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Drug Interactions. II.[1] Binding of Some Pyrazolone and Pyrazolidine Derivatives to Bovine Serum Albumin[2]

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The binding of some pyrazolone and pyrazolidine derivatives to bovine serum albumin was investigated by a dynamic dialysis method. The drugs used were aminopyrine, antipyrine, 4-aminoantipyrine, phenylbutazone, and oxyphenbutazone. Analysis of the binding data indicated that albumin possesses a single strong binding site and secondary classes of several sites with a much lower affinity for the drugs examined and the values for the association constant of the pyrazolidine derivatives are much greater than that of the pyrazolone derivatives. The affinity of binding is also shown to depend on the hydrophobic character of each drug, as expressed by a partition coefficient. The thermodynamic parameters indicated that these interactions are exothermic and occur spontaneously under the experimental conditions used. It is possible that the pyrazolone derivatives compete with pyrazolidine derivatives for plasma protein binding. However, the extended calculation for the whole body indicates that the plasma protein binding of pyrazolone and pyrazolidine derivatives probably play only a minor role in clinical medication.

Activity of one drug may be modified by prior or simultaneous administration of another, and improved therapy is sometimes possible by concurrent medication. However, serious adverse effects may also arise from drug interaction.[4] Most drugs circulate with blood by binding reversibly to serum albumin. The protein-bound portion is metabolically inactive, but the free portion is active. When highly bound drugs compete for the same binding site, weakly bound drugs may be displaced.[5] Clinically significant responses may result when the bound fraction of one drug is displaced from its binding site by a second drug of greater affinity.[4] Chen, *et al.*[6] found that a commonly prescribed anti-inflammatory agent, phenylbutazone, decreased the plasma half-life of aminopyrine in man, and stated that this observation can be explained by the ability of phenylbutazone to stimulate the metabolism of aminopyrine to 4-aminoantipyrine. However, a decrease in plasma half-life is compatible not only with enzyme induction but also with displacement of aminopyrine from plasma protein-binding sites. Phenylbutazone is a weakly acidic lipid-soluble substance highly bound (98% at therapeutic doses)[7] to the albumin molecule in the plasma. The mechanisms of drug interactions may be quite varied and complex. In some cases, more than one mechanism may be involved. The present study was undertaken to discover the significance of the plasma protein binding on the interactions between some pyrazolone and pyrazolidine derivatives.

Experimental

Materials——Bovine serum albumin (BSA), Fraction V (Armour Co., U.S.A.), was used in this study, and its molecular weight was assumed to be 69000. Aminopyrine, antipyrine, 4-aminoantipyrine, phenyl-

1) Part I: T. Tukamoto, S. Ozeki, F. Hattori, and T. Ishida, *Chem. Pharm. Bull.* (Tokyo), **22**, 385 (1974).
2) Presented at the 93rd Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April 1973.
3) Location: *Tanabe-dori, Mizuho-ku, Nagoya, 467, Japan.*
4) L.K. Christensen, L.M. Hansen, and M. Kristensen, *Lancet*, **2**, 1298 (1963); J.B. Field, M. Ohta, C. Boyle, and A. Remer, *N. Engl. J. Med.*, **277**, 889 (1967); J.A. Udal, *Clin. Med.*, **77**, 20 (1970).
5) A.H. Conney, *N. Engl. J. Med.*, **280**, 653 (1969).
6) W. Chen, P.A. Vrindten, P.G. Dayton, and J.J. Burns, *Life Sci.*, **2**, 35 (1962).
7) B.B. Brodie, *Proc. Roy. Soc. Med.*, **58**, 946 (1965).

butazone, oxyphenbutazone, and other chemicals of analytical grade were obtained from commercial sources, and used without further purification. The bags for dialysis were prepared by the previously reported method.[1] The same phosphate buffer (pH 7.45 and ionic strength 0.16) was used throughout this study.

Method——A dynamic dialysis method, similar to that described by Meyer and Guttman,[8] was used to determine the association constants of the binding of the drugs to BSA. Ten ml of drug or drug–BSA solution were placed in the dialysis bag, the bag was immersed in 200 ml of a buffer solution, and the whole thermostatted; runs were made at 5°, 22°, and 37°, under continuous stirring of both solutions. The initial concentration of aminopyrine was 4.32 mM, antipyrine 5.32 mM, 4-aminoantipyrine 4.92 mM, phenylbutazone 3.24 mM, oxyphenbutazone 3.08 mM, and BSA 0.284 mM. Every 30 min, 100 ml of the external solution was removed and immediately replaced with 100 ml of fresh buffer. Concentration of the drug in the collected sample was determined by spectrophotometry and the total concentration of drug in the protein compartment was calculated.

Partition Coefficient——Partition coefficient was determined by shaking 10 ml of the drug solution(in appropriate phosphate buffer) with 10 ml of cyclohexane for 8 hr at room temperature (20—24°). The concentration of the drug remaining in the aqueous was then determined by spectrophotometry.

Result and Discussion

Binding Data

Aminopyrine, its metabolite, 4-aminoantipyrine, and antipyrine have been reported to interact rather weakly with serum albumin.[9] It was, therefore, of interest to attempt to quantitate its binding behavior by the dynamic dialysis approach. The kinetic curves for each system obtained by the dynamic dialysis experiments were analyzed according to the previously reported method.[1] The treatment of data yielded values for the concentrations of unbound (Df) and bound (Db) species, for a number of different total drug molecule concentration (Dt). The results obtained were used to construct the Scatchard plots where $\bar{v}/Df$ ($\bar{v}$=moles of drug molecule bound per mole of protein) was plotted as a function of $\bar{v}$. Data of typical dynamic dialysis experiments for aminopyrine and BSA at 22° in a phosphate buffer of pH 7.45 is shown in Table I and plotted together with those at 1° and 37° in Fig. 1. The Scatchard plots of all the other drugs investigated also gave similar curved as in Fig. 1. Curvature of such plots may be expressed by an equation developed by Karush.[10] Assuming that there are n binding sites of two types, n_1 and n_2 with association constants k_1 and k_2, and A is the limiting value of $\bar{v}/Df$ as Df approaches zero, the equation becomes:

Table I. A Typical Dynamic Dialysis Experiment of Aminopyrine and BSA at 22° in Phosphate Buffer (pH 7.45)

Total drug concn. (mg)	Free drug concn. (mg)	Bound drug concn. (mg)	$\bar{v}$	$\bar{v}/Df \times 10^{-2}$
7.22	6.46	0.76	1.12	4.02
5.20	4.55	0.65	0.97	4.94
3.79	3.24	0.55	0.82	5.86
2.78	2.31	0.47	0.69	6.97
2.02	1.64	0.38	0.57	8.10
1.52	1.21	0.31	0.46	8.95
1.13	0.88	0.25	0.38	10.01
0.85	0.65	0.20	0.30	10.98
0.64	0.48	0.16	0.24	11.68
0.49	0.36	0.13	0.20	12.82

BSA concentration=2.84×10^{-4}M

8) M.C. Meyer and D. Guttman, *J. Pharm. Sci.*, **57**, 1627 (1968).

9) Brodie, *et al.* reported that antipyrine, aminopyrine, and 4-aminoantipyrine were bound to plasma proteins by about 10, 15, and 20% respectively. R. Soberman, B.B. Brodie, B.B. Levy, J. Axelrod, V. Hollander, and J.M. Steele, *J. Biol. Chem.*, **179**, 31 (1949); B.B. Brodie and J. Axelrod, *J. Pharmacol. Exp. Therap.*, **99**, 171 (1950).

10) F. Karush, *J. Am. Chem. Soc.*, **72**, 2705 (1950).

$$\frac{\bar{\nu}}{Df} = \frac{n_1 k_1}{1+k_1 Df} + \frac{n_2 k_2}{1+k_2 Df} \quad (1)$$

where

$$n = n_1 + n_2 \text{ and } \lim_{Df \to 0} \bar{\nu}/Df = n_1 k_1 + n_2 k_2 = A$$

Using the procedure of Rosenthal,[11] each curved line was resolved into two straight lines. Thus individual values of n_1, n_2, k_1, and k_2 were determined in the manner previously described.[1] The binding data are summarized in Table II.

The value for the association constant was the highest at the lowest temperature, showing a significant decrease with each increment in temperature. The decrease in the binding strength of albumin for the drugs with increasing temperature is characteristic of exothermic reactions. Similar decrease has been demonstrated in insulin,[12] thyroxine,[13] warfarin,[14] ascorbic acid,[1] and fatty acid ascorbyl esters.[1] It is noteworthy that the values for n_1 are identical in all

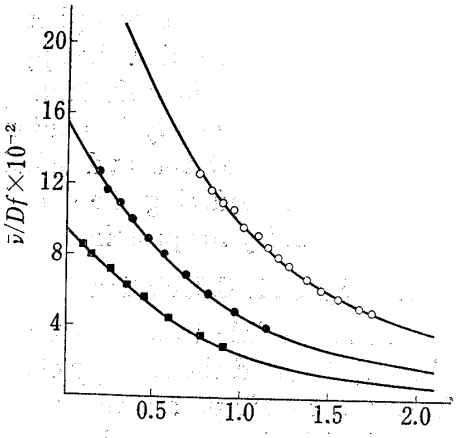

Fig. 1. Binding of Aminopyrine to BSA

—○—: at 1° —●—: at 22° —■—: at 37°

Table II. Binding Constants and Partition Coefficient (P)

Drug	P cyclohexane/buffer (pH 7.45)	Temp. (°C)	n_1	n_2	$k_1 \times 10^{-2}$	$k_2 \times 10^{-2}$
Aminopyrine	0.11	1	1	5	29.0	0.60
		22	1	5	14.0	0.24
		37	1	5	8.7	0.14
Antipyrine	0.019	1	1	5	24.6	0.32
		22	1	5	10.8	0.18
		37	1	5	6.9	0.12
4-Aminoantipyrine	0.023	1	1	5	25.5	0.18
		22	1	5	12.2	0.24
		37	1	5	7.6	0.20
Phenylbutazone	1.86	22	1	8	1300.0	18.7
		37	1	8	752.0	12.5
Oxyphenbutazone	0.82	22	1	8	730.0	12.6
		37	1	8	430.0	10.0

cases and the values for k_1 of the pyrazolidine derivatives are much greater than that of the pyrazolone derivatives. If two kinds of pyrazolidine and pyrazolone derivative are to be used together, the drugs may possibly interact by competing with each other for the same binding sites. Thus the latter with a lower affinity for protein will be displaced from the binding site. Displacement from plasma protein binding increases the amount of active (free) drug in the plasma resulting in a probable increase of its rate of metabolism and excretion. Several workers have appreciated the importance of hydrophobic interactions in the binding of drugs and other small molecules to serum albumin.[15] Efforts to quantitate the hydro-

11) H.E. Rosenthal, *Anal. Biochem.*, **20**, 525 (1967).
12) S.A. Berson and R.S. Yalow, *J. Clin. Invest.*, **38**, 1996 (1959).
13) K. Sterling, P. Rosen, and M. Tabachnick, *J. Clin. Invest.*, **41**, 1021 (1962).
14) R.A. O'Reilly and P.E. Kowitz, *J. Clin. Invest.*, **46**, 829 (1967).
15) F. Helmer, K. Kiehs, and C. Hansch, *Biochemistry*, **7**, 2858 (1968); W. Scholtan, *Arzneimittel-Forsch.*, **18**, 505 (1968).

philic or hydrophobic nature of drug molecules have involved the determination of partition coefficients aqueous buffer and organic solvents. In the present investigation, the partition of drugs between aqueous phosphate buffer and cyclohexane was measured and its results (Table II) have revealed that pyrazolidine derivatives, although they are nearly all ionized[16] at pH 7.45, have relatively high lipid/water partition coefficients, while pyrazolone derivatives, though they are nearly in nonionized form, have a considerably smaller coefficients. Thus, it was found that the hydrophobic groupings play an important role in the drug-receptor interaction considering the correlation between the high oil/water partition coefficients and the high affinity for protein.

Thermodynamic Calculation

It is possible to calculate the standard free energy change, ΔG, the standard enthalpy change, ΔH, and the entropy change, ΔS, for the binding by the previously reported method.[1] Thermodynamic parameters are reported in Table III. The negative signs for the G mean that the binding process is spontaneous. The ΔH are negative, signifying that the binding process is exothermic and that the strength of the association would decrease with an increase in temperature. The ΔS are positive, in agreement with observations on other albumin-drug

Table III. Thermodynamic Parameters of Binding

Drug	ΔG at 22° (kcal/M)	ΔH (kcal/M)	ΔS (e.u./M)
Aminopyrine	−4.244	−1.236	+10.12
Antipyrine	−4.092	−1.190	+9.83
4-Aminoantipyrine	−4.164	−1.257	+9.86
Phenylbutazone	−6.899	−1.453	+18.46
Oxyphenbutazone	−6.561	−1.411	+17.45

interaction.[1,17] It has been suggested that hydration of both drug and BSA in free state will reduce the entropy of the system, but when drug-BSA association occurs, at least some of the bound water is released, thereby decreasing the orderliness, and thus the entropy, of the system.

Clinical Significance

The extent of drug protein binding depends on the concentration of a drug and its affinity for protein. Percentage of drug to plasma protein in the plasma can be estimated by an equation (Eq. 2) developed by Martin,[18] *i.e.*, when $n=1$,

$$\alpha = \frac{[Df]}{[Dt]} = \frac{Kdp+[Df]}{[Pt]+Kdp+[Df]} \qquad (2)$$

where α is the fraction of the total drug in plasma which is not bound to the protein, Pt is the molar concentration of total protein (5.0×10^{-4}M), and Kdp is the dissociation constant ($Kdp=1/k_1$). The values of α were calculated from Eq. 2 as follows, when Dt is 5.0×10^{-4}M and at 37°: Aminopyrine 75.3%; antipyrine 78.6%; 4-aminoantipyrine 77.3%; phenylbutazone 15.4%; oxyphenbutazone 19.7%. However, the free drug in plasma is distributed into

16) The pK_a values of the drugs are as follows: Aminopyrine 5.0[a]; antipyrine 1.4[a]; 4-aminoantipyrine 4.1[b]; phenylbutazone 4.4[a], 4.5[c]; oxyphenbutazone 4.7[c]. *a*) A. Goldstein, L. Aronow, and S.M. Kalman, "Principles of Drug Action," Hoeber Medical Division, Harper & Row, New York, 1968, p. 125; *b*) T. Okano and K. Uekama, *Yakugaku Zasshi*, **87**, 1231 (1967); *c*) A.B. Gutman, P.G. Dayton, T.F. Yü, L. Berger, W. Chen, L.E. Sciam, and J.J. Burns, *Am. J. Med.*, **29**, 1017 (1960).

17) R.A. O'Reilly, *J. Clin. Invest.*, **48**, 193 (1969).

18) B.K. Martin, *Nature*, **207**, 274 (1965).

various tissues throughout the body water, and the bound drug dissociates very rapidly to maintain the equilibrium with plasma water. The body must be considered at least as a two-compartment system; plasma and a compartment composed of the remaining body water. The plasma volume and the total body water constitute typically 5 and 60%,[19] respectively, of body weight, so that on this basis the free drug in plasma is also in equilibrium with an additional volume which is 11 times that of the plasma volume. It is assumed that the free drug uniformly distributed in total body water,[20] without binding or localization of a drug in the tissues. For example, if 800 mg of aminopyrine is administered to a man weighing 60 kg with a plasma volume of 3 liter and total body water of 36 liters; the amount of aminopyrine in the body ($36Df+3Db$) is 0.096 mM, percentage of free aminopyrine ($36Df\times 100/36Df+3Db$) is 96.5, percentage of aminopyrine in plasma ($3Df+3Db\times 100/36Df+3Db$) is 11.5. On the other hand, if 800 mg of phenylbutazone is administered under the same conditions, the amount of phenylbutazone in the body is 0.072 mM, percentage of free phenylbutazone is 24.9, and percentage of phenylbutazone in plasma is 77.1. The extended calculations for the whole body indicate that the binding of a drug to the plasma protein has a prominent effect on drug elimination only when the drug has a high affinity for protein. These data show that at least 75.1% of phenylbutazone in the body has been bound. If the bound phenylbutazone is displaced by competition with each other for the same binding sites, the results would bring about rapid increase in the rate of metabolism and excretion. When only 3.5% of the dose exists in its bound form in the case of aminopyrine possessing a low affinity, the effect on the rate of elimination will be small. As a consequence, the influence of plasma protein binding of pyrazolone and pyrazolidine derivatives appears not to be of importance in accounting for the depressed plasma levels of aminopyrine observed after a long-term administration of phenylbutazone to man.

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19) W.F. Ganong, "Review of Medical Physiology," 5th, Maruzen Co., Ltd., Tokyo, 1971, p. 5.
20) Aminopyrine and antipyrine are distributed in various tissues in close proportion to their water content [R. Soberman, B.B. Brodie, B.B. Levy, J. Axelrod, V. Hollander, and J.M. Steele, *J. Biol. Chem.*, **179** 31 (1949); B.B. Brodie and J. Axelrod, *J. Pharmacol. Exp. Therap.*, **99**, 171 (1950)].