[Chem. Pharm. Bull. 32(8)3155—3163(1984)]

Binding of Hydralazine and a Major Metabolite, Pyruvate Hydrazone, to Rat Plasma Protein and Human Serum Albumin

TARO OGISO* and MASAHIRO IWAKI

Faculty of Pharmaceutical Sciences, Kinki University, Kowakae 3-4-1, Higashi-Osaka 577, Japan

(Received August 26, 1983)

The interaction of hydralazine (HP) and a major metabolite, pyruvate hydrazone (HPH), with rat plasma protein or human serum albumin (HSA) was studied both *in vivo* (by the ultrafiltration method) and *in vitro* (at 20 and 30 °C, by the equilibrium dialysis method). The binding of HP and HPH to plasma proteins was fairly high (96.0 and 81.7%. respectively) after *i.v.* administration to rats. HPH, which had an association constant higher than HP, was found to interact with a single class of site on HSA, while HP was bound to at least two heterogeneous binding sites. The interaction between HP and rat plasma protein or HSA was temperature-dependent at the secondary binding site, suggesting that a nonionic mechanism is involved in the binding, and the interaction at the primary site was ionic strength-dependent. In contrast, the interaction of HPH with HSA showed less temperature-dependence. The thermodynamic analyses of the HP binding process at the secondary site showed a negative value for ΔG°, a large contribution of ΔH° to ΔG° and a positive ΔS°, while at the primary site a large contribution to ΔG° by ΔS° was seen. Thus, these findings suggest that the main binding energies at the primary and secondary sites are derived from electrostatic and nonionic sources, respectively. For HPH binding, however, the predominantly bound species was ionic. HP did not induce competitive displacement of fluorescent probes except for a slight displacement of dansylamide in the presence of a high concentration of HP. This suggests that HP is bound weakly to sites other than sites I and II, two specific sites for acidic drugs, on HSA. The present results lead us to postulate that HP interacts with the receptor sites mainly by hydrophobic and hydrogen bonding forces and partly by electrostatic forces.

Keywords——protein binding; thermodynamic parameter; human serum albumin; rat plasma protein; hydralazine; pyruvate hydrazone

It is well known that drug binding to plasma and tissue proteins can affect the distribution, metabolism and excretion of drugs and is related significantly to pharmacological and toxicologic actions. The plasma protein binding of acidic drugs has been extensively studied, but relatively little knowledge of the binding of basic drugs is available. Hydralazine (HP) is a widely used hypotensor which acts by direct dilation of peripheral arterioles;[1] its metabolism[2,3] and pharmacokinetics[4-6] have been reviewed. However, fairly little is known on the binding of HP and its metabolites to plasma proteins; Lesser *et al.*[7] found that the binding of ^{14}C-HP is fairly high (87%) to human plasma at clinically significant concentrations, and Wagner *et al.*[8] reported that *in vitro* HP binds to human serum to the extent of 90%. However, detailed thermodynamic studies of the interactions of HP or its major metabolites with albumin have not been reported. We have, therefore, investigated the interaction of HP and a metabolite, pyruvic acid hydrazone, with rat plasma protein and human serum albumin (HSA), and carried out thermodynamic studies, in order to gain an understanding of the protein binding of HP and its major metabolite and to obtain insight into the nature of the interaction of the drugs with the receptor sites for pharmacological activity.

Experimental

Materials——Hydralazine hydrochloride and 1-hydrazino-4-methyl-phthalazine, an internal standard for gas-liquid chromatography (GLC), were kind gifts from Ciba-Geigy Co., Ltd. Human serum albumin (HSA, Fraction V, fatty acid-free) was purchased from Miles Laboratories, Inc. Dansylamide and dansyl-L-proline were obtained from Sigma Chemical. Co. Auramine-O (4,4′-carbonimidazoylbis[*N*,*N*-dimethyl]benzenamine monohydrochloride) was obtained from Eastman Kodak Co. All other chemicals used were of special or analytical grade.

Synthesis of Hydralazine Pyruvate Hydrazone——Hydralazine pyruvate hydrazone (HPH) was synthesized from HP by the method of Haegele *et al.*[9] The purity of the product was estimated by thin layer chromatography and spectroscopic analyses.

***In Vivo* Binding Study of HP and Hydrazones to Rat Plasma**——Male Wistar rats weighing 180—200 g were used throughout the study. The animals had free access to MF diet (Oriental Yeast Co., Ltd.) and water before and during the experiment. Animals received HP (7.5 mg/kg) or hydrazone (3 mg/kg) intravenously from the tail vein, and blood was taken from the jugular vein 30 min after administration. Plasma was obtained immediately by centrifugation and placed in a dialysis bag (8/32 Visking tubing; Sanko Pure Chemical Co.) which was held within a test tube as described by Kaneo *et al.*[10] The tube was centrifuged at 3000 rpm for 30 min at 4 °C. The concentration of HP or hydrazones in the filtrate and that inside the bag were determined according to the GLC method described by Zak *et al.*,[11] using a Hitachi 163 gas chromatograph with an electron capture detector.

***In Vitro* Binding Study of HP and HPH to Rat Plasma Protein and HSA**——Rat plasma containing 1 mg/ml of Na_2EDTA was delipidated with butanol–diisopropyl ether[12] and diluted with 2 volumes of 0.067 M phosphate buffer (pH 7.4). The equilibrium dialysis technique was used to determine binding of drugs to rat plasma protein and HSA by using the apparatus described by Goto *et al.*[13] with protection from light. The two compartments containing 3 ml each of drug solution (HP, 1—200×10^{-5} M; HPH, 5—100×10^{-5} M) and 5.2×10^{-5} M HSA or diluted rat plasma (17.0—20.6 mg protein/ml) were separated by a washed dialysis cellulose membrane (cellulose tubing, C-65, Sanko Pure Chemical Co.). Rat plasma protein concentration was estimated by the procedure described by Lowry *et al.*[14] All drugs and HSA were dissolved in 0.067 or 0.33 M phosphate buffer (pH 7.4). Equilibrium was reached within 12 h for HP and 28 h for HPH at 20 °C. During these experiments, 8.9 and 10.6% of HP was lost at 20 and 30 °C, respectively, and 6.8 and 27.0% of HPH was lost at 20 and 30 °C, respectively, probably due to decomposition. The tube (cellulose membrane) binding of HP during the experiment was found to be small (approximately 8.6×10^{-9} mol), while the binding of HPH was negligible. Thus, the free ligand concentration after equilibrium was normalized by correcting for the amount decomposed and lost. HP and HPH concentrations in the protein-free compartment were determined by the GLC method described above and by spectrophotometric determination at λ_{max} 340 nm, respectively.

Displacement of Fluorescent Probes from HSA by HP——Displacement of fluorescent probes from HSA by HP was determined according to the method described by Sudlow *et al.*[15] using a Hitachi 650-10 spectrofluorometer. The probes used were dansylamide, dansyl-L-proline and auramine-O. HP was added at a 1:1 or 10:1 molar ratio to HSA.

Data Analysis and Estimation of Thermodynamic Parameters——The data were analyzed by the method of Scatchard.[16] When the Scatchard plot was linear, the binding parameters were calculated by linear least-squares regression analysis according to Eq. (1):

$$r/C_f = K\cdot n - K\cdot r \qquad \text{(Eq. 1)}$$

where K and n are the association constant and the number of binding sites, respectively; C_f is the concentration of free ligand, and r is the number of moles of ligand bound per mole of protein, assuming a molecular weight of 66000.[17]

When the Scatchard plot was curved, the data from binding experiments were subjected to curve fitting according to the "multiple equilibria theory," which yields Eq. (2), by the iterative nonlinear least-squares regression procedure, MULTI,[18] written in the BASIC programing language:

$$r=\frac{n_1K_1C_f}{1+K_1C_f}+\frac{n_2K_2C_f}{1+K_2C_f} \qquad \text{(Eq. 2)}$$

where K_1 and K_2 are the association constants corresponding to n_1 and n_2, the numbers of primary and secondary class sites, respectively.

Thermodynamic parameters were calculated from the association constants according to Eqs. (3)—(5):[19-21]

$$\Delta G^\circ = -\boldsymbol{R}\cdot T\ln K \qquad \text{(Eq. 3)}$$

$$\ln\frac{K_{II}}{K_I}=\frac{\Delta H^\circ}{\boldsymbol{R}}\left(\frac{1}{T_I}-\frac{1}{T_{II}}\right) \qquad \text{(Eq. 4)}$$

$$\Delta G^\circ = \Delta H^\circ - T \cdot \Delta S^\circ \qquad \text{(Eq. 5)}$$

where ΔG° is the standard free energy change on binding; ΔH° is the standard enthalpy change for the association of 1 mol of drug with 1 mol of the binding sites; ΔS° is the entropy change; and R and T are the gas constant and absolute temperature, respectively.

Results

In Vivo Binding of HP and HPH to Rat Plasma

Total concentration and percent binding of each compound are summarized in Table I. By means of the ultrafiltration technique, both HP and hydrazone metabolite, HPH, were found to be extensively bound to rat plasma. Approximately 96% of HP and 82% of HPH in plasma were associated with plasma protein. This indicates that the level of binding of HP to rat plasma protein approximates to that reported by other workers (human serum, 87 and 90%).[7,8]

In Vitro Binding of HP to Rat Plasma Protein

Figure 1 shows the Scatchard plot of the data, where the binding of HP to rat plasma was assessed at 20 or 30 °C and at pH 7.4 by means of equilibrium dialysis. The Scatchard plot for HP binding to plasma was seen to bend sharply, indicating that at least two sets of binding

TABLE I. *In Vivo* Binding of HP and HPH to Rat Plasma Protein

Compound	Drug concentration in plasma (μg/ml)	Percent bound
HP	5.455±0.777	96.0±2.3
HPH	3.481±0.320	81.7±2.6

Experimental conditions are described in the text. The values are the means±S.D. of 5—7 experiments.

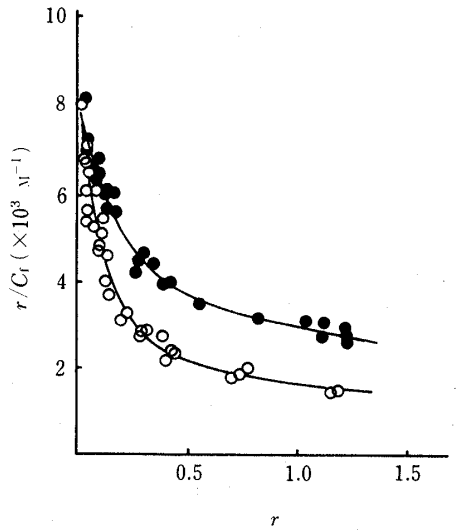

Fig. 1. Scatchard Plots for HP–Rat Plasma Protein Interaction in 0.067 M Phosphate Buffer (pH 7.4) at 20 and 30 °C

Plasma protein was estimated as albumin (molecular weight 66000). Points are observed data from 3—4 experiments and solid lines are computed from the binding parameters. ●, 20 °C; ○, 30 °C.

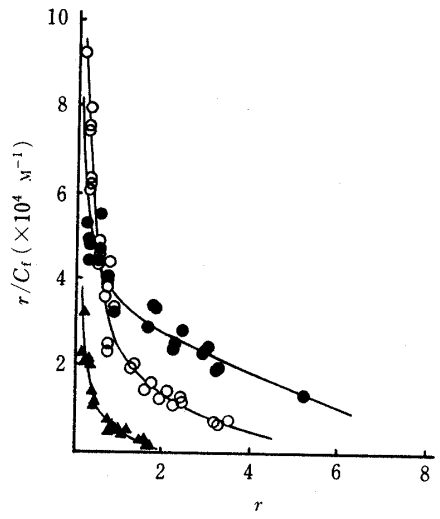

Fig. 2. Scatchard Plots for HP–HSA Interaction at 20 and 30 °C

HSA concentration was 5.2×10^{-5} M. ●, at 20 °C in 0.067 M phosphate buffer; ○, at 30 °C in 0.067 M phosphate buffer; ▲, at 20 °C in 0.33 M phosphate buffer. Other details are as in Fig. 1.

TABLE II. Binding Parameters for Interaction of HP with Rat Plasma Protein at pH 7.4 and Thermodynamic Data for Interaction with the Primary and Secondary Sites

Temp. °C	n_1 [a]	K_1 [b] ($\times 10^4$ M^{-1})	ΔG° [c]	ΔH° [c]	ΔS° (e.u.)	n_2 [a]	K_2 [b] ($\times 10^2$ M^{-1})	ΔG° [c]	ΔH° [c]	ΔS° (e.u.)
20	0.16	3.33	−6.06	+4.88	+37.33	6.5	4.40	−3.54	−2.73	+2.80
30	0.14	4.39	−6.44	+4.88	+37.33	4.5	3.77	−3.57	−2.73	+2.80

a) The number of binding sites. b) Association constant.
c) Values are expressed as kcal per mol.
Protein concentration was 17.0—20.6 mg/ml in the compartment before equilibrium.

TABLE III. Binding Parameters for Interaction of HP with HSA at pH 7.4 and Thermodynamic Data for Interaction with the Secondary Site

Conditions	n_1 [a]	K_1 [b] ($\times 10^5$ M^{-1})	n_2 [a]	K_2 [b] ($\times 10^3$ M^{-1})	ΔG° [c]	ΔH° [c]	ΔS° (e.u.)
20 °C, 0.067 M	0.16	4.34	8.7	3.81	−4.80	−2.97	+6.25
20 °C, 0.33 M	0.25	1.79	2.0	3.42	—	—	—
30 °C, 0.067 M	0.45	2.54	5.0	3.22	−4.86	−2.97	+6.25

a) The number of binding sites. b) Association constant.
c) Values are expressed as kcal per mol.
Albumin concentration was 5.2×10^{-5} M.

sites exist on the protein. The calculated binding parameters are shown in Table II. This result demonstrated that HP has two classes of binding sites, high affinity with low capacity and low affinity with high capacity, on rat plasma protein. As shown in Table II, the association constant (K_2) of HP to the secondary binding site was found to decrease moderately with increase in temperature (20 to 30 °C), while that to the primary class was increased by 31.8% under the same conditions. The increased temperature caused a 14.3% decrease in the association constant for the secondary binding site (Table II), this being characteristic of an exothermic drug–protein interaction. The numbers of secondary binding sites, n_2, are 6.5 and 4.5 at 20 and 30 °C, respectively.

In Vitro Binding of HP to HSA

The data on the binding of HP to 5.2×10^{-5} M HSA at 20 and 30 °C are shown in Fig. 2, and the associated binding parameters are summarized in Table III. These data suggest that the binding characteristics to HSA are similar to those to rat plasma protein as regards temperature dependency of the binding at the secondary site and high affinity to the primary site, except that there was a significant difference in the association constant, K_2, between rat plasma and HSA and in the binding to the primary site. As shown in Table III, decreases in the K_2 value and the number of sites of the secondary class were seen with increase in temperature from 20 to 30 °C in the HP–HSA interaction. These results also indicate that the nature of the binding process for HP and HSA was mainly exothermic. A 5-fold increase (0.33 M phosphate buffer, pH 7.4) in buffer ionic strength at 20 °C caused a significant change in the binding constant (K_1) of HP, as shown in Fig. 2 and Table III. This suggests that the binding of HP to the primary site of HSA involves ionic bonding.

The contribution of each individual class of sites to total binding in the HP–HSA interaction was compared at 20 and 30 °C by computation[22] based on the binding parameters. Figure 3 illustrates the profile for bound HP fraction *versus* free drug

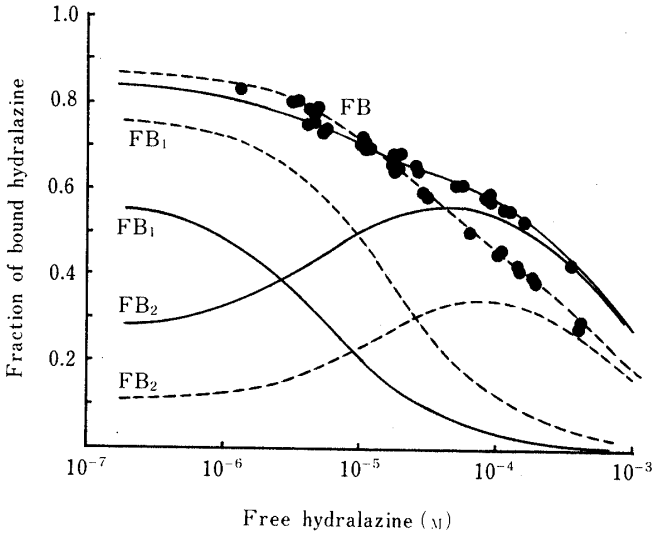

Fig. 3. Contributions of the Primary and Secondary Sites to the Total Bound Fraction in HP–HSA Interaction

The lines were computed based on the HP–HSA binding parameters. FB_1 and FB_2 are fractions bound at the primary and secondary sites, respectively. FB is the total bound fraction. The experimental values for bound fraction are shown as points. ——, at 20 °C; – – – –, at 30 °C.

concentration at each temperature. The total bound fraction was considerably altered by the change of temperature. At 20 °C the contribution of the secondary binding site appears to be relatively large at low drug concentrations. It is of particular interest that the crossover point, at which the binding at the primary and secondary classes of sites was equal, was shifted toward high free ligand concentration and the relative contribution of the secondary binding site was decreased with increasing temperature. This may result from the smaller binding capacity of the primary site at 20 °C than at 30 °C, and may reflect variation in the number of binding sites at the crossover points. At 20 °C only 2.0% of the albumin sites were occupied at the crossover point, while 14.3% of the sites were occupied at 30 °C, as calculated according to the formula $r/(n_1+n_2)$.[22]

Thermodynamic Analysis of HP–Rat Plasma Protein or HP–HSA Binding

The thermodynamic parameters calculated from the association constants for the primary and secondary binding sites at two (20 and 30 °C) temperatures, and the familiar thermodynamic relationship described in the experimental section, are presented in Table II (rat plasma protein) and Table III (HSA).

In the binding to the primary binding site of rat plasma protein, the standard change in free energy, $\Delta G°$, was negative and the entropy change was considerably positive (+37.3 e.u.), with a large contribution to $\Delta G°$ by $\Delta S°$.

In the binding to the secondary site, the standard change in free energy, $\Delta G°$, was also negative and the values at 20 and 30 °C were −3.54 and −3.57 kcal/mol, respectively. This negative sign for $\Delta G°$ indicates that the binding process under these conditions was spontaneous, as shown for albumin binding of warfarin.[23] $\Delta H°$ was negative, −2.73 kcal/mol, and thus the binding process for HP and rat plasma protein might be exothermic. Thermodynamic analysis of the HP–rat plasma protein binding indicated a large contribution of $\Delta H°$ (approximately 77%) to $\Delta G°$ and a positive entropy change of +2.8 e.u.

These results suggest, therefore, that significant portions of the binding energy at the primary and secondary binding sites were probably derived from electrostatic and hydrophobic interactions, respectively. Thermodynamic analysis of the HP–HSA interaction gave results similar to those obtained with rat plasma protein, as shown in Table III.

Binding of HPH to HSA

The binding of HPH to HSA was examined at 20 and 30 °C and at pH 7.4. The Scatchard plots of HPH–HSA interaction are shown in Fig. 4. In contrast with HP, Scatchard analysis of

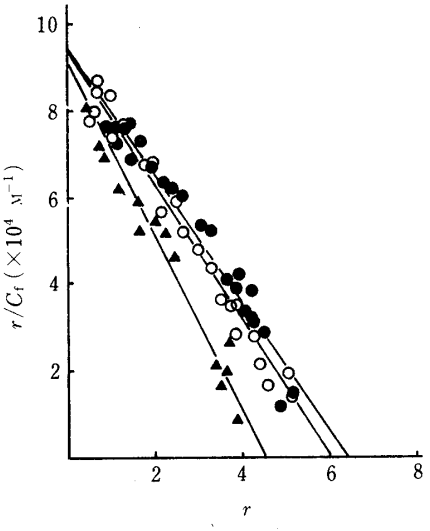

Fig. 4. Scatchard Plots for HPH–HSA Interaction at 20 and 30 °C

Details and symbols are as in Fig. 2.

TABLE IV. Binding Parameters for Interaction of HPH with HSA at pH 7.4

Conditions	n	K ($\times 10^4$ M^{-1})
20 °C, 0.067 M	6.0	1.53
20 °C, 0.33 M	4.6	1.96
30 °C, 0.067 M	6.5	1.44

The n and K values are the number of binding sites and the association constant, respectively.

the data obtained resulted in a straight line relation over the whole concentration range (5—100×10^{-5} M). This evidently suggests that HPH has a single affinity site on the HSA molecule. The most interesting result was that the binding process of HPH to HSA was much less sensitive to temperature and that similar binding capacities were observed at 20 and 30 °C, as shown in Table IV. A 5-fold increase in buffer ionic strength at 20 °C induced a significant decrease in the number of binding sites (n). Such temperature-independent and ionic strength-dependent characteristics for HPH binding suggest that the driving force of HPH association to HSA is probably ionic.

Displacement of Fluorescent Probes from HSA by HP

In order to characterize the binding sites of HP to HSA, displacement of fluorescent probes by HP was investigated. There were no changes in fluorescence intensity of site I (dansylamide) and site II binding probes (dansyl-L-proline) when HP was added up to a 1 : 1 molar ratio to albumin, and only a small change (10.5% decrease) in the fluorescence intensity of HSA-bound dansylamide was seen in the presence of HP at a 10 : 1 molar ratio to albumin. Further, no changes in fluorescence intensity of a cationic dye, auramin-O, were by HP addition. These results suggest that HP is primarily bound to sites other than sites I and II on HSA.

Discussion

The binding of HP or some metabolites to plasma and albumin have been studied by the ultrafiltration or the equilibrium dialysis method,[7] and it was shown that HP is extensively bound to human plasma[7] and serum.[8] We found that, in an *in vivo* experiment, both HP and its hydrazone metabolite, HPH, were extensively bound to rat plasma (Table I) and that HP

was more strongly bound than HPH. The percentage of HP bound to rat plasma protein was approximately the same as in the case of human plasma.

On the other hand, HP is relatively rapidly cleared from plasma after administration,[4,7,24] contrary to expectation based on the fact that this drug is extensively bound to plasma protein. This may be explained if the binding force of HP to protein is inherently weak. This possibility is supported by the observation that HP did not displace fluorescent probes for two specific binding sites and auramin-O from HSA. A similar conclusion was reached for the albumin binding of penicillin.[25]

In vitro bindings of HP and HPH to rat plasma protein or HSA were investigated at pH 7.4 and 20 or 30 °C. HP has two heterogeneous binding sites on rat plasma protein and HSA, but HPH has only a single class of binding site on HSA. The association constant K for HPH–HSA interaction was approximately 4-fold higher than that (K_2) of HP, indicating that the binding strength of HSA for HPH is higher than that for HP. The present study showed that the binding strength (K) of proteins for HPH was little affected by increasing the temperature, while HP binding at the secondary class of binding sites was significantly decreased. The latter result suggests that the binding process for HP and plasma at the secondary sites is a exothermic reaction, as has been reported for the albumin binding of warfarin,[23] thyroxine, methicillin and oxacillin.[26] The decrease in the n_2 value for both rat plasma protein and HSA with increasing temperature may be induced by the decrease in binding strength (n_2K_2) (Tables II and III).

Thermodynamic analysis of the HP binding process at the primary site (Table II) indicated that ΔG° was negative and that ΔS° was highly positive. Therefore, the main source of the ΔG° would have been the large $+\Delta S^\circ$, with a little contribution from the $+\Delta H^\circ$ factor. This suggests that the nature of the interaction at the primary binding site is largely electrostatic, as found with strongly ionized azo dye and albumin.[27] These results agree reasonably well with the data for K_1 obtained by changing the ionic strength, so the binding characteristics of HP to the primary site of HSA might depend largely on ionic bonding (Fig. 2 and Table III). In the binding to the secondary binding site of protein, the contribution of ΔH° to ΔG° was large (approximately 77 and 62% for rat plasma and HSA, respectively). In the interaction of nonionized azo dyes[27] or warfarin[23] with albumin the contribution of ΔH° to ΔG° was nearly half. These findings suggest that a significant portion of the binding energy in the HP–protein interaction at the secondary site was derived from nonionic sources. In the absence of any significant electrostatic interaction, the positive entropy change probably results from hydrophobic bonding,[28] whereas the significant evolution of heat suggests hydrogen bonding. Therefore, the negative enthalpy change and positive entropy change indicate that both hydrogen and hydrophobic bonding may occur together in the interaction of HP with the receptor sites. Cho *et al.*[21] reported that a ΔH° contribution of 83% to ΔG° is seen in the bishydroxycoumarin–BSA interaction and that the protein molecule possesses hydrophobic regions which are highly selective for the drug. Similar conclusions were reached for sulfonylurea–BSA binding[29,30] and diuretics–BSA binding.[31]

On the other hand, varying the temperature (20 and 30 °C) had little effect on the association constant for HPH, whereas an increase in ionic strength caused a significant change in K and n for the metabolite (Fig. 4 and Table IV), suggesting that the HSA binding of HPH might be ionic, since the hydrazones formed have one or two carboxyl groups that are negatively charged at pH 7.4. Such temperature-independent binding of ionic molecules has been demonstrated for sulfonamide,[32] dodecyl sulfate[33] and caprylate.[34] Chignell[35] found that in flufenamic acid–protein interaction the aromatic ring is attached to a hydophobic region on the protein molecule and the carboxyl group is bound to the surface of the protein (which is positively charged) by the electrostatic force.

The binding characteristics of HP to rat plasma protein were similar to those to HSA,

that is, the binding of HP was sensitive to temperature and the binding capacity at the primary class of binding sites was extremely small; however, the association constants, K_1 and K_2, obtained for rat plasma were about one order of magnitude smaller than that for HSA (Tables II and III). The discrepancy may be due to the interspecies difference in binding characteristics, protein concentrations and endogenous substances such as globulin and α_1-acid glycoprotein between rat and human.

The computer simulation for the contribution of the individual classes of sites to total binding revealed variation in the binding profiles at each site with temperatures, and a high temperature dependence of the secondary class of binding sites.

To clarify the nature of the binding sites of HP on HSA, we examined whether HP displaces fluorescent probes or not. HP causes little competitive displacement of the probes when added at a 1 : 1 molar ratio to HSA, and only a 10% displacement of dansylamide by HP was seen when the HP concentration was 10 times higher. Dansylamide and dansyl-L-proline are assumed to bind to two separated binding sites on the albumin molecule, site I and site II, respectively,[16] Thus, it is suggested that HP binds weakly, by hydrogen and hydrophobic interactions, at sites other than sites I and II on albumin at the physiological concentration of HP.

In conclusion, HP was extensively bound to the plasma protein *in vivo*, and the binding of HP to the proteins showed a two-phase characteristic. Thermodynamic analysis of the HP–albumin binding revealed that the nature of the interaction at the primary site was largely electrostatic, that the HP binding process at the secondary site was an exothermic reaction, and that a significant portion of the binding energy was derived from hydrophobic forces. Conversely, HPH–protein interaction might be ionic and HPH appears to have a single affinity site on HSA.

References

1) C. S. Goodman and A. Gilman (eds.), "The Pharmacological Basis of Therapeutics," 5th ed., Macmillan, New York, 1975, pp. 707–709.
2) Z. H. Israili and P. G. Dayton, *Drug Metab. Rev.*, **6**, 283 (1977).
3) K. Schmid, W. Küng, W. Riess, C. T. Dollery and S. J. Harland, *Arzneim.-Forsch, Drug Res.*, **31**, 1143 (1981).
4) T. Talseth, *Clin. Pharmacokin.*, **2**, 317 (1977).
5) A. M. M. Shepherd, T. M. Ludden, K. D. Haegele, T. Talseth and J. L. McNay, *Res. Commun. Chem. Pathol. Pharmacol.*, **26**, 129 (1979).
6) D. D. Shen, J. P. Hosler, R. L. Schroder and D. L. Azarnoff, *J. Pharmacokinet. Biopharm.*, **8**, 53 (1980).
7) J. M. Lesser, Z. H. Israili, D. C. Davis and P. G. Dayton, *Drug Metab. Dispos.*, **2**, 351 (1974).
8) J. Wagner, J. W. Faigle, P. Imhof and G. Liehr, *Arzneim.-Forsch, Drug Res.*, **27**, 2388 (1977).
9) K. D. Haegele, H. B. Skrdlant, T. Talseth, J. L. McNay, A. M. M. Shepherd and W. A. Clementi, *J. Chromatogr.*, **187**, 171 (1980).
10) Y. Kaneo, A. Nishikawa and Y. Kato, *Chem. Pharm. Bull.*, **27**, 2021 (1976).
11) S. B. Zak, G. Lukas and T. G. Gilleran, *Drug Metab. Dispos.*, **5**, 116 (1976).
12) B. Cham and B. R. Knowles, *J. Lipid Res.*, **17**, 176 (1976).
13) S. Goto, H. Yoshitomi and M. Kishi, *Yakugaku Zasshi*, **97**, 1219 (1977).
14) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1950).
15) G. Sudlow, J. Birkett and D. N. Wada, *Mol. Pharmacol.*, **11**, 824 (1975).
16) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).
17) Nihon Seikagakukai (ed.), "Seikagaku Data Book," Tokyo Kagaku Dojin, Tokyo, 1979, p. 96.
18) K. Yamaoka, Y. Tanigawara, T. Nakagawa and T. Uno, *J. Pharm. Dyn.*, **4**, 879 (1981).
19) I. M. Klotz, "The Proteins," Vol. 1, part B, H. Neurath and K. Bailey (eds.), Academic Press Inc., New York, 1953, p. 111.
20) A. N. Martin, "Physical Pharmacy," 2nd Ed., Lea and Febiger, Philadelphia, 1969, p. 343.
21) M. J. Cho, A. G. Michell and M. Pernarowski, *J. Pharm. Sci.*, **60**, 196 (1971).
22) R. F. Mais, S. Keresztes-Nagy, J. F. Zaroslinski and Y. T. Oester, *J. Pharm. Sci.*, **63**, 1423 (1974).
23) R. A. O'Reilly, *Ann. N. Y. Acad. Sci.*, **226**, 293 (1973).

24) T. Ogiso, M. Iwaki and N. Ohtsuki, *Chem. Pharm. Bull.*, **31**, 247 (1983).
25) K. Tsuda and H. Nogami (eds.), "Seizaissekei-hou (1) Biopharmacy," Chijin-Shokan, Tokyo, 1975, pp. 160–165.
26) R. A. O'Reilly, *J. Clin. Invest.*, **46**, 829 (1967).
27) I. M. Klotz and J. M. Urquhart, *J. Am. Chem. Soc.*, **71**, 847 (1949).
28) R. A. O'Reilly, *J. Clin. Invest.*, **48**, 193 (1969).
29) S. Goto, H. Yoshitomi and Y. Nakase, *Chem. Pharm. Bull.*, **26**, 472 (1978).
30) M. J. Crooks and K. F. Brown, *J. Pharm. Pharmacol.*, **26**, 304 (1974).
31) S. Goto, Y. Odawara, M. Nakano and Y. Araki, *Chem. Pharm. Bull.*, **26**, 2298 (1978).
32) B. D. Davis, *J. Clin. Invest.*, **22**, 753 (1943).
33) F. W. Putnam and H. Neurath, *J. Biol. Chem.*, **159**, 195 (1945).
34) P. D. Boyer, G. A. Ballou and J. M. Luck, *J. Biol. Chem.*, **167**, 407 (1947).
35) C. F. Chignell, *Mol. Pharmacol.*, **5**, 455 (1969).