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The transport of tetracyclines across the mouse ileum *in vitro*: the effect of cations and other agents

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The intestinal transfer of different tetracyclines dissolved in calcium- and magnesium-free Krebs bicarbonate buffer solution, pH 7·4, was studied using the everted ileum of the mouse. The rates of transfer of chlortetracycline and demethylchlortetracycline were less than those of tetracycline and oxytetracycline, the latter compounds being transferred at the same rate. Addition of calcium and magnesium to the buffer greatly reduced the transfer of tetracycline; this inhibition could be antagonized by EDTA. The presence of iron also inhibited the transfer of tetracycline. The inhibitory effect of these ions on tetracycline transfer seemed due to chelation of the drug. Glucosamine and acetylmethionine, but not acetyl glucosamine, diminished the intestinal transfer of tetracyclines. The former two agents did not influence the uptake of tissue fluids. Tetracycline was also transferred from the serous to the mucous coat in the non-everted intestinal sac of mice. The above observations suggested that the absorption of tetracyclines was not due solely to passive diffusion.

The mechanism of gastrointestinal absorption of tetracyclines is poorly understood. The degree of ionization and lipophilicity at the various pH ranges occurring in the stomach and small intestine may or may not be important (Schanker, Shore & others, 1957; Hogben, Schanker & others, 1957; Hogben, Tocco & others, 1959; Colaizzi & Klink, 1969). The conclusion of Pindell, Cull & Dickison (1959) from their studies in anaesthetized dogs that the absorption of tetracycline occurred by passive diffusion was not supported by the work of Waitzova, Squires & Levine (1971).

The studies reported here were undertaken with a simple preparation *in vitro*, to try to elucidate the mechanism of gastrointestinal absorption of tetracyclines, specially by the use of agents reported to increase (Carlozzi, 1958; Snell & English, 1960; Lugaresi, Piccinini & Colombo-corti, 1961) or decrease absorption in man (Harcourt & Hamburger, 1957; Donovan, Price & others, 1962; Greenberger, 1971; Gothoni, Neuvonen & others, 1972).

The everted intestinal sac technique of Wilson & Wiseman (1954) was used. Many published reports on drug absorption *in vitro* describe work performed with the everted intestine of rats. This preparation may lose functional and structural integrity on incubation (Levine, McNary & others, 1970; Gibaldi & Grundhoffer, 1972). The present work was carried out on the everted ileum of the mouse, which histological studies in our laboratory (to be published) have shown to suffer little disruption of the epithelial border on incubation. The viability of the mouse everted intestine incubated in Krebs bicarbonate buffer of pH 7·4 at 37° was confirmed by checking active transport of glucose against a concentration gradient for up to 1 h.

MATERIALS AND METHODS

Materials. Tetracycline hydrochloride and oxytetracycline hydrochloride (Indian Drugs and Pharmaceuticals) and chlortetracycline hydrochloride and demethylchlortetracycline hydrochloride (Lederle Laboratories) had 100% activity when determined microbiologically against reference standards. Unless otherwise stated, the buffer solution used (pH 7·4) was modified Krebs-bicarbonate, free of calcium and magnesium, as described by Mayersohn & Gibaldi (1971).

Intestinal transfer rate measurements. Swiss strain mice of either sex, 30–35 g, were fasted overnight (16 h) but water was not witheld. They were killed by stunning and decapitation. The abdomen was opened through a midline incision and 15 cm of the ileal portion of the small intestine was removed, immediately rinsed with several portions of cold normal saline and everted on a thin glass rod. After eversion, the intestine was washed in cold normal saline and cut into two segments of 7 cm length. Sacs of the everted intestine were prepared by tying one end tightly and the other end loosely with fine thread. 0·25 to 0·30 ml of buffer solution free of tetracyclines or additives was introduced inside the sac with a 1 ml syringe fitted with a blunt needle. The loose ligature was tightened and the buffer-filled sacs were then immediately transferred to 20 ml conical flasks containing 10 ml buffer mixed with a tetracycline with or without additives. The solutions were gassed with oxygen, the flasks were stoppered and transferred into a Dubnoff shaker-incubator oscillating at 1 Hz. After incubation at 37° for 1 h, the sacs were taken out and weighed after excess water had been removed. The fluid inside the sac was drained completely into a test tube and the empty sac re-weighed. The difference of the weights gave the volume of fluid inside the sac. The intestinal tissues were then dried on a weighed watch glass at 90° for 3 h and reweighed, to give the weights of dry intestine. After gassing, the pH of the mucosal solution never rose above 7·7.

Assay procedure. The antibacterial activity of the fluid inside the sac (serosal fluid) was estimated turbidimetrically using *Staphylococcus aureus* (ATCC 6538 P) as the test organism. Tetracycline, oxytetracycline and chlortetracycline were estimated by methods described by Grove & Randall (1955). Demethylchlortetracycline was estimated by the method given in the Code of Federal Regulations (1969).

Tissue fluid uptake. Tissue fluid uptake in the everted ileal sacs of mice was determined by the method of Mayersohn & Gibaldi (1971). The sacs were filled with 0·25 to 0·30 ml of buffer solution. The flasks contained 10 ml of buffer solution with or without glucosamine hydrochloride, *N*-acetyl glucosamine hydrochloride or acetyl methionine. The tissue fluid uptake was determined by subtracting the initial tissue weight from the final tissue weight after 30 min incubation.

RESULTS

Results are given in Table 1. The mean transfer of different tetracyclines, expressed as $\mu g\ g^{-1}$ dry intestine h^{-1} of incubation at 37°, through the everted sac of mice ileum when the drug was dissolved in calcium- and magnesium-free buffer solution at pH 7·4 were: tetracycline- 705 $\pm$ 86; oxytetracycline- 571 $\pm$ 60; chlortetracycline-102 $\pm$ 10 and demethylchlortetracycline- 403 $\pm$ 47. The transfer of tetracycline and oxytetra-

Table 1. *Effect of: I varying concentration of tetracycline hydrochloride, II different substances and cations, on the mean transference of the drug ($\mu g\ g^{-1}$ dry intestine h^{-1} incubation at 37°) from everted sacs of mice ileum*.*

I			II		
	Tetracycline HCl ($\mu g\ ml^{-1}$)	Tetracycline HCl transferred		Treatment ($\mu g\ ml^{-1}$)	Tetracycline HCl transferred
A	250	705 ± 86	J	tetracycline HCl 250	705 ± 84
B	250	265 ± 27 (non-everted)	K	do + glucosamine HCl 250	442 ± 28**
C	500	1584 ± 140**	L	do + glucosamine HCl 150	468 ± 28**
D	750	1907 ± 254	M	do + glucosamine HCl 100	601 ± 33
E	1000	1958 ± 212	N	do + acetyl-glucosamine HCl 250	845 ± 107
	Tetracycline HCl in KBB		O	do + acetyl-methionine 250	325 ± 97**
F	150	67 ± 8	P	tetracycline HCl 250	705 ± 84
G	250	90 ± 7**	Q	do + $CaCl_2$ 2·5 mM	74 ± 4
H	350	93 ± 6	R	do + $MgSO_4$ 1·2 mM	190 ± 22
	Oxytetracycline HCl		S	do in normal saline	506 ± 50
I	250	277 ± 32**	T	do + $FeSO_4$ 1 mM	78 ± 3
			U	do in KBB Buffer	90 ± 7
			V	do + EDTA 1060	594 ± 57
			W	do + K citrate	159 ± 18

* Intestinal sacs (including non-everted) contained only the buffer solution. The incubation medium contained the drug dissolved in the Krebs bicarbonate buffer solution either without Ca and Mg ions A-E, J-P or with these ions (KBB) F-I, U-W unless otherwise described. Values are mean of observations from 10 mice ± standard error.

** Significance A & C $P < 0{\cdot}01$; F & G $P < 0{\cdot}05$; G & I $P < 0{\cdot}001$; J & K $P < 0{\cdot}02$; J & L $P < 0{\cdot}025$; J & O $P < 0{\cdot}01$; Q & R, P & R, S & T, U & V, $P < 0{\cdot}001$; U & W $P < 0{\cdot}002$.

cycline was highest, that of chlortetracycline lowest with intermediate transfer of demethylchlortetracycline. In the above buffer the maximal transfer of tetracycline took place when the mucosal concentration of the drug was between 500 and 750 $\mu g\ ml^{-1}$. Transfer of tetracycline took place also from the sacs of non-everted ileum (Table 1-I).

Ileal transfer of tetracycline dissolved in buffer solution containing calcium and magnesium was significantly enhanced by the addition of either ethylenediamine tetraacetate (EDTA, diNa salt) or potassium citrate equimolar to the combined concentration of calcium and magnesium present and the transfer was similar to that observed when the drug was dissolved in calcium- and magnesium-free buffer. The effect of EDTA was more pronounced than that of potassium citrate.

The presence of glucosamine and acetylmethionine in the mucosal fluid in the same concentration as that of tetracycline significantly depressed the tetracycline transport through everted mice ileum. *N*-acetyl glucosamine did not influence the tetracycline transfer rate (Table 1-II).

The marked inhibitory effect of magnesium, calcium and iron on tetracycline transport through everted mouse ileum is evident from the results in Table 1 (II). The inhibitory effect of calcium was more than that of magnesium but similar to that of iron.

When tetracycline was dissolved in buffer containing calcium and magnesium, the maximal transference was observed when the mucosal concentration of the drug was

250 μg ml^{-1}. Further increase in the concentration of the drug did not increase the transport. Oxytetracycline was transported faster than tetracycline in the presence of calcium and magnesium (Table 1-I).

Tissue fluid uptake expressed as mg water g^{-1} tissue $\frac{1}{2}$ h^{-1} incubation at 37°, by everted mice ileum with varying composition of the mucosal fluid was as follows: calcium- and magnesium-free Krebs bicarbonate buffer solution 282 $\pm$ 21; the same buffer + 250 μg glucosamine hydrochloride ml^{-1} 264 $\pm$ 28; the same buffer + 250 μg acetylmethionine ml^{-1} 246 $\pm$ 26. Tissue fluid uptake was not increased by the presence of either glucosamine or acetyl methionine in the mucosal fluid.

DISCUSSION

As no reports were available on the rates of intestinal transfer of tetracycline in mice, work was undertaken to locate the site of maximal absorption in the intestinal tract. Maximal transfer was observed in the ileal portion, which was used in our experiments. Pindell & others (1959), however, showed that in anaesthetized dogs, absorption from the duodenum was double that from ileum.

The present work showed that significant amounts of tetracyclines are transported through everted sacs of mice ileum at pH 7·4–7·7 at which pH the compounds are in their ionic forms (Colaizzi & Klink, 1969). There were significant differences in the rates of intestinal transport of different tetracyclines but no correlation with corresponding n-octanol–water partition coefficients at pH 7·4–7·7 (Colaizzi & Klink, 1969). However, the order of the observed transfer rate correlates well with the order of the peak plasma concentrations of tetracyclines in man as reported by Sweeney, Hardy & others (1959).

Initially the transfer rate of tetracycline was proportional to the concentration of the drug in the mucosal fluid, but at concentrations above about 500 μg ml^{-1}, the transfer rates tended to plateau. This phenomenon is not consistent with the concept of passive diffusion. It could not be ascribed to limitations of the solubility of tetracycline hydrochloride under the conditions of our experiment because the mucosal solutions at the 4 concentrations used did not lose potency after filtration.

To investigate further the mechanism of transfer of tetracycline, studies were undertaken with the non-everted mice ileum and with substances that have been claimed to enhance absorption of tetracyclines in animals and man. Transport of tetracycline from everted sacs was greater than that from non-everted sacs of mice ileum. Benet, Orr & others (1971) also reported a difference in the permeability of the salicylate ion between the everted and non-everted rat intestine. The presence of glucosamine in the mucosal fluid retarded the tetracycline transport from everted mice ileum significantly while the acetyl derivative had no such effect. Acetylmethionine, however, inhibited the transfer of tetracycline.

Mayersohn, Gibaldi & Grundhoffer (1971) reported that the inclusion of certain cations and sugars in the mucosal fluid resulted in the uptake of water by the everted rat intestinal tissues and simultaneously inhibited significantly the transfer of polar compounds through the everted intestine. According to them, the polar compounds were transported via intercellular channels existing between adjacent mucosal epithelium. When the intestine was exposed to agents increasing tissue fluid uptake, a portion of the fluid penetrated the mucosal cell, producing swelling of the cell and narrowing of the apical portion of the intercellular channel, thereby inhibiting the transfer of the solute by the so-called pore route.

The present study indicated, however, that the inhibition of tetracycline transport through everted mice ileum by glucosamine and acetylmethionine was not due to increased tissue fluid uptake. Tesoriere, Dones & others (1972) reported that the transport of glucosamine through everted rat intestine was carrier-mediated, while the acetyl derivative was transported by simple diffusion. In the light of the lack of correlation with tissue fluid uptake and the inhibition of tetracycline transport by glucosamine, it appeared that the transfer of tetracycline through the everted mice ileum could not be explained only by passive diffusion through the so-called pore route.

Tetracycline remains predominantly as a zwitterion and anion in the pH range 7·4–7·7 (Colaizzi & Klink, 1969). The zwitterion is the most lipid soluble form of tetracycline, possibly as a result of inter-molecular ion pair formation (Colaizzi & Klink, 1969). It is possible that both the zwitterion and anion are transported through the lipid membrane of the intestinal tissues. Zwitterions are transported as the neutral ion pairs and the anions as complexes with certain molecules present in the intestinal tissues, which are able to cross the transport barrier. Formation of the absorbable complex between drugs and intestinal substances has been postulated by Levine (1963). Glucosamine and acetylmethionine might share a common route of transport with the tetracycline anions or they might form less lipid soluble, non-absorable complexes with the zwitterions and thus inhibit the transport of tetracycline.

The present investigation demonstrated that in the presence of certain cations like calcium, magnesium and iron in the mucosal fluid, a significant decrease in the transport of tetracycline through everted mice ileum took place. These observations mirror those in man (Harcourt & Hamburger, 1957; Greenberger, 1971) and in the chicken (Donovan & others, 1962). In the presence of both calcium and magnesium, oxytetracycline was better transported than tetracycline.

That the metal ions exert their inhibitory effect on tetracycline transport by means of chelation is evident from the results of the experiments conducted with the simultaneous application of the chelating agents EDTA and potassium citrate. Albert (1953) found that the tetracyclines initially formed 1:1 chelate complexes with the metal ions and as the pH rose a 2:1 complex was formed. It is, therefore, possible that the tetracycline and oxytetracycline in the different concentrations studied existed as chelate complexes and were transported.

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