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The effects of mucus glycoproteins on the bioavailability of tetracycline. III. Everted gut studies

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Summary

Everted rat intestinal sacs were used to measure the transport rate of tetracycline across gut tissue. The endogenous mucus secreted by the tissue, in vitro, was shown to have a significant effect on the rate of drug transfer with removal of this layer resulting in rate increases of between 60 and 90% depending on whether the segment was derived from the distal or proximal portion of the small intestine, respectively. A purified exogenous mucus dispersed in the sac reduced the transport rate only slightly. The bile salts taurodeoxycholate and deoxycholate increased drug transport in denuded gut sections but this was considerably reduced in the presence of the native mucus layer. Cetyltrimethylammonium bromide and sodium dodecylsulphate both reduced transport rates but to a lesser extent when the mucus layer was intact.

Introduction

The everted intestinal sac, as first described by Crane and Wilson (1958), has been widely utilised for the in vitro assessment of drug absorption. Chowan and Amaro (1977) illustrated the potential of this technique as a complementary preformulation test to dissolution rate. The technique has also been used to study the effect of various additives on the drug transport process and these have included both physiological and natural surfactants (Feldman and Gibaldi, 1969; Lovering and Black, 1974; Garcin-Salomon et al., 1981; Rubenstein et al., 1981; Allen et al., 1981), excipients and nutrients (Singh et al., 1966; Feldman and Gibaldi, 1969).

The mucus secreted by intestinal segments isolated in vivo has been shown to substantially alter the rate of drug absorption (Nakamura et al., 1978). The main component of the mucus secretion is a large glycoprotein or 'mucin' and there have been a number of studies which have used a crude 'model' mucus system in excised intestinal sacs to evaluate the effects of this material on the rate of drug transport through the gut wall (Braybrooks et al., 1975; Lovering and Black, 1974).

In this work a modified version of the perfused everted intestinal sac was used to investigate the transport rate of tetracycline hydrochloride which has been shown to interact with mucus glycoproteins (Saggers and Lawson, 1966; Marriott and Kellaway, 1975; Kearney and Marriott, 1986). Particular attention is given to the effects of the native mucus which is secreted by the tissue in vitro, and in addition a similar model mucus to that used by earlier workers was utilised but with the added refinement that the material was subjected to a purification procedure. Also, as it has

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been suggested that surface-active agents could be used to improve the varied and low absorption levels often observed with orally administered tetracycline (Allen et al., 1981), the effects of both natural and synthetic surfactants on the drug transport rate were examined.

Materials and Methods

Tris base (Trizma), sodium taurodeoxycholate (STDC), sodium cholate (SC) tetracycline hydrochloride and hog gastric mucin (type II) were obtained from Sigma Chemicals, Poole, U.K. Polyvinylpyrrolidone 700,000 (PVP), polyethylene glycol 1500 (PEG), sodium dodecyl sulphate (SDS), cetyl-trimethyl-ammonium bromide (CTAB) and all other chemicals were supplied by BDH Chemical Company, Poole, U.K. The crude mucin powder was solubilised by homogenising in phosphate buffer containing 0.22 M potassium thiocyanate, centrifuged (1 h, 27,000 *g*) and the supernatant fractionated on a column of Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The high molecular weight fraction eluted in the void volume was retained and this purified gastric mucin (PGM) concentrated in an ultrafiltration cell (Amicon Corp., U.S.A.) and stored at 4°C.

Everted intestinal sacs

Male Wistar rats, 250–350 g (Charles River UK Ltd., Margate, U.K.) were killed by cervical dislocation and the abdomen was opened by a midline incision. The whole of the small intestine (pylorus to caecum) was excised and placed in cold buffer (50 mM Tris, 42 mM HCl, 11 mM KCl, 95.5 mM NaCl, 10 mM glucose) bubbled with 95% O_2/5% CO_2. The proximal 15 cm of gut was discarded and the remainder cut, sequentially, to give two jejunal sections and two ileal sections each of 15 cm and this accounted for all but a few centimetres of the total intestine. Each section was sleeved onto separate glass rods (0.4 cm diameter, 15 cm long), everted and washed. One end of each segment was sleeved onto a length of glass tubing (0.25 cm i.d.) inserted centrally through the stopper of a 50 ml boiling tube (Fig. 1) and secured with a

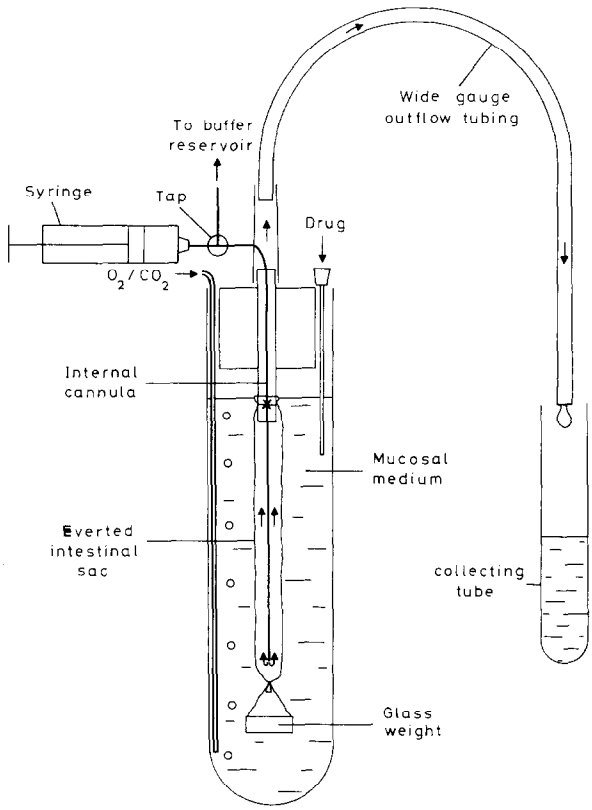

Fig. 1. Apparatus used for everted gut studies.

ligature. A silicone rubber collar on the tubing rendered the join completely water-tight. The distal end of the section was ligated to give a sac 10 cm in length and excess gut was trimmed off. The sac was weighted by a short length of thick-walled capillary tubing tied to the ligature thread (ca. 1 g). The outer end of the glass cannula was connected to a length of rigid polyethylene tubing (40 cm long, 0.15 cm internal diameter) by a 2.5-cm length of soft silicone rubber tubing (0.2 cm i.d.). The wall of the silicone rubber tube was punctured with a syringe needle and one end of a length of narrow gauge plastic tubing (30 cm long, 0.075 cm internal diameter) inserted and pushed down the glass cannula into the gut sac to within 0.5 cm of the terminal ligature. The elastic silicone rubber connection ensured that the insertion of the internal cannula was completely self-sealing. The internal cannula could then be used to fill the

gut sac with buffer from a 5-ml syringe. A volume of about 2 ml was usually needed to completely fill a typical sac. The mounted gut sac was then placed in a boiling tube containing 40 ml of buffer and was continuously bubbled with an O_2/CO_2 mixture. Temperature was maintained at 37 °C by supporting the boiling tube in a water bath.

With this arrangement the intestinal sac could be completely rinsed in one operation by flushing 5 ml of buffer through the internal cannula and collecting the perfusate from the outflow tubing. The volume collected in each case should have been 5 ml but in practice was less than this due to transudation from the serosal to the mucosal side resulting in a slightly less than full sac. The loss of perfusate was estimated by difference, from the expected volume of 5 ml and the actual volume collected which was measured by weight; in control gut sections such losses amounted to less than 3% of the total perfusate volume. The intermittent refilling of the syringe was achieved via a 3-way tap from a separate reservoir suspended in the water bath. To prevent syphonage and excessive pressures within the gut sacs, the end of the outflow tubing was supported at the same level as the surface of the mucosal solution. Holes in the stopper of the boiling tube allowed injection into, and sampling of, the mucosal medium.

The gut sacs were allowed to incubate for 1.5–2 h in buffer alone and the native mucus layer which formed was either left intact or removed by gently blotting with tissue. This layer did not form when the segments were incubated at 25 °C indicating that the material was a product of the actively metabolising epithelial cells. After the initial incubation period, the mucosal solution was replaced with fresh buffer and to commence a kinetic run, 2 ml of drug solution was added to the mucosal medium from a syringe. Accordingly, drug solutions were made up in distilled water at 20 times the required mucosal concentration which was typically 2 mM. Solutions (×20 concentration) of surfactant were also injected into the mucosal medium at specific times during the appropriate experiments. At 10-min intervals, the gut was slowly perfused with 5 ml of buffer and the pH of the collected perfusate adjusted to 1 by the addition of concentrated HCl prior to spectrophotometric analysis at 354 nm, except for solutions containing bile salt which were assayed directly at 366 nm. The rate of transport of drug into the serosal side of the sac was calculated in terms of μg transferred per mM mucosal concentration, per 10 cm gut length, per min. The loss of drug from the mucosal solution was corrected for by measuring the concentration at the beginning and end of the experiment, although such losses only amounted to a few percent of the total mucosal drug content. Protein levels in the perfusate were determined by the method of Lowry et al. (1951).

To compare the effect of the polymer additives on the transport rate of tetracycline, rates were measured first with drug solutions only in the mucosal medium before the solution was replaced with one containing tetracycline and the appropriate additive. By using the same gut segments in this way, each acted as its own control.

Results and Discussion

Fig. 2 shows the cumulative amount of drug transferred for typical proximal (jejunal) gut sections both with and without the endogenous mucus layer and similar plots were obtained for distal (ileal) sections. It is possible to calculate transport rates and lag times from these plots using the slope and intercept respectively, and these are given in Tables 1 and 2. Proximal gut sections with the endogenous mucus layer removed show 90% higher rates then sections with the layer intact. For distal sections the corresponding increase was less, amounting to only 60%. Also, the dif-

TABLE 1

Transport rates of tetracycline through the different sections of intestinal sac

Transport rate values in $\mu g \cdot ml^{-1} \cdot mM^{-1} \cdot min^{-1}$. Mean ± S.D., $n = 8$.

	Proximal	Distal
With mucus layer	12.0 ± 1.0	13.8 ± 1.8
Without layer	20.6 ± 2.1	18.4 ± 2.6

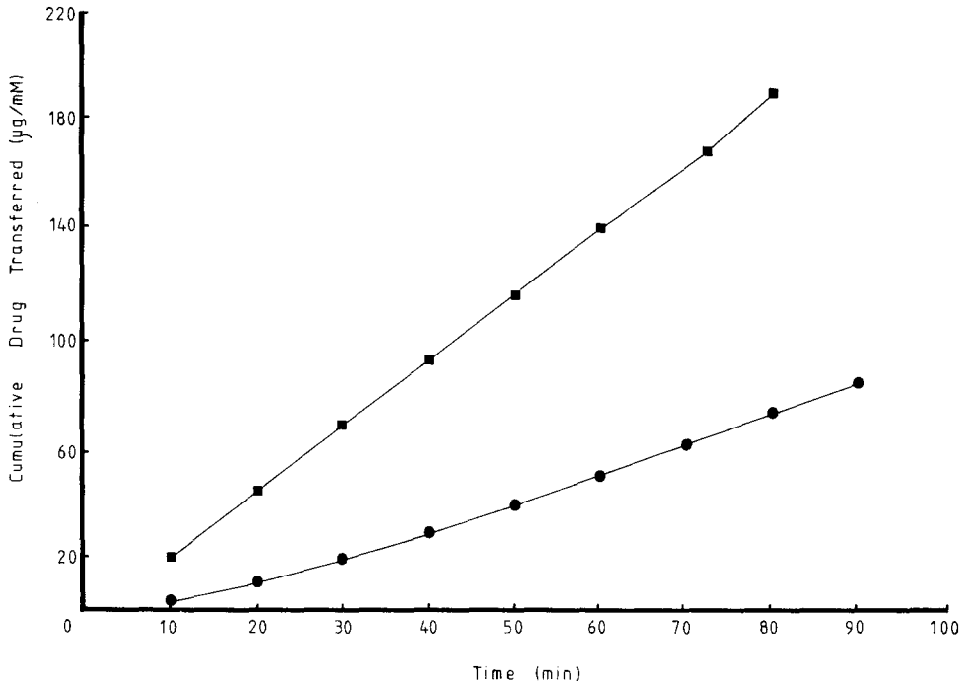

Fig. 2. Plots of cumulative amount of tetracycline transferred (μg/mM mucosal concentration/10 cm gut length) vs time for typical proximal gut sections. ●, Native mucus layer present; ■, native mucus layer removed.

ference in transport rate between the proximal and distal sections is significant both with (proximal < distal; $P = 0.014$, $n = 8$) and without (proximal > distal; $P = 0.032$, $n = 8$) the native mucus present. For both areas of gut, lag times in the absence of the mucus layer were considerably shorter than those measured when the layer was present. As with the transport rates, differences between the two areas were significant, with proximal > distal ($P = 0.025$, $n = 8$) with the layer and proximal < distal ($P = 0.01$, $n = 8$) with the layer removed. Similar differences in transport rate for the different gut sections have been reported for tetracycline in other studies (Chakrabarti and Banerjee, 1976) as have the differences observed in lag time (Chowan and Amaro, 1977). Such rate differences are in contrast with in vivo studies where absorption of tetracycline was most rapid in the proximal areas of the small intestine (Pindell et al., 1959).

TABLE 2

Lag times for tetracycline transfer through the different gut sections

Mean ± S.D., $n = 8$.

	Proximal (min)	Distal (min)
With mucus layer	12.5 ± 5.5	7.9 ± 2.2
Without layer	1.3 ± 0.8	2.5 ± 0.9

The binding of drug to the small amount of glycoprotein in the gel layer would reduce the concentration in the mucosal solution only slightly and hence would not be a major factor in the reduction of the transport rate. The reduced rates are more likely to be due entirely to the additional diffusive barrier afforded by the mucus layer and this would be a function of both the thickness and solids content. After each experiment, the surface mucus coat was removed and dried and weighed. Fig. 3 shows the observed transfer rate of TC as a function of the dry weight of secretion removed and a good negative correlation is shown ($r = -0.89$). A similar plot of lag time versus dry weight gave a positive correlation ($r = 0.76$).

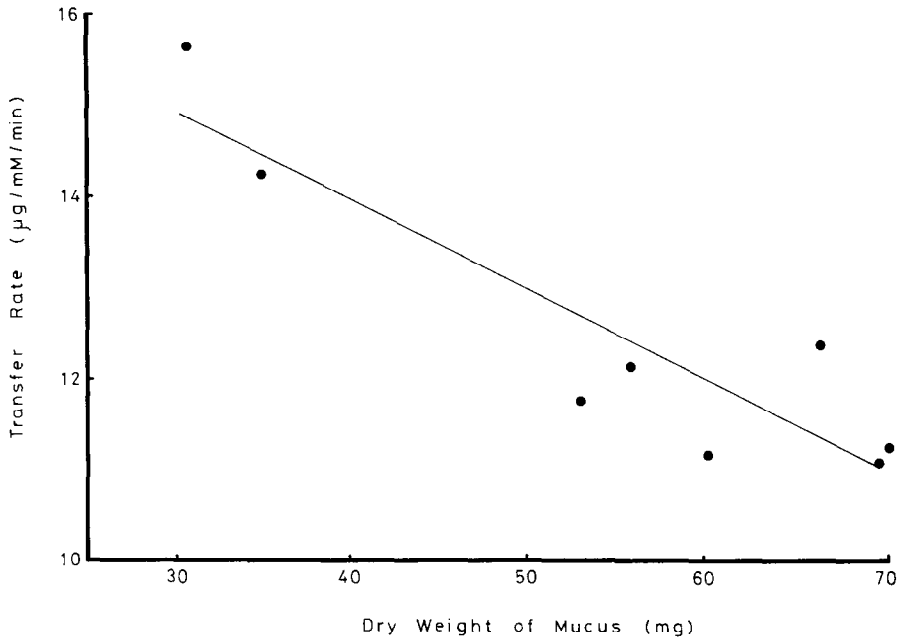

Fig. 3. Relationship of tetracycline transfer rate (μg/mM mucosal concentration/10 cm gut length/min) to the dry weight of mucus removed from the gut surface after the experiment.

An approximate estimate for the thickness of the mucus layer can be made from the wet weight of the material removed and the dimensions of the gut section. Using this approach, values of 0.58 mm and 0.54 mm are obtained for proximal and distal sections respectively, and interestingly, these values compare favourably with estimates of the diffusive mucus barrier in vivo (Smithson et al., 1981; Ryu and Grim, 1982). Thus the rate and lag time effects observed appear to be solely a function of the solid content of the secretion and not the layer thickness as this is not significantly different for the two gut regions. The more substantial jejunal secretion would explain why lower transport rates have been measured for this section of intestine in other in vitro studies (Chowan and Amaro, 1977; Chakrabarti and Banerjee, 1976). In addition, the endogenous mucus layer may explain the high variability often observed in drug transfer studies using everted intestinal sacs, where differences in handling or temperature could easily effect the integrity and thickness of this layer.

Exogenous material

Table 3 shows the percentage rate of transfer of TC (compared to control) using mucus-free sections of intestine with either PPM, PVP or PEG present in the mucosal medium at concentrations producing solutions of similar viscosity ($\simeq$ 6 cP). PVP produced no significant difference compared to control while the mucus solution depressed the transport rate by about 10%. However, with PEG, the rate was reduced to some 50% of control. This is not entirely consistent with the diffusion rates

TABLE 3

Effect of exogenous additives on the transport rate of tetracycline

Mean $\pm$ S.D., $n = 3$.

Additive	Concentration (% w/v)	Transfer rate (% of control)
Buffer only	–	100
PPM	1.0	93 $\pm$ 1
PVP	2.02	106 $\pm$ 9
PEG	29.0	47 $\pm$ 5

measured in these solutions, since in a diffusion cell, higher diffusion rates are obtained in the mucus solutions than in solutions of PVP at the same viscosity (Kearney and Marriott, 1986). This suggests that in the everted gut preparation the transport rate is not significantly affected by the viscosity of the mucosal medium. This observation is again in contrast to the effect found in vivo where solutions of methylcellulose were shown to reduce significantly the rate of drug absorption from the gastrointestinal tract (Levy and Jusko, 1965; Seager, 1968). However, in vivo, a reduction in intestinal transit rate is likely to contribute towards the lower absorption rates in addition to the diffusion effects. In the case of the everted sacs, the rate reduction in the mucus solutions may be adequately explained in terms of the binding of tetracycline to the mucus glycoprotein since as much as 10% of the drug may be bound under these conditions (Kearney and Marriott, 1987). The marked decrease in transport rate observed with the PEG solution is probably due to the transudation of solution from the serosal to the mucosal side owing to the relatively high osmolality of this polymer, since the loss of serosal medium was high in such preparations: a similar osmotic effect has been observed in vivo (Sakiya et al., 1981; Bryan et al., 1980).

It is clear that a mucus solution has a much more limited effect on drug transport than the gel layer on the gut surface. The evidence presented by other workers using a similar in vitro system has been somewhat contradictory with both an increase (Lovering and Black, 1974) and decrease (Braybrooks et al., 1975) of 50% reported for drug transfer rate from a 1% mucosal solution of a model mucus, compared to control. Both these groups used the same crude mucus source as in this work but without a purification step, and interference by the considerable amount of low molecular weight material present (Kearney, 1983) may explain this inconsistency.

Surfactants

Fig. 4 shows the effect of 10 mM STDC on the transfer rate of TC, both with and without the endogenous mucus layer. The transfer rate is expressed as a percentage of the average drug con-

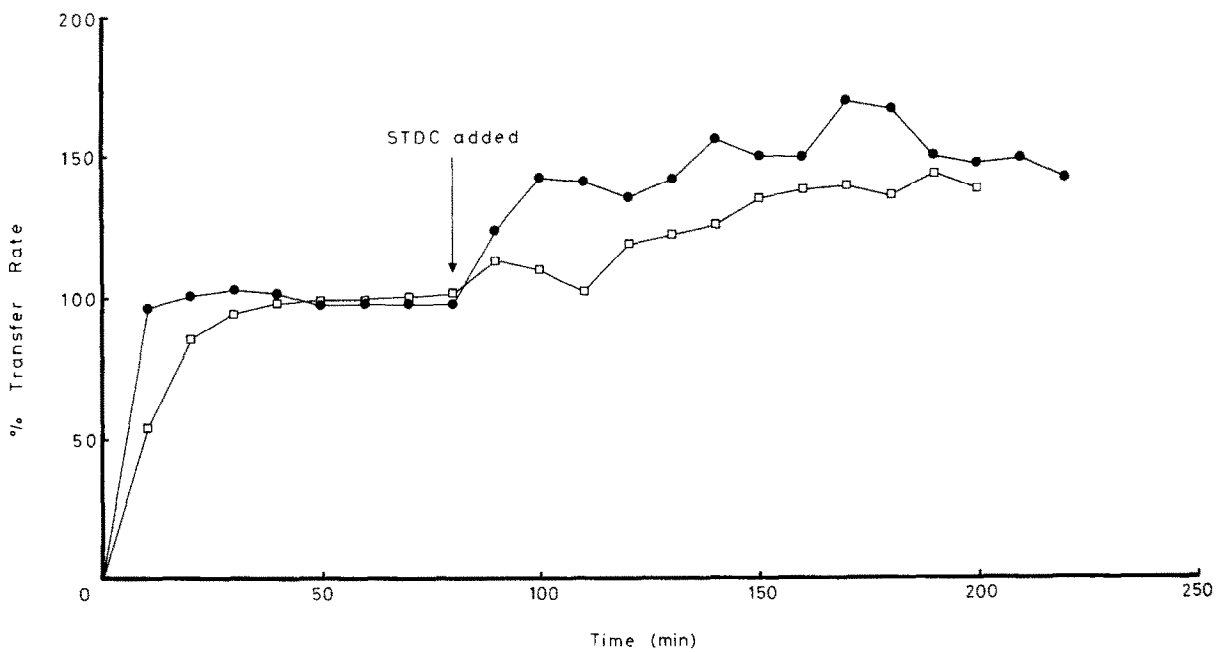

Fig. 4. Effect of 10 mM STDC on the transfer rate of tetracycline through everted intestinal sacs. □, Native mucus layer intact; ●, native mucus layer removed.

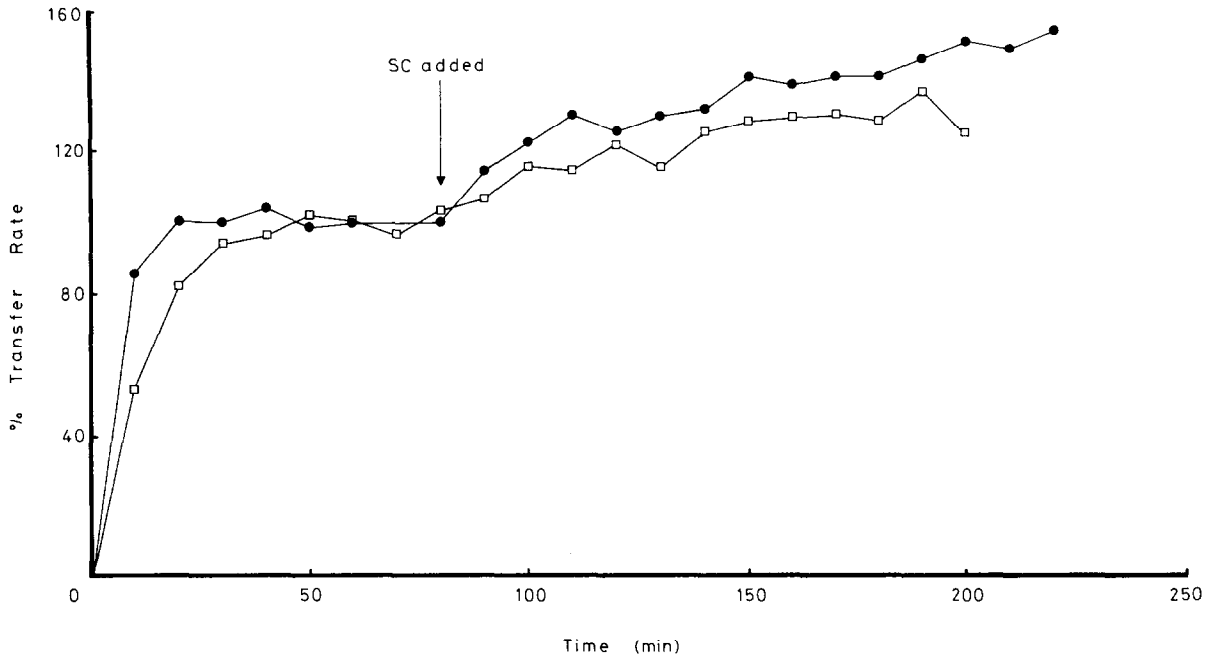

Fig. 5. Effect of 10 mM SC on the transfer rate of tetracycline through everted intestinal sacs. □, Native mucus layer intact; ●, native mucus layer removed.

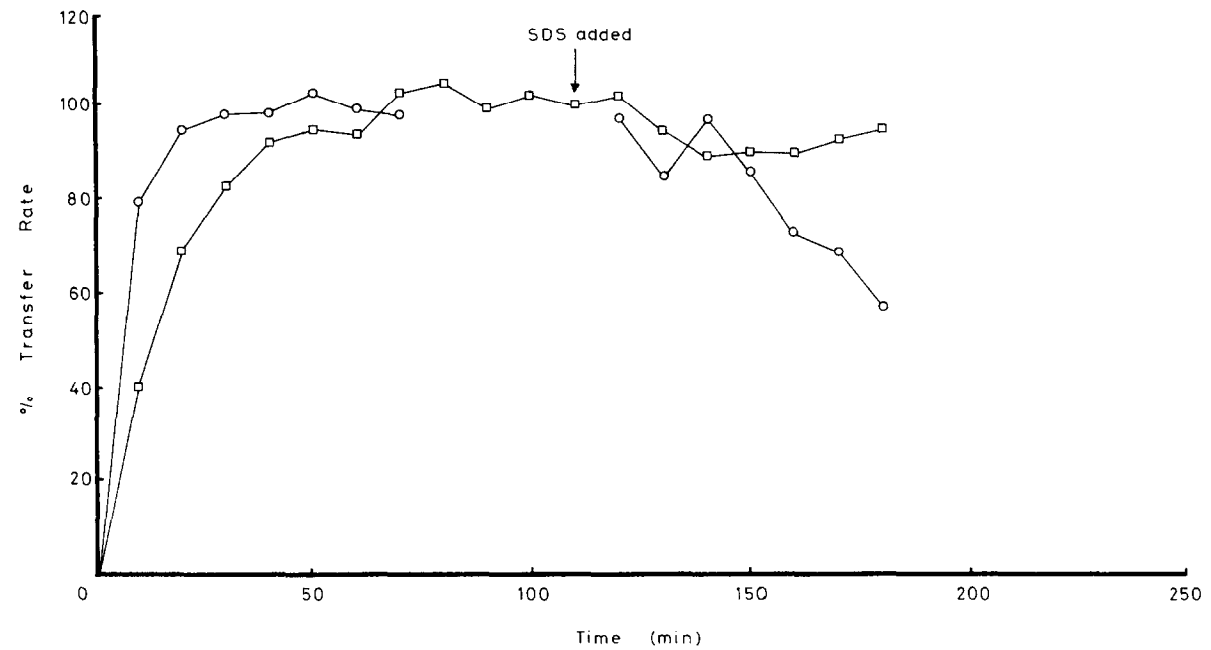

Fig. 6. Effect of 10 mM SDS on the transfer rate of tetracycline through everted intestinal sacs. □, Native mucus layer intact; ○, native mucus layer removed.

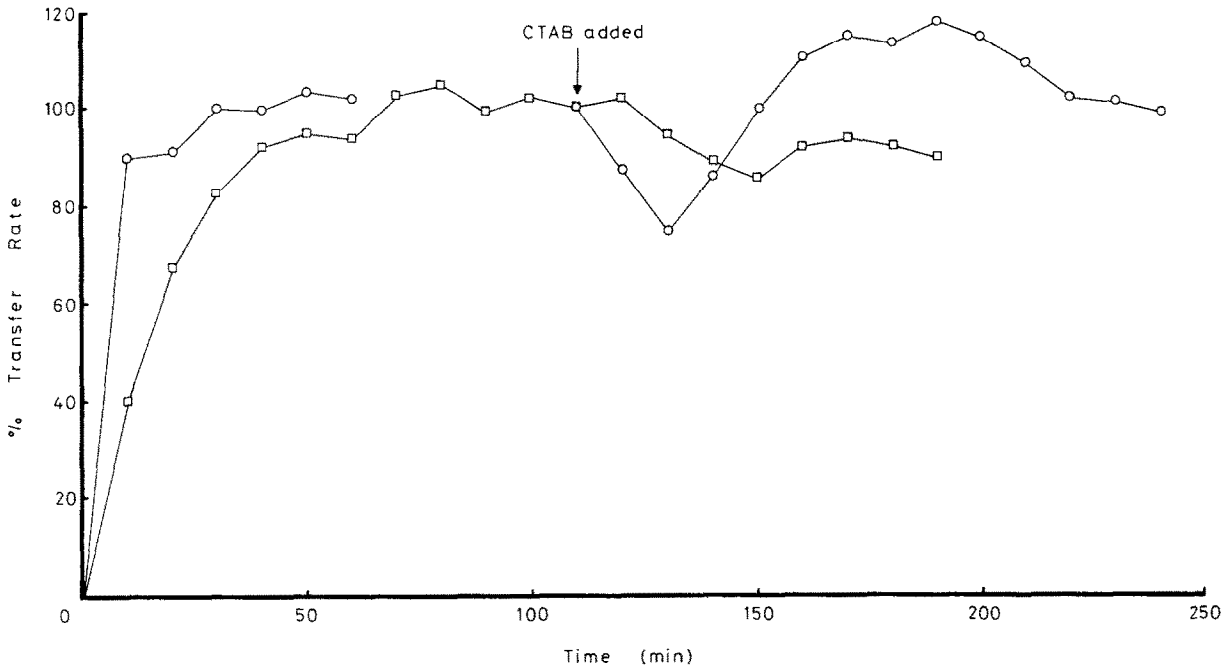

Fig. 7. Effect of 10 mM CTAB on the transfer rate of tetracycline through everted intestinal sacs. ○, Native mucus layer intact; □, native mucus layer removed.

centration in the perfusate, measured prior to the addition of the bile salt. Similar plots are shown for experiments where SC (Fig. 5), SDS (Fig. 6), and CTAB (Fig. 7) were added with the points on each plot representing the average of two experiments. The break in the curve for the SDS and CTAB controls is due to the fact that the control and test segments were incubated for different times prior to the addition of surfactant, hence the control time values have been shifted accordingly to render the point at which surfactant was added coincident on the graph.

It can be seen that both bile salts increase tetracycline transfer rate with STDC having the more marked effect and that throughout the experiment the rate continues to increase. The presence of the native mucus layer appears merely to retard this increase. In contrast, SDS decreases the measured rate but again the effect is diminished by the mucus layer. With mucus-free gut, CTAB at first sharply depresses the rate but this trend seems to be reversed after some 20 min with the rate restored to the control value. With the mucus layer intact, the addition of CTAB produced a thick precipitate on the gut surface; this was undoubtedly a CTAB-mucin complex, and the presence of this physical barrier would account for the observed decrease in rate. The fall in rate when CTAB was added to the mucus-free gut may be due to the incorporation of the drug into CTAB micelles; the subsequent rise could be explained in terms of a slower, direct effect on the membrane.

Bile salts have previously been shown to increase intestinal absorption both in vitro (Feldman and Gibaldi, 1969; Lovering and Black, 1974) and in vivo (Garcin-Salomon et al., 1981; Rose and Nahrwold, 1982). Various mechanisms have been proposed for this absorption enhancement, one of which is the disruption of the mucosal membrane with the concomitant release of protein (Feldman et al., 1973). However, after the addition of each surfactant, protein levels in the perfusate were not significantly increased over the background level of 0.22 mg/ml.

Although bile salts have been shown to break down mucus structure (Martin et al., 1978), there

TABLE 4

Perfusate loss before and after addition of surfactant

Mean ± S.D., $n \geqslant 5$

Surfactant	Before addition (ml)	After addition (ml)
STDC [a]	0.14 ± 0.05	0.20 ± 0.10
SC [a]	0.18 ± 0.10	0.14 ± 0.04
STDC [b]	0.16 ± 0.03	0.50 ± 0.10
SC [b]	0.30 ± 0.10	0.23 ± 0.07
CTAB [b]	0.26 ± 0.02	0.32 ± 0.13
SDS [b]	0.21 ± 0.04	0.41 ± 0.11

[a] With mucus layer.

[b] Without mucus layer.

was no tendency for the mucus layer to become dispersed in the mucosal solutions containing either STDC or SC.

Table 4 shows the perfusate loss before and after the addition of the surfactants both with and without the mucus layer. These values represent the average loss from a number of preceding and succeeding perfusate volumes. The increase in perfusate loss is only significant in the case of STDC ($P < 0.01$) and SDS ($P = 0.01$) hence a high transudation rate cannot be solely responsible for the rate enhancement as the addition of SDS produced decreased transport rates.

Micellar entrapment has been proposed as a mechanism for reduced drug transport rates in the presence of anionic surfactants (Lovering and Black, 1974). As this should occur irrespective of the presence of the mucus layer, the fact that a rate reduction is not observed with SDS on gut tissue with the mucus intact (Fig. 6), suggests that the rate suppression is due to an effect on the membrane directly.

In conclusion, the mucus secreted by everted rat intestinal sacs in vitro has been shown to have a significant effect on the transport rate of tetracycline and removal of this layer produces increases of up to 90% depending on the region of the gut from which the sac was derived. This has important consequences for the use of everted intestinal sacs in general, for a non-standard method of preparation could well produce differences in the endogenous mucus layer. In this respect gut sections prepared with a pre-incubation period followed by removal of the mucus layer may constitute a more reproducible model in gut transport studies. Mucus dispersed in the mucosal medium decreases transfer rate only slightly, and this can be explained in terms of drug binding to the constituent glycoproteins. Of the surfactants studied, only the bile salts produced an increase in transport rate but neither membrane disruption nor tissue transudation appear to be solely responsible for this effect, hence, an increase in the permeability of the lipoidal membrane may be the major factor. The presence of the native mucus layer does not prevent the increased transport rates but merely decreases the effect, probably due to the extra diffusional barrier presented by the layer.

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