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Binding of Commercial Diuretics with Bovine Serum Albumin[1a,b]

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The binding of seven commercial diuretics with bovine serum albumin (BSA) was investigated at three different temperatures, 5°, 30° and 37°, by the equilibrium dialysis method and at 37° by the dynamic dialysis method. The binding data of diuretics were described on the basis that the sites per BSA molecule can be divided into two classes, except that of hydrochlorothiazide, and each class characterized by the binding constants, K_1, n_1, and K_2, n_2. The thermodynamic values, $\Delta G°$, $\Delta H°$, and $\Delta S°$ for the primary site of binding were determined, and were discussed on the basis of the fact that a hydrophobic interaction is formed between diuretics and BSA. It was also shown that there are sizable negative entropy and enthalpy changes in the binding of ethacrynic acid and BSA, and that hydrochlorothiazide is bound very weakly with BSA and has a remarkable diffusion pattern through cellulose membrane in the dynamic dialysis.

Keywords——diuretics; bovine serum albumin; equilibrium dialysis method; dynamic dialysis method; thermodynamic parameter; ethacrynic acid binding; hydrochlorothiazide binding; hydrophobic interaction

Preliminary study on the stability and binding to bovine serum albumin (BSA) of seven commercial diuretics in aqueous solution was reported in our previous paper.[1b] In the present study, extensive investigation was designed to clarify the nature and possible mechanism of the binding of diuretics and BSA by analysis of the temperature dependence, and by the equilibrium and dynamic dialysis methods.

Experimental

Materials——Bovine serum albumin, fraction V (Armour Pharmaceutical Co., U.S.A.) was used, and its molecular weight was assumed to be 69000. Reagent grade KH_2PO_4 and Na_2HPO_4 were used to prepare the pH 7.4 buffer solution (1/15M). All diuretics and BSA solutions were prepared immediately before use for equilibrium and dynamic dialyses.

Equilibrium Dialysis Method——The general approach, technique, and treatment of data of this study were the same as described previously.[1b,3]

Dynamic Dialysis Method——Dialysis cell made of Teflon resin was the same as that employed in our previous experiment,[4] and the procedure in the previous paper[4] was followed to cover various concentration of BSA at 37°.

Spectrophotometric Analysis——The BSA-free compartment in equilibrium and dynamic cells was analyzed for free diuretics. A Hitachi spectrophotometer Model 181 was used for analysis, at 292 nm for chlorothiazide, at 273 nm for hydrochlorothiazide, at 274 nm for hydroflumethiazide, at 271 nm for trichlormethiazide, at 271 nm for furosemide, at 277 nm for ethacrynic acid, at 319 nm for bumetanide using 1/15M phosphate buffer as a blank.

1) *a*) This paper forms part IV of series entitled "Interaction between Drugs and Blood Components"; *b*) Part III: S. Goto, Y. Odawara, M. Nakano, and Y. Araki, *Yakugaku Zasshi*, **98**, 236 (1978).
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3) S. Goto, H. Yoshitomi, and M. Kishi, *Yakugaku Zasshi*, **97**, 1219 (1977).
4) S. Goto, T. Hara, and H. Yoshitomi, *Yakugaku Zasshi*, **96**, 565 (1976).

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Results and Discussion

Thermodynamic Analysis and Mechanism of Diuretic-BSA Binding

The standard changes in free energy, $\Delta G°$, enthalpy, $\Delta H°$, and entropy, $\Delta S°$ for the binding of one mol of diuretics were calculated by usual thermodynamic equations[3] from the data for the binding constant of primary binding site, K_1, at two (5° and 30°) or three (5°, 30° and 37°) temperatures. Binding at the primary site is regarded as of importance compared with that at secondary site as shown in the case of the ethacrynic acid–BSA binding. Therefore, discussion and calculation were performed for the binding constant at the primary site only. Because of the possible competitive role of the buffer ions in the binding, the K values are regarded as being dependent on the buffer compositions. With respect to the thermodynamic quantities this means that the standard state includes a buffer which is 1/15M phosphate, pH 7.4.

Table I. Summary of Binding Constant and Maximum Number for Diuretics at pH 7.4

Diuretics	Temp. (°C)	$K_1(\times 10^3\,M^{-1})$	$K_2(\times 10^3\,M^{-1})$	n_1	n_2
Chlorothiazide	5	56.1	6.56	1.28	1.25
	30	29.4	6.81	0.88	2.12
	37	27.4	5.45	0.82	2.47
Hydrochlorothiazide	5	—	0.57[a]	—	4.47[a]
	30	—	0.25[a]	—	7.78[a]
Hydroflumethiazide	5	33.5	2.07	0.58	1.57
	30	19.8	0.42	0.20	1.57
Trichlomethiazide	5	37.7	6.43	1.04	1.48
	30	25.7	3.40	0.62	2.77
Furosemide	5	675	5.44	0.69	4.43
	30	375	4.16	0.65	4.85
	37	355	5.13	0.61	4.98
Ethacrynic acid	5	81.8	2.91	6.37	14.8
	30	5.34	2.14	4.76	10.6
Bumetanide	5	54.1	1.04	0.80	9.37
	30	46.2	0.91	0.77	8.37

a) Hydrochlorothiazide has only one class binding site and this binding parameter could be looked as the same degree with K_2 and n_2 of another diuretics.

Table II. Thermodynamic Data for Diuretic-Bovine Serum Albumin Binding at pH 7.4

Diuretics	Temp. (°C)	For K_1		
		$\Delta G°$(kcal/mol)	$\Delta H°$(kcal/mol)	$\Delta S°$(e.u.)
Chlorothiazide	5	−6.04		
	30	−6.20	−4.33	6.17
	37	−6.30		
Hydroflumethiazide	5	−5.76		
	30	−5.95	−3.66	7.55
Trichlomethiazide	5	−5.82		
	30	−6.11	−2.57	11.7
Furosemide	5	−7.42		
	30	−7.73	−3.94	12.5
	37	−7.87		
Ethacrynic acid	5	−6.25		
	30	−5.16	−18.3	−43.4
Bumetanide	5	−6.02		
	30	−6.47	−1.06	17.9

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The values for the binding constants, K_1 and K_2, and numbers of binding sites, n_1 and n_2, are summarized in Table I, and the above thermodynamic parameters for K_1 are shown in Table II. From Table I it is noted that the values of n are not integers. This might be either due to the experimental error in the equilibrium dialysis method or to the view that BSA might not be molecularly homogeneous with respect to binding properties.

Naturally, the exact mechanism of binding of diuretics by BSA cannot be elucidated from this study. However, certain tentative conclusion may be drawn. At first, the decrease in binding constants with increasing temperature is characteristic of an exothermic reaction and has been reported for many protein interactions. The negative sign for $\Delta G°$ means that the binding process is spontaneous. $\Delta S°$ which is the molecular disorder factor, is positive except for ethacrynic acid and this fact agrees with observations for other albumin-anion interactions.[5] If the nature of these interactions is largely electrostatic, that is, if the ionic part of the diuretic molecules combines with the cationic part of the BSA molecule as shown in the previous paper,[1b] the main source of $\Delta G°$ value would be derived from a large contribution of $\Delta S°$ term with little contribution from the $\Delta H°$ factor. However, thermodynamic changes for the diuretic-BSA binding indicate a large contribution of the ΔH° factor to the $\Delta G°$ value (this contribution is approximately 20—70%). It is, therefore, very unlikely that the binding is electrostatic and significant portion of the binding energy may be derived from nonelectrostatic sources. The positive entropy change probably results from hydrophobic bonding.[6] A similar interpretation for the interaction between human serum albumin (HSA) and warfarin has been suggested by O'Reilly and Kowitz,[7,8] and by Oester and his co-workers.[9] The same conclusion was also obtained in our previous studies[3,10] on the sulfonylurea–BSA binding.

The positive $\Delta S°$ associated with many interactions involving proteins is usually attributed to disorientation and unfolding of the protein molecule. This does not offer a satisfactory explanation for the binding in the present work, because enthalpy changes observed are negative. The process of unfolding presumably requires the breaking or bending of several bonds and should result in an endothermic, not an exothermic, reaction of appreciable magnitude.[11] From the "iceberg" concept of water structure, the disordering effect due to the melting of "iceberg" structure probably exceeds the ordering effects (lose rotational and translational degree of freedom after diuretic–BSA binding and negative $\Delta S°$ will be produced) resulting in the net entropy change.

The thermodynamic parameters recorded for warfarin ($\Delta G°=-7.34$ kcal/mol, $\Delta H°=-2.55$ kcal/mol, $\Delta S°=+16.1$ e.u.),[8] sulfonylureas (for example, chlorpropamide, $\Delta G°=-7.04$ kcal/mol, $\Delta H°=-3.74$ kcal/mol, $\Delta S°=+10.9$ e.u.),[3] and diuretics except hydrochlorothiazide and ethacrynic acid are strikingly different from those of *d*-tryptophan ($\Delta G°=-5.40$ kcal/mol, $\Delta H°=-11.3$ kcal/mol. $\Delta S°=-20.0$ e.u.),[12] salicylate ($\Delta G°=-6.45$ kcal/mol, $\Delta H°=-9.8$ kcal/mol, $\Delta S°=-11.2$ e.u.)[13] and thiocyanate ($\Delta G°=-6.20$ kcal/mol, $\Delta H°=-8.8$ kcal/mol, $\Delta S°=-8.8$ e.u.).[14] The binding of the second group is characterized by high negative enthalpy and entropy and it is very interesting that ethacryicn acid–BSA binding has the same degree of thermodynamic parameters with those of the second group, especially *d*-tryptophan.

5) I.M. Klotz, *Ann. N.Y. Acad. Sci.*, **226**, 18 (1973).
6) R.A. O'Reilly, *J. Clin. Invest.*, **48**, 193 (1969).
7) R.A. O'Reilly and P.E. Kowitz, *J. Clin. Invest.*, **46**, 829 (1967).
8) R.A. O'Reilly, *Ann. N.Y. Acad. Sci.*, **226**, 293 (1973).
9) Y.T. Oester, S. Keresztes-Nagy, R.F. Mais, J. Becktel, and J.F. Zaroslinski, *J. Pharm. Sci.*, **65**, 1673 (1976).
10) S. Goto, H. Yoshitomi, and Y. Nakase, *Chem. Pharm. Bull.* (Tokyo), **26**, 472 (1978).
11) I.M. Klotz and J.M. Urquhart, *J. Am. Chem. Soc.*, **71**, 847 (1949).
12) G.F. Fairclough and J.S. Fruton, *Biochemistry*, **5**, 673 (1966).
13) J.F. Zaroslinski, S. Keresztes-Nagy, R.F. Mais, and Y.T. Oester, *Biochem. Pharmacol.*, **23**, 1767 (1974).
14) G. Scatchard and W.T. Yap, *J. Am. Chem. Soc.*, **86**, 3434 (1964).

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Binding of Ethacrynic Acid to Bovine Serum Albumin

An attempt to treat the data by plotting according to the Scatchard equation yielded the hyperbolic curve shown in Fig. 1, especially at 5°. Ethacrynic acid offers several varied potential binding sites π-rings of the phenyl ring, *etc.* The binding of ethacrynic acid to BSA was found to increase considerably with decreasing temperature, and then a sizable value of the standard $\Delta H°$ (evolution of heat on binding) was obtained for K_1 as shown in Table II.

According to Ronwin and Zacchei,[15] their results indicated that a total of 4 molecules of ethacrynic acid binds irreversibly to 1 molecule of BSA (sulfhydryl group) at pH 7.4 and that BSA can accept approximately 12 additional ethacrynic acid molecules in reversible unions, and extrapolation of this curve indicates that a maximum of 14 to 16 reversible bonds might be possible. Our data, n_1=4.8 and n_2=11 at 30°, are relatively good agreement with their findings, and a sizable $\Delta H°$ for K_1, 18.3 kcal/mol as shown in Table II, may emphasize the fact that 1 mol of BSA can bind 4 mol of ethacrynic acid strongly, probably irreversibly.[16] However, such a sizable $\Delta H°$ value leads a difficult question from the general approach of protein binding. Then this fact must be left open for further study.

As a supplement fact, the interaction of fatty acids with serum albumin was studied by Teresi and Luck,[17] and they reported that 4 to 5 molecules are strongly bound to BSA and some 27 molecules are held more weakly. The result and discussion on fatty acid-serum albumin binding might be applied to the case of ethacrynic acid.

Binding of Benzothiadiazine Derivatives to Bovine Serum Albumin

A low degree of BSA binding was obtained for hydrochlorothiazide, and subsequently, for hydroflumethiazide. The fact that both hydrochlorothiazide and hydroflumethiazide are effective in a smaller does than chlorothiazide as a natriurete agent has been reported by Young and Forrester.[18] This may suggest that there is some correlation between relative diuretic potency and degree of BSA binding for benzothiadiazine derivatives. Unfortunately, such a relationship that BSA binding alone can explain the difference in activity for these derivatives is unlikely, because trichlormethiazide was found to be an effective diuretic which,

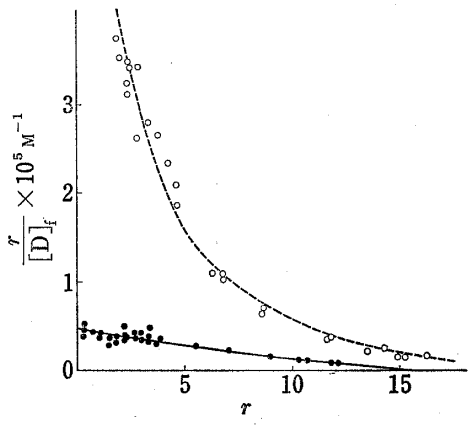

Fig. 1. Scatchard Plots for the Binding of Ethacrynic Acid to Bovine Serum Albumin (2×10^{-4} M) at pH 7.4

○ at 5°, ● at 30°.

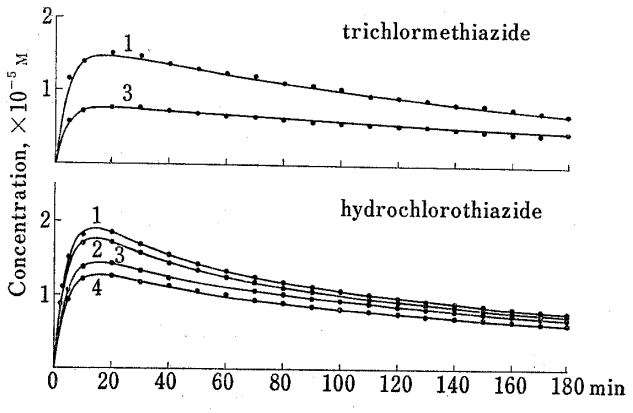

Fig. 2. Diuretic Concentration in Outer Solution-Time Curves in Dynamic Dialysis

Key: Initial concentration of diuretics: 1×10^{-3} M, Concentration of bovine serum albumin: 1, 0.0 M, 2, 1×10^{-4} M, 3, 2×10^{-4} M, 4, 5×10^{-4} M.

15) E. Ronwin and A.G. Zacchei, *Can. J. Biochem.*, **45**, 1433 (1967).
16) R. Komorn and E.J. Cafruny, *J. Pharmacol. Exp. Ther.*, **148**, 367 (1965).
17) J.D. Teresi and J.M. Luck, *J. Biol. Chem.*, **194**, 823 (1952).
18) D.S. Young and T.M. Forrester, *Lancet*, **2**, 765 (1959).

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on an oral dose basis in a dog, was 10 times more potent than hydrochlorothiazide and 60 times more potent than chlorothiazide,[19] whereas BSA binding of trichlormethiazide was found to be considerably greater than that of hydrochlorothiazide and smaller that of chlorothiazide. Beyer and Baer[20] stated that the lipid/water partition coefficient of the benzothiadiazine derivatives makes it clear that this characteristic is related to the natriuretic potency.

However, it is very interesting that saturation of the heterocyclic portion of the ring system between 3– and 4-positions to yield hydrochlorothiazide, hydroflumethiazide, and trichlormethiazide, increased the natriuretic potency but decreased the BSA binding ability compared with chlorothiazide.

Dynamic Dialysis Pattern of Diuretics

A dynamic dialysis pattern in compartment III for diuretics containing various BSA concentrations was plotted as a function of time on semilogarithmic paper. Using back-extrapolation procedures, each of the curves was resolved into three exponential conponents. The data could therefore be described by three exponential equation (1).

$$[\mathrm{III}] = Ae^{-at} + Be^{-bt} + Ce^{-ct} \quad (1)$$

Where [III] is the concentration of outer solution, and A, B, C, a, b, and c are constants. The typical curves are depicted in Fig. 2. for hydrochlorothiazide and trichlormethiazide. Also the constants are indicated in Table III. Figures 3 and 4 show the Scatchard plot

Table III. Summary of Constants in Equation, $[\mathrm{III}]=Ae^{-at}+Be^{-bt}+Ce^{-ct}$

Diuretics	A $\times10^{-5}$M.	B $\times10^{-5}$M.	C $\times10^{-5}$M.	a min^{-1}	b min^{-1}	c min^{-1}	BSA concn. $\times10^{-4}$M.
Chlorothiazide	4.46	—	−4.46	0.0057	—	0.25	0.0
	1.49	0.20	−1.69	0.0033	0.023	0.25	5.0
Hydrochlorothiazide	3.00	1.72	−4.64	0.0035	0.025	0.25	0.0
	2.80	1.48	−4.06	0.0035	0.025	0.25	1.0
	2.60	0.70	−3.26	0.0035	0.025	0.25	2.0
	2.52	0.66	−2.82	0.0035	0.025	0.25	5.0
Trichlormethiazide	3.48	—	−3.48	0.0045	—	0.27	0.0
	1.58	0.18	−1.76	0.0033	0.031	0.27	2.0
Hydroflumethiazide	4.28	—	−4.28	0.0061	—	0.24	0.0
	3.06	—	−3.06	0.0047	—	0.25	2.0
Furosemide	3.67	—	−3.67	0.0046	—	0.25	0.0
	1.56	0.20	−1.75	0.0029	0.023	0.25	2.0
Ethacrynic acid	3.50	—	−3.50	0.0043	—	0.24	0.0
	0.45	0.13	−0.58	0.0023	0.018	0.28	2.0

for chlorothiazide, furosemide-BSA bindings which are calculated from the dynamic dialysis pattern.[21] The solid line was obtained from the digital computer calculation using the data of the equilibrium dialysis method, and the solid plots in Fig. 3 and 4 represent the calculation values from the data of the dynamic dialysis method. A poor agreement was obtained between these values from two methods.

On the other hand, the diffusion curve of the diuretics alone through a cellulose membrane can be given by two exponential equation (2) except for hydrochlorothiazide.

$$[\mathrm{III}] = A'e^{-a't} + C'e^{-c't} \quad (2)$$

19) R.M. Taylor and T.H. Maren, *J. Pharm. Exp. Ther.*, **140**, 249 (1963).
20) K.H. Beyer and J.E. Baer, *Pharmacol. Rev.*, **13**, 517 (1961).
21) S. Goto, T. Ohki, S. Kiryu, and S. Iguchi, *Yakuzaigaku*, **31**, 247 (1971).

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where A', C', a' and c' are constants. However, the data for hydrochlorothiazide fitted the three-exponential equation, then, Chart 1 was considered as one of the schematic models. According to this schematic model, the following differential equations are obtained.

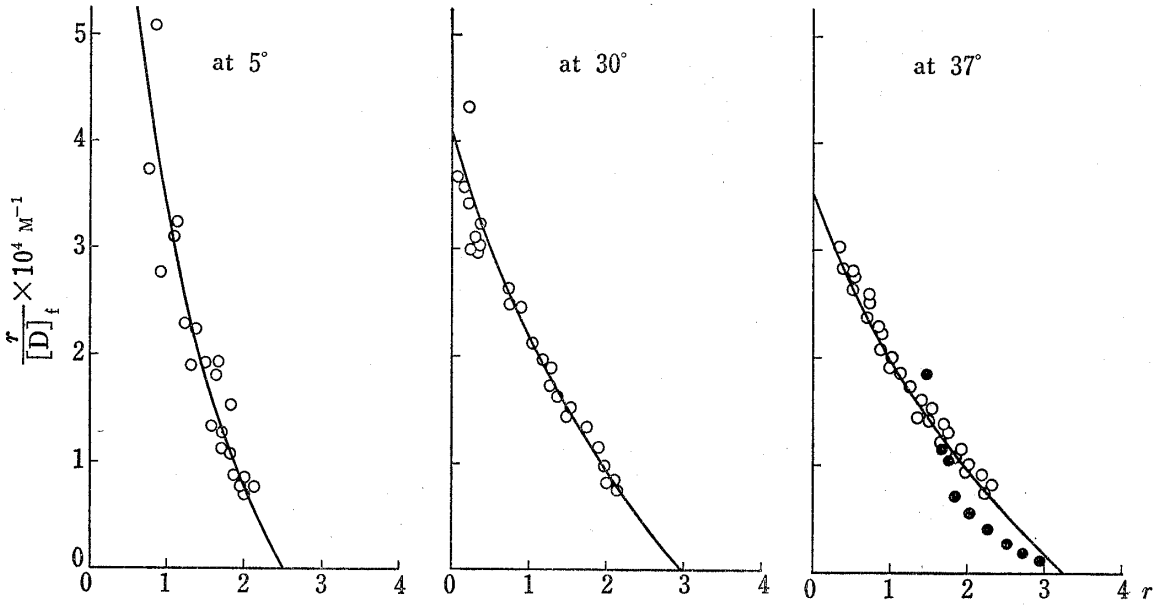

Fig. 3. Scatchard Plots for the Binding of Chlorothiazide to Bovine Serum Albumin (2×10^{-4} M) at pH 7.4

—○— according to equilibrium dialysis method,
● according to dynamic dialysis method.

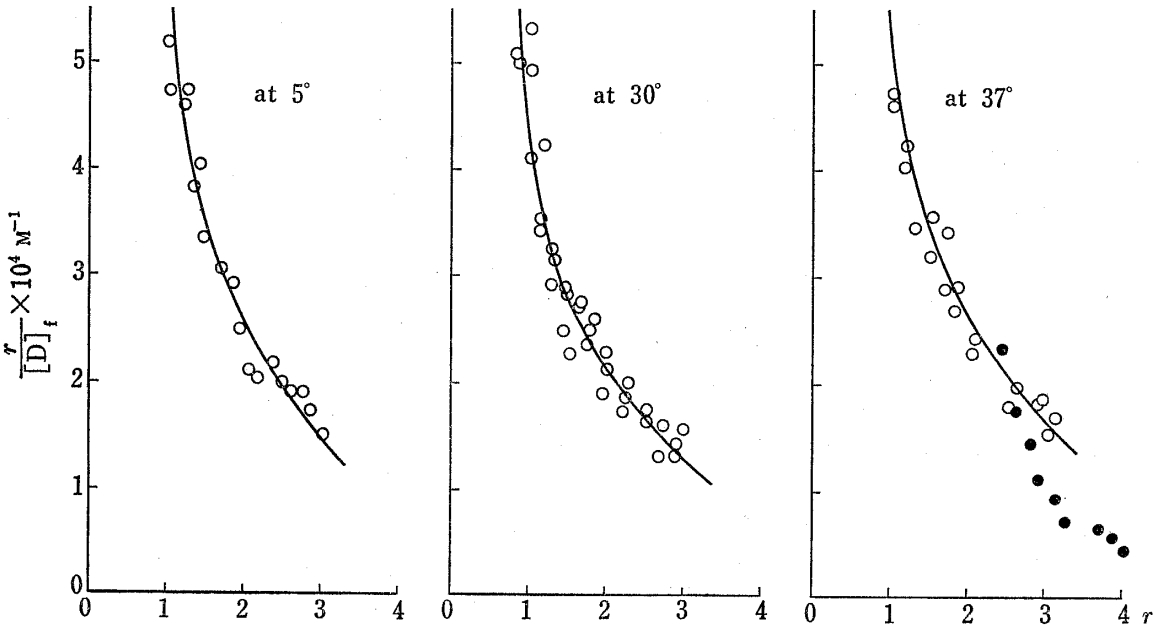

Fig. 4. Scatchard Plots for the Binding of Furosemide to Bovine Serum Albumin (2×10^{-4} M) at pH 7.4

—○— according to equilibrium dialysis method,
● according to dynamic dialysis method.

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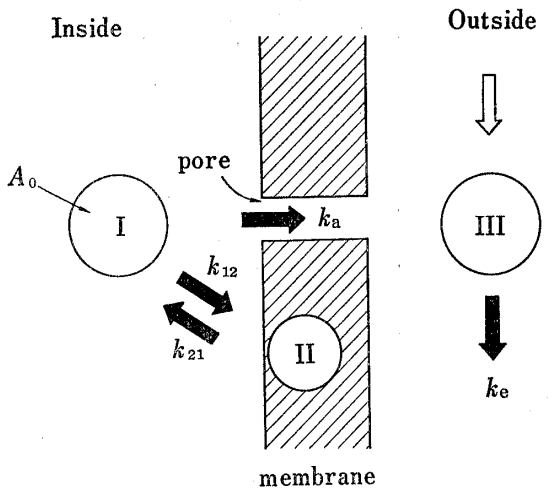

Chart 1. Hypothetical Model for Diffusion of Hydrochlorothiazide through Membrane in Dynamic Dialysis

$$d[I]/dt = k_{21}[II] - (k_a + k_{12})[I] \quad (3)$$

$$d[II]/dt = k_{12}[I] - k_{21}[II] \quad (4)$$

$$d[III]/dt = k_a[I] - k_e[III] \quad (5)$$

where k_{21}, the first-order rate constant for transfer of the diuretics from compartment II to compartment I, k_a, the first-order rate constant for diffusion of the diuretic through membrane, k_{12}, the first-order rate constant for transfer of diuretic from compartment I to compartment II, k_e, the first-order rate constant for elimination of diuretic from compartment III, [I], the concentration of diuretic in compartment I (inner solution of cell), [II], the concentration of diuretic in compartment II (inner and surface of membrane), and [III], the concentration of diuretic in compartment III (outer solution of cell). Using the method of Laplace transform, the following integral equation can be obtained from Eq. (3) to (5).

$$[III] = A_0\left[\frac{k_a(k_{21}-a)}{(a-b)(c-a)}e^{-at} + \frac{k_a(k_{21}-b)}{(a-b)(b-c)}e^{-bt} + \frac{k_a(k_{21}-c)}{(a-c)(b-c)}e^{-ct}\right] \quad (6)$$

Where $a+b=k_a+k_{21}+k_{12}$, $ab=k_ak_{21}$ and $c=k_e$.

$$K = [I]/[II] \simeq k_{21}/k_{12} \quad (7)$$

where K, the equilibrium constant.

The adsorption of hydrochlorothiazide to cellulose membrane can be estimated using Eqs. (6) and (7). The calculated K value was about 3.4 ([I]/[II]=10/2.9). Such a fact could not be observed in other diuretics, that is, hydrochlorothiazide showed a characteristic behavior in the diffusion through a membrane.

General Discussion

Generally, the roughly estimated biological half-life based on plasma levels after intravenous injection of seven diuretics is much shorter (10—60 min), regardless of the extent of their BSA binding. It may therefore be concluded that the binding ability of diuretics is not the only factor determining their plasma level and pharmacological activity.

According to Gillette,[22] the binding of a drug to serum proteins may either hasten (transport mechanism) or retard (storage depot) the elimination of drugs, depending on the mechanism of elimination. When drugs are metabolized rapidly by liver enzymes and polar drugs are rapidly eliminated by transport systems in the kidney, their clearance by an organ may approach the blood flow rate through the organ. Under these conditions the binding of drugs to serum proteins and to blood cells may enhance the rate of elimination of these drugs. Then the transport mechanism may occur in the diuretic–BSA binding and the albumin serves as an efficient carrier of diuretics between the tissue store and the kidney.

In considering the possible mechanism that limits the highly bound drugs, such as furosemide, one might think that the rate of dissociation of the drug–albumin complex limits the elimination of the bound form of the drug, but it is impossible to predict the dissociation constant with the association constant in this study only. Further clarification of the meaning of diuretic–albumin binding will be necessary.

22) J.R. Gillette, *Ann. N.Y. Acad. Sci.*, **226**, 6 (1973).

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