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Serum Protein Binding and Salivary Secretion of Salicylic Acid in Man[1)]

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The binding of salicylic acid to human serum protein was examined *in vitro* and *in vivo* using the semi-microultrafiltration method. The binding parameters of salicylic acid to human serum determined *in vitro* were: $n=2.14$ and $k=2.05\times10^4\,\mathrm{M}^{-1}$. The percentages of salicylic acid bound *in vivo* (96.5—97.1%) following oral administration of 9.3 mg/kg of sodium salicylate to normal subjects were in good agreement with the theoretical values estimated using the binding parameters described above. Furthermore, a good linear relationship between saliva and serum concentrations of salicylic acid was observed in the low range of serum concentration (—64.6 μg/ml). A theoretical approach, however, indicates that the saliva-serum concentration ratio of salicylate becomes markedly higher with increasing concentration in the serum. It is suggested that the serum protein binding of salicylic acid, which has a high binding affinity and a wide range of therapeutic concentration, must have significant effects on its distribution into the extra-vascular fraction, including secretion into the saliva.

Keywords——salicylic acid; serum protein binding; salivary secretion; serum concentration; man; semi-microultrafiltration method

In the previous work,[1)] the plasma protein binding of salicylic acid in rabbits was examined using the semi-microultrafiltration method developed by Imamura *et al.*,[3)] and it was shown that the plasma protein binding of salicylic acid *in vivo* following oral administration of sodium salicylate to rabbits is predicatable from data obtained *in vitro*. The present study was carried out in order to examine whether the same theoretical treatment would be applicable to the binding in human subjects.

It has also been suggested that drug concentrations in saliva can be used for therapeutic drug monitoring or in pharmacokinetic studies instead of the concentrations in serum. Thus, the effect of the plasma protein binding of salicylic acid on its secretion into saliva was also investigated in this study.

Experimental

Materials——Both salicylic acid and sodium salicylate of the purest reagent grade were obtained commercially. [Carboxyl-^{14}C] salicylic acid was purchased from The Radiochemical Centre Ltd., Amersham, and had a specific activity of 59 mCi/mmol. Human serum albumin (HSA fraction V, ICN Pharmaceuticals Inc.) was assumed to have a molecular weight of 69000. Blood was collected by vein puncture from 5 healthy male volunteers who were not receiving chronic medication and had not taken any drug for at least two weeks prior to the blood collections. The sera were separated by centrifugation, pooled, and stored frozen at −20° until required. The total protein concentration and the albumin concentration in the pooled human serum (HS) were 8.98 g/dl and 6.00 g/dl, respectively.

Subjects——Two healthy male subjects were used in this study.

Drug Administration and Sampling——Following an overnight fast, 9.3 mg/kg of sodium salicylate in hard gelatin capsules was orally administered with approximately 100 ml of water. Ten ml blood samples were withdrawn prior to the drug administration and at 0.5, 1, 2, 3, 4, 5, and 7 hr thereafter. The serum

1) The preceding paper: Y. Kaneo, A. Nishikawa, and Y. Kato, *Chem. Pharm. Bull.*, **27**, 2021 (1979).
2) Location: *Ichigaya Funagawara-machi, Shinjuku-ku, Tokyo.*
3) Y. Imamura, M. Sonoda, K. Arimori, and H. Ichibagase, *Chem. Pharm. Bull.*, **27**, 463 (1979).

was separated and stored frozen until analyzed. A mixed saliva sample was collected for 5 min at each blood sampling while the subject was chewing on a small piece of Teflon. The saliva was stored frozen until analyzed. The drug was administered at 10 A.M. and only a light meal was given after the sample collection at 12 A.M. The use of toothpaste and the taking of coffee, tea, and alcoholic beverages were forbidden before and during the study. The study was repeated a month later.

Determination of Salicylic Acid——a) Serum: Using the method employed previously,[1] the serum concentration of salicylic acid was determined by gas chromatography (GC).

b) Saliva: The salicylate concentration in saliva was determined by the spectrofluorometric method of Graham and Rowland.[4] A saliva sample was centrifuged to exclude mucoid sediment, then a 3 ml aliquot of the supernatant was pipetted into a centrifuge tube containing 2 ml of distilled water. The mixture was then acidified with 0.5 ml of 25% $KHSO_4$ solution and extracted with 8 ml of ethyl ether by shaking for 15 min. After centrifugation for 5 min, a 5 ml aliquot of the ethereal extract was transferred into another tube and re-extracted with 5 ml of 1/15 M phosphate buffer (pH 7.0). A trace of ether was removed from the buffer by blowing N_2 gas through the solution for 60 sec. The fluorescence of the final buffer solution was measured on a Shimadzu RF-502 spectrofluorometer. The excitation wavelength was 300 nm, while the fluorescence was measured at 350 and 400 nm.

Determination of Protein Binding——The semi-microultrafiltration was performed under the same conditions of pH, temperature, *etc.*, as described in the previous paper.[1]

Results

Bindings of salicylic acid to HSA and to HS were determined by the semi-microultrafiltration method. The concentration of HSA used was adjusted to 6.00 g/dl, *i. e.*, the same level as the albumin concentration in the pooled human serum (HS). It has been reported that salicylic acid binds mainly to the albumin fraction in plasma proteins.[5] Accordingly, in the case of HS, the binding parameters were calculated using the value of albumin concentration, which was regarded as the only protein concentration affecting the binding of salicylic acid in serum. The binding data obtained were plotted according to Klotz's equation (double-reciprocal plot), expressed as

$$1/r = 1/nk \cdot 1/C + 1/n \quad (1)$$

where r is the molar ratio of bound drug to binding protein, C is the concentration of unbound drug, and n and k are the maximum number of binding sites and the binding constant, respectively. As shown in Fig. 1, the double-reciprocal plot provided a linear relationship, from which the binding parameters, both n and k, were estimated. The results are summarized in Table I. The n values obtained from the bindings to HSA and to HS were in good agreement, being about 2. However, the binding affinity of salicylic acid to HS ($k=2.05\times10^4\,M^{-1}$) was 25% lower than that to HSA ($k=2.68\times10^4\,M^{-1}$). A similar result was obtained in the previous study in rabbits.[1] This may be due to an interaction between albumin and other plasma proteins, and/or to the influence of other endogenous substances present in plasma, such as free fatty acids, uric acid, *etc.*[6,7] Hence, the apparent binding parameters obtained from the binding to HS were adopted for prediction of the protein binding status of salicylic acid *in vivo* after the oral administration of sodium salicylate.

The time courses of salicylate concentration in serum and saliva after an oral dose of 9.3 mg/kg of sodium salicylate are shown in Fig. 2. Each point in Fig. 2 is the mean value obtained from duplicate experiments about 1 month apart in two subjects; Fig. 2 shows a good parallelism between serum and saliva levels of salicylic acid. The concentration and the observed binding of salicylic acid in serum, as well as the theoretical value of the latter, are summarized in Table II. The wide intersubject variations in serum salicylate concentrations found at 30 min after drug administration, which resulted in a high standard deviation, can be attributed

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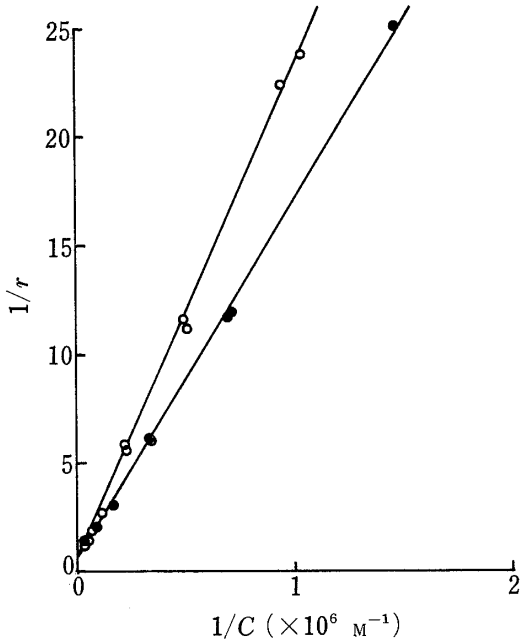

Fig. 1. Double-reciprocal Plot for the Binding of Salicylic Acid to Human Serum Albumin (HSA) and Human Serum (HS) Measured by the Semi-microultrafiltration Method

●, 8.30×10^{-4} M HSA;
○, HS (albumin concentration: 8.30×10^{-4} M).

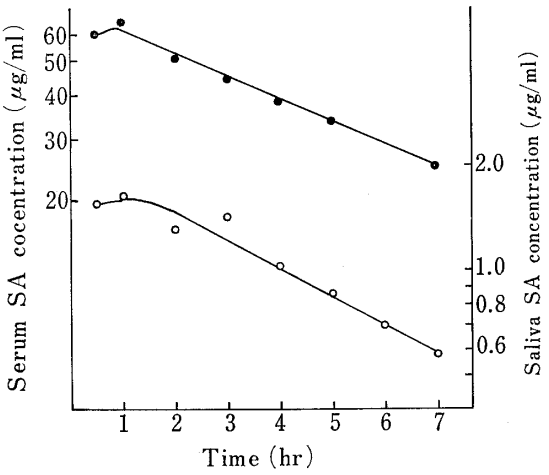

Fig. 2. Concentrations of Salicylic Acid (SA) in Human Serum (●) and Saliva (○) Following Single Administration of 9.3 mg/kg of Sodium Salicylate (Equivalent to 8 mg/kg of Salicylic Acid) in Hard Gelatin Capsules

Each point is the mean of four experimental values obtained from two subjects.

Table I. Binding Parameters for the Interaction of Salicylic Acid with 8.30×10^{-4} M Human Serum Albumin (HSA) and Human Serum (HS)[a]

Protein	n	k ($\times10^4$ M^{-1})	nk ($\times10^4$ M^{-1})
HSA	2.24	2.68	6.00
HS	2.14	2.05	4.39

a) The albumin concentration in HS was 8.30×10^{-4} M.

to individual differences in the absorption rate of the drug from the gastro-intestinal tract. However, both the intersubject and intrasubject variations of the subsequent salicylate concentrations in serum were quite small (Table II). Bindings of salicylic acid to serum protein were almost constant, ranging from 95.7% at 1 hr to 96.4% at 7 hr after drug administration; however, the binding evidently increased with decreasing salicylate concentration in the serum. The theoretical values of the percentage of bound drug, estimated using the binding parameters of salicylic acid to HS listed in Table I, were in good agreement with the experimental values (Table II). These findings indicate that the serum-protein binding of salicylic acid after oral administration in man is predictable on the basis of the binding isotherm determined by *in vitro* experiments as well as that determined in the previous study in rabbits.

The salicylate concentrations in saliva after oral administration of sodium salicylate were reproducible in each subject on repeated experiments about 1 month apart. The concentrations of salicylic acid in serum were practically equal in the two studies on Subjects 1 and 2 (Table II); however, the concentrations in saliva observed in Subject 2 were higher than those

Table II. Serum Concentration and Binding of Salicylic Acid Following Oral Administration of Sodium Salicylate[a]

Time (hr)	Serum concentration[b] (μg/ml)	Serum protein binding[b] (% bound)	
		Observed	Calculated[c]
0.5	60.1±15.5	95.8±0.3	96.6±0.3
1	64.6± 1.4	95.7±0.4	96.5±0.1
2	50.7± 1.9	95.8±0.3	96.7±0.1
3	44.3± 4.3	95.9±0.2	96.8±0.1
4	38.0± 2.3	96.1±0.2	96.9±0.1
5	33.8± 2.4	96.3±0.5	97.0±0.1
7	24.9± 2.9	96.4±0.2	97.1±0.1

a) A single 9.3 mg/kg dose (equivalent to 8 mg/kg of salicylic acid) was administered in hard gelatin capsules.
b) Results are presented as means±S.D. of four experimental values obtained from two subjects.
c) Calculated using the binding parameters obtained from the *in vitro* experiments; $n=2.14$ and $k=2.05\times10^4\ \text{M}^{-1}$.

in Subject 1 (Fig. 3). There were good linear relationships between salicylate concentrations in the two fluids (Fig. 3), and between unbound salicylate concentration in serum and salicylate concentration in saliva (Fig. 4). The half-life values shown in Table III were calculated from the salicylate concentrations in saliva and serum from 2 to 7 hr after drug administration. Elimination of salicylate appeared to be monoexponential within this period. The ratios of the concentration of salicylate in saliva to that in serum (saliva-serum ratio) and to the concentration of unbound salicylate in serum (saliva-serum unbound ratio) are also summarized in Table III. Both the saliva-serum ratio and the saliva-serum unbound ratio observed in Subject 2 were higher than those in Subject 1. In contrast to the difference of these concentration ratios between Subjects 1 and 2, the half-life values in saliva were practically equal in the two subjects, being about 3.7 (hr). The half-life in saliva, which was about 0.7 hr shorter than that in serum, was in good agreement with that

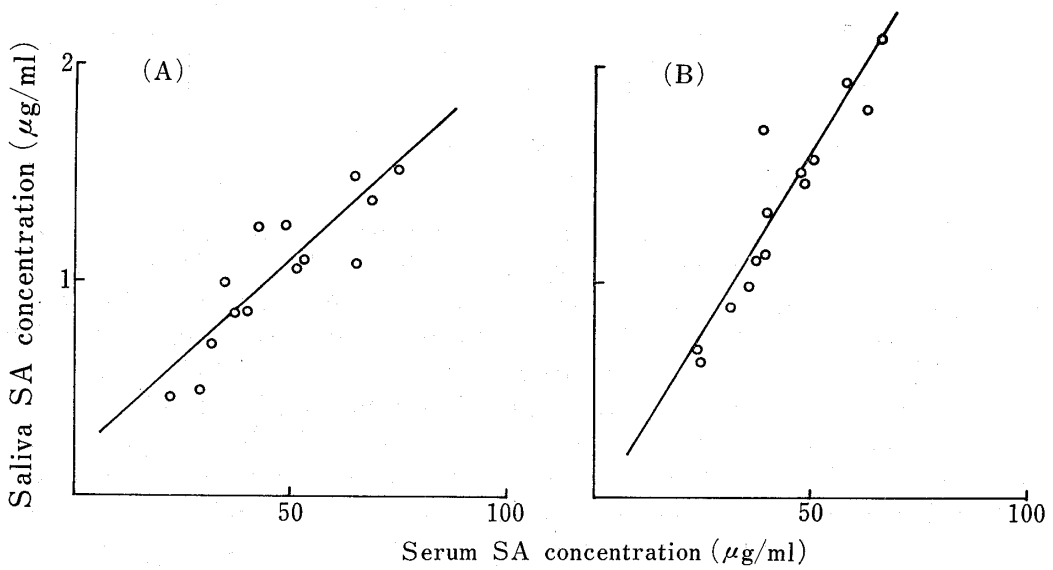

Fig. 3. Relationships between Saliva and Serum Concentrations of Salicylic Acid (SA) in Subject 1 (A) and Subject 2 (B)

(A) $Y=0.0183X+0.1714$ ($r=0.876$) ($n=14$, $p<0.01$);
(B) $Y=0.0329X-0.0680$ ($r=0.935$) ($n=14$, $p<0.01$).

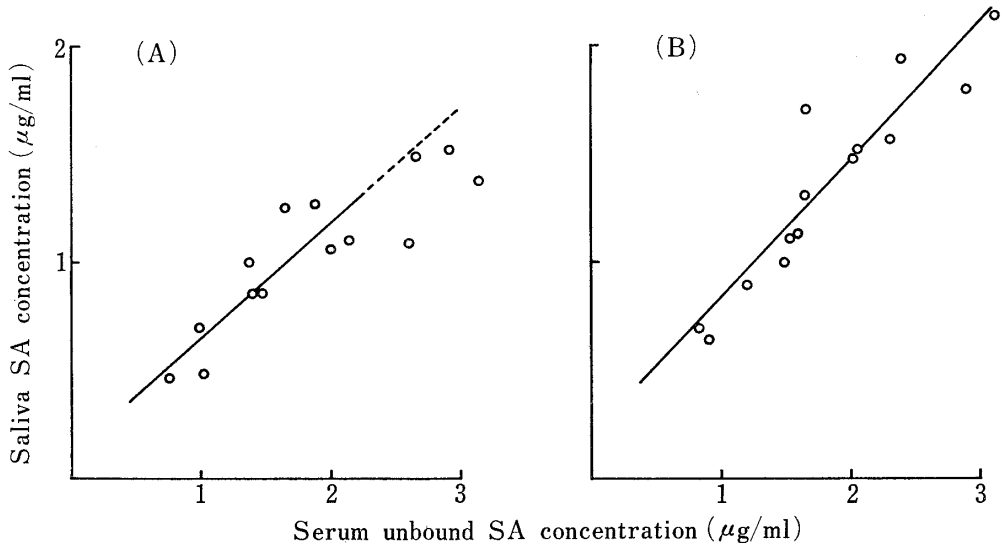

Fig. 4. Relationships between Saliva and Serum Unbound Concetrations of Salicylic Acid (SA) in Subject 1 (A) and Subject 2 (B)

(A) $Y=0.536X+0.113$ ($r=0.860$) ($n=10$, $p<0.01$);
(B) $Y=0.631X+0.195$ ($r=0.924$) ($n=14$, $p<0.01$).

Table III. Half-lives and Saliva-serum Concentration Ratios of Salicylic Acid

	Subject 1	Subject 2
Half-life, hr:		
Serum	4.41	4.44
Serum unbound	3.69	3.55
Saliva	3.71	3.73
Saliva-serum concentration ratio	0.0222 (±0.0037)	0.0311 (±0.0042)
Saliva-serum unbound concentration ratio	0.578 (±0.107)	0.748 (±0.104)

of unbound salicylate in serum (about 3.6 hr). These findings suggest that only the protein-free salicylate in serum is responsible for the secretion into saliva.

Discussion

The saliva-serum ratios of salicylate determined in this study agreed well with the value of 0.033±0.005 reported by Graham and Rowland.[4] The secretion of various drugs into saliva in man has been the subject of many investigations during recent years: a significant correlation between the levels of tolubutamide, which binds strongly to plasma protein (about 90%), in plasma and saliva was found in single dose studies, the ratio varying from 0.012 to 0.013 ($n=3$).[8] Amobarbital, a weak acidic compound which is about 40% protein bound, was found to distribute to both fluids at relatively high ratios ranging from 0.32 to 0.40 ($n=5$).[9] In contrast, the high salivary secretion of basic drugs, which usually have a relatively low binding affinity, is well known. Koup *et al.*[10] found saliva-serum ratios of 3.50±2.34 in 12 patients who had been receiving a fixed dose of procainamide hydrochloride. The values were considerably higher than those of acidic drugs and were markedly dependent on the saliva pH.

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9) T. Inaba and W. Kalow, *Clin. Pharmacol. Ther.*, **18**, 558 (1975).
10) J.R. Koup, W.J. Jusko, and A.L. Goldfarb, *J. Pharm. Sci.*, **64**, 2008 (1975).

In the case of lipid-soluble acidic compounds, the saliva-serum drug concentration ratio can be predicted from the degree of ionization in the two fluids by means of the following equation,[8]

$$R = \frac{1+10^{(pH_{sal}-pK_a)}}{1+10^{(pH_{ser}-pK_a)}} \quad (2)$$

where R is the saliva-serum unbound drug concentration ratio, ad pH_{sal} and pH_{ser} are the pH's of saliva and serum, respectively. The serum pH's of both Subject 1 and Subject 2 were 7.4, but the saliva pH's were 6.8 and 7.0, respectively. These values were nearly constant during the experimental period. The R values in Subjects 1 and 2 calculated on the basis of equation (2), 0.25 and 0.40, respectively, were lowere than those found in this study (Table III). Changes in saliva pH particularly influence the theoretical value of R in the case of salicylic acid, which has a low pK_a value ($pK_{a1}=2.98$). Therefore, the discrepancy between the experimental and the calculated values is not unexpected becuase of the difficulty in accurately determining the pH value of the saliva, which is in contact with the lipid barrier (mixed and spat saliva was collected in this study). However, a more important factor may be the binding of salicylic acid to saliva proteins,[11] as described below.

In general, saliva contains about 200 mg/dl of proteins.[12] Pohto[13] found that 40—60% of salicylate is bound to the protein fraction in saliva. Therefore, in the case of weakly acidic compounds the saliva-serum concentration ratio (R') can often be predicted on the basis of the following equation,[8]

$$R' = \frac{1+10^{(pH_{sal}-pK_a)}}{1+10^{(pH_{ser}-pK_a)}} \cdot \frac{f_{ser}}{f_{sal}} \quad (3)$$

where f_{ser} and f_{sal} are the fractions of unbound drug in serum and saliva, respectively. The reported value of f_{sal} for salicylic acid is 0.4,[13] and the value of f_{ser} is around 0.04 in the concentration range of 25 to 65 μg salicylate/ml (Table II). Thus, the predicted values of R' in Subject 1 and Subject 2 become 0.025 and 0.040, respectively. These values are in good agreement with the experimental values of 0.0222 and 0.0311 (Table III).

Borzelleca *et al.* studied the salivary secretion in dogs[14,15] and in rat submaxillary gland[16] and presented a model for the movement of salicylate across the parotid epoithelum, suggesting that the apical membrane presents the major lipid barrier at which the primary pH-dependent process occurs. Equations (2) and (3) are theoretical ones applicable to the relationship between the steady-state concentrations in the two fluids across the lipid membrance. There is some doubt as to whether these theoretical equations can be validly applied in the case of a single dose. Further detailed investigations are therefore desirable.

A theoretical binding curve for salicylic acid was estimated using the binding parameters, and is illustrated in Fig. 5. For optimal anti-inflammatory effect in patients with rheumatic diseases, salicylate concentrations of 150 to 400 μg/ml in plasma (serum) are required. Fig. 5 shows that the percentage of bound drug decreases slowly until the serum concentration of salicylate reaches about 200 μg/ml and then it decreases rapidly. This may be due to saturation of the binding sites on the protein molecule following increase of the drug concentration. This disproportionate increase in the free level of salicylate would facilitate the drug distribution into the extra-vascular fraction and would thus result in an altered saliva-serum concentration ratio, assuming that the saliva concentration is reflection of the unbound serum concentr-

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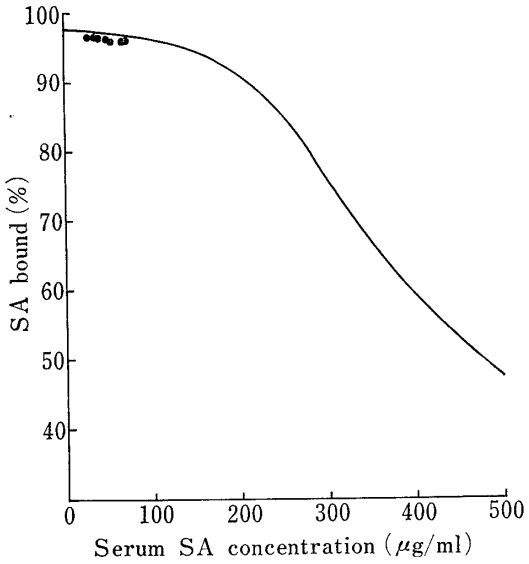

Fig. 5. Relationship between Drug Binding (%) and Serum Concentration of Salicylic Acid (SA) in Man

All points are experimental values, while the solid line is that computed from the binding parameters obtained from the *in vitro* experiments; $n=2.14$ and $k=2.05\times10^4$ M^{-1}.

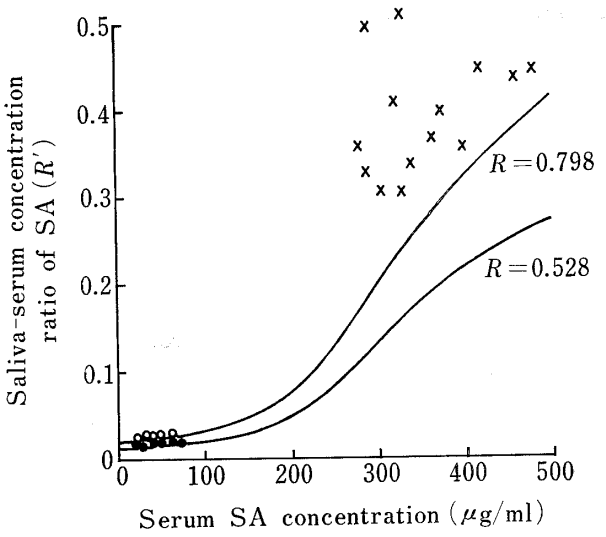

Fig. 6. Relationship between Saliva–serum Concentration Ratio and Serum Concentration of Salicylic Acid (SA)

●, subject 1; ○, subject 2.
The solid lines show the theoretical ratios calculated using the mean±S.D. values of saliva-serum unbound concentration ratios (R).
× ,experimental values determined by Leulier *et al.*[17]

ation. The mean value of the saliva-unbound serum concentration ratio obtained in this study was 0.663±0.135. Using concentration ratios of 0.528 (lower limit) and 0.798 (upper limit), theoretical curves were estimated, and are illustrated in Fig. 6. It is clear that the saliva-serum concentration ratio of salicylate increase markedly with increasing serum concentration. The symbols × in Fig. 6 represent the experimental values determined by Leulier *et al.*[17] in healthy subjects after a dose of 6 g of sodium salicylate, and these values support the validity of our theoretical approach. While only a limited number of subjects was examined in this study, it appears that the serum protein binding of salicylic acid, which has a high binding affinity and a wide range of therapeutic concentration, must have significant effects on its distribution into the extra-vascular fraction, including secretion into the saliva. Experiments are in progress to study the secretion of salicylate into saliva in the higher therapeutic concentration range in more detail.

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