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Use of the Fluorescence Probe 1-Anilino-8-naphthalenesulfonate in Predicting Interindividual Differences in the Plasma Protein Binding of Acidic Drugs in Rats

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Abstract □ The possibility that the fluorescence probe, 1-anilino-8-naphthalenesulfonate (I), might be used for predicting the interindividual differences in the plasma protein binding of acidic drugs was examined. The interindividual differences in the free fraction of I (f_I) were found not to be due to corresponding differences in plasma albumin concentration, but to those differences in binding constant. The binding constant of I to the plasma of 18 individual rats ranged from 1.75×10^6 M^{-1} to 2.3×10^7 M^{-1}. The free fraction of I had a highly significant statistical correlation with plasma concentration of free fatty acids, but had no significant correlation with the infinite fluorescence of I or the degree of the polarization of I. Each free fraction of five acidic drugs (warfarin, phenylbutazone, salicylic acid, indomethacin, and sulfaphenazole) was correlated with f_I with high statistical significance. Therefore, the simple and convenient method using I may predict the interindividual differences in the plasma protein binding of acidic drugs in rats.

Keyphrases □ 1-Anilino-8-naphthalenesulfonate—fluorescence probe, prediction of interindividual differences in plasma protein binding, acidic drugs, rats □ Fluorescence probe—1-anilino-8-naphthalenesulfonate, prediction of interindividual differences in plasma protein binding, acidic drugs, rats □ Binding, plasma protein—1-anilino-8-naphthalenesulfonate, prediction of interindividual differences, acidic drugs, rats

Serum (or plasma) protein binding can have pronounced effects on the pharmacodynamic and toxicological actions of drugs, as well as on their elimination kinetics. This effect is particularly striking with respect to the elimination of the extensively serum protein-bound drugs such as anticoagulants (warfarin, dicoumarol) by rats and humans, since there are pronounced interindividual differences in the free fraction of these drugs (1, 2). Minor interindividual changes in the degree of binding of such highly protein-bound drugs can produce significant changes in the amount of unbound drug. Differences in the structure of albumin or in the plasma concentration of endogenous inhibitory agents were suggested to be alternative reasons for such pronounced interindividual differences in the free fraction of these drugs (1); however, the details are, as yet, unresolved. Based on these facts, Levy and Yacobi (3) suggested that *in vitro* plasma protein binding tests with blood samples prior to drug administration might be useful for predicting quantitatively unusual distribution, elimination, and pharmacological effect characteristics of certain highly plasma protein-bound drugs.

It was previously reported that there are pronounced interindividual differences in binding of the anionic fluorescence probe 1-anilino-8-naphthalenesulfonate (I) to rat plasma (4). Free fatty acids were found to be one of the endogenous inhibitors, and there was a highly significant correlation between the free fractions of I and phenylbutazone (4).

In the present study, we investigated further the correlation between the free fraction of I and those of various acidic drugs extensively bound to plasma protein, and suggested the possibility that I might be used clinically for predicting the interindividual differences in the plasma protein binding of acidic drugs. Furthermore, we tried to examine whether or not differences in the structure of albumin are associated with differences in the free fraction of I.

EXPERIMENTAL

Materials—The following analytical-grade materials were used: 1-anilino-8-naphthalenesulfonate (I) as the sodium salt[1], warfarin[2], indomethacin[3], sulfaphenazole[4], sodium salicylate[5], sodium laurate[6], sodium palmitate[6], sodium stearate[6], sodium oleate[6], and rat serum albumin (fraction V)[7]. The rat serum albumin was defatted by the method of Chen (5).

Plasma Preparation—Adult male Wistar rats, weighing 290–340 g, were used. Approximately 10 min after heparinizing, blood was collected from the carotid artery under light ether anesthesia, and the plasma was obtained by centrifugation (3000 rpm for 10 min). Prepared plasma (2.5 mL) was used on the same day for measuring the bindings of drugs, and the remaining plasma (2 mL) was divided into the three aliquots, which were stored at −40°C and used within 1 week for the measurements of the concentrations of albumin, free fatty acids, and total bilirubin.

Equilibrium Dialysis—The 2-mL fresh plasma sample was diluted to 25% by the addition of 6 mL of Tris-HCl buffer (50 mM, pH 7.4). The bindings of four acidic drugs (sulfaphenazole, salicylic acid, indomethacin, and phenylbutazone) to the dilute plasma were measured by equilibrium dialysis (25°C). The details of the equilibrium dialysis techniques were described previously (4, 6).

[1] Tokyo Chemical Industries, Co., Tokyo, Japan.
[2] Eizai Co., Tokyo, Japan.
[3] Merck Banyu Co., Tokyo, Japan.
[4] Dainippon Seiyaku Co., Tokyo, Japan.
[5] Koso Chemical Co., Tokyo, Japan.
[6] Tokyo Kasei Industries, Co., Tokyo, Japan.
[7] Sigma Chemical Co., St. Louis, Mo.

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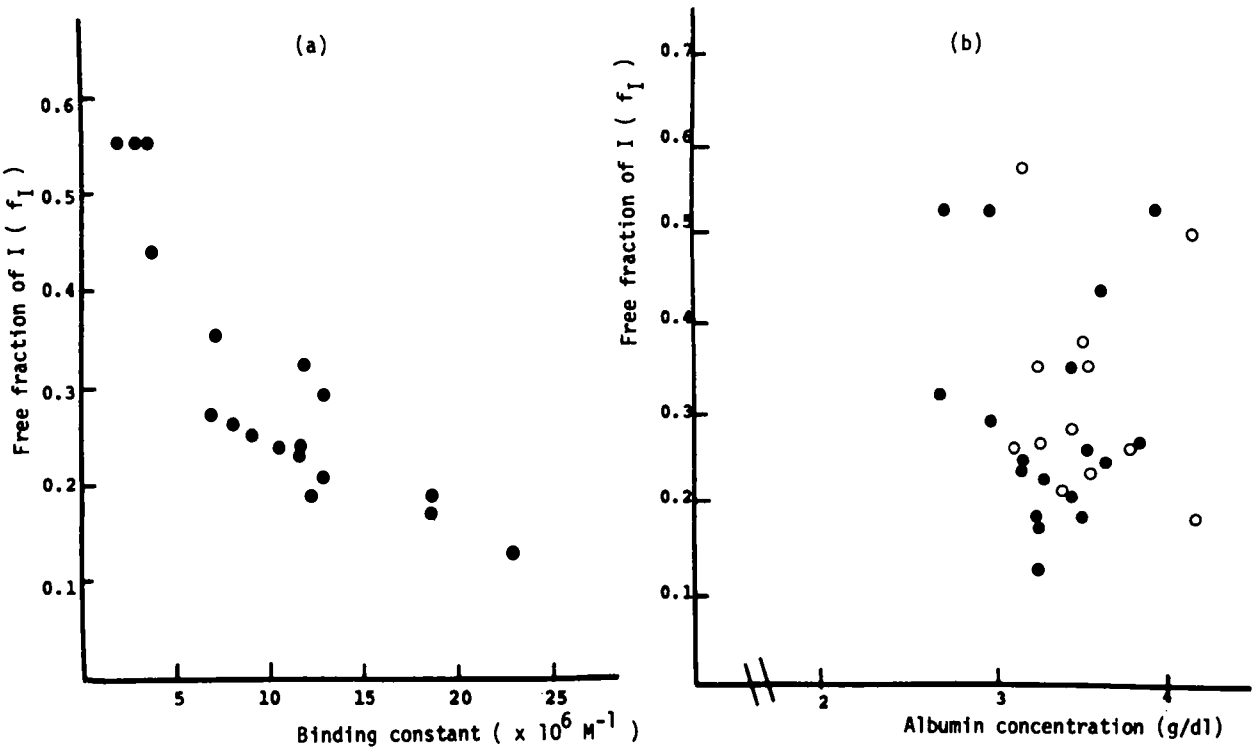

Figure 1—*Relationship between the free fraction of I and the binding constant (a) and albumin concentration (b) in the plasma of individual rats. Rat plasma diluted to 0.2% was used for the determination of f_I at a I concentration of 1 μM using the fluorescence method as described in the text. Correlation coefficient for the binding constant = −0.858, $p < 0.001$. Correlation coefficient for the albumin concentration = −0.072 (not significant). Key: (●) rats 1–18; (○) rats 19–32.*

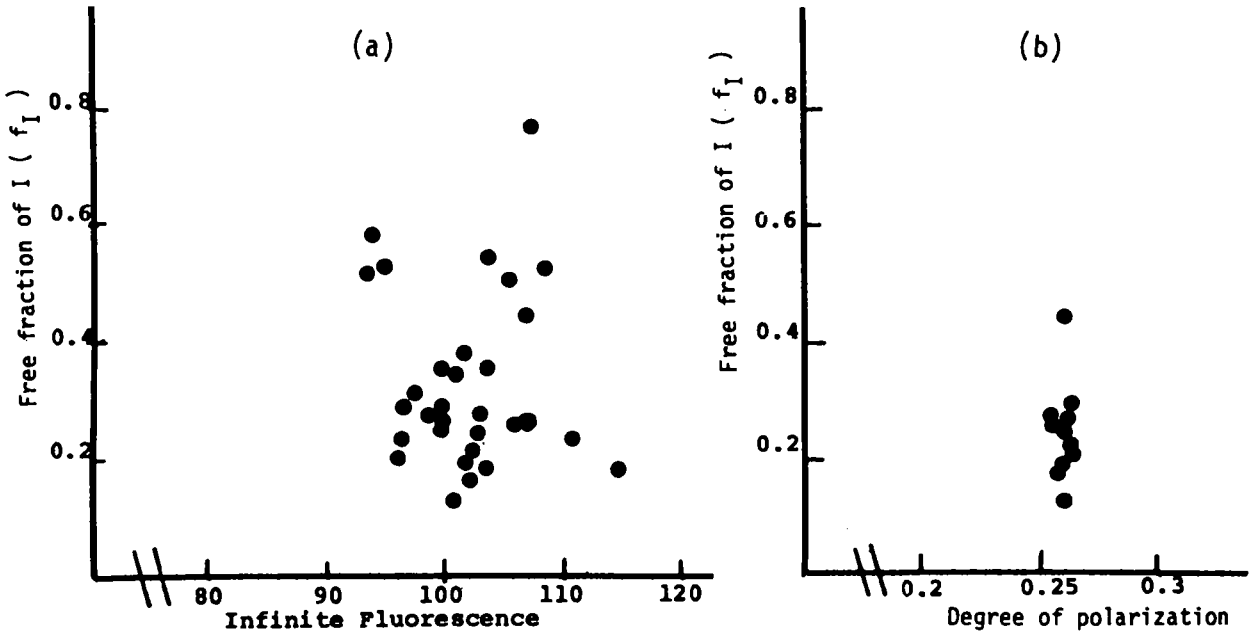

Figure 2—*Relationship between f_I and the infinite fluorescence of I (a) and the degree of polarization (b) in the plasma of individual rats. (See text for details.) Correlation coefficient for infinite fluorescence = −0.368 (not significant), using rats 1–32; correlation coefficient for degree of polarization = 0.258 (not significant), using rats 8–18.*

Fluorescence Measurement—Fluorometric measurements[8] were performed in the same buffer as above at room temperature (19–23°C). The bindings of I and warfarin to plasma were measured by the fluorescence method.

Binding of I.—The excitation and emission wavelengths were 400 and 480 nm, respectively. Titration of 1 μM of I with plasma was used to determine the infinite fluorescence when this concentration of I was completely bound. The infinite fluorescence thus obtained shows the fluorescence quantum yield of bound I and may reflect the changes in the environment at the binding site.

A reverse titration of 0.3% plasma with I was then carried out to obtain the binding parameters. Details of this titration procedure have been described previously (4, 6). The bound fraction of I (X_1) was given by:

$$X_1 = F_{obs}/F_{bound} \quad \text{(Eq. 1)}$$

where F_{obs} is the observed fluorescence and F_{bound} is the infinite fluorescence when a given concentration of I is completely bound. The free fraction of I (f_I) was then calculated by:

$$f_I = 1 - X_I \quad \text{(Eq. 2)}$$

[8] Hitachi MPF-4 fluorescence spectrometer.

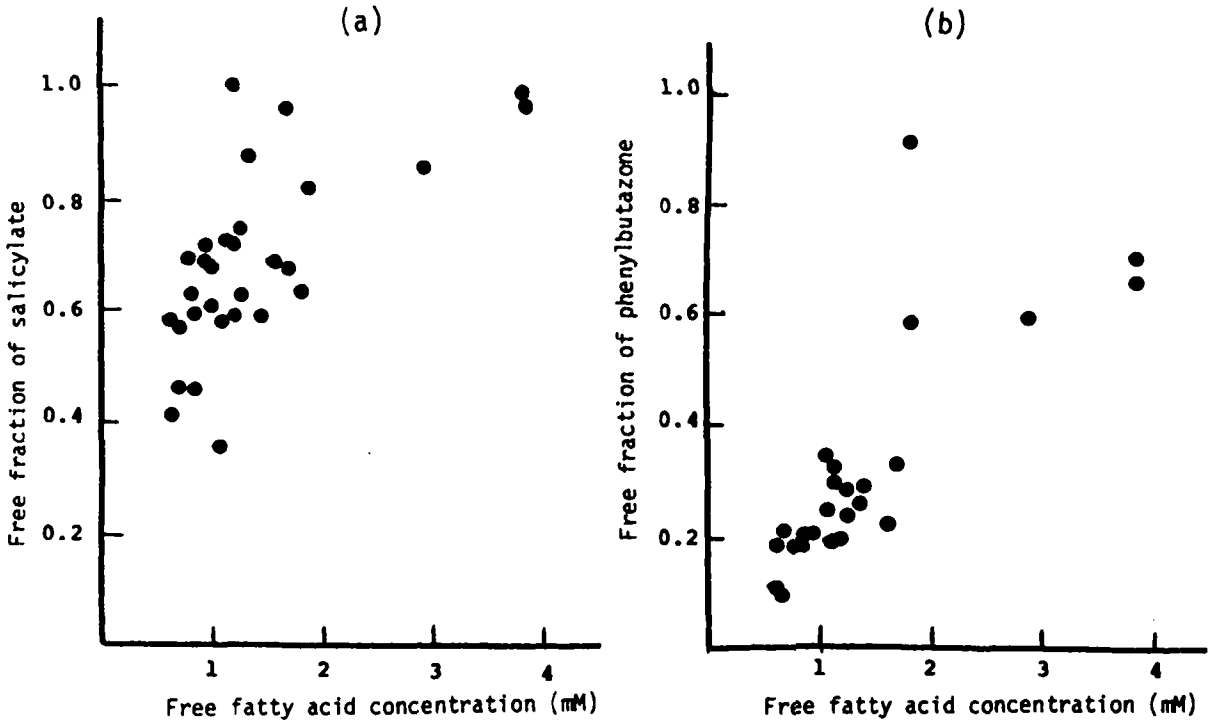

Figure 3—*Relationship between free fatty acid concentration and the free fractions of salicylate (a) and phenylbutazone (b) in the plasma of individual rats, determined by equilibrium dialysis using rat plasma diluted to 25% and initial drug concentrations of 0.1 mM. Correlation coefficient for salicylate = 0.661 ($p < 0.001$) using rats 3–32; correlation coefficient for phenylbutazone = 0.761 ($p < 0.001$) using rats 8–32.*

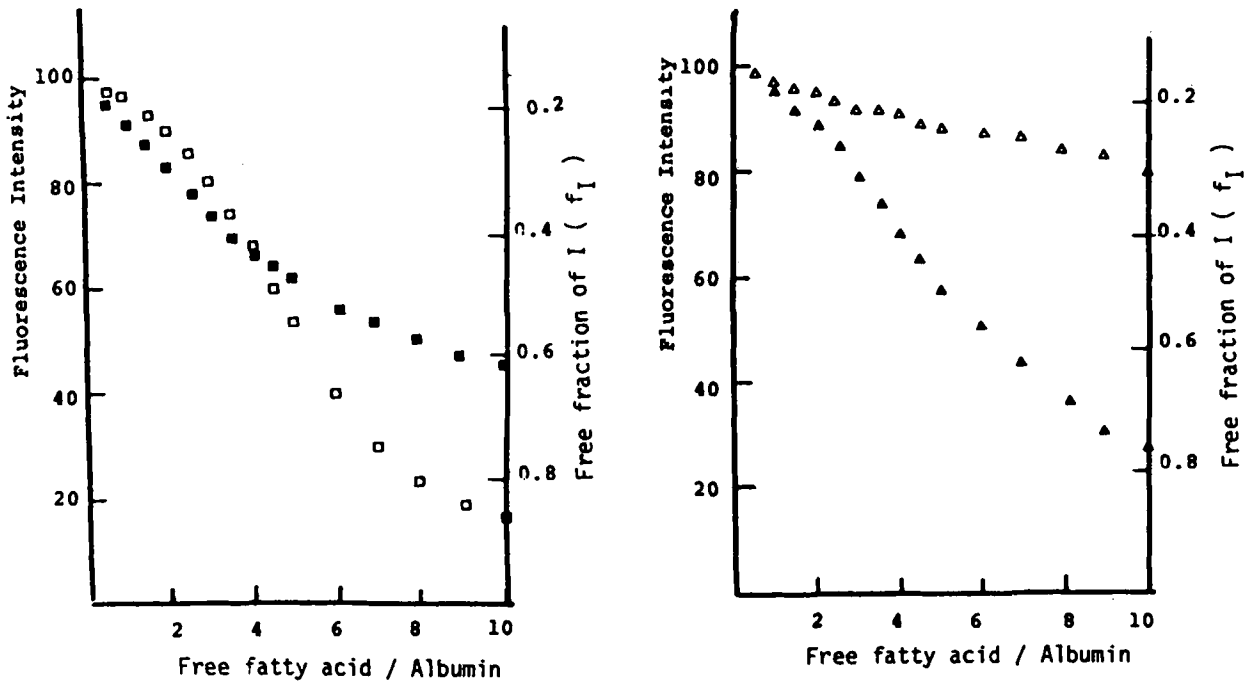

Figure 4—*Effect of various free fatty acids on the fluorescence of I with 1 μM defatted rat albumin and 1 μM of I. The fluorescence intensity in the absence of free fatty acids was set at 100 U. Fluorescence intensities (left ordinate) were converted to free fractions of I (right ordinate). Key: (□) oleic acid; (■) lauric acid; (△) stearic acid; (▲) palmitic acid.*

To study the conformational changes in the structure of plasma proteins, the polarization of the fluorescence of I bound to plasma proteins (mainly albumin) was measured using excitation and emission wavelengths of 380 and 480 nm, respectively. The degree of polarization, P, was calculated as:

$$P = (F_{par} - hF_{per})/(F_{par} - hF_{per}) \quad \text{(Eq. 3)}$$

where F_{par} and F_{per} refer to the intensity of the fluorescence light polarized parallel and perpendicular to the exciting light, respectively, and h is a correction factor which accounts for the selective transmission of the monochromatic light and the selective reflection of the sample tubes. Two film-type polarizers were used for the polarization measurements.

Warfarin Binding—The binding of warfarin to plasma was also measured by the fluorescence method previously adopted by Chakrabarti *et al.* (7, 8). The excitation and emission wavelengths were 320 and 390 nm, respectively. The bound fraction of warfarin (X_{II}) was calculated as:

$$X_{II} = (F_{obs} - F_{free})/(F_{bound} - F_{free}) \quad \text{(Eq. 4)}$$

where F_{obs} is the observed fluorescence corrected for the protein blank, F_{free}

Table I—Correlation Coefficients Between Free Fatty Acid Concentration and the Free Fractions of Various Acidic Drugs in Rat Plasma

Drug	n	Correlation Coefficient[a] (r)	Free Fatty Acid Level	
			Mean, mM	Range, mM
1-Anilino-8-naphthalenesulfonate (I)	32	0.609*[b]	1.35	0.43–3.78
Warfarin (II)	21	0.609**	1.14	0.62–1.80
Phenylbutazone (III)	25	0.761*[c]	1.14	0.62–3.78
Salicylate (IV)	30	0.661*[c]	1.39	0.62–3.78
Sulfaphenazole (V)	30	0.551** (0.687)*[d]	1.39	0.62–3.78
Indomethacin (VI)	15	0.536***	1.72	0.62–3.78

[a] Key for statistical significance: (*) $p < 0.001$; (**) $p < 0.01$; (***) $p < 0.05$. [b] Previously reported in Ref. 4. [c] Experimental data are shown in Fig. 3. [d] Among the 30 rats studied, one (rat 28) showed an abnormal free fraction (0.773), as indicated by an asterisk in Fig. 6. The correlation coefficient in parentheses was calculated excluding this abnormal datum point.

is the fluorescence of warfarin in the buffer at a given concentration, and F_{bound} is the fluorescence in the presence of a large excess of plasma proteins. The X_{II} value was obtained using 10 μM warfarin and 2% plasma, which corresponded to ~10 μM albumin.

Analytical Method—In the equilibrium dialysis experiments, the determination of drug at the buffer side was carried out spectrofluorometrically[8] or spectrophotometrically[9].

Indomethacin—The concentration was directly measured spectrophotometrically (λ_1 = 370 nm, λ_2 = 320 nm). The blank value which came from the membrane-permeable components was subtracted.

Phenylbutazone—After addition of 0.5 mL of 1 M HCl to 0.5 mL of sample, the mixture was extracted with 4 mL of *n*-heptane, and the 2.5-mL organic layer was reextracted with 2 mL of 4% sodium carbonate. The sodium carbonate layer was measured spectrophotometrically (λ_1 = 330 nm, λ_2 = 270 nm).

Salicylic Acid—After addition of 0.5 mL of 1 M HCl to 0.5 mL of sample, the mixture was extracted with 6 mL of dichloroethane, and the 4-mL organic layer was reextracted with 4 mL of 4% sodium carbonate. The drug concentration of the aqueous layer was measured spectrofluorometrically (λ_{ex} = 300 nm, λ_{em} = 410 nm) using a 390-nm cut-off filter at the emission side.

Sulfaphenazole—The concentration was determined colorimetrically (λ_1 = 650 nm, λ_2 = 550 nm) according to the method of Tsuda and Matsunaga (9). The determinations of free fatty acids[10], total bilirubin[11] (conjugated plus unconjugated), and albumin[12] in the plasma were performed using kits.

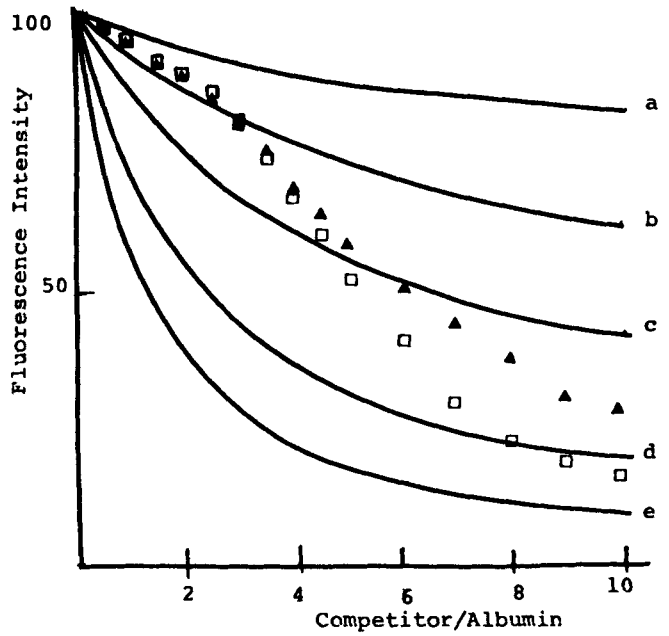

Figure 5—*Simulation of inhibition of the binding of I to rat albumin based on the assumption of simple competition. The solid lines were calculated with Eq. 5, using* $T_A = 1\ \mu M$, $P = 1\ \mu M$, $n = 1$, $K_A = 2.2 \times 10^7\ M^{-1}$, *and* K_B: *(a)* $0.3 \times 10^6\ M^{-1}$, *(b)* $1 \times 10^6\ M^{-1}$, *(c)* $3 \times 10^6\ M^{-1}$, *(d)* $10 \times 10^6\ M^{-1}$, *and (e)* $30 \times 10^6\ M^{-1}$. *(See text for details.) Data for oleic (□) and palmitic (▲) acids from Fig. 4 are shown for comparison.*

[9] Hitachi 356 dual-type spectrophotometer.
[10] Nefatest; Wako Pure Chemical Industries Co., Tokyo, Japan.
[11] Bil Set "Daiichi"; Daiichi Pure Chemical Co., Tokyo, Japan.
[12] Diagnotesta-A; Daiichi Pure Chemical Co., Tokyo, Japan.

RESULTS AND DISCUSSION

Interindividual Differences in the Plasma Binding of I—It was previously reported that the free fraction of I (f_I) showed pronounced interindividual differences ranging from 0.126 to 0.777 (4). To determine whether these interindividual differences were due to differences in the binding constant or to those in albumin concentration, the correlations between f_I and these two factors were examined. Using plasma of 18 rats, binding kinetics with changing concentration of I were examined. Although the binding constant for the high-affinity site shows some interindividual differences (ranging from $1.75 \times 10^6\ M^{-1}$ to $2.3 \times 10^7\ M^{-1}$), the number of binding sites on albumin gives values $\simeq 1$. As shown in Fig. 1a, there is a highly statistically significant negative correlation between f_I and the binding constant for the high-affinity site ($r = -0.858$, $p < 0.001$); no significant correlation between f_I and the albumin concentration was seen (Fig. 1b). Figure 2 shows the correlation between f_I and the infinite fluorescence of I and the degree of polarization, both of which have been used as indices of the conformational changes of plasma albumin (7, 10, 11). No significant correlation was observed in either case. Consequently, the conformational changes of albumin might not be associated with the interindividual differences in f_I. On the other hand, it was previously found that there was a significant positive correlation between f_I and free fatty acid concentration in plasma, which was considered to be one of the endogenous displacing agents (4). However, the correlation coefficient was not so high ($r = 0.609$) that the interindividual differences could be explained only by free fatty acids (4).

Correlation Between the Free Fractions of Various Acidic Drugs and the Free Fatty Acid Concentration—Correlations between the free fatty acid concentrations in rat plasma and the free fractions of five acidic drugs (warfarin, phenylbutazone, salicylic acid, sulfaphenazole, and indomethacin) were investigated. Results in the case of phenylbutazone and salicylic acid are shown in Fig. 3; the correlation coefficients obtained are listed in Table I. Statistically significant positive correlations were observed between the free fatty acid concentration and the free fractions of all the drugs.

Plasma albumin is generally thought to play a major role in the binding of acidic drugs to plasma. To evaluate the extent of the potency of various free fatty acids to inhibit the binding of I to albumin, the titrations of the mixture of I (1 μM) and defatted rat albumin (1 μM) with various free fatty acids were carried out (Fig. 4). If it is assumed that the quantum yield of bound I (infinite fluorescence) is not altered by the binding of free fatty acid to albumin, the decrease in the fluorescence would represent the decrease in the concentration of bound I (see Eq. 1). The free fraction of I in the absence of free fatty acids under this condition was calculated to be 0.14 using Eq. 1. This value was essentially the same as the smallest free fraction of I (0.126) among those obtained using rat plasma.

Table II—Matrix of the Correlation Coefficients Between the Free Fractions of Six Acidic Drugs[a]

Drug	I	II	III	IV	V	VI
I		0.944*[b]	0.896*[c]	0.701*[b]	0.548**[b] (0.715*)[d]	0.808*[b]
II			0.911*	0.641*	0.422[ns] (0.561**)[d]	0.797*
III				0.732*	0.721* (0.655*)[d]	0.830*
IV					0.405*** (0.638*)[d]	0.605**
V						0.810* (0.775*)[d]

[a] Key for statistical significance: (*) $p < 0.001$; (**) $p < 0.01$; (***) $p < 0.05$; (ns) not significant ($p > 0.05$). [b] Experimental data are shown in Fig. 6. [c] Previously reported in Ref. 4. [d] The explanation for the values in parentheses is given in Table I, footnote *d*.

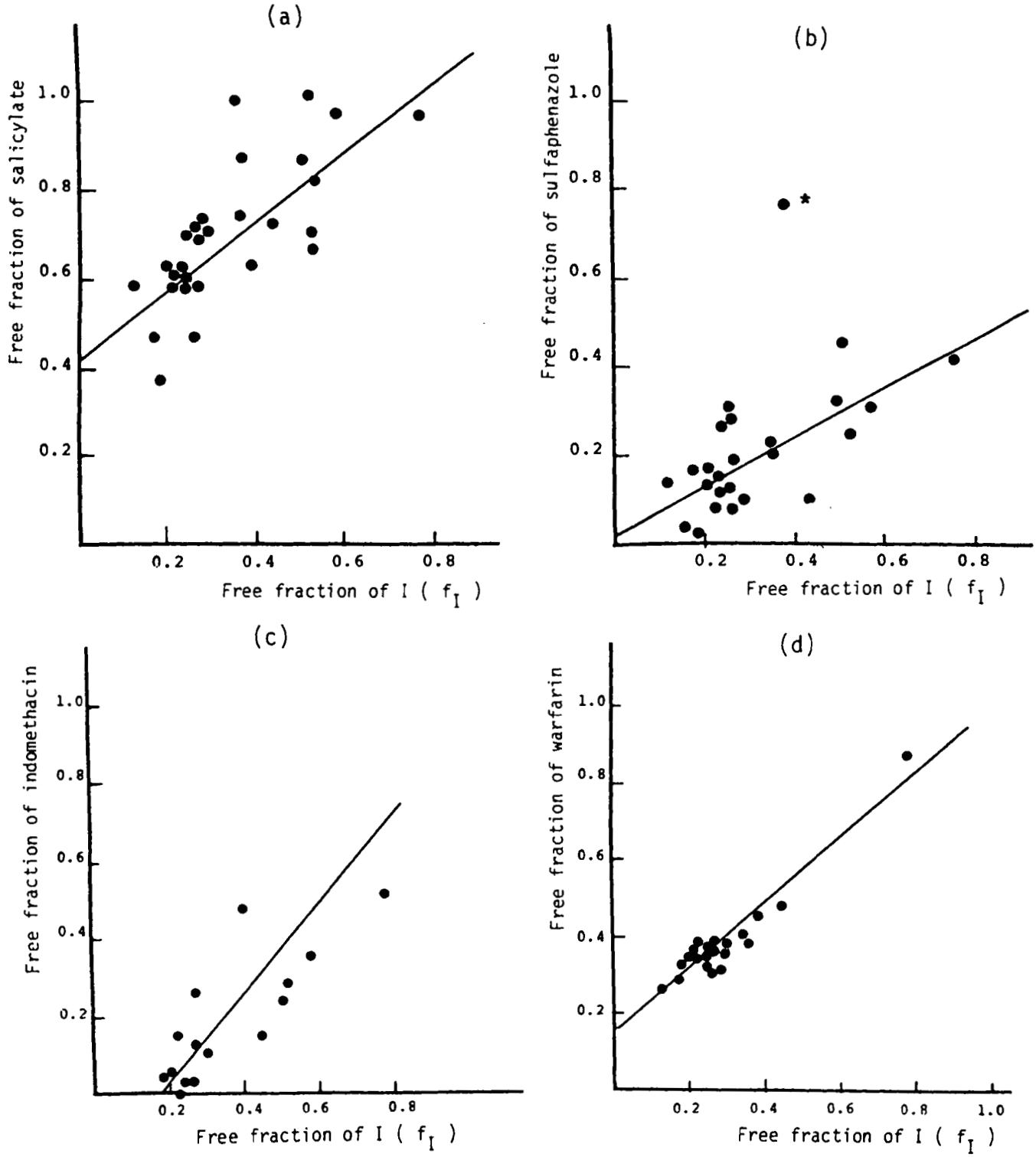

Figure 6—*Relationship between* f_I *and the free fractions of salicylate (a), sulfaphenazole (b), indomethacin (c), and warfarin (d) in the plasma of individual rats. Least-squares fits of the data yielded the solid lines. The experimental conditions are the same as described in Fig 3, and the correlation coefficients were summarized in Table II.*

Among the four free fatty acids studied, stearic acid is a weak inhibitor, while oleic acid and palmitic acid are relatively strong inhibitors. The latter acids have a similar pattern of inhibition, in which the fluorescence decrease is slow up to a free fatty acid/albumin ratio of 2 and faster thereafter. If simple competition by two ligands (I and free fatty acid in this case) for equal and independing binding sites is assumed, the following equation holds (12):

$$T_B = \left[n - r_A - \frac{r_A}{(T_A - P \cdot r_A)K_A}\right] \times \left[P + \frac{K_A(T_A - P \cdot r_A)}{r_A \cdot K_B}\right] \quad \text{(Eq. 5)}$$

where T_A and T_B are the total concentrations of I and the competitor (free fatty acid), respectively, P is the albumin concentration, r_A is the number of I molecules bound per mole of albumin, n is the number of binding sites, and K_A and K_B are the binding constants of I and competitor, respectively. In the present case, parameters used in this calculation are $T_A = 1\ \mu M$, $P = 1\ \mu M$, $n = 1$, $K_A = 2.2 \times 10^7\ M^{-1}$ (4).

Since the fluorescence intensity (F) of I is proportional to r_A, F can be calculated as a function of T_B using Eq. 5, when K_B is given. Figure 5 shows the simulation curves thus calculated. The simulation curve at any K_B value never reproduces the anomalous patterns obtained in the titration with oleic

and palmitic acids. Therefore, the slow fluorescence decrease up to the free fatty acid/albumin ratio of ~2 and the faster decrease after that could not be explained by the simple competition. Santons and Spector (13, 14) investigated the effects of free fatty acids on the binding of I to human or bovine serum albumin. They found that the fluorescence of I was enhanced when ≤2 mol of palmitate were added to 1 mol of human albumin, but larger amounts of palmitate produced a reduction in the fluorescence of I; with bovine and rabbit albumins, palmitate in all concentrations only reduced the fluorescence of I. The present result with rat albumin is similar to those with bovine and rabbit albumin.

Correlation Between the Free Fraction of I and those of Various Acidic Drugs—Figure 6 shows the correlation between f_I and the free fractions of various acidic drugs. There are highly statistically significant positive correlations in all cases. Warfarin and phenylbutazone were especially well correlated with I, $r \geq 0.9$. Such high correlations are reasonable when considering that warfarin and phenylbutazone reportedly competitively inhibit the binding of I to bovine serum albumin (15). The correlation coefficients between the free fractions of all drugs are listed in Table II; there is a statistically significant positive correlation between the free fraction of any pair of drugs.

This finding suggests that we can probably estimate the degree of plasma binding of such acidic drugs by knowing that of I. However, it must be kept in mind that the free fractions of various drugs obtained in the present study using dilute plasma samples do not necessarily represent the *in vivo* free fraction. To be able to employ this method using I as a clinical test, therefore, it would be necessary to determine the plasma bindings of various drugs using undiluted human plasma and examine the correlations between the free fractions of these drugs and that of I. However, the fluorescence method using I, which we have presented herein, has the advantages that it takes a short time (5–10 min) and needs a very small amount of plasma (<0.1 mL) to measure the free fraction of I.

Note added in proof: Heparin was used to prepare rat plasma in this study. The effect of heparin injection on plasma protein binding of I was subsequently studied and was reported (16).

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Interaction of Doxorubicin with Nuclei Isolated from Rat Liver and Kidney

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Received April 1, 1982, from the *Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunky-ku, Tokyo, 113, Japan.* Accepted for publication March 24, 1983.

Abstract □ The interaction of doxorubicin with nuclei isolated from rat liver and kidney was studied by fluorospectrometry. The nuclei had at least two different types of binding sites for the drug. Both Mg^{2+} and Ca^{2+} competitively inhibited the binding of doxorubicin to the nuclei, which showed a remarkable temperature dependency. No significant difference was observed between the numbers of binding sites ($n = 6.70 \times 10^{-2}$ mol/mol of DNA for liver; 6.41×10^{-2} mol/mol of DNA for kidney) or the affinity constants ($K_a = 4.85 \times 10^5$ M^{-1} for liver; 5.41×10^5 M^{-1} for kidney) under quasi-physiological conditions. These results obtained from *in vitro* binding experiments support previous suggestions that the differences in the *in vivo* distribution of doxorubicin among tissues are not due to differences in the nuclear binding of the drug. The amount of nuclei per gram of tissue is the primary determinant of the characteristic tissue distribution of doxorubicin.

Keyphrases □ Doxorubicin—interaction with nuclei isolated from rat liver and kidney, binding, distribution □ Binding—interaction of doxorubicin with nuclei isolated from rat liver and kidney, distribution □ Nuclei, liver and kidney—interaction with doxorubicin, rats, binding, distribution □ Distribution—interaction of doxorubicin with nuclei isolated from rat liver and kidney, binding

Doxorubicin, an anthracycline antibiotic, has cytotoxic and antineoplastic activities and inhibits both enzymatic RNA and DNA synthesis by intercalating with DNA (1). The tissue distribution of doxorubicin has been studied extensively in humans and laboratory animals, but the mechanism of its distribution in such tissues as liver, kidney, and muscle has not been elucidated (2–6). The tissue distribution of this drug is generally thought to be related to the affinity for the tissue binder, the concentration of binder, and the permeability across the plasma membrane.

The interaction of doxorubicin and other derivatives with DNA or chromatins isolated from tumor cells, calf thymus, and cultured lung cells has been studied by several methods (7–10). Although the binding of the drug to native DNA has been extensively studied, little information has been obtained on its intercalation with nuclei isolated from normal tissues. Moreover, it has been suggested that doxorubicin also interacts strongly with the negatively charged phospholipid, cardiolipin (11). In addition, it was revealed that tumor cells may have a carrier-mediated influx system and an active efflux mechanism (12). Previously, a good correlation between the *in vivo* tissue-to-plasma partition coefficients for doxorubicin in several tissues and the amounts of nuclei per gram of tissue in rats and rabbits was demonstrated, suggesting that there is little or no difference in the nuclear binding of the drug among tissues (13).

The purpose of this study was to determine the *in vitro* binding characteristics of doxorubicin with isolated nuclei from rat liver and kidney in an attempt to elucidate the mechanisms by which the *in vivo* tissue distribution of the drug occurs.

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