[Chem. Pharm. Bull. 16(11)2206—2212(1968)]

UDC 615.332.033/.034

Absorption and Excretion of Drugs. XXXVII.[1] Effect of Ca^{2+} on the Absorption of Tetracycline from the Small Intestine. (2)

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(Received March 23, 1968)

Urinary excretion of tetracycline tended to increase in the presence of EDTA. Experiments employing Wiseman's apparatus showed the accumulation of tetracycline in an intestinal membrane. Examination of fluorescence under a black lamp made possible to visualize the binding of tetracycline to mucosa both *in vitro* and *in vivo*. Using villi preparation from rat intestine the binding of tetracycline to villi in the presence of Ca^{2+} was demonstrated in a binding study, a distribution study, and a dialysis study. EDTA was found to release free tetracycline from villi-bound tetracycline both *in vitro* study using a villi preparation and *in vivo* study in man.

In the preceding paper[1] authors have reported (a) that tetracycline becomes more fat soluble in the presence of Ca^{2+} in partition studies *in vitro* and (b) that it disappears faster from recirculation solution containing Ca^{2+} in rat over the pH range above 7.5. When $CaCl_2$ was given orally with tetracycline in man, however, the percentage of excreted tetracycline decreased with increasing amounts of $CaCl_2$ administered and inter- and intra-subjects variation in the percentage of excretion disappeared. Much decrease in serum concentrations of tetracycline in rat in the presence of a calcium salt was also reported.[3] Thus the effect of Ca^{2+} on the intestinal absorption of tetracycline in man and rat seems to be similar.

Increase in fat solubility of a drug is generally considered to be favorable for absorption. Increase in disappearance rate of drugs from recirculation solution usually correlates well with increase in rate of absorption. These concepts, however, do not seem to be applicable to the absorption studies of tetracycline in the presence of Ca^{2+}, since Ca^{2+} appears to inhibit the absorption of the antibiotic. The origin of this apparent discrepancy was pursued in this study.

It is believed that binding of a drug to the gastrointestinal wall is the first step for absorption and that drugs which bound to the wall to a greater extent will be more favorably absorbed.[4] Binding to the intestinal wall, however, should not be too strong for the drug molecule to permeate through the gastrointestinal membrane. The present investigation is designed mainly to elucidate the extent and strength of binding of tetracycline to the intestinal wall.

In the earlier study[1] the decrease in urinary excretion of tetracycline in the presence of Ca^{2+} appeared to originate in the absorption process. To test this probability EDTA was administered with tetracycline and its effect on urinary excretion was examined in man.

Experiments employing Wiseman's apparatus made it possible to clarify the permeation process of the antibiotic through the intestinal membrane. Both *in vitro* and *in vivo* binding of tetracycline to the intestinal wall in the presence of Ca^{2+} was tested under a black lamp.

1) Part XXXVI: K. Kakemi, H. Sezaki, H. Ogata, and T. Nadai, *Chem. Pharm. Bull.* (Tokyo), **16**, 2208, (1968).
2) Location: *a*) *Yoshidashimoadachi-cho, Sakyo-ku, Kyoto*; *b*) *Tenpakuyagoto, Showa-ku, Nagoya.*
3) G. Tonelli, L. Alfano, and E. Takesue, *J. Pharmacol.*, **145**, 386 (1964).
4) K. Kakemi, T. Arita, R. Hori, R. Konishi, K. Nishimura, and H. Matsui, *Chem. Pharm. Bull.* (Tokyo), submitted.

In order to obtain a further insight into the tetracycline binding to the intestinal wall in the presence of Ca^{2+}, a villi preparation was used for (1) binding studies *in vitro*, (2) partition studies, and (3) dialysis through a cellophane membrane.

The ability of EDTA to release free tetracycline from the intestinal wall was examined both *in vitro* and *in vivo*.

Experimental

Urinary Excretion Studies in Man in the Presence of EDTA——Urinary excretion of tetracycline in the presence of EDTA was studied by the same procedure as described previously[1] except that varying amounts of EDTA were administered with the antibiotic instead of $CaCl_2$.

Experiments employing Wiseman's Apparatus——Absorption of tetracycline across the rat small intestine was studied with an apparatus developed by Wiseman[5] with a modification to fit a 6 cm long segment of the small intestine. Male rats, weighing 130—160 g were anesthetized with urethan and the small intestine was excised under anesthesia. A segment of about 7 cm long was obtained from the distal end of the small intestine and was tied to the apparatus. Fifty milliliter of tetracycline hydrochloride solution (400 μg/ml) either in 0.01 M Tris buffer (pH 8.0) or 0.05 M collidine buffer (pH 6.2) with varying amounts of $CaCl_2$ was placed in the mucosal part.

The serosal solution was 7 ml of a 0.9% NaCl solution. The apparatus was placed in a constant temperature bath maintained at 37°. Oxygen gas containing 5% CO_2 was bubbled into the serosal solution. After 3 hours of incubation, both the serosal and mucosal liquids were withdrawn and the tetracycline assay was carried out by the same method as described for the recirculation experiments.[1] No change in pH was observed during the incubation.

Binding to the Rat Intestinal Wall——*in Vitro* Studies: The rat small intestine was excised and cut lengthwise. The segments were then placed in tetracycline solution with and without 20 times as much $CaCl_2$ as tetracycline in molar basis at pH 8.0 and kept 5 minutes in a water-bath maintained at 37°. Each segment was then washed with distilled water and sprayed with a $CaCl_2$ solution for observation of fluorescence under a black lamp in a dark room.

in Vivo Studies: About 20 mg of tetracycline was administered to rats with 45 mg of $CaCl_2$. The animals were sacrificed 3, 6, 9, and 24 hours after the administration and the intestines were excised, frozen and sliced into rings for examination of fluorescence under a black lamp in the dark.

Binding Studies using Rat Villi Preparations——Procedures used by Rasmussen, *et al.*[6] were modified for the present study. Male rats weighing about 200 g was anesthetized with urethan and sacrificed immediately. The small intestine was immediately excised, washed with a 0.9% NaCl solution and slit open. The intestinal segment was then laid on a side glass, the mucosal side up and the mucosa was separated from the remainder of the wall by scraping with a slide glass. The villi was placed in a 0.9% NaCl solution, centrifuged for 10 minutes, and washed with a 0.9% NaCl solution. The villi were then suspended in a 0.9% NaCl solution. One milliliter portion of the suspension was dried and weighed for estimation of the amount of solid present per unit volume.

Five milliliter of tetracycline hydrochloride solution (400 μg/ml) in 0.05 M Tris buffer (pH 8.0) or 0.1 M collidine buffer (pH 6.2) with varying amounts of $CaCl_2$ and 2 ml of the villi suspension were placed in centrifuge tubes and were incubated for 15 minutes at 37°. The supernatant liquid was withdrawn after centrifuging and the concentration of tetracycline remaining in the solution was assayed.

Dialysis Studies——"Visking" tubes were boiled three times in distilled water, each boiling being followed by washing with distilled water. The tubes were tied at the center and then 5 ml of a tetracycline solution (400 μg/ml) with varying amounts of $CaCl_2$ in either 0.05 M collidine buffer (pH 6.2) or 0.05 M Tris buffer (pH 8.0) and 1 ml portion of the villi suspension were placed in both compartments. Each tube was then placed in a bottle containing 10 ml of buffer solution and kept at 4—6°. The outer buffer solution was replaced with the fresh one at 20, 25, 45, 70, and 115 hours. Tetracycline dialyzed was assayed by the method described previously.[1]

Addition of EDTA to Tetracycline Bound to Villi Preparation——Two milliliter portion of the villi preparation was placed in a tube containing 5 ml of 400 μg/ml tetracycline solution and 10 times as much $CaCl_2$ as tetracycline in 0.01 M Tris buffer (pH 8.0). The resulting solution was incubated at 37° for 15 minutes. Two milliliter portion of a EDTA solution was then added and the resulting solution was incubated at 37° for another 15 minutes. The tetracycline content was determined after centrifugation.

5) G. Wiseman, *J. Physiol.*, **120**, 63 (1953).
6) H. Rasmussen, A. Waldorf, D.D. Dziewiatkowski, and H.F. Deluca, *Biochim. Biophys. Acta*, **75**, 250 (1963).
7) K. Kakemi, T. Arita, H. Sezaki, and T. Nadai, *Yakugaku Zasshi*, **83**, 264 (1963).

Administration of EDTA following Administration of Tetracycline and $CaCl_2$ in Man——Tetracycline hydrochloride (250 mg) along with ten molar times as much $CaCl_2$ as the antibiotic in 100 ml water was administered 3 hours after a meal. Three times as much EDTA as tetracycline in 100 ml water was administered 10 hours after the administration of the antibiotic. No food was given during the study. Urine was collected at certain intervals and tetracycline excreted was estimated by the assay method described earlier.[7]

Results

Effect of EDTA on the Urinary Excretion of Tetracycline in the Presence of $CaCl_2$

EDTA is believed not to be absorbed from the gastrointestinal tract and to form a chelate in alkaline pH range with Ca^{2+} in the alimentary tract.[1] Figure 1 shows the urinary excretion data of tetracycline when EDTA is administered with it. It may be seen that the urinary excretion of tetracycline usually increases with the increasing amount of EDTA administered and that a smaller amount of EDTA is enough to bring the urinary excretion to about 60% of the administered in men who excrete a relatively large amount when the antibiotic is given alone. On the other hand in men who do not excrete much tetracycline in urine when it is administered alone, a larger quantity of EDTA is necessary to raise the urinary excretion to the same level as in the former case. In the presence of too much EDTA, however, the urinary excretion of the antibiotic tended to decrease.

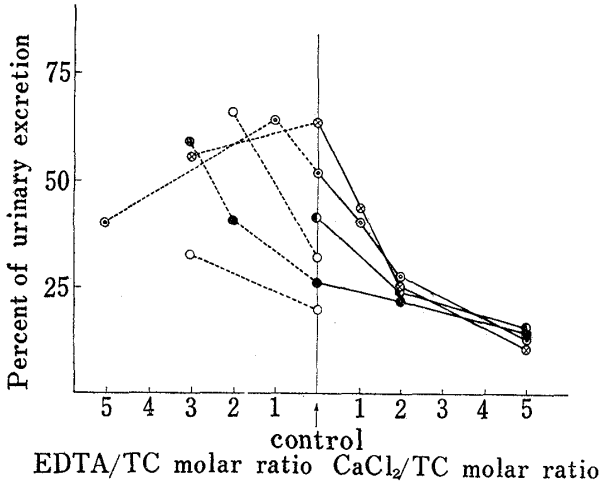

Fig. 1. Effect of $CaCl_2$ or EDTA on Urinary Excretion of Tetracycline (TC) in Man

Effect of Ca^{2+} on the Transport of Tetracycline through the Intestinal Membrane

Employing Wiseman's apparatus effect of Ca^{2+} on the transport of tetracycline through the intestinal membrane was examined depending upon the ratio of Ca^{2+}/tetracycline. Increases in tetracycline concentration in the serosal fluid during 3 hours period are plotted in

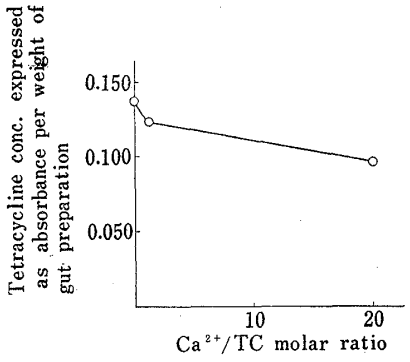

Fig. 2. Increase in Tetracycline (TC) Conc. in Serosal Side observed by Modified Wiseman's Apparatus at pH 6.2 and 37° TC conc.=400 μg/ml

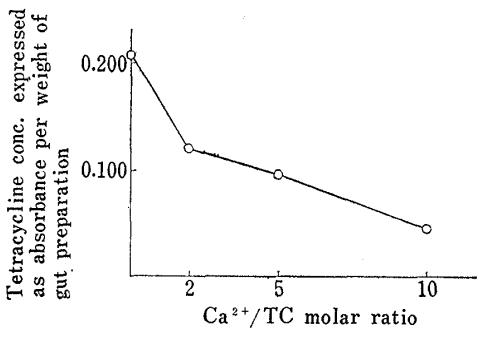

Fig. 3. Increase in Tetracycline (TC) Conc. in Serosal Side observed by Modified Wiseman's Apparatus at pH 8.0 and 37° TC conc.=400 μg/ml

Figures 2 and 3. In Figures 4 and 5 are shown its decreases in the mucosal fluid during the same period.

Results of experiments at pH 6.2 (Figures 2 and 4) show that tetracycline in the serosal liquid gradually decreases with increasing amounts of Ca^{2+} while that in the mucosal liquid increases with small amounts of Ca^{2+} and come back to the reference value with larger amounts of the ion.

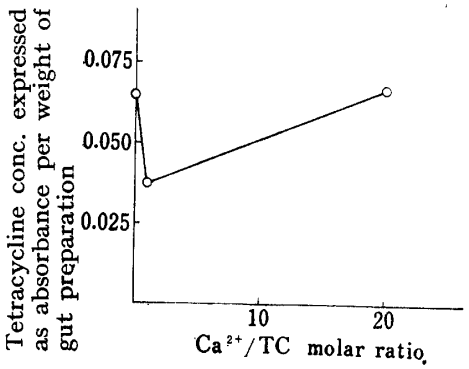

Fig. 4. Decrease in Tetracycline (TC) Conc. in Mucosal Side observed by Modified Wiseman's Apparatus at pH 6.2 and 37° TC conc. = 400 μg/ml

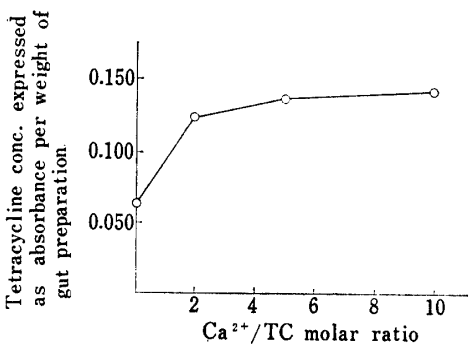

Fig. 5. Decrease in Tetracycline (TC) Conc. in Mucosal Side observed by Modified Wiseman's Apparatus at pH 8.0 and 37° TC conc. = 400 μg/ml

At pH 8.0 (Figures 3 and 5), on the other hand, tetracycline in the serosal liquid markedly decreases with increasing amounts of Ca^{2+} and at the same time that in the mucosal liquid decreases with addition of Ca^{2+}. This apparent discrepancy (the decrease in mucosal concentrations does not result in increase in serosal concentrations) may come from the fact that tetracycline which disappears from the mucosal liquid does not permeate through the membrane.

Binding of Tetracycline to Rat Intestinal Wall

In Figure 6 are shown pictures of rat small intestine segments exposed under the black lamp after immersion in tetracycline solutions with and without $CaCl_2$. Binding of tetracycline to the intestinal wall is apparent in the presence of Ca^{2+}. pictures illustrating *in vivo* binding of tetracycline to rat small intestine are shown in Figure 7. The presence of $CaCl_2$ produced intense fluorescence for the first several hours, which, however, almost disappeared in 24 hours.

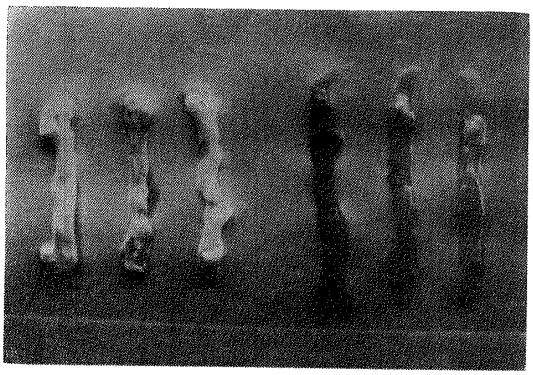

Fig. 6. A Picture showing Presence and Absence of Fluorescence under Black Lamp after Intestinal Segments were immersed in Tetracycline Solution with Ca^{2+} (No. 1—3 from left) and without Ca^{2+} (No. 4–6 from left)

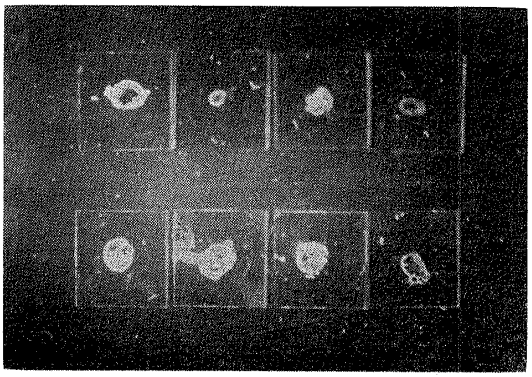

Fig. 7. A Picture showing Fluorescence under Black Lamp in Slices of Rat Intestine after Tetracycline was given with (Bottom) and without (Top) $CaCl_2$

from left 3,6,9, and 24 hours after administration

Effect of Ca^{2+} on the Binding of Tetracycline to Villi

Relationships between the percent of tetracycline bound to villi and the amount of Ca^{2+} present are shown in Figures 8 and 9. Presence of small amounts of Ca^{2+} (Ca^{2+}/tetracycline molar ratio=1:2) did not produce much binding at pH 6.2, while the same amount of Ca^{2+} caused a greater binding of tetracycline at pH 8.0.

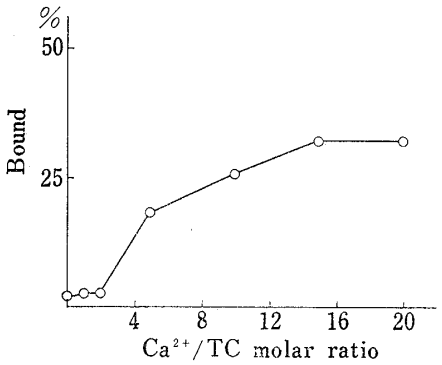

Fig. 8. Effect of Ca^{2+} on the binding of Tetracycline (TC) to the Villi Preparation at pH6.2 and 37° TCconc.=400 μg/ml

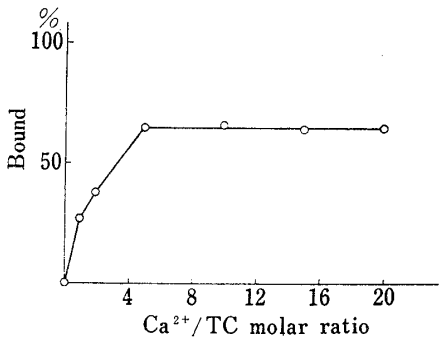

Fig. 9. Effect of Ca^{2+} on the binding of Tetracycline (TC) to the Villi Preparation at pH 8.0 and 37° TCconc.= 400 μg/ml

Effect of Ca^{2+} on the Partition Ratio of Tetracycline in the Presence of Villi

The effect of Ca^{2+} on the distribution of tetracycline between isoamyl alcohol and water was examined at pH 8.0 and 37° in the presence of the villi preparation and the results are recorded in Figures 10 and 11. Figure 10 shows the results when EDTA was added to the solution in order to eliminate the effect of Ca^{2+}. Practically no binding of tetracycline to villi was observed in the absence of Ca^{2+} and almost all tetracycline was found in aqueous layer. Addition of 10 times as much Ca^{2+} as tetracycline, however, made tetracycline much more oil soluble, tetracycline being detected mostly in the isoamyl alcohol layer. Much binding to villi was also noted in the presence of Ca^{2+}.

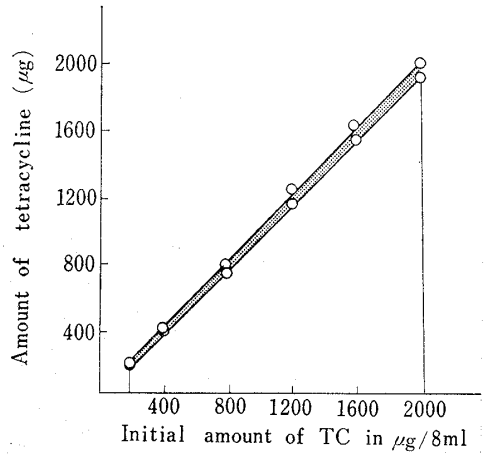

Fig. 10. Distribution of Tetracycline among Isoamyl Alcohol ■, Water □, and Villi ////, in the Absence of Ca^{2+} at pH 8.0 and 37°

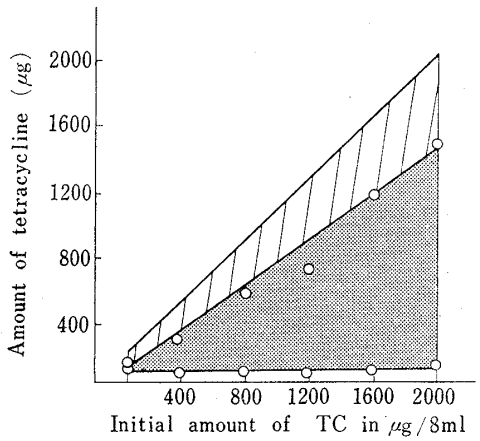

Fig. 11. Distribution of Tetracycline among Isoamyl Alcohol ■, Water □, and Villi ////, in the Presence of 10 Times at Much Ca^{2+} as Tetracycline at pH 8.0 and 37°

Effect of Ca^{2+} on the Dialysis of Tetracycline through Cellophane Membrane in the Presence of Villi Preparation

Tetracycline dialyzed is plotted against Ca^{2+}/tetracycline molar ratio in Figure 12. Slight increase in amounts of tetracycline dialyzed was observed in the presence of small amounts

of $CaCl_2$ at pH 6.2, exhibiting the similar curve as that observed in recirculation experiment *in situ*.[1] At pH 8.0, on the other hand, was observed a remarkable decrease in amount of the drug dialyzed. Binding to the villi preparation seems to be reversible since tetracycline dialyzed through the membrane over the considerable period.

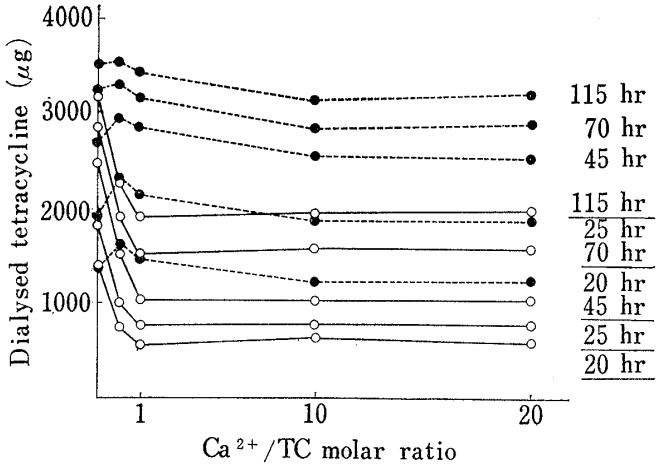

Fig. 12. Effect of Ca^{2+} on the Dialysis of Tetracycline (TC) through Cellophane Bag in the Presence of Villi Preparation at pH 8.0 ——○—— and pH 6.2 ------●------ at 4°

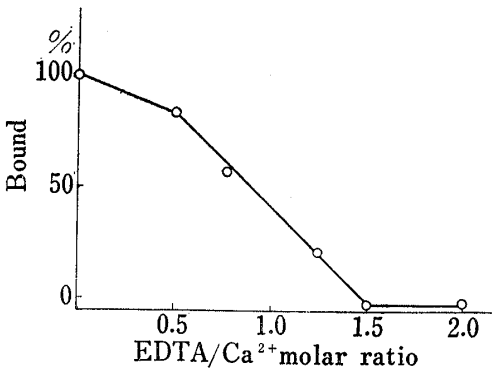

Fig. 13. Effect of EDTA on the Binding of Tetracycline to Villi Preparation in the Presence of Ca^{2+} at pH 8.0 and 37°

Effect of EDTA on Tetracycline binding to Villi Preparation

Relationship between percent of tetracycline bound and EDTA/Ca^{2+} molar ratio is plotted in Figure 13. Increase in EDTA/Ca^{2+} molar ratio tended to decrease the binding, practically no binding being observed above EDTA/Ca^{2+} molar ratio=1.5.

Effect of Administration of EDTA after Administration of Tetracycline with $CaCl_2$ in Man

Effect of Administration of EDTA on the urinary excretion of tetracycline in man is recorded in Figure 14. Increase in the rate of excretion of tetracycline is apparent about 1 hour after the administration of EDTA.

The urinary excretion curve would have followed the dotted line without EDTA. The discontinuity in the curve may be rationalized by the absorption of tetracycline through the intestinal tract in the presence of EDTA.

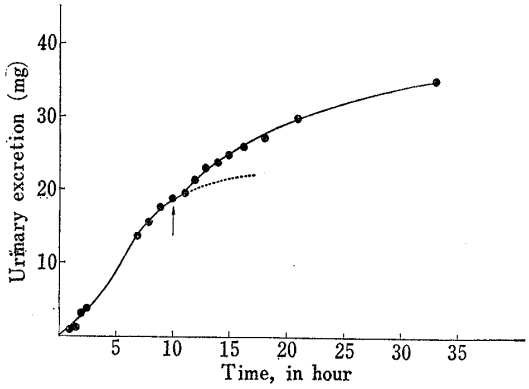

Fig. 14. Effect of EDTA on the Urinary Excretion of Tetracycline in Man

EDTA was given 10 hr after the oral administration of tetracycline and $CaCl_2$
Dose=TC-HCl 250 mg
TC-$CaCl_2$-EDTA=1:10:3 in molar basis

Discussion

When it is administered orally, tetracycline is expected to be bound to some protein substances in the intestinal wall in the presence of endogenous divalent metal ions. Tetracycline was postulated to be bound to protein molecules *via* Ca^{2+} bridge.[8] Bound tetracycline may be eventually removed from the intestinal wall to come into feces. Loss in feces results

8) J.T. Doluisio and A.N. Martin, *J. Med. Chem.*, **16**, 20 (1963).

in poor absorption of the antibiotic and its poor urinary excretion. This may rationalize poor physiological availability of tetracycline as often reported.

When EDTA is administered with tetracycline, it will form chelates with metal ions, leaving the antibiotic in absorbable form. Increased urinary excretion of tetracycline in the presence of EDTA may be rationalized on this basis. Too much EDTA, however, tended to decrease physiological availability of tetracycline. The decrease in absorption may result from change in gastric empting time in the presence of much EDTA as pointed out by Sögnen.[9] In other report,[10] on the other hand, EDTA was found to be without noticeable effect.

In lower pH region (pH 6—7), in the presence of a small amount of Ca^{2+}, the absorption of tetracycline tended to increase as observed in recirculation experiments in rats, in permeation experiments using Wiseman's apparatus, and dialysis studies employing cellophane membrane at pH 6.2.

In higher pH region, on the other hand, binding of tetracycline to intestinal wall substances increased in the presence of even small amounts of Ca^{2+}.

If tetracycline bound to the intestinal wall were present at the site for longer period, it would be gradually released and elongated absorption would result, the blood level of the antibiotic being kept constant for extended period.

Since little tetracycline was detected in the intestinal wall 24 hours after the administration of the antibiotic in rats, it may be expected that bound tetracycline eventually comes into feces as mucosa sloughs from underlying layers.

Implication of the results shown in Figure 14 is now discussed. No drug may be expected to be found in the upper region of the gastrointestinal tract 10 hours after its administration. Absorption of tetracycline after the administration of EDTA, then, can only take place by the release of tetracycline bound to intestinal wall in the presence of EDTA. Effect of EDTA on absorption behavior of tetracycline in the presence of $CaCl_2$ was demonstrated *in vitro* as shown in Figure 13. The result shown in Figure 14 demonstrated the same effect in intact man.

The present finding may suggest that tetracycline bound to the intestinal wall is hardly absorbed and that some substances which inhibit the binding of drugs to the intestinal wall may be useful to increase their physiological availability.

It may be concluded that tetracycline forms chelate with endogenous metal ions in an alimentary tract, is bound to mucosa and comes off into feces as mucosa comes off from underlying layers, resulting in poor absorption.

Acknowledgement This investigation was supported in part by grants-in-aid from Takeda Science Foundation.

9) E. Sögnen, *Acta pharmacol. et toxicol.*, **22**, 31 (1965).
10) G.M. Eisenberg, W. Weiss, and H.F. Flippin, *J. Lab. & Clin. Med.*, **52**, 895 (1958).