BBA 75727

INHIBITION OF AMINO ACID TRANSPORT IN RAT INTESTINAL RINGS BY TETRACYCLINE

VICTOR LING AND CLAUDE L. MORIN

Section de gastroentérologie, Département de pédiatrie, Hôpital Sainte-Justine, Université de Montréal, Montréal (Canada)

(Received June 1st, 1971)

SUMMARY

1. Tetracycline inhibited the uptake of L-alanine, L-lysine and a non-metabolized amino acid, cycloleucine (1-aminocyclopentane carboxylic acid), into rat intestinal rings. Protein synthesis was inhibited in this tissue within 4 h after intraperitoneal injection of the antibiotic, but transport of the amino acids was significantly impaired only after 12 h.

2. Time course studies of the uptake of cycloleucine showed that influx was reduced by the drug. The efflux was unaffected. Kinetic studies over a 20-fold range of alanine concentration showed a saturable transport mechanism with a v_{max} that was decreased by the drug. The apparent affinity of this amino acid for the transport mechanism was unchanged.

3. Our findings suggest that a transport protein(s) which turns over rapidly and which is essential or auxiliary to the translocation of amino acids becomes reduced in amount and acts as a rate limiting factor.

INTRODUCTION

The tetracycline group of drugs have been found to inhibit protein synthesis in *in vitro* systems derived from microbial as well as mammalian cells[1-3] and also to reduce the *in vivo* incorporation of amino acids into mammalian protein[4,5].

However, the effect of these antibiotics on transport has received little attention except for studies showing inhibition of iron and fat absorption[6,7].

The present study was carried out to investigate the effects of tetracycline on amino acid transport in rat intestinal rings. This report presents evidence that tetracycline is an effective inhibitor of intestinal amino acid transport.

MATERIALS AND METHODS

Male Sprague–Dawley rats weighing between 140 and 180 g were deprived of food but given free access to water for 20 h before sacrifice.

The radioactive compounds used, L-[^{14}C]alanine (specific activity 123 mC/

Abbreviation: Cycloleucine, 1-aminocyclopentane carboxylic acid.

mmole), L-[^{14}C]lysine (specific activity 255 mC/mmole), 1-aminocyclopentane [^{14}C]carboxylic acid (cycloleucine, specific activity 6.7 mC/mmole) and [*carboxy*-^{14}C]-inulin were obtained commercially (New England Nuclear). The amount of radioactive amino acid added to each flask was 0.3 μC in a volume of 2 ml. Unlabelled L-amino acids of analytical grade were used to bring the concentration of the amino acid studied to a fixed level at the beginning of the incubation period. This initial concentration, unless otherwise specified, was 0.8 mM for alanine, 0.065 mM for lysine and 0.5 mM for cycloleucine.

Administration of tetracycline

Animals were given intraperitoneal injections of 400 mg/kg of tetracycline·HCl at 4, 8 and 12 h before study. The solution of the antibiotic was prepared immediately before injection from a pure tetracycline powder. The solution injected contained 30 mg of tetracycline per ml and the final pH was 2.14. Control animals received an intraperitoneal injection of 2.0 ml of a normal saline solution adjusted to pH 2.14 with HCl.

Preparation and incubation of intestinal rings

The animals were killed by decapitation and a section of the intestine at 15 cm from the pylorus was removed, everted and cut into intestinal rings weighing 7–10 mg. The rings were kept in chilled Krebs–Ringer bicarbonate buffer (pH 7.4), until incubation, which had been gassed for 30 min with 95 % O_2–5 % CO_2.

Each ring was then transferred to a 10-ml erlenmeyer flask containing 2 ml of freshly gassed Krebs–Ringer bicarbonate buffer (pH 7.4) *plus* the labelled inulin or amino acid. Incubation was at 37°. At the end of the incubation, the rings were removed from the flasks, dipped twice in isotonic saline, blotted, weighed and dissolved in a counting vial containing 1 ml of NCS (Nuclear Chicago Solubilizer). A 0.1-ml aliquot of the incubation medium was also placed into 1 ml of NCS for assessment of radioactivity. The following day, 9 ml of a solution containing 4 g/l of Omnifluor (New England Nuclear) in toluene was added to the NCS solution. The vials were then sealed, shaken and counted in a liquid scintillation spectrometer at about 75 % efficiency. Quenching was accounted for by the channel ratio method.

Calculation

Disint./min per ml of intracellular fluid were calculated from measurements of radioactivity, total tissue water and extracellular space as described by ROSENBERG *et al.*[8]. Total tissue water, expressed as a percentage of weight was determined from the difference between wet weight and the weight after drying for 24 h at 105°. Extracellular space was measured with [*carboxy*-^{14}C]inulin as described by ROSENBERG *et al.*[9] except that the tissue was dissolved with NCS solution.

The transport of the labelled amino acids studied was expressed as the distribution ratio:

Distribution ratio = (disint./min per ml intracellular fluid)/(disint./min per ml incubation medium).

A distribution ratio greater than 1.0 is presumed to represent uptake in excess of simple diffusion.

Incorporation of L-[^{14}C]*lysine into protein of intestinal rings*[10]

6 intestinal rings were incubated as described above for 1 h in freshly gassed Krebs–Ringer bicarbonate buffer (pH 7.4) containing 0.6 μC of L-[^{14}C]lysine at 0.065 mM.

At the end of the incubation, the intestinal rings were removed from the flasks, washed twice in isotonic saline, blotted, weighed and homogenized in physiologic saline. An aliquot was used for protein determination by a modified LOWRY's method[11] while the rest was precipitated in 15% trichloroacetic acid. After standing in a cold room overnight, the precipitate was centrifuged and extracted with ethanol–diethyl ether (2:1, by vol.) at 60° and with 10% trichloroacetic acid at 90° and washed again in ethanol–ether mixture. It was then dried and dissolved in 1.5 ml of NCS and counted after addition of 9 ml of counting solution as described above.

Mitotic index

The mitotec index, *i.e.* the percentage of epithelial cells of the crypt of Lieberkühn in mitosis was evaluated from 8 drug-treated and 8 control animals. For this purpose 5-μm thick sections of intestine were taken at 15 cm from the pylorus and stained with hematoxylin–eosin. The sections were coded and reviewed independently by the two authors. Ten properly oriented crypts of Lieberkühn from each animal, and 50 cells of each crypt were examined for mitotic figures. All mitotic figures from the latter stages of prophase to the initial stages of telophase were counted. These criteria, when applied by the two observers gave an average difference in counts of slightly over 10%. The results are expressed as the percentage of epithelial cells in mitosis and analyzed statistically by Student's "*t*" test.

RESULTS

Effect of tetracycline on protein synthesis in rat intestinal rings

The effect of tetracycline on protein synthesis by rat intestinal rings is shown in Table I. The tetracycline was given 4 h before the study. The results show that

TABLE I

EFFECT OF TETRACYCLINE ON INCORPORATION OF L-[^{14}C]LYSINE INTO PROTEIN BY RAT INTESTINAL RINGS *in vitro*

6 intestinal rings from each rat were incubated for 1 h in freshly gassed Krebs–Ringer bicarbonate buffer (pH 7.4) containing 0.6 μC of L-[^{14}C]lysine at 0.065 mM. Values are for mean ± S.E.; number of rats is given in parentheses. The dose of tetracycline was 400 mg/kg body weight given intraperitoneally 4 h previous to time of study.

Experimental group	*Incorporation of* L-[^{14}C]*lysine into protein*		*Inhibition* (%)
	disint./min per mg tissue / *disint./min substrate* × 10^{-5}	*disint./min per 100 µg protein* / *disint./min substrate* × 10^{-5}	
Control	82.2 ± 3.7 (11)	88.1 ± 5.8 (11)	
Tetracycline	58.3 ± 4.2 (10)	62.0 ± 5.6 (10)	29.5
Significance of difference *	$P < 0.01$	$P < 0.01$	

* Student's "*t*" test.

incorporation of L-[^{14}C]lysine into trichloroacetic acid-precipitable protein was significantly decreased in tetracycline treated rats when compared with controls (per cent of inhibition: 29.5 %). These results are similar to those reported by GREENBERGER[4] while using L-[^{14}C]leucine and indicated that the drug inhibits new protein synthesis very rapidly. In this study, after 1 h of incubation, 6.1 % of the absorbed L-lysine was incorporated into protein for the controls against 4.3 % for the treated animals.

Effects of tetracycline on amino acid transport

Table II shows the distribution ratios developed by intestinal rings after 40–50 min incubation with the amino acids indicated. Compared to control, rats that received tetracycline 4 and 8 h before study showed no significant decrease in the intracellular accumulation of lysine, alanine and cycloleucine. However, in animals injected with tetracycline 12 h before study the tissue accumulation of the three amino acids was significantly reduced as compared to that in control rats. The percentage of inhibition of the distribution ratios for the amino acids studied averaged 28.5 %.

Kinetic experiments

Fig. 1 presents data on the accumulation of cycloleucine at different intervals by intestinal rings of controls and animals injected with tetracycline 12 h previously. Tissue accumulation was lower in treated than in control animals as early as 5 min after the start of incubation. The reduced uptake at 5 min indicates that influx of cycloleucine was slowed in intestinal rings of treated animals since efflux has a relatively insignificant effect on rate of accumulation at so early a point. However,

TABLE II

TISSUE ACCUMULATION OF ALANINE, LYSINE AND CYCLOLEUCINE BY RAT INTESTINAL RINGS

Incubations were carried out in Krebs–Ringer bicarbonate buffer (pH 7.4) at 37°. Period of incubation was 40 min for alanine, 45 min for lysine, and 50 min for cycloleucine. Values given are mean ± S.E.; all observations were in duplicate and the number of animals is given in parentheses. Tetracycline·HCl was given intraperitoneally at 400 mg/kg body weight; control was injected with normal saline adjusted to pH 2.14 with HCl.

Amino acid	*Concn. (mM)*	*Distribution ratio*			
		Control	*Tetracycline 4 h after injection*	*Tetracycline 8 h after injection*	*Tetracycline 12 h after injection*
Alanine	0.80	12.1 ± 0.5 (10)	12.4 ± 0.5 (11)	12.0 ± 1.2 (5)	8.6 ± 0.8 (9)
	5.00	4.3 ± 0.3 (10)	4.7 ± 0.5 (10)		
Lysine	0.065	19.0 ± 0.7 (10)	19.4 ± 0.8 (11)	20.5 ± 1.5 (5)	13.7 ± 1.0 (9)
	1.00	4.0 ± 0.2 (10)	4.2 ± 0.5 (10)		
Cycloleucine	0.50	8.8 ± 0.6 (13)	9.1 ± 0.4 (11)	9.2 ± 1.3 (5)	6.3 ± 0.7 (9)
	5.00	3.8 ± 0.3 (10)	4.2 ± 0.4 (10)		
Significance of difference from control *			Not significant	Not significant	$P < 0.01$ **

* Student's "*t*" test.
** $P < 0.05$ for cycloleucine.

the lower distribution ratio of cycloleucine in treated animals at later intervals could be the result of faster efflux in addition to lower influx.

To verify this latter hypothesis, intestinal rings from treated and control animals were loaded with [^{14}C[cycloleucine by incubation for 30 min. The rings were then transferred to a volume of 25 ml of fresh buffer containing no cycloleucine

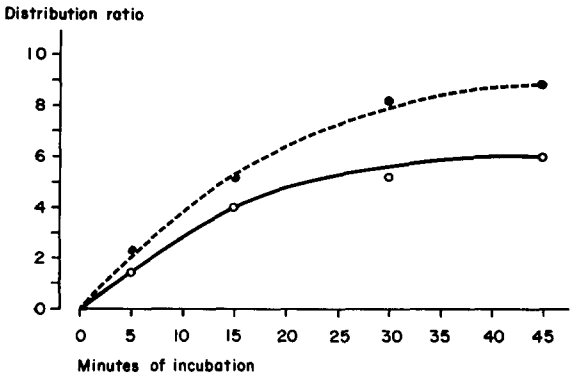

Fig. 1. Time course of cycloleucine uptake 12 h after tetracycline at 400 mg/kg was injected. Cycloleucine was at 0.50 mM in Krebs–Ringer bicarbonate buffer (pH 7.4) at 37°. The points shown represent the mean of 6 observations from 3 animals. ●---●, control; ○—○, tetracycline-treated animals.

The decline of residual intracellular radioactivity with time was measured. The amount of radioactivity in the intracellular fluid at the start of the efflux study was considered as 100 %. Fig. 2 shows the results of that study. No significant difference was found between intestinal rings from controls and treated animals.

The relationship of substrate concentration to initial transport rate was studied using L-alanine. The data in Fig. 3 show that the velocity of uptake of alanine was slowed by tetracycline throughout the 20-fold range of concentrations used. As higher concentrations were reached, the rate of increase slowed, consistent with the presence of a saturable transport mechanism. When the Lineweaver–Burk transformation was applied to the data, it was found that the least squares lines differed in slope and ordinate intercept, but the extrapolated abscissa intercept was unchanged. Thus the K_m for alanine was unaltered (about 5 mM), but the v_{max} was decreased in the intestinal rings from treated animals by 19 %.

Absence of an effect of tetracycline on tissue water spaces, intestinal histology and mitotic index

The extracellular fluid space and total tissue water of intestinal rings were determined for both drug treated and control rats. The total tissue water was much the same at 82.9±0.12 % for control and 82.7±0.41 % for treated animals. The extracellular space was somewhat increased in the treated animals at 14.0±1.16 % as compared to 10.6±0.86 % in controls. The appropriate data were used in the calculation of distribution ratio in each case. These values are from a minimum of 12 determinations taken at different times (4, 8 and 12 h) and compare well with previously reported results[12].

Sections of jejunum were taken for histological examination 4 and 12 h after treatment with tetracycline. The surface epithelium, villi, lamina propria and crypts all appeared normal upon examination by light microscopy. At 12 h, the mitotic index for control animals was 4.2 ± 0.36 % while that for drug treated animals was 3.9 ± 0.32 %. The difference between the two groups was not significant ($p > 0.5$).

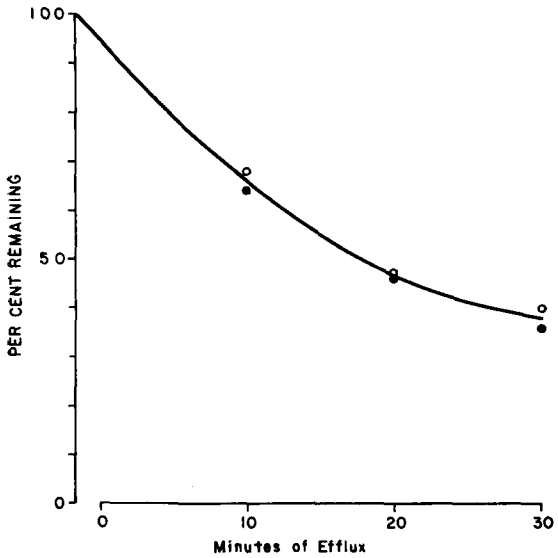

Fig. 2. Absence of effect of tetracycline on the efflux of cycloleucine. Intestinal rings were incubated with cycloleucine at 0.50 mM in Krebs–Ringer bicarbonate buffer (pH 7.4) at 37° for 30 min. Radioactivity per ml of intracellular fluid assayed at the end of this incubation period was regarded as 100%. Tissue rings were washed in 0.9% saline and transferred to 25 ml of buffer containing no amino acid. At the intervals shown, tissue was withdrawn and assayed for radioactivity remaining. Each point is the mean of 6 observations from 3 animals. ●—●, control; ○—○, tetracycline-treated animals.

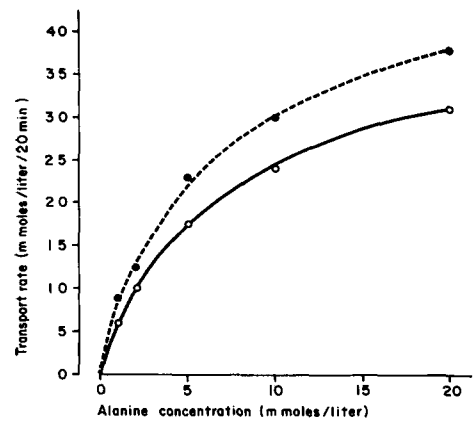

Fig. 3. Effect of increasing substrate concentration on the uptake of alanine. Incubation of 20 min. Each point represents the mean of 6 observations from 3 animals. ●---●, control; ○—○, tetracycline-treated animals.

DISCUSSION

Many investigators have postulated that transport proteins are located in the plasma membrane and are responsible for the entry of amino acids and other polar solutes into microbial and mammalian cells. This postulate is reinforced by the fact that membrane constituents of transport systems are genetically controlled[13] and that in recent years an increasing number of reports have appeared on the isolation and partial characterization of transport proteins[14–16].

In these studies we have used three different animo acids. L-Alanine and L-lysine are actively transported amino acids using different intestinal transport systems[17] and were chosen as representative of neutral and basic amino acids, respectively. Another reason for this choice is that in rats treated with actinomycin D, a known inhibitor of protein synthesis, the absorption of neutral and basic amino acids has been shown to be inhibited to different extents[18]. In animals 4 h after injection of actinomycin D, the percentage of absorption of neutral amino acids was significantly less than those of the control while that of the basic amino acids was only slightly decreased. Cycloleucine was chosen as an actively transported amino acid which is not incorporated into protein[19,20]. Therefore, the effect of tetracycline on the intestinal transport mechanism for this substrate could be dissociated from its influence on protein synthesis.

We have shown in this study, as previously reported[4,5], that there is decreased incorporation of ^{14}C-labelled amino acids into intestinal proteins in tetracycline treated rats as compared with controls. Moreover, this inhibition of protein synthesis occurred within 4 h after the administration of tetracycline.

These findings in addition to the delay noted in inhibition of transport suggest that a transport protein(s) which turns over rapidly and which is essential or auxiliary to the translocation of amino acids becomes reduced in amount and acts as a rate limiting factor. In this regard, it is interesting to note that the percent decrease in the uptake of the three amino acids at 12 h was similar to the 29.5 % reduction in the incorporation of L-[^{14}C]lysine into trichloroacetic acid-precipitable proteins. Very similar to our observations, YAMADA *et al.*[18] in studying the effect of actinomycin D noted a delayed effect on amino acid absorption from rat jejunal loops. Similar observations were also made by ELSAS and ROSENBERG[21] while observing the *in vitro* effect of puromycin on protein synthesis and transport of amino acids by rat kidney cortex slices.

Other mechanisms whereby treatment with tetracycline results in impaired amino acid absorption might be considered: (1) non specific damage to the intestinal mucosa; (2) generalized toxic condition in the intestinal tissue, which interferes with all absorption processes; (3) impairment of the intestinal epithelial cell turnover. It appears unlikely that the observed effects of tetracycline were due to non-specific mucosal damage or a generalized toxic condition in the intestinal tissue since tetracycline treatment was not associated with evident structural changes in the intestinal mucosa upon examination by light microscopy. Similar results[6,7] including electron microscopy[7] were reported earlier. Moreover in the study done by YEH and SHILS[6] tetracycline administration to adult rats was associated with impaired intestinal absorption of fat and radioactive iron while D-xylose and vitamin B_{12} absorption was not affected, thus indicating that the antibiotic does not produce generalized damage of

small bowel mucosa. The lack of non-specific damage is further documented in this study by unchanged total tissue water and inulin space. Changes would be expected if gross cell shrinkage or swelling had been produced by direct membrane damage. Finally, the mitotic index which expresses numerically the rate at which cell reproduction balances the loss of cells that have completed their life span[22–24], was not different in control and tetracycline treated animals. This indicates that the inhibition of the amino acid transport is not related to inhibition of cell renewal.

The data obtained in the present study clearly indicate that treatment of rats with tetracycline results in impaired intestinal amino acid absorption. However, the mechanism of this inhibitory effect could not be defined here. The possibility that tetracycline has a primary effect on the synthesis of enzymes catalyzing reactions providing energy for transport mechanisms is not eliminated here.

ACKNOWLEDGMENTS

These studies were supported by the Medical Research Council of Canada MA-3320.

Tetracycline as a pure powder was kindly supplied by Mr. R. K. Marwood of Cyanamid of Canada Ltd., Montreal.

REFERENCES

1 T. J. FRANKLIN, *Biochem. J.*, 87 (1963) 449.
2 T. J. FRANKLIN, *Biochem. J.*, 90 (1964) 624.
3 M. HIEROWSKI, *Proc. Nat. Acad. Sci. U.S.*, 53 (1965) 594.
4 N. J. GREENBERGER, *Nature*, 214 (1967) 702.
5 S. D. J. YEH AND M. E. SHILS, *Proc. Soc. Exp. Biol. Med.*, 121 (1966) 729.
6 S. D. J. YEH AND M. E. SHILS, *Proc. Soc. Exp. Biol. Med.*, 123 (1966) 367.
7 N. J. GREENBERGER, R. D. RUPPERT AND F. E. CUPPAGE, *Gastroenterology*, 53 (1967) 590.
8 L. E. ROSENBERG, A. BLAIR AND S. SEGAL, *Biochim. Biophys. Acta*, 54 (1961) 479.
9 L. E. ROSENBERG, S. J. DOWNING AND S. SEGAL, *Am. J. Physiol.*, 202 (1962) 800.
10 J. S. HIRSCHFIELD AND F. KERN, *J. Clin. Invest.*, 48 (1969) 1224.
11 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
12 S. SEGAL, L. M. LOWENSTEIN AND A. WALLACE, *Gastroenterology*, 55 (1968) 386.
13 C. R. SCRIVER, *Pediatrics*, 44 (1969) 348.
14 C. F. FOX AND E. P. KENNEDY, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 891.
15 A. B. PARDEE, *J. Biol. Chem.*, 241 (1966) 5886.
16 J. R. PIPERANO AND D. L. OXENDER, *J. Biol. Chem.*, 241 (1966) 5732.
17 D. M. MATTHEWS AND L. LASTER, *Gut*, 6 (1965) 411.
18 C. YAMADA, A. J. CLARK AND M. E. SWENDSEID, *Science*, 158 (1967) 129.
19 L. BERLINGUET, N. BÉGIN, L. M. BABINEAU, F. MARTEL, R. VALLÉE AND R. O. LAFERTÉ, *Can. J. Biol. Chem.*, 40 (1962) 425.
20 K. AHMED AND P. G. SCHOLEFIELD, *Can. J. Biol. Chem.*, 40 (1962) 1101.
21 L. J. ELSAS AND L. E. ROSENBERG, *Proc. Natl. Acad. Sci. U.S.*, 57 (1967) 371.
22 C. P. LEBLOND AND C. E. STEVENS, *Anat. Rec.*, 100 (1948) 357.
23 F. D. BERTALANFFY, *Acta Anat.*, 40 (1960) 130.
24 O. BRUNSER, A. REID, F. MÖNCKEBERG, A. MACCIONI AND I. CONTRERAS, *Pediatrics*, 38 (1966) 605.