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New Fluorescence Probes for Drug-Albumin Intereaction Studies

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Some new and known coumarincarboxylic acids were synthesized for use as fluorescence probes. The fluorescence properties of these compounds were examined in various solvents and albumin solutions. The fluorescence of 7-anilinocoumarin-4-acetic acid (I) was significantly enhanced in nonpolar solvents and in the presence of human serum albumin (HSA). The binding parameters of I were estimated from the fluorescence enhancement and spectral change of I bound to HSA. The Scatchard plots and the continuous variation plots indicated that only the primary site was capable of enhancing the fluorescence and causing the spectral changes of I. Digitoxin displaced I from its primary site on HSA, but site 1 and site 2 drugs on the basis of Sudlow's classification did not significantly displace the probe. The present data indicate that I may be useful as a third site marker.

Keywords——human serum albumin; coumarincarboxylic acids; 7-anilinocoumarin-4-acetic acid; protein binding fluorescence probe; coumarincarboxylic acid binding site on serum albumin; coumarincarboxylic acid fluorescence quantum yield; digitoxin; phenylbutazone; ibuprofen

Fluorescence probes[1] including coumarin derivatives have been extensively used for studying drug-protein binding sites and binding parameters, because fluorescence spectroscopy is a sensitive and convenient method for the study of protein-ligand interaction. In general, the binding sites and the binding parameters of drugs were determined by competitive binding. Probes with a single binding site or two sites with widely different binding constants should be used as specific probes of human serum albumin (HSA) binding sites. For example, 1-anilino-8-naphthalenesulfonate (ANS)[2] has several high affinity sites on HSA, and this might further complicate the interpretation of the data.

Sudlow *et al.*[3] have suggested two distinct binding sites on HSA, site 1 and site 2, for anionic drugs using two specific fluorescence probes; site 1 of HSA showed affinity for warfarin, phenylbutazone, *etc.* (referred to as site 1 drugs) and site 2 for ibuprofen, flufenamic acid, *etc.* (referred to as site 2 drugs). Recently, Ozeki *et al.*[4] and Sjöholm *et al.*[5] have suggested that HSA has a third high affinity site for drugs in addition to site 1 and site 2. Therefore, several probes having one high affinity site seem to be required for the detailed study of certain binding sites because of the complex nature of the binding process.

Thus, in order to obtain new fluorescence probes we synthesized several water-soluble coumarincarboxylic acids and examined the fluorescence characteristic of these compounds in various solvents and HSA solutions. Furthermore, the binding specificity of the probes was investigated in connection with Sudlow's classification[3] of the binding sites in the hope of finding a new binding site.

Experimental

Materials——7-Aminocoumarin derivatives were synthesized by the method of Dey[6] for 7-dimethylaminocoumarin-4-acetic acid (III) and their structures were confirmed by elemental analysis and nuclear magnetic resonance (NMR) spectroscopy. 7-Methoxycoumarin derivatives were synthesized according to the literature.[7] HSA (Lot. No. A-2386) was obtained from Sigma Chemical Company, St. Louis, Mo. Phenylbutazone (Ciba-Geigy Co.), ibuprofen (Kakenyaku Kako Co.), and digitoxin (Mitsubishi Yuka Pharma-

ceutical Co.) were gifts of the manufacturers. Organic solvents for spectra were commercial spectro grade products. All other chemicals used were of reagent grade, and deionized and distilled water was used throughout.

Syntheses 7-Anilinocoumarin-4-acetic Acid (I)——A mixture of 3-hydroxydiphenylamine (6.8 g), redistilled ethyl acetonedicarboxylate (7.0 g), and anhydrous $ZnCl_2$ (6.0 g) in absolute EtOH (20 ml) was refluxed for 10 h. After cooling, the reaction mixture was poured into ice-water under stirring. The resulting separated dark oil slowly solidified on being left in contact with cold EtOH. Recrystallization from EtOH gave 3.5 g of ethyl 7-anilinocoumarin-4-acetate as pale yellow needles, mp 150—151°C. *Anal.* Calcd for $C_{19}H_{17}NO_4$: C, 70.57; H, 5.30; N, 4.33. Found: C, 70.69; H, 5.38; N, 4.36. This ester (1.0 g) obtained above was hydrolyzed by the method described in the previous paper.[8] Recrystallization from EtOH gave 0.4 g of I as pale yellow needles, mp 186—189°C (dec.). *Anal.* Calcd for $C_{17}H_{13}NO_4$: C, 69.14; H, 4.44; N, 4.74. Found: C, 69.37; H, 4.39; N, 4.76. NMR (DMSO-d_6) δ: 3.80 (2H, s, C_4–CH_2), 6.12 (1H, s, C_3–H), 6.8—7.7 (8H, m, aromatic H), 7.52 (1H, d, C_5–H), 8.84 (1H, s, C_7–NH), and 12.67 (1H, broad, COOH).

7-Anilino-4-methylcoumarin-3-acetic Acid (II)——Treatment of 3-hydroxydiphenylamine (5.0 g) with diethyl acetosuccinate (5.8 g) in absolute EtOH (20 ml) in the presence of anhydrous $ZnCl_2$ (4.5 g) according to the method described above gave ethyl 7-anilino-4-methylcoumarin-3-acetate. Recrystallization from EtOH gave 2.5 g of the ester as pale yellow needles, mp 177—178°C. *Anal.* Calcd for $C_{20}H_{19}NO_4$: C, 71.20; H, 5.68; N, 4.15. Found: C, 71.61; H, 5.65; N, 4.15. After hydrolysis of this ester (1.0 g), recrystallization of the product from EtOH gave 0.2 g of II as pale yellow needles, mp 210—215°C (dec.). *Anal.* Calcd for $C_{18}H_{15}NO_4$: C, 69.89; H, 4.89; N, 4.53. Found: C, 69.90; H, 4.86; N, 4.60. NMR (DMSO-d_6) δ: 2.32 (3H, s, C_4-CH_3), 3.50 (2H, s, C_3–CH_2), 6.8—7.4 (7H, m, aromatic H), 7.60 (1H, d, C_5–H), 8.77 (1H, s, C_7–NH), and 12.3 (1H, broad, COOH).

7-Dimethylamino-4-methylcoumarin-3-acetic Acid (IV)——A mixture of *m*-dimethylaminophenol (2.0 g), diethyl acetosuccinate (3.3 g), and anhydrous $ZnCl_2$ (2.5 g) in absolute EtOH (10 ml) was refluxed for 6 h. The reaction mixture was poured into ice-water containing a small amount of hydrochloric acid and allowed to stand overnight in a refrigerator. The resulting dark red crystals were collected by filtration and air-dried. Repeated recrystallization from EtOH with the aid of animal charcoal gave 0.5 g of ethyl 7-dimethylamino-4-methylcoumarin-3-acetate as colorless needles, mp 120—121°C. *Anal.* Calcd for $C_{16}H_{19}NO_4$: C, 66.42; H, 6.62; N, 4.84. Found: C, 66.23; H, 6.39; N, 5.06. After the hydrolysis of this ester (0.5 g) with 6 N HCl (2 ml), the reaction mixture was diluted with water (100 ml) and allowed to stand in a refrigerator. The resulting precipitates were collected by filtration and washed with water. Recrystallization from EtOH gave 0.2 g of IV as colorless needles, mp 210—213°C (dec.). *Anal.* Calcd for $C_{14}H_{15}NO_4$: C, 64.36; H, 5.79; N, 5.36. Found: C, 64.31; H, 5.80; N, 5.52. NMR (DMSO-d_6) δ: 2.30 (3H, s, C_4–CH_3), 3.00 (6H, s, C_7–$N(CH_3)_3$), 3.52 (2H, s, C_3–CH_2), 6.53 (1H, d, C_8–H), 6.70 (1H, dd, C_6–H), and 12.23 (1H, broad, COOH).

Apparatus and Methods——All melting points were measured with a Yanagimoto micro-melting point apparatus and are uncorrected. ^1H NMR spectra were obtained with a JEOL C60-H spectrometer, employing tetramethylsilane as an internal reference. The abbreviations used are as follows: s, singlet; d, doublet; dd, double doublet; m, multiplet. Absorption and absorption difference spectra were measured with a Jasco UVIDEC-1 spectrophotometer, and a Hitachi 556s dual-wavelength spectrophotometer, respectively. Fluorescence spectra were measured with a Hitachi MPF-3 fluorescence spectrophotometer. Fluorescence quantum yields were determined according to the method of Parker and Rees,[9] and quinine sulfate in 1 N H_2SO_4 was used as the standard. All HSA and I solutions were prepared in 0.1 M phosphate buffer of pH 7.4 at 25±1°C. HSA solutions of $0.5—0.9\times10^{-5}$ M (M.W. 69000) were used, and a wide concentration range of $0.05—10\times10^{-5}$ M of I and drug was used.

Fluorometric titrations: HSA solution of appropriate concentration was titrated by successive additions of a solution of I (to give a final concentration of 0.5×10^{-6} M) and the fluorescence intensity was measured (excitation at 370 nm and emission at 398 nm).

Absorbance difference titrations: Absorption difference spectra were obtained by using a pair of split-compartment-tandem mixing cells. The contents of the buffer solution cell in the reference beam and HSA solution cell in the sample beam were titrated by successive additions of a solution of I (to give a final concentration of $0.1—1.0\times10^{-4}$ M in cell) and the absorbance differences were measured at 398 nm.

Equilibrium dialysis: Equilibrium dialysis experiments were carried out using Visking cellulose tubing. Aliquots of 2 ml of 1.45×10^{-4} M HSA solution in 0.1 M phosphate buffer, pH 7.4, were placed in dialysis bags, and the bags were placed in 20 ml flasks containing 10 ml of solution of I ($0.2—6.0\times10^{-4}$ M). The flaks were shaken for 15 h at 25°C. After equilibrium, the concentration of I outside the bag was determined by measurement of the absorbance at 375 nm.

Data treatment: The fraction of I bound, X, is usually determined by using equation (1) or (2),

$$X = \frac{F_p - F_o}{F_b - F_o} \quad (1)$$

$$X = \frac{\Delta A_p}{\Delta A_b} \quad (2)$$

where F_p and F_o are the fluorescence intensities of a given concentration of I in a solution of low HSA concentration and in a solution without HSA, and F_b is the fluorescence of the same concentration of fully bound I. ΔA_p and ΔA_b are the absorbance differences of a given concentration of I in solutions of low and high HSA concentrations. To determine the values of F_b and ΔA_b for a given concentration of probe, fluorescence and absorbance difference titrations were carried out for several albumin concentrations. F_b is taken to be the fluorescence intensity of the substrate in the presence of excess albumin. ΔA_b is obtained by extrapolation of plots of $1/\Delta A$ *versus* $1/[P]$, where $[P]$ represents the total concentration of HSA. After values for the fraction of bound probe had been found for all points along the titration curve, the results were plotted according to the Scatchard equation:

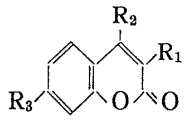
$$r/D_f = nK - rK \qquad (3)$$

where r is the number of mol of I bound per mol of protein, n is the number of binding sites, K is the binding constant and Df is the concentration of free I.

Results and Discussion

Table I summarizes the absorption and fluorescence spectral data of coumarincarboxylic acids in water, ethanol, and cyclohexane. The absorption maxima for I were near 270 and 375 nm in those solvents. The absorption maximum near 375 nm remained almost unchanged in more polar and more nonpolar solvents, whereas the absorption peak near 270 nm shifted to shorter wavelength with increasing polarity of the solvent. Compound I was practically nonfluorescent in water and ethanol but was brightly fluorescent in cyclohexane. Compound II also showed spectral behavior similar to that of I. In contrast to I and II, two absorption peaks for III and IV were shifted to longer wavelength as the polarity of the solvent was increased. Compounds III and IV showed relatively intense fluorescence in different solvents, and their emission maxima shifted toward the blue as the polarity of the solvent was decreased. The absorption and emission maxima for V and VI were not affected by the polarity of the solvent. However, the fluorescence intensities of V and VI increased with increasing polarity of the solvent.

TABLE I. Absorption and Fluorescence Spectral Data for Coumarincarboxylic Acids



Compd. No.	R_1	R_1	R_3	UV λ_{max} nm (log ε)			Fλ_{max} nm (Quantum yield)[a]		
				H_2O	EtOH	Cyclo-hexane	H_2O	EtOH	Cyclo-hexane
I	H	CH_2COOH	NHC_6H_5	262(4.15) 375(4.35)	269(4.22) 376(4.22)	272(4.16) 378(4.44)	459 (<0.01)	458 (0.01)	404 (0.32)
II	CH_2COOH	CH_3	NHC_6H_5	264(4.18) 365(4.43)	270(4.17) 372(4.49)	272(4.03) 363(4.42)	450 (<0.01)	457 (0.02)	412 (0.47)
III	H	CH_2COOH	$N(CH_3)_2$	248(4.14) 371(4.28)	244(4.24) 368(4.37)	239(4.19) 353(4.37)	473 (0.18)	453 (0.82)	399 (0.27)
IV	CH_2COOH	CH_3	$N(CH_3)_2$	247(4.28) 378(4.28)	245(4.16) 373(4.35)	240(4.24) 356(4.29)	470 (0.61)	451 (0.80)	400 (0.31)
V	H	CH_2COOH	OCH_3	325(4.16)	323(4.15)	323(4.15)	387 (0.49)	384 (0.09)	378 (0.00)
VI	CH_2COOH	CH_3	OCH_3	324(4.27)	323(4.23)	321(4.21)	390 (0.77)	384 (0.43)	381 (0.07)

a) I—IV: Ex. at 370 nm. V and VI: Ex. at 340 nm.

The interactions of coumarincarboxylic acid derivatives with HSA were also studied. The fluorescence spectra of I in the presence of HSA in pH 7.4 phosphate buffer are shown in Fig. 1, and the spectral data and quantum yields are summarized in Table II. The addition

of HSA resulted in large increases in the quantum yields of I an II accompanied by a slight blue shift of the emission maxima. On the other hand, the fluorescence intensities of V and VI were quenched, as might be expected from the solvent effect. The fluorescence properties observed for I and II in different solvent and in albumin mixture indicate that I and II should be useful as probes for drug-protein binding studies. However, preliminary experiments revealed that the mole ratio of II-HSA complex was different from that of I-HSA and was not clear-cut. Compound I seemed to be more suitable than II for studies of the binding process. Therefore, the interaction of I and HSA was also examined by other methods. Fig. 2 shows typical absorption difference spectra at constant concentration of I with several HSA concentrations. These spectra have a maximum at 398 nm, and the absorbance difference increases with the concentration of I or HSA.

Table II. Quantum Yield of 7-Anilinocoumarin-4-Acetic Acid (I) in the Presence of HSA in pH 7.4 Phosphate Buffer[a)]

HSA concentration M	Emission nm	Quantum Yield
6×10^{-6}	475	0.06
9×10^{-6}	475	0.07
6×10^{-5}	470	0.12
9×10^{-5}	468	0.13

a) Concentration of I: 4×10^{-6} M. Excitation wavelength: 370 nm.

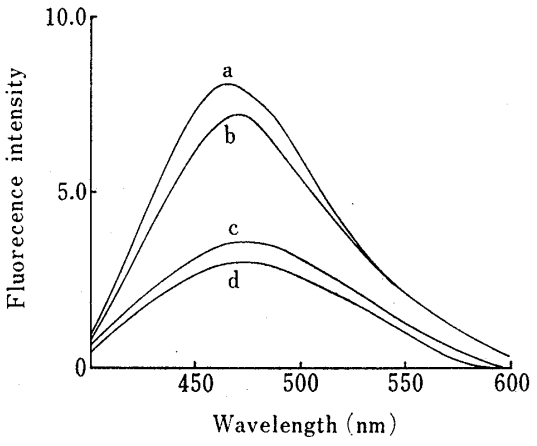


Fig. 1. Fluorescence Spectra of I (4×10^{-6} M) in the Presence of HSA

HSA concentration: a: 9×10^{-5} M, b: 6×10^{-5} M, c: 9×10^{-6} M, d: 6×10^{-6} M.

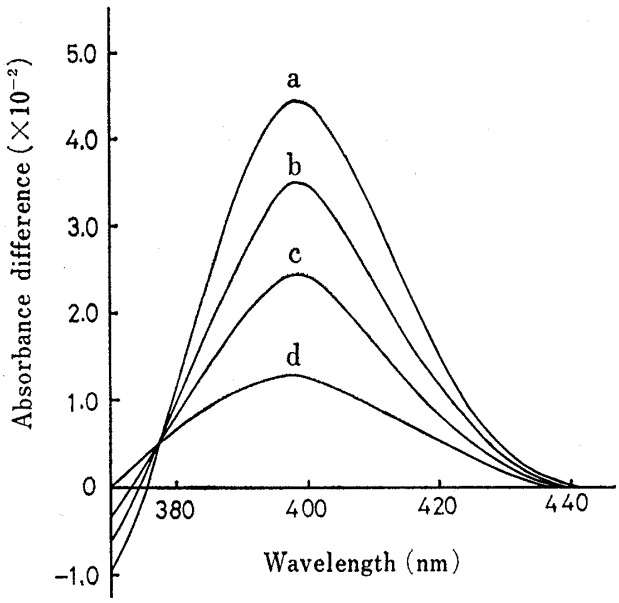


Fig. 2. Absorption Difference Spectra of I (5×10^{-5} M) in the Presence of HSA

HSA concentration: a: 5×10^{-5} M, b: 3×10^{-5} M, c: 2×10^{-5} M, d: 1×10^{-5} M.

The fluorometric and absorbance difference titrations of I with HSA are shown in Figs. 3 and 4. The fluorescence intensities for two titrations with high HSA concentrations (7.0×10^{-5} and 9.0×10^{-5} M) were identical (straight line a), suggesting that I added was fully bound. For this treatment to be valid, the fulorescence intensity of the bound probe must be a linear function of concentration. This is the case only when the absorbance of the complex at the exciting wave length is low. A correction for this absorption can be made by the method of Naik *et al.*,[10] and was made for all the data when the absorbance at 370 nm was greater

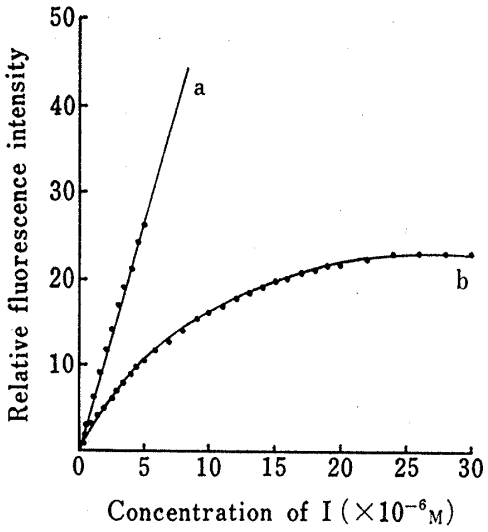


Fig. 3. Relative Fluorescence Intensity for the I-HSA Interaction as a Function of the Concentration of I

HSA concentration: a: 7×10^{-5} and 9×10^{-5} M, b: 9×10^{-6} M.

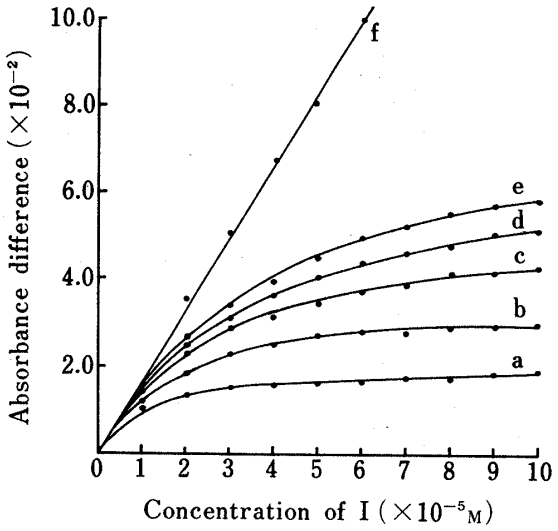


Fig. 4. Absorbance Difference for the I-HSA Interaction as a Function of the Concentration of I

HSA concentration: a: 1×10^{-5} M, b: 2×10^{-5} M, c: 3×10^{-5} M, d: 4×10^{-5} M, e: 5×10^{-5} M, f: the theoretical line drawn by using calculated values of absorbance difference for high HSA concentrations.

than 0.02. At low HSA concentrations, I was only partially bound (curve b). The absorbance difference data measured at 398 nm for the I-HSA system are plotted as a function of the concentration of I in Fig. 4. The values of the points on the theoretical line (straight line f) are taken from the extrapolated plots of $1/\Delta A$ *versus* 1/[HSA] (see "Data treatment").

To determine the maximum number of binding sites, Job's plots were prepared for the I-HSA system by keeping the total concentration of I and HSA at 1.0×10^{-5}M. These plots are shown in Fig. 5. The inflection points for the two plots are near 0.5, the value expected for 1:1 complex formation. Therefore, it is reasonable to conclude that the maximum numbers of binding sites contributing to the fluorescence and the absorbance difference of I-HSA are one.

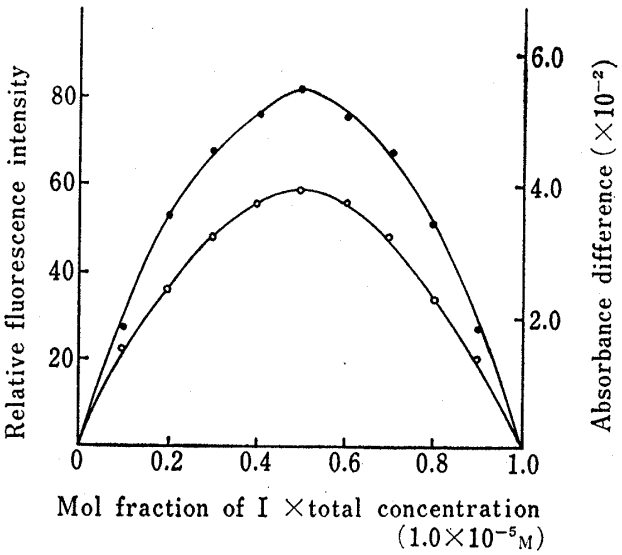


Fig. 5. Job's Plots of Relative Fluorescence Intensity (●) and Absorbance Difference (○) for the I-HSA Interaction

The total concentration of [HSA]+[I] was kept at 1.0×10^{-5} M

Protein binding equilibria have traditionally been evaluated by means of the Scatchard equation. Fig. 6 shows the Scatchard plots for the I-HSA system obtained by several methods. The linearity of the Scatchard plots obtained by the fluorescence and absorption difference methods indicates that I binds to one class of sites on HSA. However, the curvature of the Scatchard plots obtained by the equilibrium dialysis method shows that I binds to more than

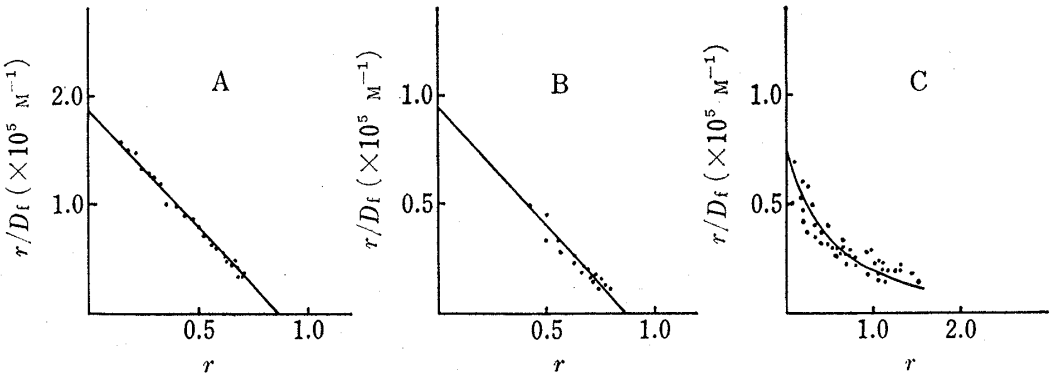


Fig. 6. Scatchard Plots of I-HSA Interaction

A: fluorescence, B: spectrophotometr, C: dialysis.

TABLE III. Binding Parameters for I-HSA Interaction at pH 7.4

Method	Parameter			
	n_1	K_1	n_2	K_2
Fluorescence	0.85	2.23×10^5		
Spectrophotometry	0.86	1.04×10^5		
Dialysis	0.81	1.06×10^5	1.54	0.12×10^5

one class of sites. The primary binding constants obtained by the fluorescence and spectrophotometric methods are in good agreement with that obtained by equilibrium dialysis, suggesting that the fluorescence and spectrophotometric techniques detect only the primary binding sites which enhance fluorescence and cause spectral change (Table III).

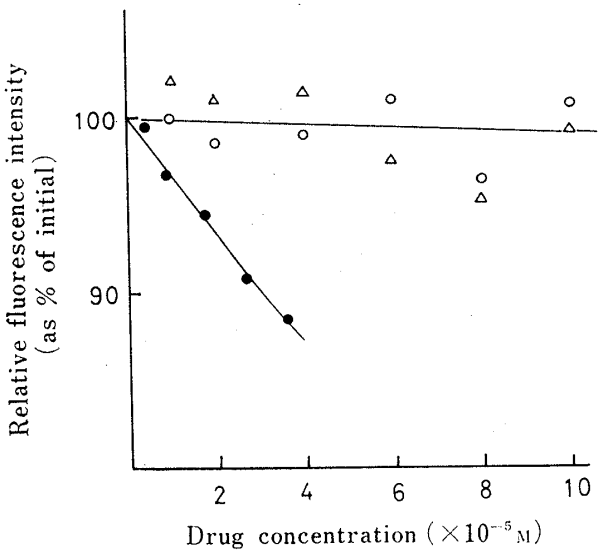


Fig. 7. Effects Various Drugs on the Fluorescence Intensity of I (2.0×10^{-5}M) in the Presence of HSA (1.0×10^{-5}M)

○: phenylbutazone, △: ibuprofen, ●: digitoxin.

The effect of drugs on the fluorescence of probes bound to HSA was also investigated to determine the specificity of the probe binding sites, in connection with Sudlow's classification of the binding sites. Fig. 7 shows the changes in fluorescence of I bound to HSA on the addition of drugs. Compound I was not significantly displaced by phenylbutazone (a site **1** drug) or by ibuprofen (a site **2** drug). However, digitoxin gave significant displacement of I at lower concentrations, suggesting that digitoxin binds to a further site (a third site). The present data support the suggestion of Sjöholm *et al.*[5] that the digitoxin binding site is independent of site **1** and site **2**.

The limited data obtained here suggest that I may be useful as a specific marker for the third site on albumin.

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