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Transfer of metformin across monolayers of human intestinal Caco-2 cells and across rat intestine

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Abstract

Intestinal absorption of the antihyperglycaemic agent metformin (dimethylbiguanide) was examined in vivo in rats, and using cultured monolayers of Caco-2 human intestinal epithelial cells. After intrajejunal administration of metformin (50 mg/kg) to overnight fasted rats there was a rapid (within 1 min) appearance of the drug in the plasma, and there were consistently higher concentrations of metformin in the hepatic portal vein than the inferior vena cava during the period of absorption. The transfer of metformin across Caco-2 monolayers was concentration dependent, non-saturable and non-polar. Transfer was linear for > 1 h, and the apparent permeability coefficient (P_{app}) was 5.5×10^{-6} cm/s at pH 7.4. Approximately 19% of a 1 mM metformin solution was transferred from the apical to the basolateral chamber in 1 h. The P_{app} was decreased by 50% when the pH was decreased from 7.4 to 5.5. Metformin was not transported by the imino transport system. The results provide evidence that transepithelial transfer of metformin in the intestine is at least partly by passive permeation, possibly involving a paracellular route. Metformin drains from the intestine via the hepatic portal vein.

Keywords: Metformin; Intestinal absorption; Caco-2 cells; Permeability coefficient

1. Introduction

The antihyperglycaemic agent metformin (dimethylbiguanide hydrochloride) is used in the treatment of non-insulin-dependent diabetes mellitus (NIDDM) (Bailey, 1992; Bailey, 1993). Under physiological conditions it is singly protonated on the central imino group $[(CH_3)_2NC(=NH_2^+)NHC(=NH)NH_2]$ (Hariharan et al., 1989). The molecule is stable, not metabolized in man, rat or mouse, and excreted unchanged in the urine. Metformin is absorbed mainly in the small intestine with an estimated absorption half-time of 0.9–2.6 h and a bioavailability of 50–60% (Noel, 1979; Pentikainen et al., 1979; Tucker et al., 1981; Vidon et al., 1988). The process by which metformin is transferred from the intestinal lumen into the circulation is not known. The present study investigates this process in normal rats and using a cultured human intestinal epithelial (Caco-2) cell line.

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2. Materials and methods

2.1. Chemicals and materials

Culture medium was from Gibco (Paisley) and culture plastic-ware was from Sterilin (Hounslow). Chemicals and their sources were: L-[5-^{3}H]proline (26 Ci/mmol) from Amersham International plc (Amersham); D-[1-^{3}H]mannitol (19.1 Ci/mmol) from Sigma Chemical Company Ltd (Poole), ^{14}C-metformin hydrochloride (0.5 mCi/mmol), ^{3}H-metformin hydrochloride (1.9 mCi/mmol) and unlabelled metformin hydrochloride from Lipha Pharmaceuticals (West Drayton); α-methylaminoisobutyric acid (MeAIB) from Aldrich Chemical Company (Gillingham); phosphate-buffered saline (PBS) tablets from Unipath (Basingstoke); sodium pentobarbitone (Sagatal) from RMB Animal Health Ltd (Dagenham); and Optiphase Hisafe III scintillant from Fisons (Loughborough). Other chemicals were from Sigma Chemical Company Ltd (Poole) and BDH (Poole).

2.2. Distribution coefficient and osmolarity

The distribution coefficient (D) of metformin was determined in a mixture of equal volumes of n-octanol and McIlvaine's citric acid-Na_2HPO_4 buffer at different pH values (5.5, 6.5, 7.0, 7.4). The octanol-buffer mixtures were pre-equilibrated by continuous agitation for 18 h at 37°C; 1.5 ml of each phase was transferred to a bottle containing 0.5 μCi ^{14}C-metformin, vortexed for 1 min and incubated for 1 h at 37°C with continuous agitation. After separation of the two phases the radioactivity in each phase was determined by tranfer of 200-μl aliquots to 10 ml scintillant.

The osmolarity of metformin (5–100 mM) was determined by freezing point depression using a Roebling micro-osmometer (Camlab, Cambridge).

2.3. Caco-2 cells

Caco-2 cells, originally derived from a human colon adenocarcinoma (Fogh et al., 1977), were kindly provided by Professor Colin Hopkins, Imperial College, University of London. Cells were grown in 150-cm^2 plastic flasks containing Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum, 1% non-essential amino acids and 1% glutamine. Flasks were maintained at 37°C in a humidified atmosphere of 10% CO_2 and 90% air. The medium was changed every second day, and sub-cultures (1:3) were made weekly. Cells were detached from the flasks with 0.25% trypsin and 0.2% EDTA in PBS. For experimentation passages 95–115 were used.

Cells obtained by trypsinisation of 2–3-day post-confluent monolayers were resuspended in the medium described above supplemented with penicillin (100 units/ml) and streptomycin (10 μg/ml). Cells, 10^6 in 2ml of medium, were added to apical chambers (4-ml plastic wells fitted with aluminium oxide membranes, pore size 0.2 μm, diameter 2.3 cm; Anocell, Anotec Separations, Banbury). The chambers were placed in six-well plates containing 3 ml of medium (basolateral chamber). The inserts were not disturbed for 72 h and thereafter the medium was changed every second day. Cells were maintained for 17–21 days under the conditions described above. Cell growth, differentiation and monolayer formation followed the characteristic pattern described elsewhere (Pinto et al., 1983; Artursson, 1990; Nicklin et al., 1992b). At the time of experimentation the monolayer density was 2.6×10^6 to 3.2×10^6 cells per insert. Previous studies in this laboratory have confirmed the integrity of the monolayers under these culture conditions (Nicklin et al., 1992b; Nicklin, 1993).

Caco-2 monolayers have been well characterized as a model to investigate the transport properties of human intestinal epithelium. Bile acids, amino acids, vitamins and various drugs have previously been examined, and the monolayers have offered useful predictive value for transport properties in vivo (Hidalgo et al., 1989; Artursson, 1990; Wilson et al., 1990; Karlsson and Artursson, 1991; Nicklin et al., 1992a; Nicklin et al., 1992b).

Table 1
Octanol-phosphate buffer distribution coefficient for metformin over the pH range 7.4–5.5 (values are mean of triplicate determinations)

Distribution coefficient	pH			
	7.4	7.0	6.5	5.5
D	0.0515	0.0501	0.0490	0.0393
log$D$	−1.228	−1.300	1.310	−1.406

2.4. Transfer studies with Caco-2 cells

Measurements of metformin transfer were conducted at 37°C in humidified 10% CO_2 and 90% air using medium pre-equilibrated for 1 h. The medium comprised Hanks balanced salt solution containing 0.1% bovine serum albumin (BSA), 0.01% phenol red, 5 mM D-glucose, and buffered with 14 mM Hepes (pH 7.0 and 7.4) or Mes (pH 6.5 and 5.5). The pH was 7.4 except where otherwise stated. Agents were added to the apical chamber or basolateral chamber according to the direction of transfer to be investigated. The medium was changed every 20 or 30 min, and samples (2 or 3 ml) of medium were added to scintillant to count the radioactivity. Results were corrected for changes in volume.

The transport of L-proline was measured as previously (Nicklin et al., 1992a,b). Amino acid-free buffer was used, consisting of 1% BSA, 3.2 mM calcium chloride, 1.2 mM magnesium chloride, 4 mM potassium chloride, 150 mM sodium chloride and 5 mM D-glucose in double distilled water, buffered to pH 7.4 with Hepes. Fifty μM L-proline containing 2 μCi L-^{3}H-proline was added to the apical chamber. After 20 min the appearance of ^{3}H in the basolateral chamber gave total transport.

Transfer data are expressed either as nmoles transferred per hour or as the apparent permeability coefficient (P_{app}) calculated during 30-min incubations when $< 10\%$ of the agent was transferred and linear kinetics occurred. The calculation was made according to Schoenwald and Huang (1983): $dQ/dt \times 1/(A \cdot C_o)$ (cm s^{-1}) where dQ/dt is the transfer rate (nmol s^{-1}), A is the surface area of the membrane (4.2 cm^2) and C_o is the initial concentration (nmol/ml) of the agent in the donor chamber.

2.5. Animal studies

Male Wistar rats weighing 350–400 g were maintained at 22 ± 2°C with a 12-h light-dark schedule (08:00–20:00h light). A standard pellet diet (Rat breeding diet, Heygate and Sons Ltd., Northampton) and tap water were supplied ad libitum. Rats were fasted overnight for 18 h before experimentation, and anaesthetised with sodium pentobarbitone (60 mg/kg i.p.). The abdomen was opened and the jejunum was ligated at the ligament of Treitz and 20 cm distally. A solution of ^{14}C-metformin (10 μCi/50 mg/kg body weight) in PBS (5 ml/kg body weight), pH 7.4, was introduced into the ligated segment of jejunum. Blood samples (100 μl) were taken at 1, 2, 5, 10, 15 and 30 min through a fine needle inserted into the hepatic portal vein (HPV) adjacent to the liver. Samples were also taken at 5, 10, 15 and 30 min through a fine needle inserted into the inferior vena cava (IVC) adjacent to the ilio-lumbar veins. Plasma was separated immediately and ^{14}C radioactivity was counted in scintillant using a Packard 1800 TR liquid scintillation analyser.

3. Results

3.1. Distribution coefficient and osmolarity

The distribution of metformin in octanol and phosphate buffer showed strong hydrophilicity over the pH range 7.4–5.5, although there was a 20% reduction of hydrophilicity at pH 5.5 compared with pH 7.4 (Table 1).

The osmolarity of incubation medium was increased linearly by increasing concentrations of

metformin from 5 to 100 mM (Fig. 1). However, the increase in osmolarity was nominal at metformin concentrations < 10 mM which were used to evaluate the rate of drug transfer across the Caco-2 monolayers.

3.2. Studies with Caco-2 cells

Transfer of metformin (1 mM containing 1 μCi ^{14}C-metformin) from the apical to basolateral chamber was linear for 60 min, but declined by 20% at 180 min (Fig. 2). A 50-fold molar excess of unlabelled metformin (50 mM) did not significantly alter the transfer of the ^{14}C-metformin.

A linear association was observed between the concentration of metformin (0.1–25 mM) and the amount of metformin transferred from the apical to basolateral chamber during 30 min incubations (Fig. 3). At metformin concentrations of 50 and 100 mM there was an excessive and variable transfer of the drug (see inset to Fig. 3) possibly reflecting an alteration in the normal integrity of the Caco-2 monolayers at very high metformin concentrations.

The apparent permeability coefficient (P_{app}) for the transfer of metformin (0.1 mM containing 0.1 μCi ^{14}C-metformin during 30 min) from the apical to basolateral chamber was 5.5×10^{-6} cm s^{-1}. The rate of transfer was similar in the reverse direction. The P_{app} for metformin was more than 10-fold greater than the passive permeability marker mannitol under the same conditions (Fig. 4).

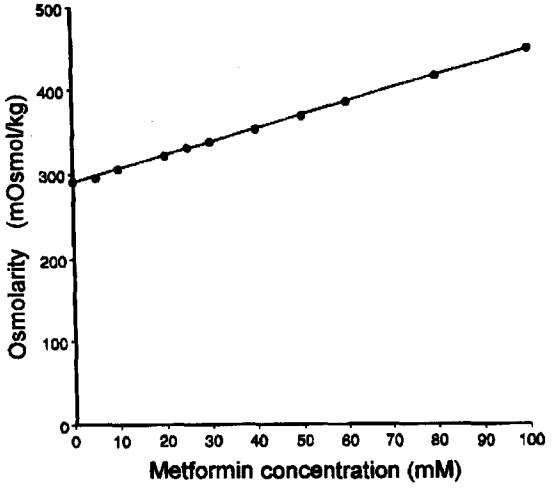

Fig. 1. Osmolarity of incubation medium supplemented with metformin (5-100 mM).

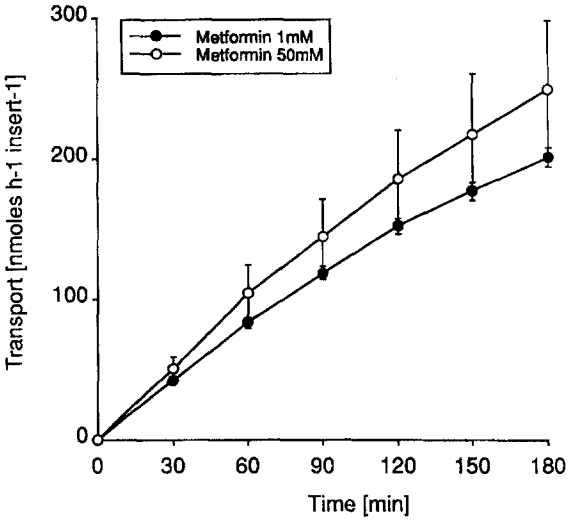

Fig. 2. Transfer of ^{14}C-metformin by Caco-2 monolayers from the apical to the basolateral chamber in the presence of 1 mM (●) and 50 mM (○) unlabelled metformin. Values are mean ± SD, $n = 6$.

The rate of transfer of metformin from the apical to the basolateral chamber declined by 50% when the pH was decreased from pH 7.4 to 5.5, whereas the transfer of mannitol was not significantly affected (Fig. 5).

Possible interaction of metformin with the active imino acid transport system was investigated using L-proline. Transport of 50 μM L-^{3}H-proline during 20 min incubations was inhibited by a 100-fold molar excess of unlabelled L-proline, L-alanine or MeAIB, whereas excess metformin did not exert an inhibitory effect (Fig. 6). Moreover, transport of 50 μM ^{3}H-metformin was not significantly affected by a 100-fold excess of unlabelled metformin, L-proline, L-alanine or MeAIB (Fig. 7).

3.3. In vivo studies in rats

Administration of ^{14}C-metformin (10 μCi/50 mg/kg body weight) into a 20-cm segment of jejunum of overnight starved rats resulted in a

rapid (within 1 min) appearance of radioactivity in the HPV (Fig. 8). The plasma concentration of metformin was consistently greater in the HPV than the IVC, reaching values of 200 and 70 μM respectively ($p < 0.01$, Student's t-test) by 30 min.

4. Discussion

Pharmacokinetic studies in human subjects have shown that oral dosages of 500–1500 mg in tablet form (equivalent to about 5–25 mg/kg body weight) gave peak metformin concentrations of 1–3 μg/ml (10–20 μM) in peripheral venous plasma at 1–3 h (Noel, 1979; Pentikainen et al., 1979; Tucker et al., 1981). The present in vivo study in rats has shown that administration of a higher dosage of a metformin solution (50 mg/kg body weight) into the jejunum predictably produced higher plasma concentrations of the drug:

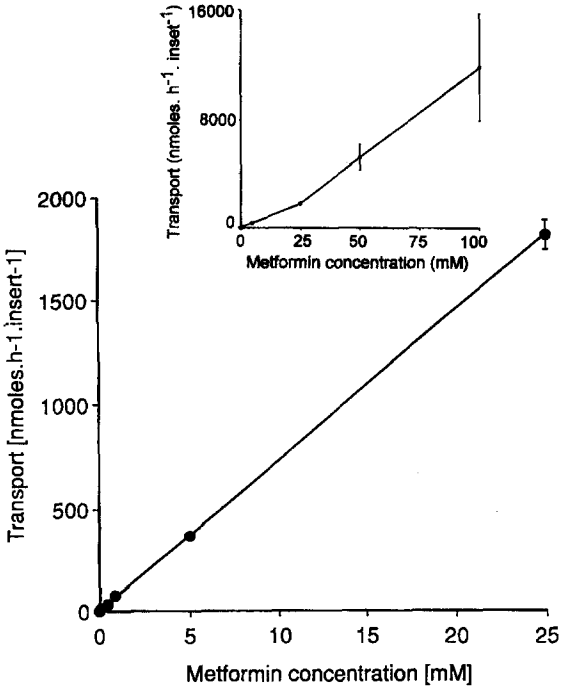

Fig. 3. Transfer of different concentrations of metformin (0.5–100 mM) by Caco-2 monolayers from the apical to the basolateral chamber. Values are mean ± SD, $n = 6$. For values at 0.5–25 mM, $r = 1.0$.

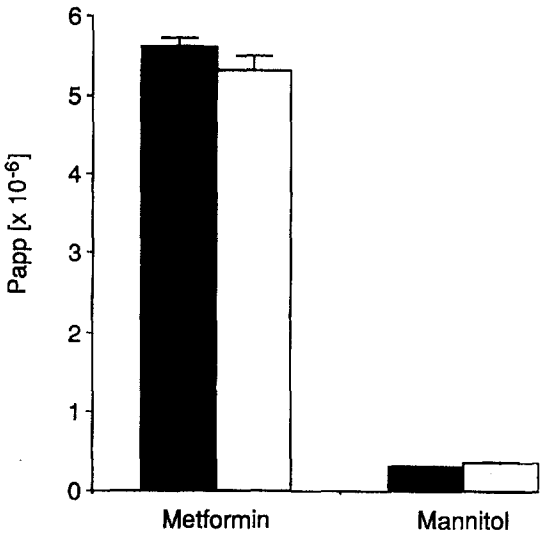

Fig. 4. Transfer of metformin (1 mM) and mannitol (1 mM) by Caco-2 monolayers from the apical to the basolateral chamber (solid bars) and from the basolateral to the apical chamber (open bars). Data are expressed as the apparent permeability coefficient (P_{app}). Values are mean ± SD, $n = 6$.

up to 70 μM at 30 min. By 30 min the plasma concentration of metformin will be about maximal (Wilcock and Bailey, 1994). The consistently higher concentrations of metformin in the HPV than the IVC during this study support the view that metformin is transferred from the lumen of the small intestine mainly into the hepatic portal drainage rather than the lymphatic drainage (Wilcock et al., 1991; Wilcock and Bailey, 1994).

The human intestinal Caco-2 monolayers used to investigate the mechanism of transepithelial transfer of metformin were grown on microporous aluminium oxide filters to minimise non-specific drug adsorption (Nicklin et al., 1992b). Consistent with the in vivo data, metformin was rapidly transferred from the apical to the basolateral chamber. At an apical metformin concentration of 1 mM, 19% of the drug was transferred in 1 h. Since the transfer of metformin was not saturable and the rate of transfer increased in proportion to concentration, it would appear that a passive mechanism of transfer was involved.

The greater than expected transfer of a very high concentration of metformin (100 mM) raises

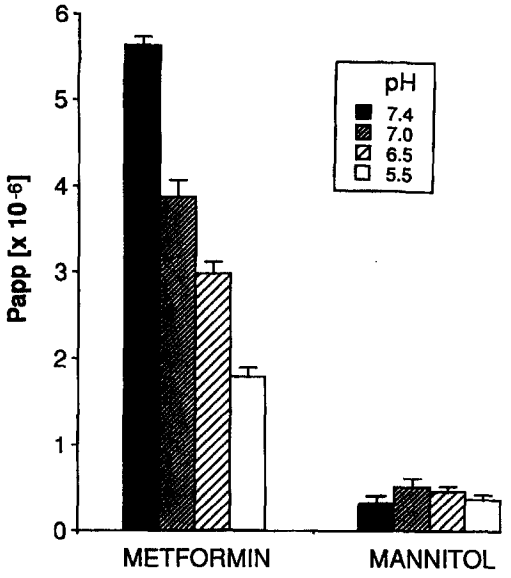

Fig. 5. Transfer of metformin (1 mM) by Caco-2 monolayers from the apical to the basolateral chamber at pH 7.4, 7.0, 6.5 and 5.5. Data are expressed as the apparent permeability coefficient (P_{app}). Values are mean ± SD, $n = 6$. All groups are significantly different ($p < 0.05$) from each other (Student's t-test).

the possibility of increased permeability due to disruption of normal Caco-2 monolayer integrity. The osmolarity of incubation medium containing 100 mM metformin was 445 mOsm/kg. Medium

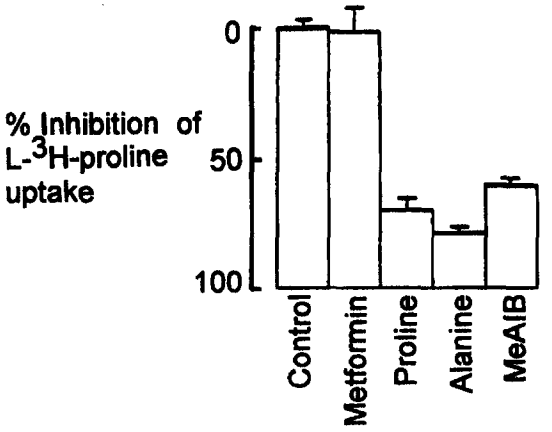

Fig. 6. Transport of L-^{3}H-proline (50 μM) by Caco-2 monolayers in the presence of a 100-fold molar excess (5 mM) of unlabelled L-proline, L-alanine, α-MeAIB and metformin. Data are expressed as percentage inhibition of L-^{3}H-proline uptake. Values are mean ± SD, $n = 6$.

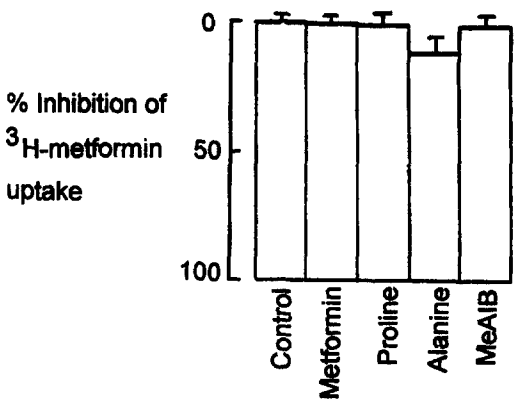

Fig. 7. Transport of ^{3}H-metformin (50 μM) by Caco-2 monolayers in the presence of a 100-fold excess (5 mM) of unlabelled metformin, L-proline, L-alanine and α-MeAIB. Data are expressed as percentage inhibition of ^{3}H-metformin uptake. Values are mean ± SD, $n = 6$.

of this osmolarity has been shown to have no significant effect on the integrity of Caco-2 monolayers during short-term (up to 1 h) incubation studies, as indicated by the passive transfer of mannitol and polyethylene glycol 4000 (Nicklin et al., 1992b). Nevertheless 100 mM metformin may have a toxic effect on the Caco-2 cells, since very

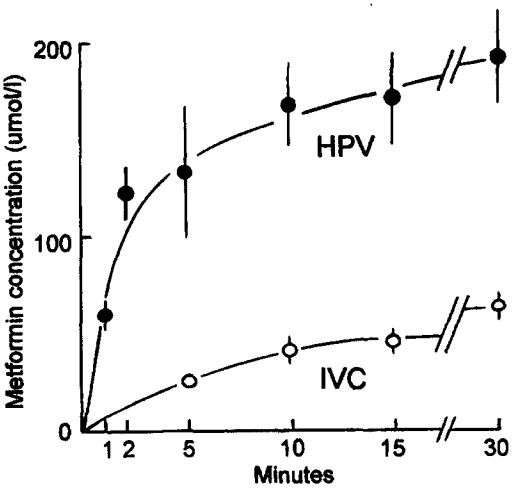

Fig. 8. Appearance of metformin in the hepatic portal vein (HPV) and inferior vena cava (IVC) of overnight starved anaesthetised rats after administration of ^{14}C-metformin (10 μCi/50 mg/kg body weight) into the jejunum. Values are mean ± SE, $n = 5$. All values in the HPV were significantly higher than in the IVC at the same time ($p < 0.05$; Student's t-test).

high concentrations of the drug can have a toxic effects in other cell preparations (Jalling and Olsen, 1984).

A passive transfer mechanism for metformin across Caco-2 monolayers is supported by the observation that the drug can be transferred across the monolayer in either direction at a similar rate. Given the size (molecular weight 129) and hydrophilicity of metformin under physiological conditions, the apparent permeability coefficient (5.5×10^{-6} cm s^{-1}) is comparable with other agents of similar size and hydrophilicity studied using Caco-2 monolayers (Karlsson and Artursson, 1991). Non-specific endocytosis is not adequate to account for this apparent permeability coefficient (Lundin and Artursson, 1990).

According to the correlation described by Karlsson and Artursson (1991), the apparent permeability coefficient for metformin across Caco-2 monolayers would predict an oral bioavailability approaching 100%. Since the oral bioavailability of metformin is 50–60% in man it is possible that an accumulation of metformin within the intestinal wall could reduce the concentration gradient for passive absorption and retard absorption in vivo. Alternatively the positively charged metformin might bind to intestinal mucins. This cannot be evaluated with the present Caco-2 monolayer preparation which does not have a mucus layer. The pH within the lumen of the upper small intestine is typically slightly acidic. Although the rate of metformin transfer was reduced at an acid pH, this was not sufficient to predict a substantial reduction in bioavailability.

The reduced rate of metformin transfer in acidic medium cannot be explained by a change in hydrophilicity (Table 1). The charge associations of proteins in the tight junctions become altered at lower pH values, and can reduce the permeability of positively charged molecules. This is consistent with the transfer of metformin via the paracellular route, which is a major route for passive transfer of many other small ionized molecules (Jackson, 1987).

It is possible that a major passive paracellular route of transfer could mask a minor carrier-mediated route. To investigate this possibility we examined the imino system of active transport. As shown previously in this laboratory the transport of L-proline from the apical to basolateral chamber was inhibited by L-alanine and MeAIB (Nicklin et al., 1992a). The lack of an effect of a 100-fold molar excess of metformin precludes the involvement of this transport system. Indeed, failure of a 100-fold molar excess of L-proline and L-alanine to inhibit the transfer of metformin substantiates this view, although it does not preclude the possibility that another active transport system could make a minor contribution to the transfer of metformin.

It is concluded that metformin is transferred across Caco-2 monolayers mainly by a non-saturable and non-polar process. The rate is comparable with other small hydrophilic molecules suggesting that transfer is at least partly due to passive permeation, and may involve a paracellular route.

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