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Report

Intestinal 5-Fluorouracil Absorption: Use of Ussing Chambers to Assess Transport and Metabolism

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We have employed an *in vitro* system to study transport and metabolism of organic molecules by gastrointestinal tissues. Such a system would aid in the evaluation of the potential for oral delivery of organic molecules. Transport and metabolism of 5-fluorouracil (5-FU) were studied using rabbit intestinal preparations. Unidirectional fluxes and metabolism were measured *in vitro* in Ussing chambers under short-circuit conditions. Results from these studies reveal that in ileum, proximal, and distal colon, steady-state fluxes of 5-FU (10 μM added to both bathing solutions) are established after 30 min and remain constant for at least 110 min. Transport of 5-FU under "sink" conditions with 10 μM 5-FU present in the mucosal or serosal bathing solution alone demonstrated similar rates of transport as under "nonsink" conditions. The concentration dependence of 5-FU fluxes indicates that the mucosal (m)-to-serosal (s) flux is composed of both a saturable and a linear component over the range of 1–100 μM in the ileum, whereas the s-to-m flux in the ileum and both fluxes in the colon are linear functions of concentration. Over the concentration range employed and the time course of these studies, 5-FU had no effect on the electrical properties of the ileum or colon. In the ileum, the m-to-s but not the s-to-m flux of 5-FU was reduced by (1) serosal ouabain (0.1 mM); (2) reduction of the bathing solution Na concentration; and (3) addition of uracil, thymine, thymidine, uridine, 2-deoxyuridine, or uridine-5′-monophosphate. These results indicate that 5-FU absorption in the ileum occurs by a Na-dependent mechanism that is inhibited by uracil and structurally related compounds. In distal colon, no evidence for an active transport mechanism was obtained. High-performance liquid chromatography (HPLC) analysis reveals that both ileum and distal colon metabolize 5-FU to more polar compounds. Metabolism in ileum is quantitatively greater than in distal colon. Metabolites are found predominantly on the side to which transport has occurred, suggesting that metabolism occurs concomitantly with transport. Since the intestinal cells metabolize 5-FU to more polar compounds and active absorption is inhibited in a competitive manner by related compounds, these results may provide an explanation for the variable oral activity reported for 5-FU.

KEY WORDS: 5-fluorouracil; intestine absorption; transport; metabolism.

INTRODUCTION

The suitability of a compound for oral delivery is often assessed by monitoring plasma levels subsequent to intragastric administration. In cases where compounds exhibit a low bioavailability, alternate techniques are required to determine the source(s) of the problem. Since the luminal environment and the intestinal epithelial cell layer are the first major barriers to absorption encountered by a xenobiotic, an understanding of the mechanisms and sites of absorption and metabolism is a critical first step in designing orally active compounds.

In this study, we describe the use of Ussing chambers (1) as a method for evaluating transepithelial transport and metabolism of 5-fluorouracil (5-FU),[3] a compound with variable oral absorption (2). Advantages of this technique include (1) the ease and rapidity with which absorption and metabolism by the gut wall can be evaluated; (2) the ability to assess the site specificity of absorption and metabolism; and (3) the ability to examine effects of xenobiotics on the "basal" physiologic characteristics of the intestine. We chose 5-FU for this study since it has been previously studied by other *in vivo* and *in vitro* techniques (3–6), thereby allowing comparison of results obtained in this system with results reported by others and to determine what factors contribute to its variable oral absorption and efficacy. The emphasis of this study was to demonstrate the utility of the Ussing chambers for the study of transport of compounds across the intestine and to demonstrate that this preparation maintains the ability to metabolize foreign com-

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[3] Abbreviations used: m, mucosal; s, serosal; J, flux; I_{sc}, short-circuit current; G_t, transepithelial conductance; 5-FU, 5-fluorouracil; PD, transepithelial potential difference; HPLC, high-performance liquid chromatography.

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pounds. However, we did not attempt to identify or quantitate the metabolites of 5-FU in this study.

MATERIALS AND METHODS

Materials. Ouabain, 5-FU, uracil, uridine, 2-deoxyuridine, thymine, thymidine, and uridine-5'-monophosphate were obtained from Sigma Chemical Co. (St. Louis, Mo.). Radiolabeled compounds were from New England Nuclear (Boston Mass.).

Tissue Preparation. Distal ileum and proximal (0–6 cm distal to the ampulla caecalis coli) or distal colon were obtained from New Zealand white rabbits (2–3 kg), opened along the mesenteric border and rinsed with a solution containing (m*M*): Na^+, 141; K^+, 5; Ca^{2+}, 1.2; Mg^{2+}, 1.2; Cl^-, 122; HCO_3^-, 25; HPO_4^{2-}, 1.6; and $H_2PO_4^-$, 0.4. At 37°C this solution has a pH of 7.4 when gassed with 95% O_2–5% CO_2.

Isotope Flux Measurements. Ileal and colonic mucosa were then stripped of underlying muscle as described previously (7–9). Segments of stripped mucosa were mounted in Ussing chambers (1.13-cm^2 exposed surface area) and bathed on both tissue surfaces with 10 ml of bicarbonate Ringer solution containing 10 m*M* glucose in the serosal bath and 10 m*M* mannitol in the mucosal bath. Solutions were circulated by gas lift with 95% O_2–5% CO_2 and maintained at 37°C by water-jacketed reservoirs.

Tissues were allowed to equilibrate for 30–45 min while monitoring transepithelial potential difference (PD), short-circuit current (I_{sc}), and transepithelial conductance (G_t) as previously described (7). Steady-state fluxes (Period I) were calculated from four samples taken at 15-min intervals starting 30–45 min after addition of isotope. Immediately after Period I, test agents were added or bathing solution changes made and flux measurements determined over three 15-min intervals (Period II) after a 30- to 45-min reequilibration period. Both ^{14}C and ^3H were analyzed on a Packard Tri-Carb liquid scintillation spectrometer (Model 4640). Counts per minute were converted to disintegrations per minute using the external standard channels ratio.

HPLC Analysis. 5-Fluorouracil and metabolites were analyzed by HPLC according to the methods described by Gelijkens and DeLeenheer (10). Aliquots from the Ussing chambers were injected with an auto injector (SIL-6A, Shimadzu) directly onto a spheri-5, RP-18 column (5 μm, 220 × 4.6 mm, Brownlee Labs, Santa Clara, Calif.). The column was eluted with 20 m*M* KH_2PO_4 (pH 5.0) with 5% (v/v) methanol containing 1.5 m*M* tetrabutylammonium phosphate (TBA) and a flow rate of 0.8 ml/min on a Shimadzu isocractic chromatographic system consisting of a model LC-6A pump and a model SPD-6AV UV–vis detector (270 nm). Samples were collected at 0.4-min intervals for up to 45 min and counted in a scintillation counter to determine the elution profile of radioactivity. These elution profiles were compared with the UV absorbance profiles of 5-FU.

Statistics. Results are presented as means ± 1 SE. Statistical significance was evaluated using a paired *t* test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Steady-state fluxes of 5-FU were determined under short-circuit conditions using tracer quantities of either 5-[6-^{3}H]fluorouracil or 5-[6-^{14}C]fluorouracil. In initial experiments with 5-[6-^{3}H]fluorouracil (Fig. 1), we determined that steady-state fluxes were achieved after approximately 30 min in both ileum and colon. For these studies, 5-FU (10 μ*M*) was added to both bathing solutions (nonsink conditions). In two experiments, the mucosal (m)-to-serosal (s) and s-to-m fluxes of 5-FU were examined in the proximal colon and were nearly equal in magnitude (m-to-s flux = 0.54 ± 0.05 and s-to-m flux = 0.44 ± 0.03 nmol/hr · cm^2). Since the results with proximal colon were qualitatively similar to those obtained with distal colon, subsequent studies

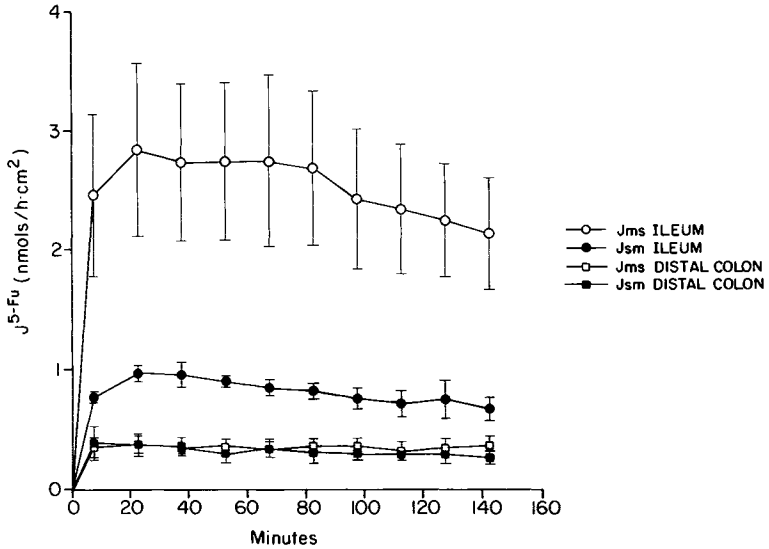

Fig. 1. Time course of 5-FU fluxes in rabbit ileum or distal colon. At time zero, 5-FU (10 μ*M*) was added to both bathing solutions, with tracer quantities of ^{3}H-5-FU added to either the mucosal (for m-to-s fluxes) or the serosal (for s-to-m fluxes) bathing solution. The flux (*J*) of 5-FU at 15-min intervals was then determined. Results are means ± 1 SE for five or six animals.

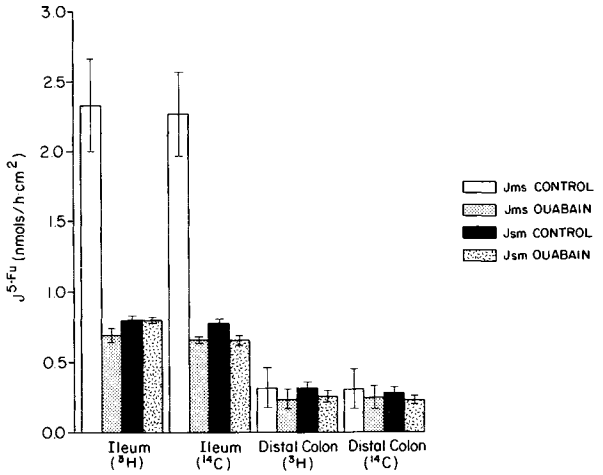

Fig. 2. Simultaneously measured fluxes of 5-[^{14}C-6]fluorouracil or 5-[^{3}H-6]fluorouracil across rabbit ileum or distal colon. 5-FU (10 μM) was added to the mucosal or serosal bathing solution 30-45 min after mounting tissues *in vitro*. Thirty minutes later, a 30-min flux was determined and ouabain (0.1 mM) was then added to the serosal bathing solution. After a 45-min reequilibration, a second 30-min flux was determined. Results are means ± 1 SE for four animals.

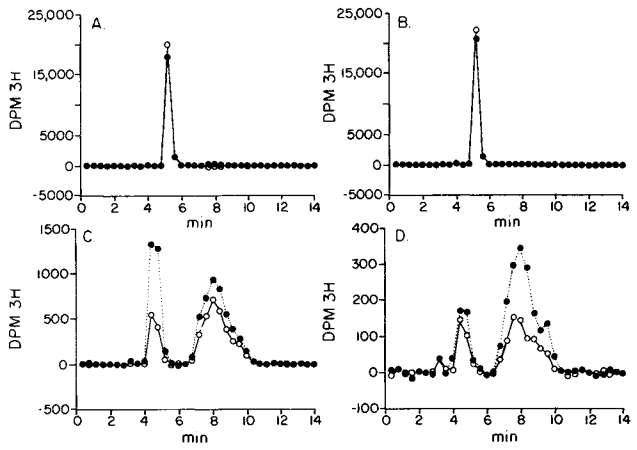

Fig. 3. HPLC analysis of the mucosal or serosal bathing solution of rabbit ileum from tissues studied in Fig. 2. 5-FU, as determined from radioactivity, was measured at 3 min (○) and 60 min (●) after adding to the mucosal (A) or serosal (B) bathing solution and the radioactivity appearing in the serosal (C) or mucosal (D) bathing solution was determined at 30 min (○) and 60 min (●). Results are means from two experiments.

were conducted with ileum and distal colon only and ^{3}H- or ^{14}C-5-fluorouracil was added 30–45 min prior to the measurement of transepithelial fluxes. Unidirectional fluxes of 5-FU in the ileum and distal colon determined with either 5-[^{3}H-6]fluorouracil or 5-[^{14}C-6]fluorouracil are depicted in Fig. 2. We compared fluxes measured with ^{3}H-5-FU to those measured with ^{14}C-5-FU to determine whether ^{3}H exchange could be influencing our flux measurements. In these studies, 5-FU (10 μM) was added to the mucosal (for mucosal-to-serosal fluxes) or the serosal (for s-to-m fluxes) bathing solution only (sink conditions) and the rate of appearance of ^{3}H and ^{14}C on the opposite side of the tissue was measured. Samples from these experiments were also assayed by HPLC to assess metabolism (Figs. 3 and 4).

Rates of 5-FU transport measured with either ^{3}H or ^{14}C are identical (Fig. 2). Addition of ouabain (0.1 mM) to the serosal bathing solution abolishes net absorption of 5-FU in the ileum ($J_{net} = J_{ms} - J_{sm}$) due entirely to inhibition of the m-to-s flux of 5-FU, with no effect on the s-to-m flux (Fig. 2). Addition of ouabain (0.1 mM) to the serosal bathing solution of distal colon had no effect on either unidirectional flux of 5-FU (Fig. 2). In both the ileum and the colon, ouabain (0.1 mM) abolished the short-circuit current (I_{sc}), indicating that active ion transport had been abolished (data not shown) (11). Comparison of the results presented in Fig. 1 with those in Fig. 2 reveal that transport of 5-FU in the presence (sink conditions) or absence (nonsink conditions) of a concentration gradient gives nearly identical flux rates.

HPLC analysis of the radioactivity added to the luminal or serosal bathing solution of either ileum or colon reveals that greater than 90% coelutes with 5-FU. In the ileum, accumulation of radioactivity is three- to fourfold greater in the serosal solution after mucosal addition than in the mucosal solution after serosal addition (Figs. 3C and D). In the absence of the ion-pairing agent TBA, the radioactivity transported to the mucosal or serosal bathing solution coelutes with the solvent front (data not shown). However, in the presence of TBA, radioactivity elutes with two broad peaks at 4–5 and 7–10 min, with no clearly defined peak for 5-FU (Figs. 3C and D). This elution profile is qualitatively identical at both the 30- and the 60-min collection points when samples are taken from either the serosal (for m-to-s transport) or the mucosal (for s-to-m transport) bathing solution.

With rabbit colon, the accumulation of radioactivity is the same in the mucosal or serosal bathing solution after serosal or mucosal addition, respectively (Figs. 4C and 4D). In the absence of the ion-pairing agent TBA, greater than 85% of the radioactivity transported to the serosal bathing solution coelutes with the solvent front, while 10–12% coelutes

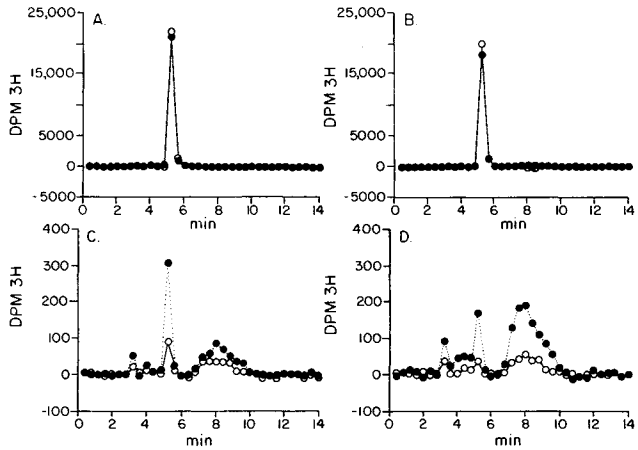

Fig. 4. HPLC analysis of the mucosal or serosal bathing solution of rabbit colon from tissues studied in Fig. 2. 5-FU, as determined from radioactivity, was determined from samples taken at 3 min (○) and 60 min (●) after adding to the mucosal (A) or serosal (B) bathing solution and the radioactivity appearing in the serosal (C) or mucosal (D) bathing solution was determined at 30 min (○) and 60 min (●). Results are means from two experiments.

with 5-FU (data not shown). In the presence of TBA, there are three peaks of radioactivity, which elute at 3, 5 (the 5-FU peak), and between 6 and 10 min (Figs. 4C and D). Qualitatively similar patterns of elution on HPLC are seen whether 5-FU is added to the mucosal or serosal bathing solution. Although we have presented the data for 5-[^{3}H-6]fluorouracil in these figures, qualitatively and quantitatively identical results were obtained with 5-[^{14}C-6]fluorouracil. In this study, we have not attempted to identify the metabolites formed by the intestinal mucosa. However, from Figs. 3 and 4 it is clear that this technique for studying intestinal mucosa maintains its metabolic capability.

The concentration dependence of 5-FU fluxes in the ileum is presented in Fig. 5. In the ileum, it can be seen that the m-to-s flux of 5-FU is not a linear function of concentration over the range of 1 to 100 μM 5-FU but is composed of a saturable and a linear component (Fig. 5). However, the s-to-m flux of 5-FU and the m-to-s and s-to-m fluxes of 5-FU in the presence of serosal ouabain (0.1 mM) are linear functions of 5-FU concentration over this same range (Fig. 5). The slopes of the s-to-m and m-to-s fluxes in the presence of ouabain are 0.089 ± 0.004 cm/hr(r = 0.997) and 0.067 ± 0.001 cm/hr (r = 0.999), respectively, and the intercepts are indistinguishable from zero. The difference in the slopes for the "basal" s-to-m flux and the s-to-m flux in the presence of ouabain may be due to the decrease in transepithelial conductance (G_t) that results from treatment of the tissue with ouabain (G_t in "basal" = 36.6 ± 1.8 mS/cm^2 vs G_t with ouabain = 30.4 ± 1.1 mS/cm^2; $P < 0.01$). Over the concentration range and time course of these studies, 5-FU did not alter I_{sc} or G_t (data not shown).

In distal colon, both unidirectional fluxes of 5-FU are linear functions of 5-FU concentration over the range of 1 to 100 μM. The slope and intercept of the m-to-s flux are 0.0222 ± 0.0001 cm/hr and −0.037 ± 0.037 nmol/hr · cm^2 (r = 0.999), respectively, and those for the s-to-m flux are 0.0196 ± 0.002 cm/hr and 0.099 ± 0.084 nmol/hr · cm^2, respectively.

Figure 6 demonstrates that uracil added to both bathing solutions reduces 5-FU absorption (10 μM 5-FU in both bathing solutions) in a concentration-dependent manner, with a maximal effect occurring at 60 μM and a half-maximal effect occurring at 15.5 μM uracil. The addition of 100 μM uracil to both bathing solutions had no significant effect on the s-to-m flux of 5-FU (Fig. 6), and in parallel control tissues, both unidirectional fluxes were constant over the time periods examined (data not shown).

Figure 7 shows that inhibition of 5-FU absorption (10 μM in both bathing solutions) is also produced by the addition of thymine, thymidine, uridine, 2-deoxyuridine, and uridine-5′-monophosphate (all at 100 μM concentrations in both bathing solutions). With the exception of uridine-5′-monophosphate, the addition of these analogues had no effect on either I_{sc} or G_t. With uridine-5′-monophosphate, I_{sc} increased by 1.2 ± 0.4 μEq/hr · cm^2 ($P < 0.05$). In parallel time controls, the m-to-s flux of 5-FU did not change, nor did I_{sc} or G_t (data not shown). Also shown in Fig. 7 is the effect of removing Na^+ from the luminal bathing solution.

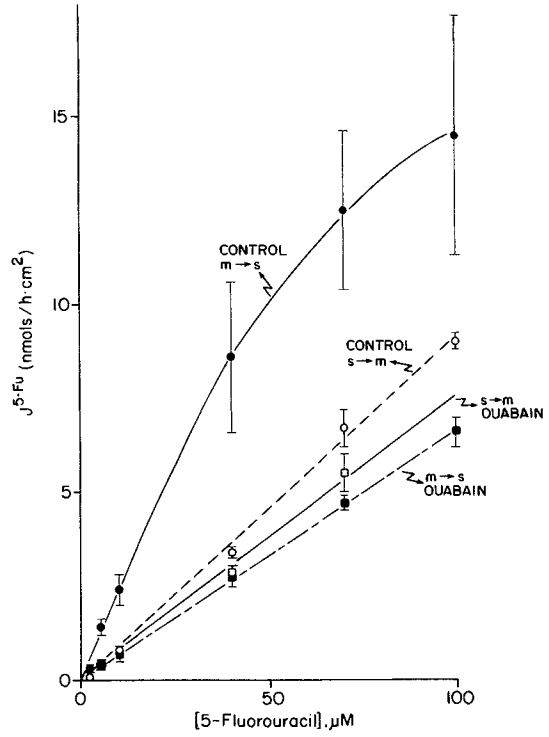

Fig. 5. Concentration dependence of 5-FU fluxes across rabbit ileum. 5-FU was added to the mucosal and serosal bathing solutions 30–45 min after mounting tissues *in vitro* together with tracer quantities of 5-[^{3}H-6]fluorouracil added to the mucosal bathing solution (for m-to-s fluxes) or the serosal solution (for s-to-m fluxes). Thirty minutes later, a 30-min flux was determined and ouabain (0.1 mM) was then added to the serosal bathing solution. After a 45-min reequilibration, a second 30-min flux was determined. Results are means ± 1 SE for five animals at each point.

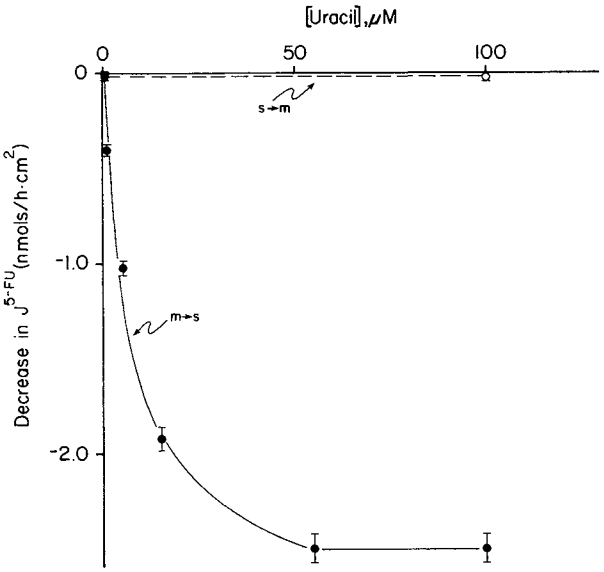

Fig. 6. Concentration-dependent inhibition of 5-FU absorption by uracil. 5-FU (10 μM) was added to the mucosal and serosal bathing solutions 30–45 min after mounting tissues *in vitro*. Thirty minutes later, three 15-min fluxes were measured. Uracil at the appropriate concentration was then added to the mucosal and serosal bathing solutions and 30 min later a second set of three 15-min fluxes was measured. Results are the means ± 1 SE for four or five animals at each uracil concentration.

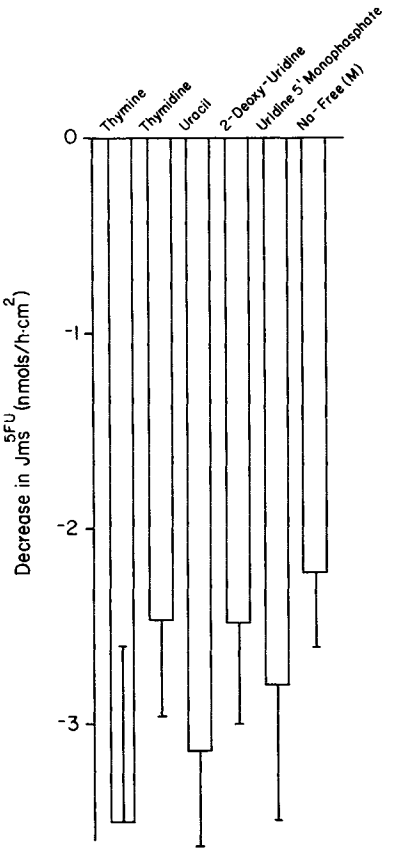

Fig. 7. Inhibition of 5-FU absorption by related structures or luminal Na^+ replacement. 5-FU (10 μM) was added to the mucosal and serosal bathing solutions 30–45 min after mounting tissues *in vitro*. Thirty minutes later, three 15-min fluxes were measured. Inhibitor compounds (100 μM) were then added to the serosal and mucosal bathing solutions or Na^+ Ringer was replaced with choline Ringer in the luminal bathing solution and 30 min later a second set of three 15-min fluxes was measured. Results are means ± SE for four animals with each condition.

Replacement of luminal Na^+ with choline reduced the m-to-s flux of 5-FU by approximately the same degree as the analogues of 5-FU.

DISCUSSION

In this study, we have presented data comparing the rates of transport of 5-FU in rabbit ileum and distal colon. We have also shown that the Ussing chamber technique allows assessment of metabolism by the intestinal segment being studied. These results together with results from prior studies allow us to construct a model for 5-FU absorption illustrated in Fig. 8. According to this model, 5-FU added to the luminal bathing solution in contact with the ileum is taken up at the brush border membrane by a Na^+-dependent mechanism similar to that described for the uptake of sugars and amino acids by the ileum (13). Thus, the uptake of 5-FU is driven by the concentration gradients for both 5-FU and for Na^+. Support for this uptake mechanism is provided by the findings that the absorption of 5-FU is inhibited by the Na^+/K^+-ATPase inhibitor, ouabain, and by replacement of Na^+ with choline in the luminal bathing solution. The postulation of a carrier mechanism is further supported by the findings that the absorption of 5-FU is a saturable function of the bathing solution concentration and that it is inhibited by the analogues, thymine, thymidine, uridine, uracil, 2'-deoxyuridine, and uridine-5'-monophosphate. Transepithelial transport of 5-FU is accompanied by metabolism. Whether metabolism occurs within the cell or by brush border processes and the identity of the metabolites has not been determined in these studies. However, studies with rat hepatocytes demonstrate that metabolism of 5-FU results in the formation of dihydro-fluorouracil, α-fluoro-β-ureidopropionic acid, and α-fluoro-β-alanine (18). If passage across the ileum resulted from an entirely passive process confined to the paracellular spaces, metabolism would not be expected.

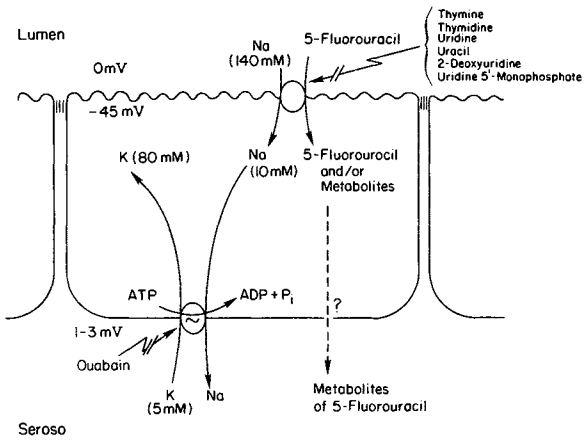

Fig. 8. Proposed model for 5-FU absorption by rabbit small intestine.

In the distal colon, the presence of concentration-dependent transport, lack of effect of ouabain on the absorptive flux, and equal rates of transport in both the m-to-s and the s-to-m direction suggest that in this segment, transepithelial transport of 5-FU is entirely via a passive process. However, as in the ileum, the finding that the majority of the transported material is the form of more polar metabolites suggests that transport occurs through a cellular pathway.

Previously, Schanker and Tocco (3,5) reported on the absorption of thymine and uracil from *in vivo* perfusion studies and from *in vitro* studies with everted sac preparations from rat small intestine. Their studies indicate that thymine and uracil are highly stable in the intestinal lumen and that these two pyrimidines are concentrated by both active and passive processes (3–5). Furthermore, these studies demonstrated that the absorption of thymine could be inhibited by uracil, cytosine, 6-azothymine, and 6-azouracil and that the purine, hypoxanthine, inhibited thymine absorption almost as well as uracil (3–5). Subsequently, Schanker and co-workers reported that the antimetabolite, 5-FU, was concentrated to the same extent as uracil, while 5-bromouracil was concentrated to a value approximately 30% less (3–5). Furthermore, accumulation of uracil in the hamster and frog small intestine is active and inhibited by thymine, 5-FU, 5-bromouracil, 6-azauracil, 6-azathymine, and barbital (3–5). Bronk and Hastewell (6) have recently reported that 5-FU transport into rat small intestinal rings is not accompanied by metabolism and that this process is Na^+ -dependent and

inhibited by thymine and uracil. Thus, these studies employing *in vivo* perfusion or *in vitro* techniques have found similar mechanisms for small intestinal absorption of pyrimidines including 5-FU and support the use of the Ussing chamber for evaluating transepithelial transport of organic molecules. However, advantages of the Ussing chamber include the ability to monitor tissue viability through measurement of electrical potential difference and transepithelial conductance, the determination of local intestinal effects of agents being investigated again through measurement of electrical potential difference and transepithelial conductance, and the ability to determine the metabolic capability of the intestinal mucosa. Further support for the use of the Ussing chamber technique is provided by the studies of Lauterbach (14), who has investigated the passive permeabilities of the guinea pig intestine and, in collaboration with Sund (15,16), determined the metabolism and transport of 1-naphthol in this preparation. Although the studies of Lauterbach and co-workers (14–16) did not allow for measurement of transepithelial electrical parameters, tissue viability was assessed from histologic studies and impermeability to large molecules such as inulin and polyethylene glycol. In addition to these studies, Jackson and co-workers (17) have used the Ussing chamber technique to investigate the transport of a series of weak acids and bases and have proposed a three-compartment model to explain the differences in absorptive and secretory fluxes determined with these compounds.

As with other animal studies, transport rates and metabolism of 5-FU in rabbit intestine have not been directly compared to those in human intestine. However, there is still a need for a test system that can reproducibly be employed to compare the rates and metabolism of new drugs to allow a prediction of their potential for oral activity in humans. In addition to confirming that the Ussing chamber technique is a useful method for evaluating drug absorption, segmental differences in absorption, and metabolism, these results confirm that 5-FU is absorbed by an active mechanism in the small intestine. However, from these results as well as those of Bronk and Hastewell (6), the active transport mechanism appears to be saturated at concentrations well below those employed in the oral delivery of 5-FU (19,20). Under these conditions the absorption of 5-FU will be dominated by the passive absorptive mechanisms, which as shown in Figs. 3 and 4, result in metabolism to and export of more polar compounds. Thus, the oral antitumor activity of 5-FU will be reduced since little or no 5-FU is absorbed intact.

Future studies will be directed at determining the transport rates and metabolism of other "standard" compounds and at investigating the effects of permeability enhancers on the absorption rates and the basal physiology of the gastrointestinal tract.

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