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The Classification of Drugs on the Basis of the Drug-binding Site on Human Serum Albumin

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The classification of 9 antiinflammatory agents, as well as warfarin and diazepam, on the basis of the binding site on human serum albumin (HSA) was studied by means of competition experiments with bilirubin. The displacement effect was evaluated through kinetic measurements of the free bilirubin concentration by oxidation with hydrogen peroxide and horseradish peroxidase. Bilirubin is a suitable marker for the localization of binding sites, because it has one high-affinity site which is on the loops 3–4 (including Lys-240) in the model of the secondary structure of HSA. Oxyphenbutazone, mefenamic acid and flufenamic acid displaced bilirubin strongly (first group). Sulfinpyrazone, warfarin, ibuprofen, phenylbutazone and aminopyrine showed moderate effects (second group). Ketophenylbutazone, antipyrine and diazepam showed the lowest displacing activity (third group). These results suggest that the binding sites of the first group were the same as or very close to the bilirubin site. The second group may be bound in the neighborhood of the bilirubin site or in the overlapping region of the bilirubin and warfarin sites. The third group may be bound to a different site(s) from bilirubin.

Keywords——binding site; human serum albumin; bilirubin; warfarin; diazepam; phenylbutazone; horseradish peroxidase

Serum albumin has the important function of reversible binding with various substances in the circulatory system, notably fatty acids, bilirubin, tryptophan and many acidic drugs.[1] In the case of drugs, the binding to albumin is a critical factor affecting the intensity and duration of drug action. The binding competition between drugs also affects the pharmacokinetic properties of drugs.[2] It is thought that the competitive reaction between drugs occurs on the same or adjacent sites on the albumin molecule. Therefore, the characterization and localization of drug-binding sites are important.

Recently, the specific drug binding sites of human serum albumin (HSA) have been divided into various groups by using various spectroscopic methods, specific fragmentation of HSA and chemical modification of specific amino acid residues. The use of substances showing specific binding should be helpful for deducing the binding sites of other substances by means of competitive binding experiments.

Bilirubin binds to HSA with one high affinity site.[3] It was confirmed by Jacobsen[4] that the location of this binding site is part of loops 3–4 (containing Lys-240) in the model of the secondary structure of HSA.[5] Brodersen *et al.*[6] have shown that peroxidase and hydrogen peroxide oxidize bilirubin to green products including biliverdin. However, the albumin-bilirubin complex is not oxidized under these circumstances. When a drug that binds competitively with respect to bilirubin is added to this complex, the amount of free bilirubin is increased. The free bilirubin can be determined by the use of peroxidase and hydrogen peroxide, simply by measuring the absorbance change. Therefore, bilirubin is one of the most suitable probes for the characterization of drug-binding sites on HSA. Brodersen[7] examined the competitive binding between bilirubin and several drugs by means of the methods mentioned above.

In this paper, we studied the displacement of bilirubin by various drugs according to the methods of Jacobsen[3] and Brodersen,[7] and classified the drugs on the basis of binding site.

Experimental

Materials——HSA (Sigma Chem. Co., Fraction V, essentially fatty acid-free) was used without purification. The molecular weight of HSA was assumed to be 66200 and the concentration was determined by using an extinction coefficient $E_{1cm}^{0.1\%}$ of 0.531 at 279 nm.[8] Bilirubin and horseradish peroxidase were also Sigma products. Horseradish peroxidase was of type I and the molecular weight was assumed to be 40200.[9] Phenylbutazone, aminopyrine, antipyrine and warfarin were purchased from Sigma Chem. Co. Oxyphenbutazone was kindly provided by Nihon Sieber Hegner, flufenamic acid and mefenamic acid by Sankyo, sulfinpyrazone by Nihon Ciba-Geigy, ibuprofen by Kaken Kagaku, ketophenylbutazone by Kyowa Hakko Kogyo and diazepam by Yamanouchi Seiyaku. All other chemicals used were obtained commercially. For all experiments, 1/15 M phosphate buffer of pH 7.4 was used.

Kinetic Procedures——The concentration of albumin was 3.0×10^{-5} M in 1/15 M phosphate buffer (pH 7.4), and that of bilirubin was 0.5 times the albumin concentration in all experiments.

Bilirubin is bound almost exclusively to one high affinity site on the albumin molecule with a binding constant of about $10^8\ M^{-1}$,[8] while the binding constants of drugs to albumin are $10^5\ M^{-1}$ at most. Bilirubin bound to albumin is not oxidized by peroxidase and hydrogen peroxide, and the Michaelis constant is high compared with the free bilirubin concentration. Accordingly, the rate is proportional to the free bilirubin concentration in the system. The process was monitored by recording the absorbance at 455 nm, which is close to the spectral maximum of albumin-bilirubin complex, in a Hitachi type 557 spectrophotometer with a thermostated cell holder at 37°C. The velocity of oxidation was determined with and without drug. The ratio of these velocities was equated with the ratio of free bilirubin concentration.

$$\frac{V}{V_0} = \frac{b}{b_0} = K_D D + 1 \qquad \text{Eq. 1}$$

Subscript 0 denotes the absence of drug. V/V_0 is the ratio of initial velocities and b/b_0 is that of free bilirubin concentration in the presence and in the absence of the displacer. K_D is the binding constant of the drug to the bilirubin-binding site and D is the total concentration of drug.

The time, $t_{0.2}$, required for completion of the fraction 0.2 of the total process was measured in each experiment. The enzyme concentration was adjusted to give $t_{0.2(0)}$ values of about 4 min. Hydrogen peroxide concentration was 1.0×10^{-4} M. The ratio of time periods with and without drug is consequently equal to the inverse ratio of initial velocities.

$$\frac{t_{0.2(0)}}{t_{0.2}} = \frac{V}{V_0} \qquad \text{Eq. 2}$$

As the values of $t_{0.2}$ decrease with increasing concentration of competitive drug, the horseradish peroxidase (HRP) concentration was lowered to obtain $t'_{0.2}$ of about 4 min.

$$t_{0.2} = t'_{0.2} \times \frac{[\text{HRP}]}{[\text{HRP}]_0} \qquad \text{Eq. 3}$$

The results of determination with and without drug were described by the following expression.

$$\frac{t_{0.2(0)}}{t'_{0.2}} \times \frac{[\text{HRP}]_0}{[\text{HRP}]} = K_D D + 1 \qquad \text{Eq. 4}$$

The left side of Eq. 4 was plotted on the ordinate against the drug concentration, D, as the abscissa. The line gave an intercept of 1 on the ordinate. The slope of the line is K_D, the binding constant of the drug to the bilirubin-binding site.

Control experiments without albumin were performed at a bilirubin concentration of 1.2×10^{-6} M (peroxidase 2.4×10^{-11} M, 5 cm optical cell) to test the effect of the drug on enzyme activity, formation of bilirubin-drug complex or oxidation of the drug by hydrogen peroxide and peroxidase. No such effects were detected.

Results and Discussion

The time course of the oxidation of free bilirubin with hydrogen peroxide and peroxidase is shown in Fig. 1. The optical density at 455 nm was recorded during 3 min before addition of the enzyme and remained constant, indicating that the system is stable. On addition of the enzyme, the optical density initially decreased due to the dilution and then proceeded to decline with slightly decreasing velocity. Oxidation yielded products with less absorbance

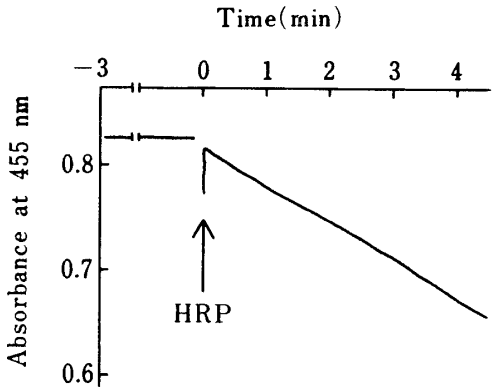

Fig. 1. Time Course of the Extinction at 455 nm, before and after Addition of Peroxidase at Zero Time

The concentrations of bilirubin and HSA were 3.0×10^{-5} M and 1.5×10^{-5} M, respectively.
The measurement was done in 1/15 M phosphate buffer, pH 7.4, at 37°C, with a 1 cm optical cell.

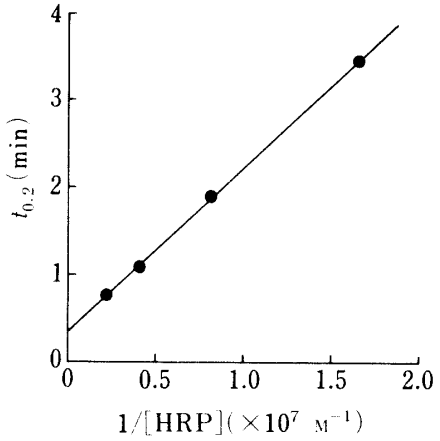

Fig. 2. Plots of $t_{0.2}$ against 1/[HRP] for Determination of the Kinetic Parameters of the Reaction of Bilirubin–HSA Complex with HRP

at this wavelength. Variation of the oxidation velocity with the enzyme concentration was examined, at constant concentrations of bilirubin and albumin (Fig. 2). If the dissociation of bilirubin from the complex with albumin is very fast compared with the oxidation, the velocity should be proportional to the enzyme concentration, as the latter process is rate-limiting. Therefore, $t_{0.2}$ should be proportional to the inverse enzyme concentration. This was the case, to a good approximation. In the absence of a competitive drug, 6.0×10^{-8} M peroxidase took 3.45 min to cause a fraction of 0.2 of the total oxidation ($t_{0.2(0)}$) (Fig. 2).

The competitive effect of 9 antiinflammatory agents, as well as warfarin and diazepam, on bilirubin binding was examined. As shown in Fig. 3, a linear relationship was obtained for the velocity ratio as a function of drug concentration. These decreased the binding of bilirubin to its high-affinity site on HSA. The increase of free bilirubin concentration may be ascribed to competitive binding of the drug to the same binding site. The slope of the line gives K_D, which is the binding constant of the drug to the bilirubin site. The calculated K_D values are summarized in Table I. As shown in Fig. 3, the displacement of bilirubin by antiinflammatory agents was grouped into three classes. Oxyphenbutazone,

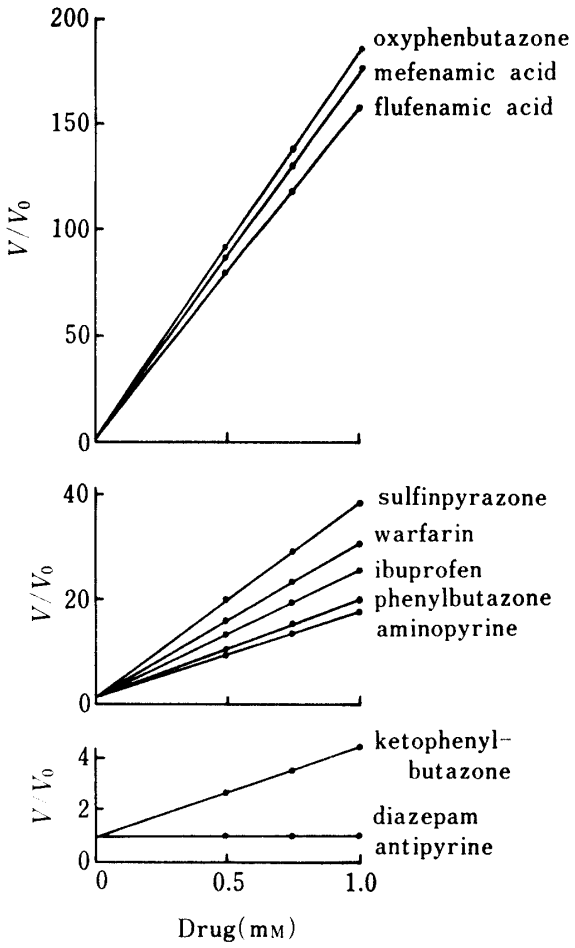

Fig. 3. Relative Velocity (V/V_0) of Oxidation of Free Bilirubin in Bilirubin–HSA Complex (0.5 : 1) with and without Drugs as a Function of Drug Concentration

V/V_0 corresponds to b/b_0 according to Eq. 1.
The values represent means of 3 to 5 runs

Table I. Binding Constants of Drugs to the High-affinity Bilirubin-binding Site of HSA

Drug	K_D (M^{-1})
Oxyphenbutazone	1.80×10^5
Mefenamic acid	1.75×10^5
Flufenamic acid	1.60×10^5
Sulfinpyrazone	3.80×10^4
Warfarin	3.10×10^4
Ibuprofen	2.70×10^4
Phenylbutazone	1.80×10^4
Aminopyrine	1.75×10^4
Ketophenylbutazone	3.30×10^3
Antipyrine	0
Diazepam	0

mefenamic acid and flufenamic acid displaced bilirubin from its binding site on HSA strongly (first group). Sulfinpyrazone, ibuprofen, phenylbutazone and aminopyrine showed moderate effects (second group). Ketophenylbutazone and antipyrine were the least effective displacers among these drugs (third group). Warfarin and diazepam belong to the second group and third group, respectively.

In the case of derivatives of phenylbutazone, the order of displacement effect was as follows: oxyphenbutazone>sulfinpyrazone>phenylbutazone>ketophenylbutazone. The K_D value of oxyphenbutazone is about ten times that of phenylbutazone (Table I). It is known that oxyphenbutazone is a metabolite of phenylbutazone *in vivo*, and the binding parameters of phenylbutazone and oxyphenbutazone are $5.06\times10^5\ M^{-1}$ and $3.53\times10^5\ M^{-1}$,[11] respectively. It is suggested that bilirubin is displaced by oxyphenbutazone from the same binding site, that is, the bilirubin-binding site.

Bilirubin binds to HSA on one high affinity and one or two low affinity sites. Jacobsen[4] has obtained evidence that Lys-240 in HSA may be involved in the high-affinity binding of bilirubin, based on the results of convalent coupling of bilirubin to HSA with carbodiimide. This finding, together with results on bilirubin binding to albumin fragments, indicates that the bilirubin-binding site is mainly associated with loops 3–4 in the model of the secondary structure of HSA. Muller *et al.*[10] reported that the binding sites of warfarin and diazepam on the HSA molecule were different by using the chemical modification of specific amino acids in conjunction with circular dichroism (CD) spectroscopy. The observation of bilirubin, warfarin and diazepam binding to HSA indicated that the binding sites on HSA are independent of each other. However, it is suggested that parts of the bilirubin- and warfarin-binding sites overlap each other. It is well known that phenylbutazone competes with warfarin for binding to HSA. Accordingly the binding sites of phenylbutazone and the warfarin site may be almost identical. The displacement of bilirubin by warfarin or phenylbutazone may be due to binding at the overlapping site. Similarly, sulfinpyrazone, aminopyrine and ibuprofen may also be bound to this overlapping area, while ketophenylbutazone and antipyrine may not bind to the bilirubin and warfarin site. Diazepam did not displace bilirubin (Fig. 3). This result is in good agreement with the observation that the diazepam binding site is independent of the other two sites.

Sudlow *et al.*[12] have suggested two specific binding sites on HSA, site I and site II, for anionic drugs on the basis of fluorescence techniques using two specific probes. Ikeda *et al.*[13] have also classified binding sites into R-site and U-site on the basis of the reaction rate of *p*-nitrophenyl acetate with HSA. Site I showed affinity for bulky heterocyclic molecules with a negative charge and site II for aromatic carboxylate anions. Phenylbutazone, oxyphenbutazone, sulfinpyrazone and warfarin are site I drugs and flufenamic acid and ibuprofen

are site II drugs according to them. The classification of these drugs in the present work was not quite in accordance with that shown by Sudlow *et al.* Though the primary site of bilirubin may overlap with site I, this experiment was carried out with a much higher molar ratio of drug to HSA, so drugs bound to secondary or tertiary sites might affect the binding of bilirubin competitively. This may account for the discrepancy.

Bilirubin is a suitable binding site marker, because it has one high-affinity site, whose location within HSA is known (*i.e.*, on loops 3–4, including Lys-240), and the binding sites of other drugs can thus be studied by means of competitive binding experiments with bilirubin.

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References and Notes

1) T. Peters, "The Plasma Proteins," Vo. 1, ed. by F.W. Putnam, Academic Press, New York, 1975, p. 133.
2) J.J. Vallner, *J. Pharm. Sci.*, **66**, 447 (1977).
3) J. Jacobsen, *FEBS Lett.*, **5**, 112 (1969).
4) C. Jacobsen, *Biochem. J.*, **171**, 453 (1978).
5) P.Q. Behrens, A.M. Spiekerman and J.R. Brown, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, **34**, 591 (1975).
6) R. Brodersen and P. Bartels, *Eur. J. Biochem.*, **10**, 468 (1969).
7) R. Brodersen, *J. Clin. Invest.*, **54**, 1353 (1974).
8) G.E. Means and M.L. Bender, *Biochemistry*, **14**, 4989 (1975).
9) "SEIKAGAKU DATA BOOK," Vol. 2, ed. by the Japanese Biochemical Society, 1980, p. 212.
10) W.E. Muller and U. Wollert, *Pharmacology*, **19**, 59 (1979).
11) A.A. Elbary, J.J. Vallner and C.W. Whitworth, *J. Pharm. Sci.*, **71**, 241 (1982).
12) G. Sudlow, D.J. Birkett and D.N. Wade, *Mol. Pharmacol.*, **12**, 1052 (1976).
13) Y. Ozeki, Y. Kurono, T. Yotsuyanagi and K. Ikeda, *Chem. Pharm. Bull.*, **28**, 535 (1980).