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Report

Mechanism of Acyclovir Uptake in Rat Jejunum

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The intestinal uptake mechanism of the purine analogue, acyclovir, was investigated in rat jejunum using *in vitro* and *in situ* methods. The pyrimidine, uracil, was used as a reference compound for carrier-mediated transport, while the purine analogue, caffeine, served as the reference compound for passive diffusion. With the *in vitro* intestinal ring method, acyclovir uptake was linear in the concentration range 0.01–5 m*M*. No significant competition for uptake was observed with uracil, 6-mercaptopurine, hypoxanthine, caffeine, or adenine. In addition, use of 2,4-dinitrophenol (DNP), ouabain, or K^+ substituted buffer did not reduce the rate of acyclovir uptake. The *in situ* single-pass perfusion method yielded a wall permeability of ~0.2, which did not vary consistently with increasing concentration. Coperfusion of acyclovir with DNP did not decrease the wall permeability. None of the data provided evidence of a carrier-mediated transport system, and it was concluded that the uptake mechanism of acyclovir in the rat jejunum is predominantly via passive diffusion.

KEY WORDS: intestinal absorption; acyclovir; purines; rat jejunum.

INTRODUCTION

Acyclovir {9-[(2-hydroxyethoxy)methyl] guanine sodium} is a synthetic acyclic purine nucleoside analogue with *in vitro* and *in vivo* inhibitory activity against herpes simplex (HSV-1 and HSV-2), varicella-zoster, Epstein–Barr, and cytomegalovirus (1). Herpes simplex virus-coded thymidine kinase converts acyclovir into acyclovir monophosphate in infected cells. Cellular guanylate kinase and other enzymes further convert the monophosphate to the triphosphate which then interferes with viral DNA polymerase and inhibits viral DNA replication (2). Acyclovir has pK_a's of 2.3 and 9.2, an octanol-to-water partition coefficient of 0.018, and an aqueous solubility of 0.13%. In humans, the oral bioavailability is low (~20%) and can be highly variable (3). Oral absorption, however, is species dependent (4), with rats exhibiting values similar to humans' (20%). Rhesus monkeys have very low oral absorption (4%), while dogs and mice have higher absorption (75 and 43%, respectively).

While an active transport system for the pyrimidine bases has been established (5–7), the mode of absorption of purine bases has not been determined and no comprehensive gastrointestinal uptake study has been performed for acyclovir. Passive diffusion has been suggested as the major route of oral absorption for purine analogues such as allopurinol (8,9) and caffeine (10,11). Studies of other purines have produced conflicting results. The uptake of uric acid has been reported both as passive diffusion (12–14) and as carrier-mediated transport (15). The intestinal uptake of hypoxanthine and xanthine has been shown by some investigators to involve active transport (10,16,17) and by others to involve only passive diffusion (18). Likewise, the uptake of 6-mercaptopurine, which like acyclovir displays low and variable oral bioavailability, has been reported as active transport (19), while other investigators could not find any evidence of a carrier-mediated transport system (20).

The existence of a saturable process in the oral absorption of acyclovir by mice, rats, and dogs has been proposed (4) based on a decline in the fraction of dose absorbed with rising dosage levels. The same suggestion has been made for humans (21) based on a decline in percentage acyclovir urinary recovery with increasing dosage. The high degree of species variation and interanimal variability, along with the fact that some investigators have reported active transport for other purines, also seems to suggest that carrier-mediated transport may be involved in the intestinal uptake of acyclovir. On the other hand, the low oral bioavailability may simply be a function of poor membrane permeability due to the low partition coefficient of acyclovir.

This study was designed to investigate the uptake mechanism of acyclovir. The small intestine of the rat was chosen as the animal model since the fraction absorbed orally in rats is similar to that in humans. An *in vitro* intestinal ring method and an *in situ* single-pass perfusion technique were used to determine whether the uptake consists predominantly of passive diffusion or carrier-mediated transport.

MATERIALS AND METHODS

Chemicals

Acyclovir sodium was obtained from Burroughs Wellcome Co., Research Triangle Park, NC (lot No. 3E2755), caffeine was obtained from MCB (Cincinnati, OH), and 6-

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mercaptopurine, 2,4-dinitrophenol, hypoxanthine, adenine, cytosine, uracil, guanosine, and ouabain were all obtained from Sigma (St. Louis, MO). ^{3}H-Acyclovir, [6-^{3}H]uracil, ^{14}C-polyethylene glycol, and [1-*methyl*-^{14}C]caffeine were obtained from New England Nuclear (Boston, MA). The purity of radiolabeled materials was 98% or greater.

In Vitro Intestinal Ring Method

Both the ring studies and single-pass perfusion were based on the methodology of Amidon *et al.* (22). Male Wistar rats (280–400 g) were fasted 18–20 hr with water *ad libitum*. Urethane (150 mg/100 g body weight) was administered i.m., a midline incision was made, and 20 cm of the upper jejunum was excised. The jejunal segment was everted on a glass rod and placed in 0.9% saline solution. Segments (rings) approximately 2 mm thick were cut from the intestine and placed in oxygenated McIlvaine buffer (citric acid/sodium phosphate dibasic) at pH 6.8 which was adjusted to an osmolarity of 310 ± 5 mOsm/kg (Wescor 5500 vapor pressure osmometer, Logan, VT) with water. The rings were either individually added to test tubes or added corporately to a beaker containing the radiolabeled drug solution (2–10 μCi/50 ml solution) and placed in a shaker bath (90 cycles/min) at 37°C. After a designated amount of time, the jejunal ring was removed from solution, rinsed with ice-cold saline solution, and blotted dry. The wet tissue weight was determined and 500 μl of Scintigest (Fisher Scientific, Fair Lawn, NJ) along with 100 μl water was added to each ring in a scintillation vial before radioactive counting. A Beckman LS 9000 scintillation counter (Beckman Instruments, Irvine, CA) with automatic quench correction and external standards was used to calculate decays per minute (dpm). Uptake is expressed as nanomoles per gram wet tissue weight after subtracting background radiation from control rings not exposed to the radioactive solution.

To characterize the transport system for each compound, a systematic series of experiments was performed. First, timed uptake experiments were completed to determine the linear period of uptake at a given concentration. All subsequent experiments were performed within the determined linear region. Sixty-second incubations were utilized for uracil studies, and 30-sec incubations were utilized for acyclovir and caffeine studies. Second, a series of concentrations was used to determine whether the uptake was linear over the concentration range or showed evidence of saturation. Third, various compounds of similar chemical structure were studied as potential competitors for the compound of interest. Finally, a metabolic inhibitor (2,4-dinitrophenol; DNP), an ATP-ase pump inhibitor (ouabain), and a K^+-substituted buffer system were used to detect evidence of active transport.

In Situ Single-Pass Perfusion

Male Wistar rats (280–400 g) were fasted for 18–20 hr with water *ad libitum*. The rats were anesthetized with an i.m. urethane dose of 150 mg/100 g body weight. A midline incision was made and a cannula was inserted into the jejunum approximately 10 cm distal to the ligament of Treitz. The inlet cannula was attached to a Harvard infusion pump (Harvard Apparatus Co., South Natick, MA). A second cannula along with outlet tubing was inserted 10 cm distal to the first. The nonabsorbable water marker, ^{14}C-polyethylene glycol (PEG), was made into a stock solution as 45 mg/50 ml (0.65 mCi/mg) and added to the buffer at a concentration of 4 ml/L. The rats were preperfused with buffer alone at a flow rate of 0.0764 ml/min and the perfusate was saved for making HPLC standards. The animals were then perfused with drug solution for a 30-min equilibration period followed by sampling every 30 min for 120 min. Samples were then analyzed by HPLC.

For water flux determinations, 300 μl of each sample was added to 10 ml of scintillation cocktail (Safety-Solve, Research Products, Inc., Mt. Prospect, IL). The Beckman LS9000 scintillation counter was used for counting and the water flux was calculated as

$$\frac{\text{dpm (inlet sample)} - \text{dpm (outlet sample)}}{\text{dpm (inlet sample)}} \times 100$$

Data for which the water flux was greater than 1%/cm of intestinal length were excluded from the analysis of results. Dimensionless wall permeabilities, P_w*, were calculated from the fraction of drug absorbed using the method of Elliot *et al.* (23). Concentrations were corrected for water flux as follows:

$$\frac{C_{out}}{C_{in}} \times \frac{\text{dpm (inlet sample)}}{\text{dpm (outlet sample)}}$$

For each rat, the corrected C_{out}/C_{in} concentrations were averaged to calculate one permeability per animal.

HPLC Assay for Acyclovir

Reverse-phase high-performance liquid chromatography, adapted from the methodology of deMiranda *et al.* (24), was performed with a Micromeritics autoinjector with a Valco valve actuator (Anspec, Ann Arbor, MI), Kratos Spectroflow 773 pump (Ramsey, NJ), Kratos Spectroflow 400 ultraviolet detector, and Hitachi D-2000 integrator (Hitachi, Tokyo, Japan). A Whatman 10-μm ODS column was used with a mobile phase of 96% 50 m*M* KH_2PO_4:4% MeOH. The wavelength was 254 nm and retention time was 7.5 min. An external standard was used and concentrations were determined by peak height.

Statistical Analysis

In most cases, the unpaired two-sample *t* test was used for statistical analysis with two-tailed rejection criteria at a level of $\alpha = 0.05$. When *n* is given, it includes the number of samples and the number of animals [e.g., $n = 24$ (3) indicates 24 samples, 8 each from 3 rats]. Error bars represent standard deviations in all figures.

RESULTS AND DISCUSSION

Results from Intestinal Ring Experiments

Since the existence of an active transport system for pyrimidine bases has been well documented (5–7), uracil was chosen as a control compound for uptake involving carrier-

mediated transport. Likewise, caffeine was chosen as the control for uptake involving passive diffusion.

Initial timed uptake experiments were performed for uracil, acyclovir, and caffeine to determine the region of linear uptake at concentrations of 5, 0.5, and 0.05 m*M*. Typical data are given in Fig. 1. At all three concentrations, the linear region for uracil was less than 1 min and the linear uptake region for caffeine and acyclovir was approximately 30 sec. Subsequent experiments were performed within these time frames.

The uptake of uracil, acyclovir, and caffeine was then determined over a broad range of concentrations. Over a 3000-fold range from 0.01 to 30 m*M*, uracil uptake was nonlinear and involved a saturable component. Figure 2A shows representative data which have been normalized by dividing the uptake by the concentration. For linear data, this will give a constant value for the normalized uptake as the concentration is increased. For nonlinear data involving a saturable component, the normalized uptake will decrease with increasing concentration. In the case of uracil, evidence of a saturable component is seen since the normalized uptake decreases as the concentration of uracil is increased from 0.01 to 0.5 m*M*.

The Michaelis–Menten kinetic parameters for uracil were determined from the concentration data by plotting an Eadie–Scatchard plot of uptake/[uracil] vs uptake (25). This plotting method can be used to separate the active and passive components of uptake. Linear regression of the active component (lower concentrations) yields a slope equal to $-1/K_m$ with V_{max} as the y intercept. The passive component, J_m, is calculated by subtracting V_{max} from the uptake for the higher concentrations (10, 20, 30 m*M*) where passive diffusion dominates and then dividing by the concentration. This calculation results in a concentration-normalized J_m which can then be compared with V_{max}/K_m to assess the relative importance of the saturable and non-saturable components of transport. The results are given in Table 1.

The K_m from our data is higher than the K_m of 0.05 m*M* determined by Katgely *et al.* (26). Katgely's methodology was much different since they used everted sacs with a sampling time of 60 min. With such long sampling times, their data probably were not based on initial uptake rates. The same is likely true for the K_m of 1.06 m*M* determined by Sasaki *et al.* (19). Their sampling time was 90 min and they used an *in situ* recirculating technique.

Figure 2B shows representative normalized data for caf-

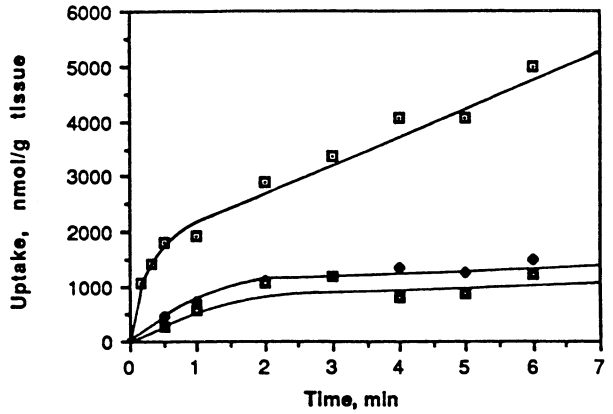

Fig. 1. Intestinal ring uptake vs time for 5 m*M* uracil (♦), caffeine (□), and acyclovir (■); n = 4 (1).

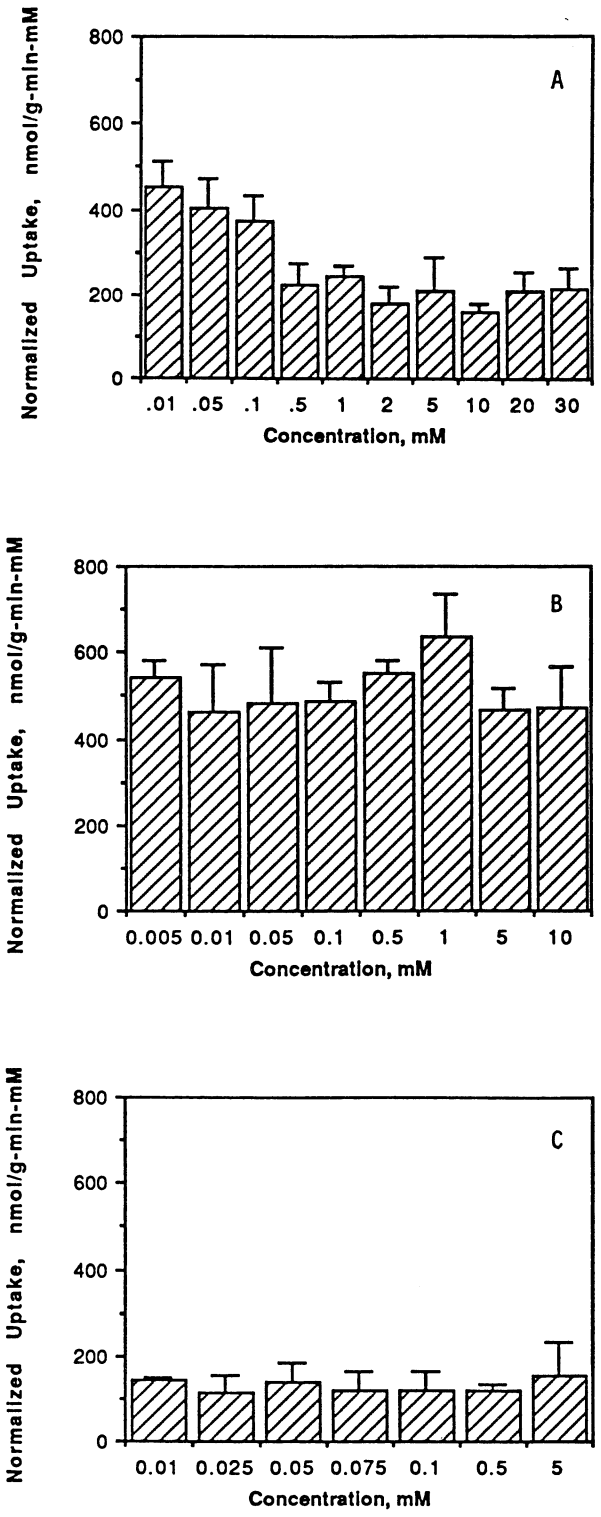

Fig. 2. Dependency of uptake in intestinal rings on drug concentration. Representative data from one rat are given for each compound, presented as [uptake/concentration] vs drug concentration. (A) Uracil, n = 4 (1); (B) caffeine, n = 4 (1); (C) acyclovir, n = 6 (1).

Table I. Kinetic Parameters for Uracil Transport

	Rat 1	Rat 2	Average
K_m, mM	1.5	0.90	1.2
V_{max}, nmol/g − min	630	460	550
J_m, nmol/g − min/mM	160	120	140
V_{max}/K_m	430	510	470
n	40	40	

feine uptake over the concentration range 0.001–10 mM. The data do not provide evidence for a saturable uptake process and appear linear throughout the entire range (n = 3 rats; r = 0.940, 0.976, 0.982). The concentration uptake experiment for acyclovir over the range 0.01– 5 mM gives data similar to that of caffeine. The normalized data show a constant normalized uptake with increasing concentration (Fig. 2C), and thus uptake appears to be linear with respect to concentration (n = 3 rats; r = 0.967, 0.979, 0.954).

Compounds of similar structure were chosen for the series of competition experiments. The uptake of each of the three compounds was compared to its uptake in the presence of 6-mercaptopurine, hypoxanthine, adenine, acyclovir, uracil, and caffeine. In addition, the pyrimidine, cytosine, was used as a competitor in the uracil experiments, and the purine nucleoside, guanosine, was used as a competitor in the caffeine and acyclovir experiments. Competition data are given in Table II. The carrier-mediated uptake of uracil showed the greatest sensitivity to the presence of competitors. Hypoxanthine inhibited the uptake of uracil to the greatest extent (70%). 6-Mercaptopurine and caffeine also produced an inhibition in uptake (50%), while cytosine, acyclovir, and adenine caused a lesser inhibition (<30%). To compare directly the competitive properties of the above purines, it would be necessary to determine the inhibition constant, K_i, of each of the above compounds of the uracil carrier. However, the results indicate that several purines do interfere with the carrier's ability to transport uracil.

Unlike uracil, the competition data for acyclovir did not show any appreciable decrease in uptake of the compound in the presence of other purines or pyrimidines. The exception was a small reduction of uptake in the presence of adenine.

Table II. Competition Study: Percentage Inhibition of Uracil, Acyclovir, and Caffeine Uptake by Various Pyrimidine and Purine Analogues

	Compound (0.025 mM), %		
Competitor	Uracil	Acyclovir	Caffeine
20 mM uracil	—[a]	NS[b]	NS
10 mM cytosine	21	—	—
10 mM hypoxanthine	70	NS	+20
5 mM acyclovir	31	—	NS
10 mM caffeine	48	NS	—
10 mM adenine	20	30	NS
5 mM 6-MP	51	NS	NS
3 mM guanosine	—	NS	NS
n	12 (2)	18 (3)	18 (3)

[a] Not performed.
[b] Not significant.

Likewise, the uptake of caffeine was not affected by the presence of other purines, as expected in the case of passive diffusion.

Since the carrier-mediated uptake of uracil involves active transport, the uptake of uracil should be reduced in the presence of metabolic inhibitors. In addition, the uptake has been shown to involve sodium ion transport (7) and, therefore, should be reduced in the presence of a potassium substituted buffer. A series of inhibition experiments was performed with 0.05 mM uracil and acyclovir using the metabolic inhibitor, 2,4-dinitrophenol, which uncouples oxidative phosphorylation, the ATP-ase pump inhibitor, ouabain, and potassium-substituted McIlvaine buffer. Rings for the metabolic inhibitor experiments were preincubated for 15 min in 2 mM DNP and 2 mM ouabain buffer solutions. Figure 3A and B show representative data. The uptake of uracil was decreased by each of the inhibitors and with potassium buffer. Acyclovir uptake, on the other hand, was not affected by DNP, ouabain, or K^+ buffer.

Results from Single-Pass Perfusion

When using the single-pass perfusion method for intestinal uptake studies, one determines the wall permeability, P_w*, for a given compound at various concentrations. For drugs absorbed predominantly by passive diffusion, P_w* should remain constant over a concentration range because the fraction of drug absorbed is independent of concentration. However, if the uptake mechanism involves carrier-mediated transport, the fraction absorbed decreases as the

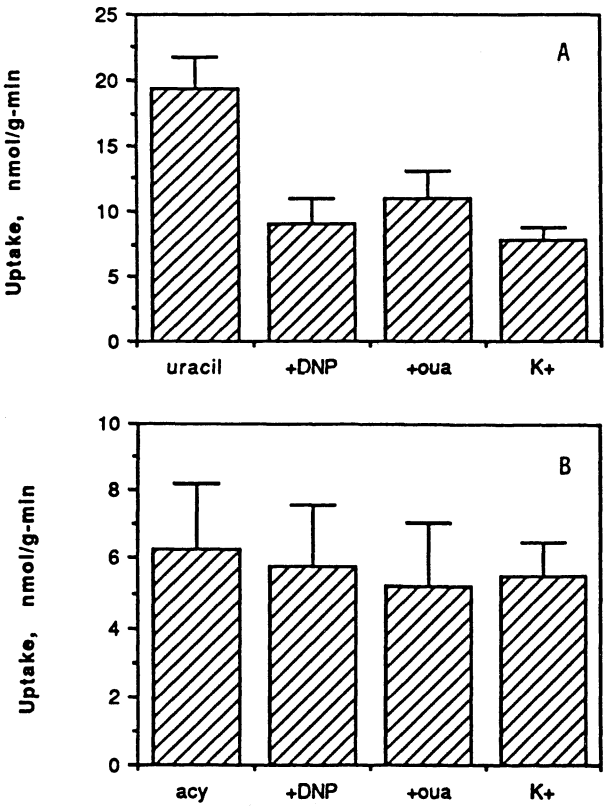

Fig. 3. Inhibition of intestinal ring uptake by DNP, ouabain, and K^+-substituted buffer. (A) Uracil, n = 4 (1); (B) acyclovir, n = 18 (3).

concentration increases, therefore, the wall permeability will also decrease.

The single-pass perfusion was performed for acyclovir over a 500-fold concentration range of 0.01–5 m*M* (Fig. 4). The dimensionless wall permeabilities ranged from 0.1 to 0.3 and no overall decrease of permeability was seen with increase in initial perfused drug concentration. However, the standard deviation was quite high (e.g., for 0.01 m*M*, $P_w^* = 0.18 \pm 0.13$) for each concentration. Comparing the overall data and standard deviations obtained from the single-pass and the ring method, for compounds like acyclovir which have low and variable absorption, the ring method is probably a more efficient means of evaluating the transport mechanism.

Although the single-pass concentration data gave no evidence of a carrier transport system, the variability in the data led us to verify the lack of energy-dependent carrier involvement by perfusing acyclovir in the presence of dinitrophenol. If active transport were involved, the wall permeability of acyclovir when perfused with DNP should decrease since the amount of drug absorbed would decrease. Coperfusion of 0.05 m*M* acyclovir with 1 m*M* DNP resulted in an elevation in P_w^* from 0.098 ± 0.031 ($n = 4$) to 0.212 ± 0.052 ($n = 6$), indicating that there is no significant active component to uptake.

CONCLUSIONS

Based on the data from both the *in vitro* intestinal ring method and the *in situ* single-pass perfusion method, it is concluded that the major uptake mechanism of acyclovir in the rat jejunum is passive diffusion. Concentration studies with both methods gave linear uptake data and showed no evidence of saturation. Incubation of rings in solutions of acyclovir with a series of purine competitors also did not cause significant decrease in uptake. The use of DNP with both intestinal rings and single pass did not decrease the uptake of acyclovir; likewise the use of ouabain and K^+ buffer with the rings did not demonstrate any decrease in acyclovir uptake. If there is a carrier-mediated component, it has a very minor contribution to the overall transport of acyclovir.

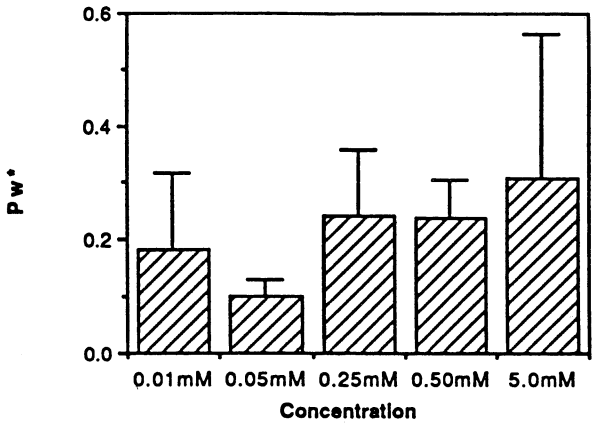

Fig. 4. Wall permeability as a function of acyclovir concentration. P_w^* was calculated from single-pass perfusion experiments; $n = 4$–6 rats.

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