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Absorption and Excretion of Drugs. XXXIII.[1] The Correlation between the Absorption of Barbituric Acid Derivatives from the Rat Small Intestine and Their Binding to the Mucosa[2]

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In order to examine the fundamental problems concerning the contribution of the binding to the mucosa in the absorption of barbituric acid derivatives from the rat small intestine, some *in situ* and *in vitro* experiments were carried out. It was clarified that the accumulation in the small intestine was negligible during the *in situ* continuous perfusion, and therefore the absorption rate constants computed from the amount decreased in the perfusion solution express almost quantitatively the penetration into the vascular system. The degree of binding to the mucosa determined *in vitro* was correlated to the absorption characteristics. The importance of the binding process in the absorption was also confirmed by *in vitro* uptake experiments. The uptake was classified into two processes; the adsorption on the mucosal surface and the accumulation in the tissue. Of these two processes, the adsorption was ascertained as a determining step in the entire absorption process from the rat small intestine.

In the previous paper,[1] it was shown that some discrepancies from pH-partition hypothesis developed by Brodie and his colleagues[4] exist in the absorption of barbituric acid derivatives from the rat small intestine *in situ*, (1) pH-profile of the absorption has an optimal range from 6.5 to 7.5, and (2) compared with the corresponding non-methylated derivatives, the absorption of N-methylated derivatives was almost identical or slow despite of their higher lipid solubilities, and furthermore, (3) as a possible factor reconciling these particular phenomena, the interaction with the mucosal preparations *in vitro* was examined and related fairly well to the absorption characteristics observed. The present investigations were undertaken to examine further the fundamental problems concerning the contribution of the binding process in the absorption of barbituric acid derivatives from the rat small intestine.

Experimental

Materials——All barbituric acid derivatives investigated were obtained as previously.[1,5] Nomenclature and the abbreviation are also same as described previously.[1,5] Crystalline bovine serum albumin was purchased from the Armour Pharmaceutical Co., Ltd. The membranes used in the binding experiments were cut from the cellulose dialyzer tubing from Visking Co., Ltd. (8/32 inch, 0.7 cm diameter). All other materials were of analytical grade.

Animal——Male Wistar rats weighing 130—170 g were used in all the experiments.

1) Part XXXII: K. Kakemi, T. Arita, R. Hori, and R. Konishi, *Chem. Pharm. Bull.* (Tokyo), **15**, 1883 (1967).
2) Presented in part to the 84th Annual Meeting of the Pharmceutical Society of Japan, Tokyo, April 1964.
3) Location: *a*) *Yoshida-shimoadachi-cho, Sakyo-ku, Kyoto*; *b*) *Nishi-7-chome, Kita-15-jo, Sapporo.*
4) L.S. Schanker, D.J. Tocco, B.B. Brodie, and C.A.M. Hogben, *J. Pharmacol. Exptl. Therap.*, **123**, 81 (1958); C.A.M. Hogben, D.J. Tocco, B.B. Brodie, and L.S. Schanker, *J. Pharmacol. Exptl. Therap.*, **125**, 275 (1959).
5) K. Kakemi, T. Arita, R. Hori, and R. Konishi, *Chem. Pharm. Bull.* (Tokyo), **15**, 1534 (1967); *idem, ibid.*, **15**, 1705 (1967).

Determination of the Accumulation in the Small Intestine during the *in Situ* Continuous Perfusion——Barbital and thiopental were used as the typical derivatives in this experiment. The perfusion method used here was almost identical with that in the absorption experiments described in the previous paper,[1] except the following procedure. After a given perfusion period during which samples for the drug and phenol red were taken at an arbitrary interval, the perfusion solution remained in the small intestine was withdrawn as completely as possible by air infusion from the pyloric cannula, and washed with 20 ml of the fresh buffer. The washings were combined to the effluent and made up to 50 ml by the same buffer. Immediately after the washing, the whole part of the small intestine was isolated tearing off the mesentery, and the serosal surface was then blotted by filter paper. After weighing, the small intestine was cut into small pieces and homogenized in a Potter–Elvehjem type teflon homogenizer with water to make 30% (w/v, wet weight) homogenates and an aliquot of the homogenates was analyzed. From the difference in amount between the initial perfusion solution and that of the combined effluent in which the amount decreased by sampling was corrected, the eliminated amount of the drug from the perfusion solution was calculated.

The amount of the accumulation in the tissue was separately determined according to the method described below.

Determination of the Binding of Barbituric Acid Derivatives to the Mucosa of the Rat Small Intestine and Bovine Serum Albumin——i) Equilibrium Dialysis Method: The binding of several derivatives to the mucosa of the rat small intestine was determined at pH 5.8 by the same technique as employed in the previous paper.[1] In determining the binding of barbituric acid derivatives to bovine serum albumin, the same method was used with 1% (w/v) buffered protein solution (citric acid–Na_2HPO_4 buffer, $\mu=0.05$, pH 5.8).

ii) Ultrafiltration Method: Since the mucosal homogenates contained micellaneous insoluble debris, the mixture of the mucosal homogenates and drug solution was centrifuged before the proper ultrafiltration. This procedure is different from the usual method for the soluble protein.[6] Preparation of the mucosal homogenates was same to the dialysis method above. Drugs were dissolved in pH 5.8 citric acid–Na_2HPO_4 buffer ($\mu=0.05$) to approximately 3.3 mM solution. Twenty ml of this drug solution was added to 20 ml of 1% mucosal homogenates, and the mixture was stirred gently for 60 min, and then submitted to the centrifugation at $2500\times g$ for 60 min. Five ml of the supernatant fraction was taken in the cellulose tube devised as previously reported.[6b] This tube was hanged in the centrifuge tube and centrifuged at $4000\times g$ for 3 hr. All these procedures were carried out at 5°. The ultrafiltrate was then analyzed. The fraction bound to the mucosal homogenates was determined by comparing the concentration of the drug in the ultrafiltrate with that in the original mixture. It was ensured that the cellulose membrane did not significantly adsorb the drug or impede the passage relative to water (less than 1%) by equilibrating or ultrafiltrating the drug solution free of mucosal preparations according to these experimental conditions.

The *in Vitro* Uptake by the Everted Sacs of the Small Intestine——The rats were treated similarly as in the *in situ* absorption experiments. The small intestine was washed with 50 ml of saline and isolated. The serosal surface was then washed with sufficient volume of saline. To examine the difference in the uptake by part of the small intestine, the isolated intestine was cut into three segments of approximately same length; upper, intermediate, and lower part. Each segment was everted with a glass rod so that the mucosal surface is on the outside,[7] and ligated at both ends without filling any medium into the serosal lumen. Immediately after the ligation, the everted sacs were placed in a flask containing 30 ml of the isotonically buffered drug solution (50 μg/ml, pH 5.5 citric acid–Na_2HPO_4 buffer), and incubated with gentle shaking at 37° in an atmosphere of air for 30 min. Samples of the incubation medium were taken at 5, 10, and 30 min after the beginning of incubation. And in the experiments other than described above, the segment of 4 cm length was prepared similarly. The uptake by the whole small intestine was determined from the incubation with the pooled segments from an animal as described above. And in order to determine the adsorption and accumulation, the pooled segments were divided into three groups by mixing at random, and each group was incubated in 10 ml of the buffered drug solution. At the end of incubation, an aliquot of the medium was taken, and the segments were rinsed with 4 ml of 0.1N HCl at 37° for 2 min. This rinsing procedure was carried out three times successively with the same group of segments. The rinsings and the residual incubation medium were combined and made up to 25 ml with water. An aliquot of this combined solution was taken for analysis. The amount desorbed from the segments into the rinsing medium, which was refered as the adsorption in this paper, was calculated by the following equation.

$$A=(C_2\times 25-C_1\times(10-S))/T$$

In this equation, A is the amount of adsorption (μg/g wet tissue), C_1 is the concentration of drug in the incubation medium at the end of incubation, C_2 is that in the incubation medium to which the rinsings was added, S is the volume of sample for the determination of C_1, and T is total wet weight of the group

6) *a*) L.G. Goldbaum and P.K. Smith, *J. Pharmacol. Exptl. Therap.*, **111**, 197 (1954); *b*) K. Kakemi, T. Arita, H. Yamashina, and R. Konishi, *Yakugaku Zasshi*, **82**, 536 (1962); *c*) P.M. Keen, *Brit. J. Pharmacol.*, **26**, 704 (1966).

7) T.H. Wilson and G. Wiseman, *J. Physiol.* (London), **123**, 116 (1954).

of segments incubated. The amount remained in the rinsed segments, which was as the accumulation in this paper, was determined by the method described below.

Analytical Methods——All analytical determinations were carried out by the ultraviolet spectrophotometric method. For aqueous samples, the similar methods to the previously described methods[1,5] were used except for N-methyl series; metharbital and N-methylallobarbital which were determined as follows. Samples were acidified with 0.3 ml of 1N HCl, and extracted with 10 ml of the mixed solvent of chloroform and carbon tetrachloride (3:7, v/v) for 30 min, then 8 ml of the lower solvent layer was separated. The extractant was reextracted with 5 ml of pH 11.5 Kolthoff buffer and the alkaline layer was read at 245 mμ and the concentration was calibrated according to the usual manner. For the tissue samples, the modified methods of Goldbaum,[8] and Brodie, *et al.*[9] were used for oxy series and thio series respectively.

Results and Discussions

In the previous paper,[1] the absorption from the rat small intestine was estimated solely from the disappeared amount from the perfusion solution, therefore, this apparent disappeared amount is assumed to contain both the amount of accumulation by the whole small intestine and the absorbed amount into the vascular system. If the accumulation in the tissue was significantly large, the observed characteristics in the previous paper can not be attributed exactly to the intrinsic absorption process. In order to examine the ability of the rat small intestine to accumulate barbituric acid derivatives under the *in situ* conditions of the continuous perfusion, the accumulation was determined directly at various time during the perfusion with barbital and thiopental. The effect of pH was also examined. These are shown on Fig. 1, and

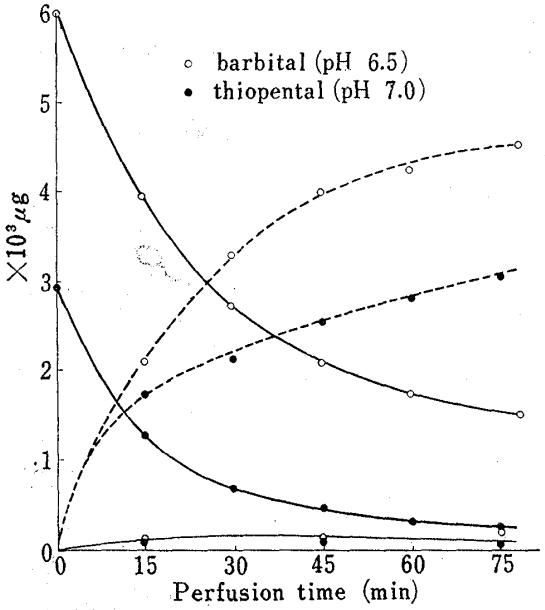

Fig. 1. Absorption and Tissue Accumulation[a] during *in Situ* Perfusion

—— accumulated amount in the whole small intestine
—— remaining amount in the perfusion fluid
------ calculated absorbed amount

a) The accumulated amount at each time is from one rat for barbital, a mean from two rats for thiopental, and the remaining amount is a mean from at least three rats for both barbiturates.

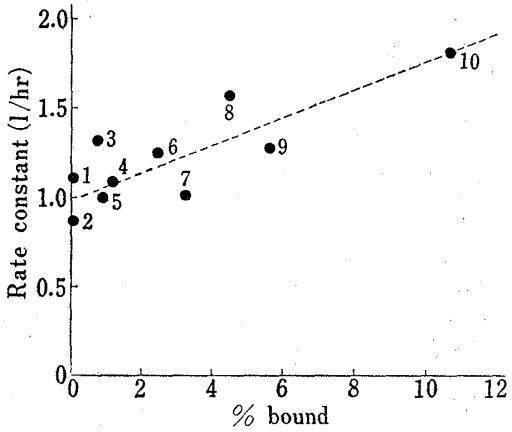

Fig. 2. Relationship between the Binding to the Rat Mucosa[a] and the Absorption Rate Constant[b]

a) The binding was determined by the equilibrium dialysis method.
b) The illustrated data are taken from those reported previously.[1]

1. mephobarbital
2. hexobarbital
3. allobarbital
4. cyclobarbital
5. metharbital
6. amobarbital
7. barbital
8. phenobarbital
9. pentobarbital
10. thiopental

8) L. Goldbaum, *Anal. Chem.*, **24**, 1604 (1952).
9) B.B. Brodie, L.C. Mark, E.M. Papper, P.A. Lief, E. Bernstein, and E.A. Rovenstine, *J. Pharmacol. Exptl. Therap.*, **98**, 85 (1950).

Table I. Accumulation[a] of Thiopental in the Whole Small Intestine during *in Situ* Continuous Perfusion

Perfusion time (min) / pH of perfusion fluid[b]	15	45	75
3.8	3.1	3.0	4.9
5.5	3.0	3.4	4.0
7.0	3.1	3.7	1.4

a) All values are expressed as the percentages of the accumulated amount to the inital total amount, and mean values of duplicate experiments.
b) Buffers used are isotonic citrate–phosphate buffers.

Table I. From these results, it was clarified that the accumulation in the small intestine was negligible during the *in situ* perfusion, and not affected by pH of the perfusion solution and the perfusion time. Thus the absorption rate constants previously reported[1] imply almost quantitatively the transfered amount into the body, and they are irrelevant to the accumulation in the tissue. From the similarity of the binding to the small-intestinal mucosa to the absorption, it seems that the binding is a limiting factor in the absorption process. In respect to the effect of the buffer systems as an experimental problem, some preliminary experiments were undertaken with citric acid–Na_2HPO_4 isotonic buffer which was used exclusively, but no significant effect of phosphate or citrate ion was observed, and the details can not be clarified because of the buffer capacity. To develope the concept that the binding to the mucosa is a limiting factor in the absorption from the small intestine, the binding abilities of the derivatives other than those reported previously were determined at pH 5.8 and correlated with the absorption rate constants. The results are shown in Fig. 2. It is apparent that N-methyl series did not exhibit any significant binding to the mucosa in this condition, which is in good agreement to the phenomena of absorption. The non logarithmic relationship of the binding to the absorption is depicted, that is, a linear correlation exists between the binding and the absorption from the small intestine and this is one of the evidences supporting the considerations suggested. These results necessarily led to the analysis of the active components involved in the binding. From the similarity of pH-profile of the binding to that with bovine serum albumin (BSA) observed by Goldbaum and Smith,[6a] the active site for the binding to the mucosal preparations has been presumed to be on the protein moiety of the mucosal surface.

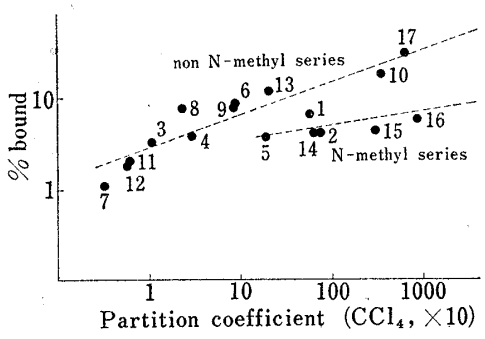

Fig. 3. Relationship between the Binding to BSA[a] and the Partition Coefficient[b]

a) The binding was determined by the equilibrium dialysis method.
b) The data are taken from the reported data. The numbers correspond to those in Fig. 2, except the followings.
11. dormin 12. probarbital
13. secobarbital 14. N-methylallobarbital
15. N-methylcyclobarbital
16. N-methylamobarbital 17. thiamylal

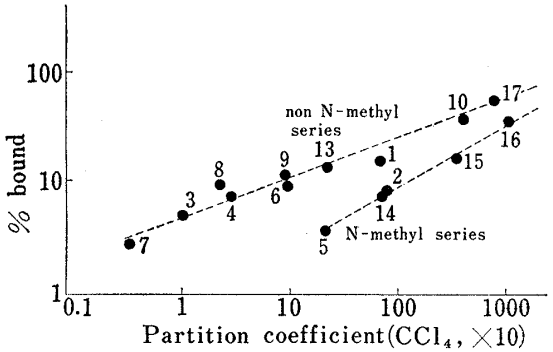

Fig. 4. Relationship between the Binding to the Rat Mucosa[a] and the Partition Coefficient[b]

a) The binding was determined by the ultrafiltration method.
b) The data are taken from the reported data. The numbers correspond to those in Fig. 2, and Fig. 3.

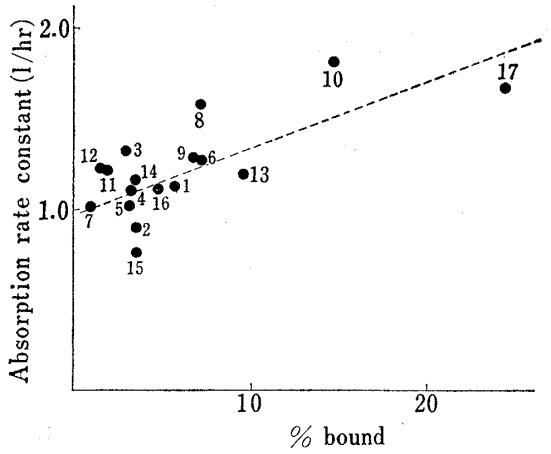

Fig. 5. Relationship between the Binding to BSA and the Absorption Rate Constant

The numbers correspond to those in Fig. 2, and Fig. 3.

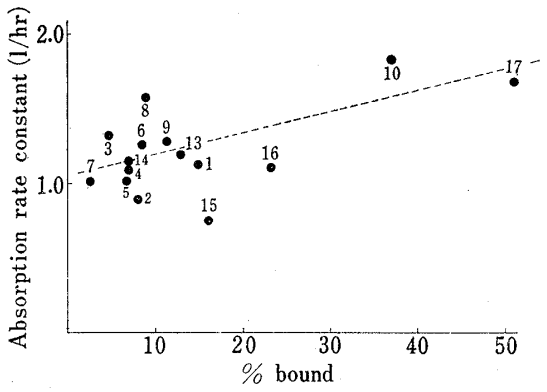

Fig. 6. Relationship between the Binding to Rat Mucosa and the Absorption Rate Constant

The numbers correspond to those in Fig. 2, and Fig. 3.

Therefore it was felt to be of interest to compare the binding to the mucosa with the binding characteristics to BSA which is commonly used in the studies on drug–protein interactions as a standard protein.[10] The binding abilities of barbituric acid derivatives to BSA were determined at pH 5.8 according to the equilibrium dialysis method. The relation[11] of the observed binding to the partition coefficient was represented in Fig. 3, the binding to the rat mucosa is shown in Fig. 4. The plots of the absorption rate constants versus these bindings were also

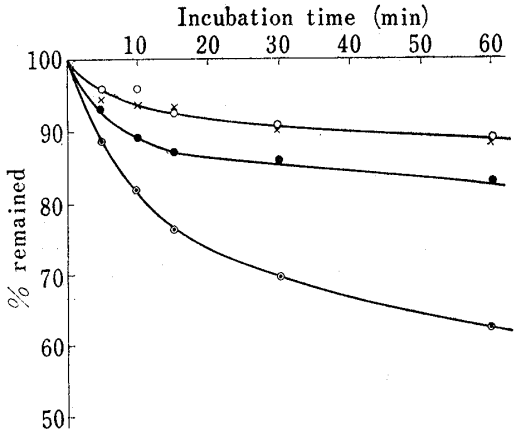

Fig. 7. Time Course of *in Vitro* Uptake[a] by Everted Rat Small Intestine

a) Incubated at pH 5.5.
—○— barbital
—×— metharbital
—●— pentobarbital
—⊙— thiopental

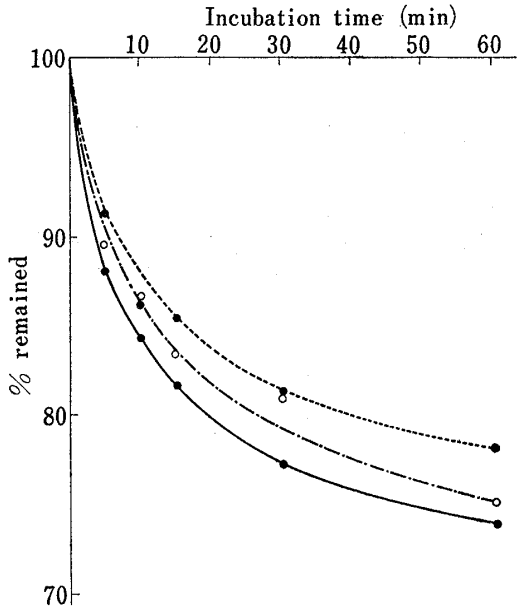

Fig. 8. Comparison of Uptake Ability[a] by Different Segment of Rat Small Intestine: Thiopental

a) Incubated at pH 5.5.
·····●····· lower part of small intestine
– – –○– – – intermediate part of small intestine
——●—— upper part of small intestine

10) A. Goldstein, *Pharmacol. Revs.*, **1**, 102 (1949); I.M. Klotz, "The Proteins," Chapter 8, ed. by H. Neurath and K. Bailey, Academic Press, New York, N. Y., 1952; J.M. Thorp, "Absorption and Distribution of Drugs," ed. by T.B. Binns, E & S Livingstone Ltd., Edinburgh and London, 1964, p. 64.

11) These plots (log – log type) are empirical, and analogous to those employed previously[1] in relation to the absorption.

shown in Fig. 5 and Fig. 6. These relations are in accord with the comparable findings[1] in the absorption from the small intestine, and also suggest that the participant supramolecular moiety for the binding to the mucosa is predominantly a structural protein. The importance of the binding process in the absorption *in situ* was also demonstrated by *in vitro* uptake experiments using the everted sacs technique. The incubation medium was the isotonic buffer used in the absorption experiments without special considerations on whether the preparations remain fully active or not. The uptake of four derivatives by pooled segments from an animal was determined at pH 5.5. The time course of uptake is shown in Fig. 7. The uptake of barbital is almost identical to that of metharbital and smaller than pentobarbital and thiopental. These were found to be correlated with the absorption rate constants previously reported. The effect of the incubation pH was also examined with thiopental, which will be discussed later in the present paper (see Fig. 10). The uptake abilities of the segments prepared from different part of the rat small intestine were examined as a preliminary experiment for those in which the segments from an animal were divided at random into three or four groups. As shown in Fig. 8, the outstanding difference in the uptake was not observed. It is generally accepted that the uptake determined by the method as employed here, contains essentially two processes, the adsorption on the mucosal surface and the accumualtion in the small intestine. It is difficult to separate experimentally these processes, but an approximate estimation was made by utilizing the successive rinsing of the incubated sacs in 0.1N HCl which was adapted after preliminary experiments as to facilitate the desorption of drug from the mucosal surface and limit the efflux of drug accumulated in the tissue. The amount rinsed out, and the amount remained in the tissue were refered as the adsorption and the accumulation respectively in this paper. All these experiments were undertaken with thiopental as a typical derivative. Time course of the adsorption and the accumulation are shown in Fig. 9. The ad-

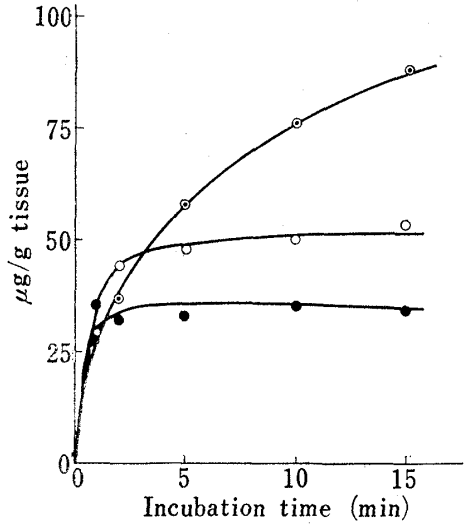

Fig. 9. Time Course of Adsorption and Accumulation of Thiopental

—●— adsorption at pH 4.5
—○— adsorption at pH 6.5
—⊙— accumulation at pH 6.5

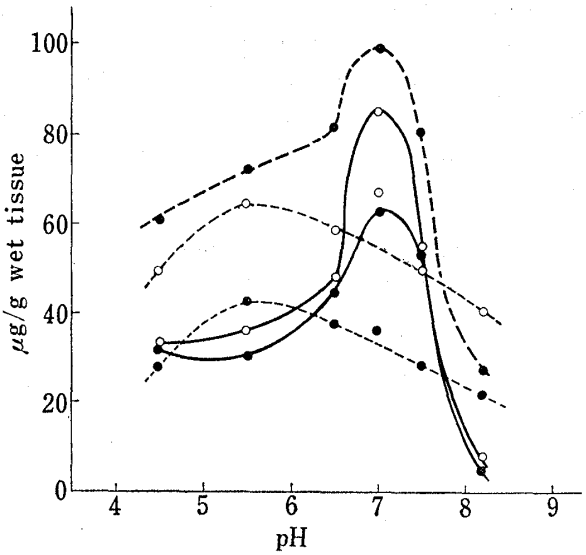

Fig. 10. pH–Profiles of Adsorption and Accumulation of Thiopental

– – – – total uptake ——— adsorption
····· accumulation
●: incubated for 2 min ○: incubated for 5 min

sorption process was saturated after 5 min of incubation and thereafter a plateau was observed. On the other hand, the accumulation increased with the lapse of incubation time. The adsorption constitutes an important fraction of the over-all uptake at the earlier stage of incubation. Since it is already verified above that the accumulated drug in the tissue was transfered rapidly into the body at the *in situ* physiological conditions, it seems that the adsorption process is a possible decisive factor in regulating the absorption from the small intestine. On these lines,

the experiments were made concerning the effect of pH of the incubation medium on the adsorption and the accumulation. The results are shown in Fig. 10. Both the processes were affected by pH of the incubation medium but the optimal pH values were not same, for the adsorption it was about 7, and for the accumulation about 4.5. As shown in Fig. 10, the total uptake depended exclusively on the adsorption. The pH-profile of the adsorption or the total uptake resembled that of the *in situ* absorption reported in the previous paper. The adsorption of N-methyl series, metharbital, was compared with other non N-methylated derivatives in Table II. The mechanism involved in the binding or the adsorption can not be

Table II. Relation of Absorption to Partition Coefficient and Adsorption to Mucosa

Barbiturates	Absorption rate constant (1/hr)	Partition coeff. (CCl_4, $\times 10$)	Adsorption[a] (μg/g)
Thiopental	1.807	378	48
Amobarbital	1.260	9.4	22
Metharbital	1.014	20.2	3

a) Incubated for 5 min, pH 6.5.

elucidated and correlated quantitatively to the chemical structures or the physico-chemical properties of drugs from these results. Some informations, however, on the forces involved were drawn from the results obtained. The comparison of the binding with the partition coefficient as represented in Fig. 3 or 4 indicates that hydrophobic bonding[12] is partially mediating, although the contribution of the interaction between the polar functional groups, such as ionic[6c] or hydrogen bonding[13] was also suggested by the specific behavior of N-methyl series in the adsorption and the binding. In view of the results above, the most reasonable conclusion to be drawn is that the process of the adsorption or the binding to the mucosal surface is an essential, determinant part of the whole absorption process of barbituric acid derivatives from the rat small intestine. The further extension of this hypothesis to various drugs which structures are not related to barbituric acid derivatives, and whether these phenomena observed in the small intestine can be demonstrated in the other part of the intestinal tract will be discussed in the following paper.

Acknowledgement The authors express their thanks to Dr. H. Sezaki, Kyoto University, for his continuous instructions during the course of this work and for the reviewal of this manuscript.

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13) G. Nemethy and H.A. Scherage, *J. Phys. Chem.*, **66**, 1773 (1962); T, Nash and A.C. Allison, *Biochem. Pharmacol.*, **12**, 601 (1963).