determination—Partition coefficients of the carboxylic acid analogs were determined according to the method of Hansch[12]. 1-Octanol was purified by washing with 10% sodium hydroxide, 10% sulfuric acid, and, finally, 10% sodium bicarbonate solution. After drying over magnesium sulfate, the octanol was distilled under reduced pressure and then saturated with distilled water. Analogs were dissolved in octanol, and 10 ml of the resulting solution was placed in a 250-ml glass-stoppered centrifuge bottle. Two hundred milliliters of the appropriate buffer solution (pH two units below the pKa of the analog) was added; the bottle was stoppered and the phases were mixed by inverting and shaking by hand for 2 min. After centrifugation for 1 hr, samples of the octanol phase were removed by pipet and analyzed spectrophotometrically. The wavelengths used for these measurements and the ϵ values for the various analogs are listed in Table III.

RESULTS

Hydrolysis of Esters by Rat Plasma *In Vitro*—The hydrolysis of esters I, III, VII, XI, and XVI by rat plasma *in vitro* was carried out at 37°. All of these esters were rapidly hydrolyzed, in accord with the results obtained by Thorp (3) for clofibrate. The mean percent and standard deviation of the ester hydrolyzed in 5

[11] Baird-atomic well counter, model 810C, with a Baird-atomic spectrometer, model 530.

[12] C. Hansch, Department of Chemistry, Pomona College, Claremont, CA 91711, personal communication.

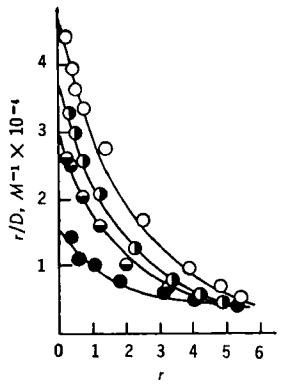

Figure 2—*Scatchard plot for the binding of XVIII to bovine serum albumin (1.0×10^{-4} M) in the presence of various total amounts of II. Key: ○, XVIII alone; ◑, 1.0×10^{-4} M II; ◒, 2.0×10^{-4} M II; and ●, 5.0×10^{-4} M II.*

min at 37° were as follows: I, 98.6 ± 0.2%; III, 98.1 ± 0.1%; VII, 97.8 ± 0.5%; XI, 96.3 ± 0.5%; and XVI, 99.0 ± 0.3%.

Binding of Carboxylic Acid Analogs to Bovine, Rat, and Human Serum Albumins—At the pH of the binding studies (7.4), all of the analogs exist primarily in the anionic form since their pKa's range from 3.90 to 4.46 (Table II). It is apparent that the species bound must be the charged form for each analog. A Scatchard plot for the binding of each analog to the various serum albumins gives the same general nonlinear relation, excluding analog X which binds to only one site and, therefore, gives a linear Scatchard plot. A typical or representative result is shown in Fig. 1 for the binding of XII to human serum albumin. The solid line in Fig. 1 is the computer fit for the data using the binding parameters generated assuming two independent classes of binding sites exist on serum albumin. It is apparent that this assumption adequately describes the binding of this analog to human serum albumin. Similar computer fits were obtained for each of the other analogs with all serum albumins used. The computer-generated values for n_1, K_1, n_2, and K_2 best describing the binding of the analogs to bovine, rat, and human serum albumins are given in Table IV. The parameters are related by the well-known equation:

$$r = \frac{n_1 K_1 D_f}{1 + K_1 D_f} + \frac{n_2 K_2 D_f}{1 + K_2 D_f} \qquad \text{(Eq. 2)}$$

in which r is the number of moles of analog bound per mole of albumin; n_1 and n_2 are the maximum number of sites in the first and second class of mutually independent sites, respectively; K_1 and K_2 are the binding constants in each class, and D_f is the concentration of free analog in equilibrium with bound analog.

For all analogs and a particular albumin, the binding constant for the first class of sites is larger than that for the second binding class. With bovine serum albumin, analogs L(S)-IV, VI, and XIV have the larger K_1 values, ranging from four to five times the K_1 value for II. With rat serum albumin, XIV has a primary binding constant approximately twice that of the other analogs. With human serum albumin, XIV has a K_1 less than the one observed for II, but again differences in binding constants for the various

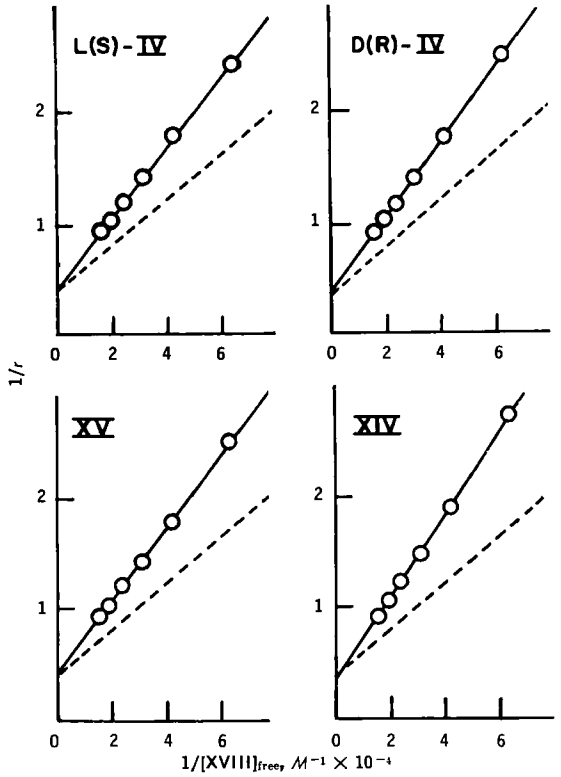

Figure 4—*Relationship between 1/r (moles bovine serum albumin per mole XVIII) and $1/[XVIII]_{free}$ at low r values in the presence of 1.0×10^{-4} M L(S)-IV, D(R)-IV, XIV, and XV. The broken line is XVIII alone, having an intercept of 0.38.*

analogs are comparatively small. It is apparent that there are minor species differences in the albumin binding parameters for a given analog. From these data, determined *in vitro* for human serum albumin, one can calculate the percent of II (derived from the *in vivo* hydrolysis of clofibrate) that would be expected to be bound to human serum albumin *in vivo* by using a computer program developed by Garten and Wosilait (21). Assuming a total therapeutic serum concentration of 170 mg/liter for clofibrate (22) and a total albumin concentration of 6.0×10^{-4} *M*, 93% of the free acid II is predicted to be bound to serum proteins *in vivo*. This value is in agreement with results obtained by Thorp (3) who found that II was 96% bound to 4% bovine serum albumin (6.0×10^{-4} *M*) *in vitro*. Whereas the therapeutic serum concentrations for all experimental drugs in humans (or animals) are not known, the relatively small differences in log *P* values observed for these analogs leads to the prediction that similar serum concentrations would be attained if equivalent oral doses were administered. When using the same therapeutic serum concentra-

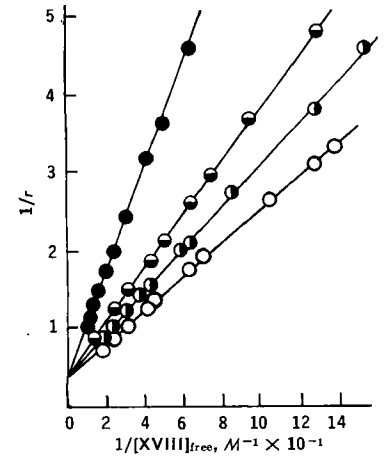

Figure 3—*Relationship between 1/r (moles bovine serum albumin per mole XVIII) and $1/[XVIII]_{free}$ at low r values in the presence of II. Key: ○, no II; ◑, 1.0×10^{-4} M II; ◒, 2.0×10^{-4} M II; and ●, 5.0×10^{-4} M II.*

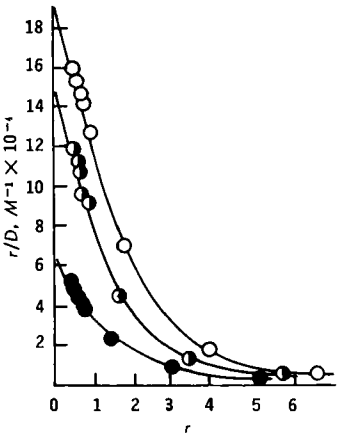

Figure 5—*Scatchard plot for the binding of XVIII to rat serum albumin in the presence of various total amounts of II. Key: ○, XVIII alone; ◑, 2.0×10^{-4} M II; and ●, 5.0×10^{-4} M II.*

Table IV—Binding Parameters of Analogs to Bovine, Rat, and Human Serum Albumins (1.0×10^{-4} *M*) at pH 7.4 (0.1 *M* Phosphate Buffer)

Albumin	Parameter	Analog II	L(*S*)-IV	D(*R*)-IV	VI	VIII	X	XII	XIV	XV	XVII
Bovine	n_1	4.0	1.5	2.1	2.0	3.3	—	3.1	2.1	3.7	2.6
	n_2	9.7	7.8	15.1	4.3	6.1	—	11.4	10.4	7.6	9.0
	K_1, $M^{-1} \times 10^{-4}$	1.28	5.97	2.64	4.56	1.16	—	3.78	6.46	1.55	2.64
	K_2, $M^{-1} \times 10^{-2}$	3.3	3.8	1.4	6.3	2.5	—	2.3	2.3	2.9	1.7
Rat	n_1	2.6	2.3	2.2	2.2	3.6	2.8	4.1	1.6	3.3	3.3
	n_2	18.0	3.5	6.9	10.2	13.8	—	19.2	5.8	8.7	8.0
	K_1, $M^{-1} \times 10^{-4}$	2.94	1.81	2.43	2.27	1.83	0.34	1.88	5.06	2.93	2.41
	K_2, $M^{-1} \times 10^{-2}$	4.4	5.0	6.5	2.1	1.7	—	1.1	5.9	4.6	1.7
Human	n_1	1.8	1.3	1.7	1.5	2.3	—	3.1	2.4	2.8	2.7
	n_2	9.0	6.2	8.0	10.0	10.2	—	3.4	12.9	3.6	2.7
	K_1, $M^{-1} \times 10^{-4}$	2.47	2.40	1.56	3.18	2.59	—	1.52	1.09	1.35	2.19
	K_2, $M^{-1} \times 10^{-2}$	4.7	4.8	4.1	1.5	3.9	—	8.8	0.96	6.2	5.3

Table V—Statistical Analysis of Linear Regression Intercepts at $p = 0.01$

System	Number of Observations	Variance of Intercept[a], s_I^2	Mean Square Error	*F* Ratio for Mean Square Error	Theoretical *F* Ratio[b]	Theoretical *t* Value[b]	Calculated *t* Value[a]
Bovine serum albumin:							
XVIII alone	11	8.73×10^{-4}	3.06×10^{-3}	—	—	—	—
XVIII + II (1.0×10^{-4} *M*)	10	8.20×10^{-4}	2.51×10^{-3}	1.22	5.47	2.90	0.74
XVIII + II (2.0×10^{-4} *M*)	14	7.80×10^{-4}	3.94×10^{-3}	1.29	4.39	2.83	1.22
XVIII + II (5.0×10^{-4} *M*)	13	3.86×10^{-4}	2.30×10^{-3}	1.33	4.63	2.84	0.19
XVIII + L(*S*)-IV (1.0×10^{-4} *M*)	9	8.44×10^{-4}	2.60×10^{-3}	1.77	6.72	2.92	1.39
XVIII + D(*R*)-IV (1.0×10^{-4} *M*)	9	1.94×10^{-3}	6.01×10^{-3}	1.96	6.72	2.92	0.23
XVIII + XIV (1.0×10^{-4} *M*)	9	3.32×10^{-4}	1.02×10^{-3}	2.99	5.61	2.92	2.06
XVIII + XV (1.0×10^{-4} *M*)	9	6.47×10^{-4}	2.00×10^{-3}	1.53	6.72	2.92	1.10
Rat serum albumin:							
XVIII alone	6	1.55×10^{-4}	1.04×10^{-4}	—	—	—	—
XVIII + II (2.0×10^{-4} *M*)	6	1.33×10^{-4}	1.27×10^{-4}	1.22	16.00	3.36	4.80
XVIII + II (5.0×10^{-4} *M*)	6	3.77×10^{-4}	2.40×10^{-4}	2.30	16.00	3.36	3.35
XVIII + XII (2.0×10^{-4} *M*)	6	6.75×10^{-4}	6.91×10^{-4}	6.62	16.00	3.36	2.72
XVIII + XIV (2.0×10^{-4} *M*)	8	4.49×10^{-4}	8.80×10^{-4}	2.04	15.20	3.17	1.20
Thyroxine and rat serum albumin:							
Thyroxine alone	6	1.19×10^{-3}	1.23×10^{-3}	—	—	—	—
Thyroxine + II (1.0×10^{-4} *M*)	4	9.68×10^{-4}	8.08×10^{-4}	1.52	18.00	3.71	1.43
Thyroxine + XII (2.0×10^{-4} *M*)	5	1.16×10^{-3}	9.76×10^{-4}	1.26	28.70	3.50	1.13
Thyroxine + XII (1.0×10^{-4} *M*)	5	1.52×10^{-3}	1.07×10^{-3}	1.15	28.70	3.50	0.39
Thyroxine + XIV (1.0×10^{-4} *M*)	7	8.04×10^{-4}	7.56×10^{-4}	1.63	11.40	3.25	2.25

[a] Calculated by a reported method (11). [b] From "Scientific Tables," K. Diem and C. Lentner, Eds., J. R. Geigy S. A., Basle, Switzerland, 1970.

tion employed for clofibrate, computer analysis of data obtained *in vitro* using human serum albumin predicted that all analogs would be 85–95% bound to serum proteins in humans.

Competitive Binding between XVIII and II, L(*S*)-IV, D(*R*)-IV, XIV, and XV on Bovine Serum Albumin—The binding of XVIII to bovine serum albumin was studied in the presence of a constant total concentration of II where three levels of II were used (1.0×10^{-4}, 2.0×10^{-4}, and 5.0×10^{-4} *M*). The results represented as Scatchard plots are given in Fig. 2. As the concentration of II increases, displacement of XVIII from its binding sites also increases. By using values of *r* (moles XVIII bound per mole bovine serum albumin) of approximately 1 and less, where the first class of sites is primarily affected, the data of Fig. 2 were replotted as $1/r$ *versus* $1/[\text{XVIII}]_{\text{free}}$ to determine if II and XVIII are competing for the same binding site on the albumin. If the same extrapolated $1/r$ intercept is obtained for each concentration of II as found for XVIII alone, the conclusion can be reached that the same site is involved (23). Figure 3 shows the effect of the three different total concentrations of II on the binding of XVIII to bovine serum albumin. The linear relationships obtained at the three levels of II extrapolated to essentially the same intercept on the $1/r$ axis as found for XVIII alone (*i.e.*, 0.38). Linear regression analysis of the data yielded $1/r$ intercepts of 0.35, 0.43, and 0.39 for II at 1.0×10^{-4}, 2.0×10^{-4}, and 5.0×10^{-4} *M*, respectively. The values for the intercepts are not statistically different at $p = 0.01$ as shown by the Student *t* test (Table V). The validity of the use of the test was described previously (11).

Four other analogs [L(*S*)-IV, D(*R*)-IV, XIV, and XV] were studied to determine if they also competed with XVIII at the same binding site on bovine serum albumin. Reciprocal plots using data at low *r* values gave the results presented in Fig. 4 where a constant total level of analog was used (1.0×10^{-4} *M*). The intercepts obtained for L(*S*)-IV, D(*R*)-IV, XIV, and XV by regression analysis were 0.44, 0.39, 0.30, and 0.42, respectively. The values for these intercepts also are not statistically different at $p = 0.01$ as shown by the Student *t* test (Table V).

Moriguchi Method—The binding of the analogs by bovine serum albumin using Moriguchi's method gave the results presented in Table VI. For purposes of comparison, the K_1 values obtained for binding of the analogs to bovine serum albumin using ultrafiltration are also presented in Table VI. In terms of decreasing K_1 values, the results obtained using Moriguchi's method rank the analogs as follows: XII, XVII, XIV, VIII, XV, II, D(*R*)-IV, and L(*S*)-IV. The results obtained using ultrafiltration, on the other hand, rank the analogs as follows: XIV, L(*S*)-IV, XII, XVII, D(*R*)-IV, XV, II, and VIII. There is no apparent similarity in the ordering of the analogs using the two methods.

Competitive Binding between XVIII and II, XII, and XIV to Rat Serum Albumin—The binding of XVIII to rat serum albumin was studied in the presence of a constant total concentration of II where two levels of II were utilized (2.0×10^{-4} and 5.0×10^{-4} *M*). The results, represented as Scatchard plots, are given in Fig. 5. To determine if the same binding site is involved in the displacement, reciprocal plots were constructed for those binding data taken at $r = 1$ or less, where the strong primary site for XVIII would likely be involved. Plots of $1/r$ *versus* $1/[\text{XVIII}]_{\text{free}}$

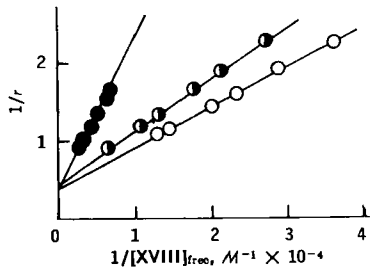

Figure 6—*Relationship between 1/r (moles rat serum albumin per mole XVIII) and 1/[XVIII]free at low r values in the presence of II. Key: ○, no II; ◑, 2.0 × 10⁻⁴ M II; and ●, 5.0 × 10⁻⁴ M II.*

at the different concentrations of II are given in Fig. 6. Linear regression analysis of the data yielded $1/r$ intercepts of 0.37 for XVIII alone and 0.45 for II at levels of 2.0×10^{-4} and 5.0×10^{-4} M. The value for the intercept is statistically different at $p = 0.01$ for II at a level of 2.0×10^{-4} M, but it is not statistically different for II at a level of 5.0×10^{-4} M as shown by the Student t test (Table V).

Two additional analogs, XII (an active hypolipemic agent) and XIV (an inactive analog), were studied to determine if they also competed with XVIII at the same binding site on rat serum albumin. Reciprocal plots using data taken at low r values gave the results presented in Fig. 7, where a constant total level (2.0×10^{-4} M) of analog was used. The $1/r$ intercepts obtained for XII and XIV by regression analysis were 0.46 and 0.36, respectively. The values for the intercepts are not statistically different at $p = 0.01$ as shown by the Student t test (Table V).

Binding of Thyroxine to Rat Serum Albumin—The binding of thyroxine to rat serum albumin gave results plotted in Fig. 8. The solid line in Fig. 8 is the computer fit for the data using the binding parameters generated assuming that two independent classes of binding sites exist. It is apparent that this assumption adequately describes the binding of thyroxine to rat serum albumin. The computer-generated values are: $n_1 = 0.49$, $K_1 = 1.51 \times 10^6$ M^{-1}, $n_2 = 3.5$, and $K_2 = 8.2 \times 10^4$ M^{-1}.

Competitive Binding between Thyroxine and II, XII, and XIV on Rat Serum Albumin—The binding of thyroxine to rat serum albumin was studied in the presence of a constant total concentration of XII where two levels of XII were employed (1.0×10^{-4} and 2.0×10^{-4} M). The results, represented as Scatchard plots, are given in Fig. 9. As the concentration of XII increases, displacement of thyroxine also increases. To determine if the same binding site is involved in the displacement, reciprocal plots were constructed for those binding data taken at low r values, where the strong primary site for thyroxine would most likely be involved. Plots of $1/r$ *versus* $1/[\text{thyroxine}]_{free}$ at the different concentrations of XII are given in Fig. 10. The linear relationships obtained at the two levels of XII extrapolated to essentially the same intercept on the $1/r$ axis as that found for thyroxine alone (0.57). Linear regression analysis of the data yielded $1/r$ intercepts of 0.59 and 0.62 for XII at levels of 1.0×10^{-4} and 2.0×10^{-4} M, respectively. The values for the intercepts are not statistically different at $p = 0.01$ as shown by the Student t test (Table V).

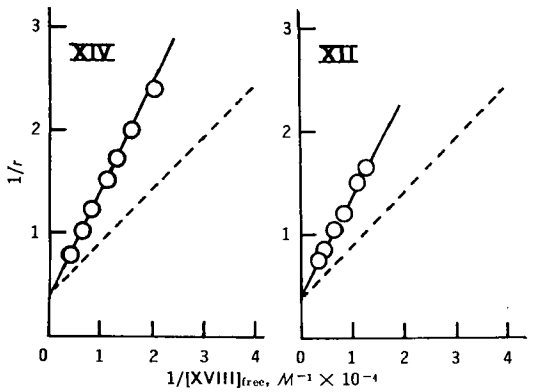

Figure 7—*Relationship between 1/r (moles rat serum albumin per mole XVIII) and 1/[XVIII]free at low r values in the presence of 2.0 × 10⁻⁴ M XII and XIV. The broken line is XVIII alone, having an intercept of 0.37.*

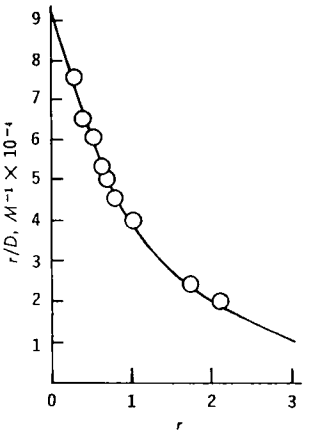

Figure 8—*Scatchard plot for the binding of L-thyroxine to rat serum albumin (7.5×10^{-6} M) at pH 7.4. Key: ○, experimental values; and ——, computer fit assuming two independent classes of binding sites exist on serum albumin.*

Two other analogs (II and XIV) were studied to determine if they also competed with thyroxine at the same binding site on rat serum albumin. Reciprocal plots using data at low r values gave the results presented in Fig. 11, where a constant total level (1.0×10^{-4} M) of analog was used. The intercepts obtained for II and XIV by regression analysis were 0.50 and 0.67, respectively. The values for the intercepts are not statistically different at $p = 0.01$ as shown by the Student t test (Table V).

Octanol-Water Partition Coefficients of Analogs—The octanol–water partition coefficients of the analogs were determined at a pH two units below the pKa of each analog. The log P values for the analogs are presented in Table III. The experimental log P values are in fairly good agreement with the calculated log P values, using the Fujita *et al.* (24) log P value for phenoxyacetic acid (1.21) as a reference.

DISCUSSION

Clofibrate is rapidly hydrolyzed by tissue and serum esterases *in vivo* and *in vitro* to the active hypolipemic acid II (3). Like I, esters III, VII, XI, and XVI also rapidly undergo hydrolysis by rat plasma *in vitro*. Therefore, the differences in hypolipemic activity observed previously (10) cannot be explained on the basis of differential rates of hydrolysis to the active free acids.

To explore further the possibility that the differential hypolipemic effects of these analogs are related to the physicochemical properties of the free acids, the pKa's for a number of these analogs were determined. As expected, the pKa's of the analogs do not differ greatly and fall in the 3.90–4.46 range (Table II). Hence, there is no simple relationship between biological activity and pKa among these structurally modified analogs of clofibrate. The log P values for the carboxylic acid analogs [II, D(*R*)-IV, VI, VIII, XII, XIV, XV, and XVII], experimentally determined at a pH two units below their respective pKa's, are shown in Table III. These values are in agreement with those values calculated from the experimentally determined log P value for phenoxyacetic acid (24) and may be used to predict distribution of drugs *via* a random-walk process to sites of action (25). While the two most

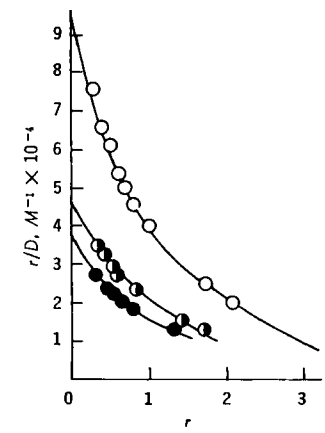

Figure 9—*Scatchard plot for the binding of L-thyroxine to rat serum albumin (7.5×10^{-6} M) in the presence of various total concentrations of XII. Key: ○, L-thyroxine alone; ◑, 1.0×10^{-4} M XII; and ●, 2.0×10^{-4} M XII.*

Table VI—Comparison of K_1 Obtained by the Moriguchi Method with K_1 Obtained by Ultrafiltration for Binding of Analogs to Bovine Serum Albumin

Analog	K_1, $M^{-1} \times 10^{-4}$ (Moriguchi Method)	K_1, $M^{-1} \times 10^{-4}$ (Ultrafiltration Method)
XII	7.94	3.78
XVII	6.31	2.64
XIV	6.31	6.46
VIII	2.51	1.16
XV	2.00	1.55
II	1.59	1.28
D(*R*)-IV	1.00	2.64
L(*S*)-IV	0.63	5.97

active hypotriglyceridemic and hypocholestrolemic drugs (I and XI) also have the largest log P values for their corresponding carboxylic acids, the relative activity of the other analogs shows no apparent relationship to partition coefficients. Because of their similar pKa's and the lack of correlation of log P with hypolipemic activity, it seems that differences in activity observed *in vivo* are not due to major differences in absorption and distribution but rather to structural differences that either enhance or decrease the affinity of the molecule for certain enzymes at sites of action or loss.

Several mechanisms of action have been proposed for the hypolipemic effect of clofibrate. Of immediate interest is the thyroxine displacement mechanism proposed by Platt and Thorp (26). In this hypothesis, the free acid hydrolysis product of clofibrate is proposed to exert its effect by displacing thyroxine from its binding proteins in the plasma and the liver. The result is presumed to be increased lipid metabolism with concomitant lowering of serum lipid levels because of the hyperthyroid effect in the liver.

To explore the hypothesis that these compounds exert their action indirectly by displacing thyroxine from plasma proteins, the binding parameters for these analogs as well as the nature of the sites to which they are bound were investigated, and these results were compared with binding studies involving thyroxine and dye XVIII which, as previously shown, mirrors the binding of thyroxine (11). Since prevous studies in these laboratories detected species differences in the binding of dye XVIII and acids II, L(*S*)-IV, and D(*R*)-IV (27), a thorough investigation of the binding of the compounds to bovine, rat, and human serum albumins was in order before any further conclusions concerning the relationship between albumin binding parameters and biological activity could be made. Bovine serum albumin has been used by several workers for studying the binding of thyroxine (13, 28, 29) and its displacement by drug entities (27, 30). Studies utilizing rat serum albumin are important since the biological evaluation of these compounds in rats has been performed. Studies utilizing human serum albumin are important since such studies may ultimately reflect or be predictive of hypolipemic activity in humans (9). The results show that thyroxine binds to rat serum albumin at site n_1 with $K_1 = 1.51 \times 10^6\ M^{-1}$ and that this value is similar to the binding of thyroxine at site n_1 on bovine serum albumin ($K_1 = 1.6 \times 10^6\ M^{-1}$) (29). Similarly, the results obtained for the binding of the analogs to bovine, rat, and human serum albumins (Table IV) also show only minor species differences. For all studies involving albumin derived from these three species, both active and inactive analogs bind similarly at site n_1. Since the primary site n_1 is the major site for thyroxine binding, the possibility that these compounds may be able to displace the hormone *in vitro* was investigated.

To test differences in the ability of the analogs to displace thyroxine, the optical probe 2-(4′-hydroxybenzeneazo)benzoic acid (XVIII) was used as a convenient means to mirror thyroxine binding to serum albumin in order to determine whether earlier reported work (27) had biological relevance. In a previous paper (11), it was shown that thyroxine competed with XVIII at the same binding site on bovine and rat serum albumins. Competitive binding studies between XVIII and five analogs [II, L(*S*)-IV, D(*R*)-IV, XIV, and XV] to bovine serum albumin were carried out to determine whether the five analogs compete with XVIII at the same binding site on the albumin. The results (Figs. 3 and 4) show that all analogs compete with XVIII at the same binding site on

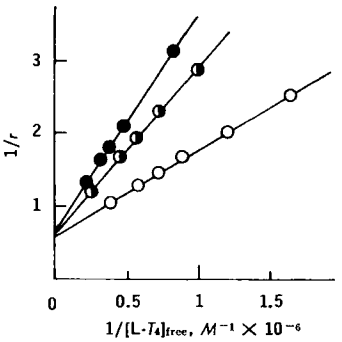

Figure 10—*Relationship between 1/r (moles rat serum albumin per mole* L-*thyroxine) and the reciprocal of free* L-*thyroxine concentration* (L-T_4) *at low r values in the presence of XII. Key:* ○, *no XII;* ◑, *1.0* × 10^{-4} M *XII; and* ●, *2.0* × 10^{-4} M *XII.*

bovine albumin. It may be concluded, therefore, that these acids could theoretically displace thyroxine from its binding site on the albumin. However, the primary binding constant for thyroxine is much greater than the primary binding constant for any analog studied. Similar results were obtained for rat serum albumin using analogs II, XII, and XIV; however, binding studies in greater detail were carried out with bovine serum albumin owing to the high cost of rat serum albumin.

The slope of a plot of $1/r$ *versus* $1/[\text{XVIII}]_{\text{free}}$ at low r values is $1/nK$, where n would approximate the number of primary sites for XVIII on bovine serum albumin and K is the apparent primary binding constant for XVIII. The reciprocal plot for XVIII alone, having an intercept of 0.38, shows n to be 2.6. The computer fit of the Scatchard curves shows n_1 to be 1.7 ± 0.2 (average ± average deviation of five determinations). Reciprocal plots assuming one class of sites normally generate a high value for n as compared to a multiple-class model, so a high n value in the reciprocal treatment was expected (31). The slope of the reciprocal plot for XVIII alone predicts K_1 to be 18,800 M^{-1}, whereas the computer fit for the Scatchard treatment of the two class assumption gives $K_1 = 23{,}000 \pm 3000$ (average ± average deviation of five determinations). A lower value for K in the reciprocal treatment assuming only one class of sites is to be expected (31).

In the presence of an inhibitor, the binding constant, K, obtained for the binding of XVIII should be related to the binding constant in the absence of the inhibitor, K', by Eq. 3 (31):

$$K = \frac{K'}{1 + K_c(C_f)} \qquad \text{(Eq. 3)}$$

Thus, K for XVIII in the presence of the inhibitor depends on the binding constant, K_c, and the free concentration of the inhibitor, (C_f). Therefore, the greater the value of $K_c(C_f)$, the greater is the slope of the reciprocal plot. The slopes of the reciprocal plots for XVIII in the presence of 1.0×10^{-4} *M* II, L(*S*)-IV, D(*R*)-IV, XIV, and XV are 2.7×10^{-4}, 3.2×10^{-4}, 3.4×10^{-4}, 3.7×10^{-4}, and 3.4×10^{-4} *M*, respectively. The effectiveness of these compounds

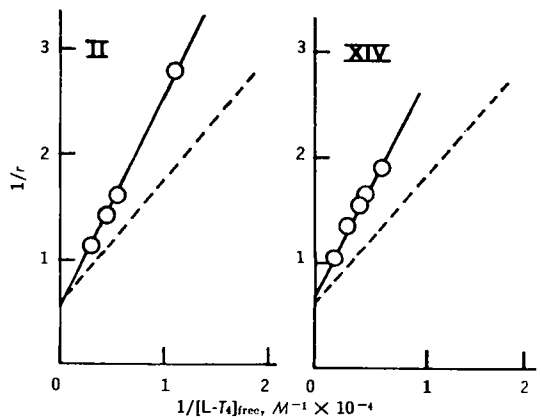

Figure 11—*Relationship between 1/r (moles rat serum albumin per mole* L-*thyroxine) and the reciprocal of free* L-*thyroxine concentration* (L-T_4) *at low r values in the presence of 1.0* × 10^{-4} M *II and XIV. The broken line is* L-*thyroxine alone, having an intercept of 0.57.*

to displace thyroxine from bovine serum albumin stand in the order XIV, XV, D(*R*)-IV, L(*S*)-IV, and II based on the assumption that the concentration of the inhibitor free (C_f) is the same in each case and that the small differences in the observed slopes are significant.

Moriguchi and coworkers (19, 20) also used XVIII in their binding studies with bovine serum albumin. These studies were carried out at low concentrations of XVIII, and the maximum r value that was obtained was about 1.5. They indicated that their data were best fitted by the equation:

$$\log \{r/(n - r)\} = m \log A + m \log K \qquad \text{(Eq. 4)}$$

where r represents the moles of bound drug A per mole of total albumin, A is the concentration of unbound A, K is the binding constant, m is an empirical parameter, and n is the number of binding sites on a single molecule of albumin. This equation is based on the assumption that binding to serum albumin is affected by electrostatic interactions altering binding affinity. Such an assumption differs from the one used in this study, which assumes that all binding sites are independent but that more than one class of sites exist. The validity of Eq. 4, where n had to assume a value of 2.0, was based solely on best fit of data. This assumption is not valid since the results of previous investigations (11) showed that r values much greater than 2.0 can be obtained. The results obtained for the binding of the analogs to bovine serum albumin using Moriguchi's method are listed in Table VI; comparison of these values with those derived from ultrafiltration showed differences in rank ordering of the analogs. From these data, it was concluded that Moriguchi's method is not applicable.

Recognizing that binding to human serum albumin is pertinent and that binding might depend on species differences, the binding of the analogs to human serum albumin was also studied. In humans, three carrier proteins for thyroxine were identified: *(a)* thyroxine binding prealbumin, which has an anodal mobility greater than that of serum albumin; *(b)* thyroxine binding globulin, with an electrophoretic mobility between α_1- and α_2-globulin; and *(c)* serum albumin (32). When tracer quantities of ^{131}I-thyroxine are added to serum and electrophoresis is performed in a glycine–acetate buffer at pH 8.6 (33), approximately 30% of the radioactivity is associated with the prealbumin, 60% with thyroxine binding globulin, and the remaining 10% with serum albumin (34). The prealbumin and binding globulin preparations are not available commercially, so the present studies were done with human serum albumin. The results obtained (Table IV) show no marked differences in K_1 among the analogs; *i.e.*, active and inactive analogs bind similarly. The percent of each analog that would be expected to be bound to serum albumin was calculated using the parameters listed in Table IV through the use of a published computer program (21), as described in the *Results* section. There is no dramatic difference in the amount of each analog that would be expected to be bound to human serum albumin, although active analogs II, VIII, and XII showed a slightly larger percent bound than analog XIV which was inactive in the hyperlipemic rat model (10). Furthermore, the differential hypocholesterolemic and hypotriglyceridemic activity observed for VIII could not be predicted on the basis of these albumin binding studies.

In the rat, thyroxine is bound to both an α-globulin and albumin (35), and it seemed that attempts to correlate hypolipemic activity *in vivo* using rats with rat serum binding parameters would have greater validity. The results obtained for the binding of analogs to rat serum albumin (Table IV) show that both active and inactive analogs have similar binding parameters. There are no marked differences in K_1 among the analogs except X and XIV. Analog X differs from the other analogs in that it binds to only one site and has a low K_1 value. Why this analog is the only one that binds in this fashion is not understood. Analog XIV has a primary binding constant that is approximately twice that of the other analogs. Newman *et al.* (10) showed that both X and XIV are biologically inactive in hyperlipemic rats *in vivo*. Therefore, it would seem that Thorp's displacement mechanism cannot be invoked to differentiate the varying activity among the analogs, since most analogs essentially bind to the same extent to rat serum albumin and differences in binding by X and XIV do not account for their inactivity *in vivo*. Furthermore, the differential hypocholesterolemic effects of I, D(*R*)-III, and L(*S*)-III in normocholesterolemic Swiss Webster rats (15) cannot be accounted for on the basis of differential binding of the corresponding free acids to rat serum albumin *in vitro*. In fact, D(*R*)-IV binds to a slightly greater extent than L(*S*)-IV, but ester L(*S*)-III is active *in vivo*.

To substantiate this conclusion, the binding of thyroxine to rat serum albumin was determined by equilibrium dialysis at 37° using ^{125}I-thyroxine. These studies were necessary to confirm that data obtained using the optical probe XVIII indeed can be used to determine thyroxine binding to, and displacement from, serum albumin. Competitive binding studies between thyroxine and three analogs (II, XII, and XIV) were carried out to determine if the three analogs do indeed displace thyroxine at the same binding site on rat serum albumin. The results (Figs. 10 and 11) show that the three analogs compete with thyroxine at the same binding site on rat serum albumin. From earlier studies (11) utilizing the optical probe XVIII and rat serum albumin, it was shown that thyroxine displaces XVIII from the same binding site on rat serum albumin; from the present studies, it was shown that the analogs used also displace XVIII from the same binding site on rat serum albumin. Therefore, it was concluded that the analogs could displace thyroxine from its binding site on this albumin. The results of competitive binding studies between thyroxine and the analogs to rat albumin show that the analogs do displace thyroxine from the same (primary) binding site on the albumin. This finding confirms the results obtained using the probe XVIII and substantiates the validity of the use of the probe XVIII to mimic thyroxine binding to rat serum albumin. Analogs II and XII are biologically active while analog XIV is inactive in hyperlipemic rats (10); since all three analogs displace thyroxine from its binding site on rat serum albumin under the experimental conditions *in vitro*, and the ester XIII of analog XIV is inactive but readily undergoes hydrolysis by serum esterases *in vitro*, it seems that thyroxine displacement determined *in vitro* is not related to activity *in vivo*. Rather, albumin binding *in vivo* likely is important for drug transport, and the mode of action of these compounds involves their selective influence on lipid regulating enzymes. Work designed to probe the differential enzymatic mechanisms of action of clofibrate and related analogs is continuing in these laboratories.

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Simple and Specific Assay of Penicillins by IR Spectrophotometry in Deuterium Oxide and Dimethyl Sulfoxide Solutions

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Abstract □ An IR spectrophotometric method for the qualitative and quantitative analysis of penicillins is described. The method is based on the inspection of the carbonyl region of the IR spectrum of penicillins dissolved in deuterium oxide or dimethyl sulfoxide solution and on the measurement of the absorbance of the β-lactam band at about 1760 cm^{-1}. Most common natural and synthetic penicillins are amenable to the present IR analysis in solution, without previous derivatization. The accuracy of the method is better than ±2% and is thus comparable to that usually attainable by current iodometric procedures. Since the solution spectra in the analytical region are characteristic for the individual penicillins, the described method is superior to iodometry as far as specificity is concerned. A single analysis usually requires no more than 15 min. The method allows a direct evaluation of the stability of penicillins in aqueous (deuterium oxide or deuterium chloride) solutions. Usual salts or buffers generally do not interfere with the analysis. Some qualitative and quantitative aspects of the IR spectra of penicillins in solution are discussed.

Keyphrases □ Penicillins—IR spectrophotometric analysis in deuterium oxide, deuterium chloride, or dimethyl sulfoxide □ Deuterium oxide—solvent for IR spectrophotometric analysis of penicillins □ Dimethyl sulfoxide—solvent for IR spectrophotometric analysis of penicillins □ IR spectrophotometry—analysis, penicillins, deuterium oxide, deuterium chloride, or dimethyl sulfoxide solutions

Current chemical methods for penicillin assay, such as those based on iodometric (1, 2) and acidimetric (3) titrations, are indirect and nonspecific. UV spectrophotometry is of limited applicability in penicillin analysis. The aminopenicillanic moiety of penicillins displays only end-absorption in the UV region. Only the degradation products of penicillins and a few compounds related to penicillins (including cephalosporins) display characteristic UV absorption bands (4). Because of the lack of specificity of the iodometric and acidimetric methods, it is also a common practice to characterize penicillins from their IR spectra. Such spectra, usually obtained on solid samples, are excellent "fingerprints" for individual penicillins, the elucidation of the structure of penicillins having actually been one of the oldest applications of IR spectroscopy (5).

It should be attractive to use the IR spectra for both the qualitative and quantitative analysis of penicillins. However, the IR spectra run on solid samples are not easily amenable to quantitative measurements. In fact, the absorbance of the IR bands is strongly affected by scattering and crystallinity effects (6). The logical approach to an IR quantitative analysis is to work on samples in solution. An IR assay in chloroform solution of a number of semisynthetic penicillins in the acid form was reported (7). Since most important penicillins are in the salt form, this method requires acidification and solvent extraction. Moreover, the chloroform method does not apply to ampicillins (7).

In principle, water could be taken into consider-