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Effect of Complex Formation on the Intestinal Absorption of Tryptophan[1,2]

Kiichiro Kakemi, Hitoshi Sezaki, Masahiro Nakano, and Etsuko Suzuki

Faculty of Pharmaceutical Sciences, Kyoto University[3]

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Complexation influence on the rate of absorption of actively transported drug was investigated in the perfused rat small intestine employing L-tryptophan as a model drug and caffeine, 8-chlorotheophylline, 8-methoxycaffeine, 7-hydroxyethyltheophylline, and flavin mononucleotide as complexing agents. Preliminary experiment on the absorption of L-tryptophan by the *in situ* intestinal perfusion method revealed that the kinetical pattern of the absorption of L-tryptophan approximated to the Michaelis–Menten relationship, D-isomer was also absorbed from the small intestine with apparently minor contribution by a specialized amino acid transport system, and that little absorption occured for both compounds in the rectum. Among the complexing agents studied, 7-hydroxyethyltheophylline and flavin-mononucleotide (FMN) reduced the absorption of L-tryptophan considerably. In the former, mutual effect in the reduction of absorption was observed. For the latter, reduction of the rate of absorption of L-tryptophan was caused neither by direct effect on the absorptive membrane nor by imparing effect on the neutral amino acid transport system but by the decrease of thermodynamic activity of L-tryptophan resulting from molecular complexation with FMN in solution.

There are several ways in which the rate of absorption of a dissolved, stable and freely diffusible drug can be modified. One such method would be the formation of a soluble complex with some other compound in the gastrointestinal fluid.

Some drug complexes penetrate biologic barriers more readily than the free drug itself. On the other hand, nonintended complexation of a drug with other components may reduce the effectiveness of the preparation due to the slower absorption of the drug.

In recent years, studies on the effect of complex formation on the gastrointestinal absorption are considerable interest and have been investigated extensively.[4]

What appears to be conspicuously absent, however, is an attempt to study complexation influence on the absorption rates of compounds which can be absorbed by specialized or active transport processes.

The present communication deals with the effect of various complexing agents on the active intestinal transport of the neutral amino acid, tryptophan. This is of practical importance in the light of recent biopharmaceutical interest in the applications of such phenomenon to the absorption modification of a drug in the presence of another.

Experimental

Materials——L- and D-tryptophan (Try), L-methionine, 8-methoxycaffeine(8-MeO-Caf), 7-hydroxyethyltheophylline (7-HET), and flavin–mononucleotide (FMN) were obtained from commercial sources. Caffeine (Caf) was recrystallized from distilled water. 8-Chlorotheophylline (8-Cl-Theo) was recrystallized from methanol.

1) This paper forms Part XLV of "Absorption and Excretion of Drugs," Preceding paper, Part XLIV: K. Kakemi, H, Sezaki. S. Muranishi, and A. Yano, *Chem. Pharm. Bull.* (Tokyo), **18**, 1563 (1970).
2) Presented at the 88th Annual Meeting of Pharmaceutical Society of Japan, Tokyo, April, 1968.
3) Location: *Yoshida-shimoadachi-cho, Sakyo-ku, Kyoto.*
4) G. Levy and R.H. Reuning, *J. Pharm. Sci.*, **53**, 1471 (1964); S. Goto, R. Takamatsu, M. Shibao, and S. Iguchi, *Chem. Pharm. Bull.* (Tokyo), **16**, 332 (1968).

Absorption Experiments——Small Intestine: Male Wistar rats, weighing 150—180 g, were fasted overnight prior to the experiments. Water was given *ad* libitum. The small intestine was cannulated under pentobarbital anesthesia and the intestinal absorption was determined using *in situ* perfusion technique, as reported from our laboratory[5]. Amino acids and other drugs were dissolved in 0.9% NaCl solution except 8-Cl-Theo. The latter compound was dissolved in pH 7.0 isotonic KH_2PO_4–$NaHCO_3$ buffer solution of its solubility. Thirty milliliter test solutions were circulated through the intestine for 20 min at 37°. At the end of this period, the drug remaining in the intestine was washed out with an aid of a syringe and made to 100 ml with 0.9% NaCl solution and the amount absorbed was calculated by the difference in amount of a drug between the initial and the final solutions. Rectum: Male Wistar rats, weighing 180—220 g, were anesthetized with pentobarbital. The rectum was cannulated and the absorption was determined using *in situ* perfusion technique, as reported from our laboratory.[6] After one hour recirculation, amount absorbed was calculated in the same manner as described before.

Analytical Method——Unless otherwise specified, ultraviolet or visible spectrophotometric methods were employed. All spectrophotometric analyses were performed with a Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer.

Tryptophan was determined by procedure A of the method of Spies and Chambers.[7] Complexing agents used in this study did not interfere with the assay procedure.

7-Hydroxyethyltheophylline was determined as Follow: Two ml aliquot of the sample solution was diluted to 20 ml with 0.9% NaCl solution and shaken with 20 ml of chloroform for 20 min and centrifuged. A 10 ml aliquot of the chloroform phase was shaken with 10 ml of 0.9% NaCl solution for 20 min and optical density of the separated aqueous layer was determined at 274 mμ.

Methionine: Details of the chemical method have been described previously.[8] One ml of 5N NaOH and 0.1 ml of 10% aqueous solution of sodium nitroprusside was mixed with one ml of methionine sample solution. After 10 min of frequent shaking, 2 ml of 3% aqueous solution of glycine was added. After shaking the mixture for 10 min, concentrated phosphoric acid was added and made to 10 ml. After 20 min shaking, optical density was determined at 515 mμ. FMN interfered with methionine assay and had to be removed by ion exchange. For this purpose, Dowex-1×8 (100—200 mesh) was placed in a glass tube of 8 mm diameter with a stopcock, making a bed depth 40 mm.[9] After conversion of the resin to salt-type with 0.05M sodium borate, 3 ml of the perfusion solution was run through a column of the resin to remove FMN. After the solution passed below the upper level of the resin, the column was washed with 0.05M sodium borate to a volume of 20 ml. Ten ml aliquot of this solution was evaporated to dryness on a water-bath. Five ml of distilled water was added to dissolve the residue and 4 ml of the solution was used for methionine assay as described before.

Determination of Stability Constants for Tryptophan Complexes——Stability constants for Caf (2×10^{-2}M), 8-Cl-Theo (2×10^{-2}M), 7-HET (2×10^{-2}M) and 8-Meo-Caf (10^{-2}M) were determined by partition method using cyclohexane–chloroform mixture or cyclohexane as an organic phase. As for tryptophan, 2×10^{-2}M was used for Caf, 8-Cl-Theo and 7-HET, and 10^{-2}M for 8-Meo-Caf. Volume ratios of organic phase were equal for 7-HET and 8-Cl-Theo, 9 volumes of cyclohexane and 1 volume of chloroform for Caf. Organic phase for 8-Meo-Caf was cyclohexane only. Ten ml each of organic solvent and aqueous drug solution were shaken at room temperature for 20 min followed by shaking in a constant temperature bath at 25° for 1 hour. After the separation of two phases the drug concentration of the organic phase was determined and stability constants of tryptophan complexed were calculated according to the equation.[10] The partition coefficients should be determined at the temperature of the absorption experiment. At higher temperature, however, both phases tend to become miscible. For this reason, they were determined at 25°.

5) T. Koizumi, T. Arita, and K. Kakemi, *Chem. Pharm. Bull.* (Tokyo), **12**, 421 (1964).
6) K. Kakemi, T. Arita, and S. Muranishi, *Chem. Pharm. Bull.* (Tokyo), **13**, 861 (1965).
7) J.R. Spies and D.C. Chambers, *Anal. Chem.*, **20**, 30 (1948).
8) T.E. McCarthy and M.X. Sullivan, *J. Biol. Chem.*, **141**, 871 (1941).
9) K. Yagi, T. Okuda, and Y. Matsuoka, *Nature*, **175**, 555 (1955).
10) When two species, A and B, interact reversibly only in the aqueous phase to form 1:1 complex, the stability constant, K, in the concentration unit is expressed by

$$K=\frac{c_c}{(c_a-c_c-c_{aob})\,(c_b-c_c-c_{boa})}$$

$$(pc)_a=\frac{c_a-c_{ao}}{c_{ao}}$$

$$c_c=c_a-c_{aob}\{1+(pc)_a\}$$

where $(pc)_a$ is the partition coefficient of A, c_a and c_b are the initial concentration of A and B in the aqueous phase respectively and c_c is the concentration of the complex at equilibrium, c_{ao} is the concentration of A in the organic phase, c_{aob} and c_{boa} are the concentration of A and B in the organic phase when B and A are present.

Stability constant of tryptophan–FMN complex was determined by the visible spectrophotometric method. The concentration of FMN was kept constant at 10^{-4}M in 0.9% NaCl solution and the concentration of tryptophan was varied between 4×10^{-3}M and 16×10^{-3}M in 0.9% NaCl solution. The stability constant was calculated by the following equation.[11]

Result and Discussion

Absorption of Tryptophan in the Absence of Complexing Agents

The purpose of the present investigation was to study the effect of various complexing agents on the rate of intestinal absorption of a drug which is absorbed mainly by an active transport process. For this purpose, tryptophan was chosen as a model drug on the ground that being one of the neutral amino acids which are absorbed rapidly from the intestine by means of neutral amino acid transport system, it has an unique characteristics in complexation in solution. The method, as described in previous reports from this laboratory, seemed to obviate most of the difficulties encountered in the studies of the absorption of such an actively transported drugs like tryptophan. Apparent lack of information on the intestinal absorption of tryptophan in rat by the perfusion method, however, led to a detailed investigation on the absorption characteristics of the compound such as effect of stereo isomer, site specificity, and the ionic nature of the perfusion media.

(1) Absorption of L-Tryptophan——The active intestinal transport of tryptophan has been studied in detail by Spencer and Samiy.[12] Their experimental technique was based mostly on *in vitro* method using isolated rat intestinal segment. In the present investigation, tryptophan absorption was studied mainly by the perfusion method. Since substance like

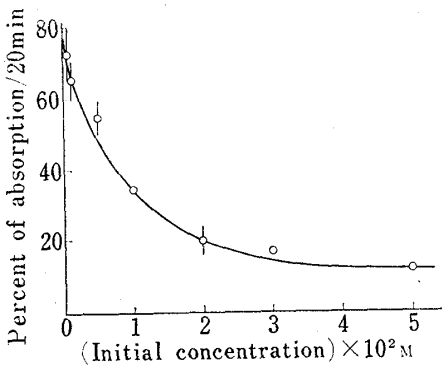

Fig. 1. Absorption of L-Tryptophan from the Small Intestine of the Rat at Various Initial Concentrations

Each point represents the mean value of at least five experiments. Vertical bars indicate S.D.

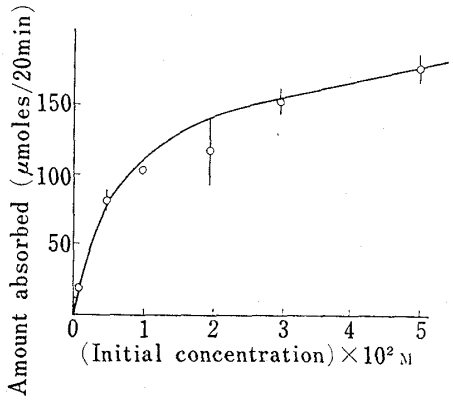

Fig. 2. Absorption of L-Tryptophan from the Small Intestine of the Rat at Various Initial Concentrations

Each point represents the mean value of at least five experiments. Vertical bars indicate S.D.

11) If two species, A and B, interact reversibly in solution to form a 1:1 complex C, the stability constant, K, in the concentration unit can be expressed by

$$K=\frac{c_c}{(c_a-c_c)(c_b-c_c)}$$

$$\frac{c_a c_b}{\Delta A}=\frac{1}{K\cdot l\cdot \Delta a}+\frac{c_a+c_b-c_c}{l\cdot \Delta a}$$

where ΔA is the difference between measured absorbance and that expected if no interaction takes place, Δa represents $(a_c-a_a-a_b)$, l is the pathlength of the cell in cm, and a_a, a_b, and a_c are the molar absorptivities of A, B, and C respectively.

12) R.P. Spencer and A.H. Samiy, *Am. J. Physiol.*, **199**, 1033 (1960).

tryptophan is transported by an active transport process and the rate of absorption observed under these conditions was so high, it seemed more desirable to measure the initial rate of absorption and 20 minutes was selected for the length of absorption study. The results of the experiment with L-tryptophan alone are shown in Fig. 1. It is clear from the figure that the rate of absorption of L-tryptophan is not proportional to the initial concentration of the amino acid. At higher concentrations, increase in the percentage of absorption fails to keep pace with the increase in initial concentration. This saturative phenomenon is also shown in Fig. 2 in terms of amount absorbed *vs.* initial concentration. These values were plotted in Fig. 3 to show the reciprocal of transport rate as a function of the reciprocal of concentration, a procedure similar to that proposed by Lineweaver and Burk for the analysis of enzyme kinetics.

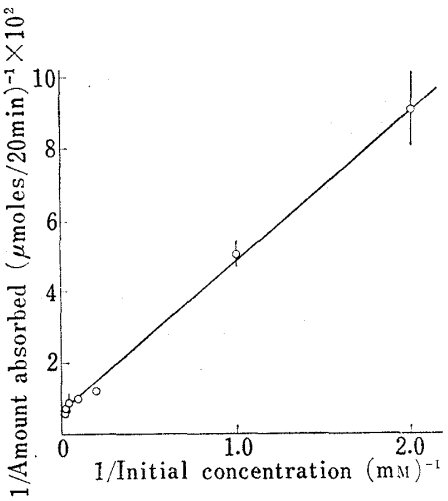

Fig. 3. Lineweaver–Burk Plots for L-Tryptophan

Each points represents the mean value of at least five experiments. Vertical bars indicate S.D.

The kinetics of absorption of L-tryptophan approximate to the Michaelis–Menten relationships. The values were determined from the graph for V_{max}, the apparent ilmiting rate of transport and K_m, the concentration at which half this rate was attained. These values were 200 μmole/20 min and 8.7 mM respectively, and were approximate to the values of methionine, obtained by the similar *in situ* perfusion method.[13]

(2) Absorption of D-Tryptophan——One of the striking features of the amino acid transport system in the mammalian intestine is its preference for L-stereo-isomer. This, however, has not been confirmed by the tryptophan absorption from the rat small intestine. Fig. 4 and Fig. 5 show the results of absorption studies concerning D-isomer. It is apparent that quantity of D-tryptophan absorbed by the specialized transport process is not so large as L-isomer.

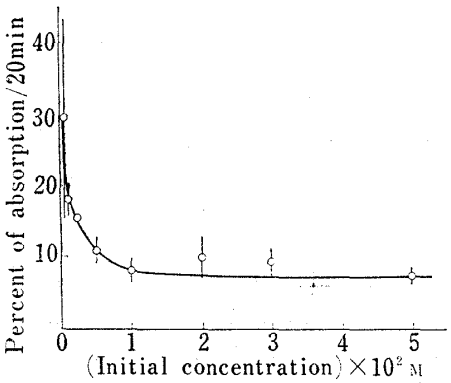

Fig. 4. Absorption of D-Tryptophan from the Small Intestine of the Rat at Various Initial Concentrations

Each point represents the mean value of at least five experiments. Vertical bars indicate S.D.

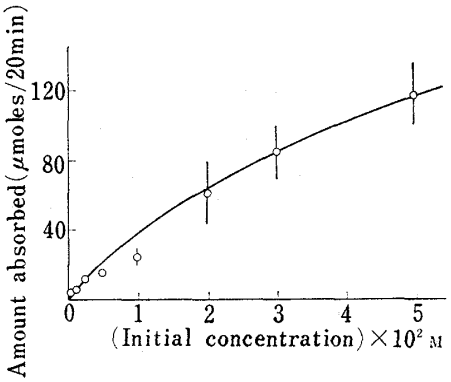

Fig. 5. Absorption of D-Tryptophan from the Small Intestine of the Rat at Various Initial Concentrations

Each point represents the mean value of at least five experiments. Vertical bars indicate S.D.

13) E.L. Jervis and D.H. Smyth, *J. Physiol.*, **149**, 433 (1959).

In the case of L-tryptophan, percentage absorbed values approach constant at very high concentrations, whereas that of D-isomer decreases rapidly at about the concentration of 5×10^{-3}M, much smaller than L-tryptophan. This suggests the posibility that although the absorption of D-tryptophan involves the same carrier system as the L-enantiomorph, the latter possesses a much greater affinity for the specialized transport system.[13]

(3) Absorption of Tryptophan from the Rectum——If the complex formation influence on drug absorption was merely a matter of decrease of the thermodynamic activity of a diffusible drug in perfusion media, the rectum would be a better site of absorption study. Previous papers from this laboratory reveal that in the rectum, lipid pore theory holds much better than in the small intestine and shift of pH or dilution effect during experiment can usually be neglected.[6] It seemed worthwhile, therefore, to investigate the site specificity of amino acid transport from this site of absorption. The percentage absorbed values for L- and D-tryptophan after one hour perfusion experiment in the rat rectum were $4.2\pm1.3\%$ and less than 3.3% respectively. Since the presence of endogenous tryptophan in the perfusate was proved to be negligible in these procedures, the rectum is considered not to be a main site of active as well as of passive transport of tryptophan in the rat.

(4) Effect of the Composition of Perfusion Media on the Absorption of L-Tryptophan——It is well documented that the rate of absorption of actively transported drugs are highly influenced by the composition of perfusion media. Table I shows the effect of the compositions of perfusion media on the absorption of L-tryptophan. Although the absorption of L-tryptophan was slightly inhibited by glucose, small varience of the concentration of NaCl did not affect the absorption of amino acid at all. Therefore, 0.9% solution of NaCl was used throughout the experiment.

Taebl I. Effect of Ionic Nature of Perfusion Medium of L-Tryprophan Absorption (Tryptophan Concentration=10^{-3}M)

Medium	% Absorbed	Medium	% Absorbed
Control (0.9% NaCl)	65.1 ± 4.9	5.0% Glucose	53.2 ± 6.4[a]
1.0% NaCl	58.7 ± 7.7	0.9% NaCl: 5.0%-glucose (volume ratio=1:1)	55.1 ± 12.5
0.8% NaCl	67.8 ± 4.7		

a) significant as compared to the control ($p<0.05$).

Absorption of Tryptophan in the Presence of Complexing Agents

Effect of various complexing agents on the absorption of tryptophan was investigated using readily absorbed L-isomer. Small intestine was chosen as the site of experiment because of its absorptive characteristics. Table II shows the percentage absorption of tryptophan in 20 minutes with or without complexing agents.

Among the complexing agents studied, 7-HET and FMN decreased the absorption of tryptophan remarkably whereas rest of the compounds exhibited little influence. In the case of 7-HET, absorption of the complexing agent, 7-HET itself, was also decreased in the presence of tryptophan. In the combination of tryptophan–FMN, decrease of absorption of tryptophan by FMN may not be due to the complex formation effect but to the direct action of FMN to the absorptive membrane or to the active transport system of L-tryptophan. With the above in mind, it became desirable to determine the effect of FMN pretreatment on tryptophan absorption and the effect of FMN on the absorption of a marker amino acid. Pretreatment of rat intestine with isotonic NaCl FMN solution of 5×10^{-2}M for 20 minutes did not significantly alter the absorption of tryptophan. This suggests that the decrease of absorption of L-tryptophan complexed with FMN is not due to the direct effect of the latter to the absorptive membrane. As FMN might influence on the carrier system of L-

Table II. Effect of Some Complexing Agents on L-Tryptophan Absorption

Compound	Try. Concn. (M)	% Absorbed	
		Control	Sample
Caf(1.8×10^{-2}M)	10^{-3}	65.1±4.9	59.9±10.4
8-Cl-Theo (1.8×10^{-2}M)	10^{-3}	65.1±4.9	63.2± 2.8
8-Meo-Caf (5.0×10^{-3}M)	5×10^{-4}	72.9±7.9	62.9± 0.9
7-HET (5.0×10^{-2}M)	5×10^{-4}	72.9±7.9	36.7± 6.7[a]
FMN (5.0×10^{-2}M)	10^{-3}	65.1±4.9	32.5± 1.9[a]

Effect of Tryptophan on 7-HET Absorption

Compound	7-HET Concn. (M)	% Absorbed	
		Control	Sample
Try (4×10^{-2}M)	4×10^{-3}	54.6±3.9	45.8±1.8[a]

a) significant as compared to the control ($p<0.01$).

tryptophan, methionine, one of the neutral amino acids and shares the common transport system with L-tryptophan, was selected as a marker amino acid and the absorption rates of methionine alone and with FMN were determined. It seemed likely by spectrophotometry that methionine did not interact with FMN. The results of the two absorption experiments were identical and it was concluded that contrary to L-tryptophan, absorption of methionine was hardly affected at all by the presence of FMN.

Since FMN gives no damage on the membrane as well as on the carrier system of neutral amino acid, the decrease of L-tryptophan absorption in the presence of FMN could be attributed to the decreased thermodynamic activity of L-tryptophan resulting from the complex formation. In the case of complexing agents other than FMN and 7-HET, little complexation effect on the absorption of L-tryptophan was observed. The exact cause of the phenomenon is uncertain at present. It may be ascribed to the difference in stability constants or in the rates of absorption of such complexing agents themselves from the intestine as well as the rapidity of the absorption of the parent compound, L-tryptophan. As shown in Table II and III, FMN, having the largest stability constant of all, is hardly absorbed under the experimental condition, whereas 7-HET, having the K value of 22.4, is moderately, and other complexing agents are absorbed very rapidly.

Table III. Stability Constants of Tryptophan complexed with Some Compounds

Compound	Stability constant	Method
Caf	30.6 (25°)	partition
8-Cl-Theo	16.9 (25°)	partition
8-MeO-Caf	34.6 (25°)	partition
7-HET	22.4 (25°)	partition
FMN	56.8 (37°)	spectroscopy

7-HET was absorbed 54.6% in 60 minutes, whereas Caf, 8-MeO-Caf, and 8-Cl-Theo were absorbed 43.2%, 45.7%, and 56.6% respectively in 20 minutes. Although it has been

reported that drug complexes having stability constants of the magnitude of 20—30 significantly altered the absorption pattern of the component drugs from the small intestine,[14] complexing agents used in this study, having about equivalent K values, could not influence the absorption of the rapidly absorbed amino acid. During the 20 minute perfusion period, rapidly absorbed complexing agents are absorbed themselves to a considerable extent thereby reducing the fraction of complexed amino acid which is also absorbed very rapidly.

14) I. Sugimoto, *Chem. Pharm. Bull.* (Tokyo), **16**, 1527 (1968); *Idem, ibid.*, **17**, 994 (1969).