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S-adenosyl-L-methionine: transcellular transport and uptake by Caco-2 cells and hepatocytes

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Abstract

S-adenosyl-L-methionine (SAMe) is an endogenous molecule that is known to be protective against hepatotoxic injury. Although oral SAMe appears to be absorbed across the intestinal mucosa, its systemic bioavailability is low. The reason for this is unknown. Using the Caco-2 cell culture model for enterocyte absorption, we determined the mode by which SAMe is transported across this cell monolayer. We also determined the extent it is taken up by both Caco-2 cells and hepatocytes. In Caco-2 cells transport was observed in both apical to basolateral and basolateral to apical directions. The apparent permeability coefficients (P_{app}) appeared to be concentration independent and were similar in both directions (0.7×10^{-6} and 0.6×10^{-6} cm s^{-1}, respectively), i.e. identical to that of the paracellular transport marker mannitol (0.9×10^{-6} and 0.7×10^{-6} cm s^{-1}). This mode of transport was supported by a four-fold increase in the P_{app} for SAMe transport in Ca^{++}-free buffer. Cellular uptake of SAMe was examined in both Caco-2 cells and cultured rat hepatocytes. Uptake by hepatocytes was not saturable in a concentration range of 0.001–100 μM. Accumulation by both cell types was very low, with a cell:medium ratio at equilibrium of only 0.2–0.5. This low cell accumulation supports the finding of paracellular transport as the only mode of cell membrane transport. Increased hepatocellular protection for SAMe may be accomplished by converting SAMe to a more lipid-soluble prodrug.

Introduction

S-adenosyl-L-methionine (SAMe) is an important and ubiquitous molecule in cells (Lu 2000). It serves several key biochemical functions: as the primary biological methyl donor, as the precursor of aminopropyl groups used in polyamine biosynthesis and as a precursor for glutathione (GSH) synthesis.

Because of its role in GSH synthesis, SAMe has been used as a treatment for liver diseases, where reduced hepatocellular GSH levels have been associated with hepatotoxicity (Lu 2000). In animal studies it has been shown to be effective in protecting from a variety of chemical-induced liver injuries (Stramentinoli et al 1978; Friedel et al 1989; Lieber et al 1990; Bray et al 1992; Corrales et al 1992; Wu et al 1996) and from chemical-induced hepatocarcinogenesis (Pascale et al 1992). Recent human studies have indicated that daily oral SAMe can attenuate alcoholic liver disease and improve survival (Friedel et al 1989; Mato et al 1999). SAMe has also been used in clinical studies for the treatment of neurological and affective disorders (Echols et al 2000). In addition to these proposed clinical uses of SAMe, it is widely used as a nutritional supplement. It is taken prophylactically for the prevention and relief of the symptoms of depression, osteoarthritis, liver disease and fibromyalgia. The recommended daily oral dose to achieve therapeutic efficacy is 400 mg three to four times per day.

The pharmacokinetics of exogenous SAMe have been studied in animals and humans (Stramentinoli et al 1979; Bombardieri et al 1983; Stramentinoli 1987). SAMe is absorbed across the intestinal mucosa; however, its systemic bioavailability is low (0.5–1.0%). The reason for the low systemic bioavailability is still unknown and the mechanism of intestinal absorption has not been determined. Based on the chemical structure of SAMe (Figure 1), it is an amino acid and carries a positively charged sulfonium group as well as a sugar moiety, thus, as a highly polar molecule, it is not likely to penetrate lipid membranes. On the other hand, because of these functional

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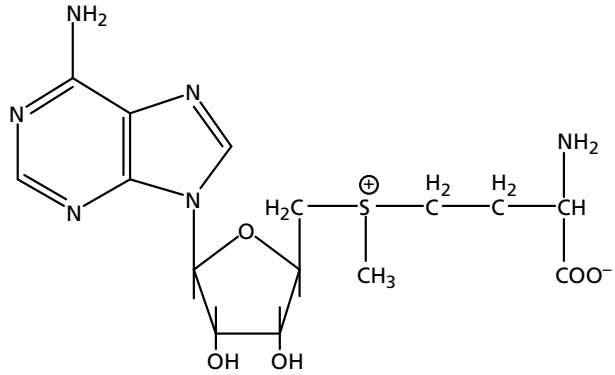

Figure 1 Structure of S-adenosyl-L-methionine.

groups, it might be a substrate for one or several membrane transporters. The low systemic oral bioavailability and the cost of treatment serve to limit the use of SAMe as a therapeutic agent.

In the present studies we used the Caco-2 human colonic adenocarcinoma cell line to determine the transepithelial transport of SAMe. Our data indicate that SAMe is transported across the intestinal epithelium by a strictly paracellular mechanism, without any evidence of the involvement of membrane transporters. In addition we observed that both Caco-2 cells and hepatocytes showed very low cellular uptake of SAMe.

Materials and Methods

Materials

^{14}C-(carboxyl)-SAMe (0.025 mCi mL^{-1}) and ^{14}C-mannitol (0.2 mCi mL^{-1}) were purchased from Amersham Pharmacia (Piscataway, NJ). α-Minimum Essential Medium (α-MEM), Hank's balanced salt solutions without phenol red (HBSS) and penicillin/streptomycin were purchased from Gibco-Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS), SAMe, S-adenosylhomocysteine, HEPES, collagen, William's Medium E, gentamicin sulfate and insulin were obtained from Sigma Chemical Co. (St Louis, MO). Collagenase type II was purchased from Worthington Biochemicals (Freehold, NJ).

Culture of Caco-2 cells

Caco-2 cells obtained from the American Type Culture Collection were cultured in α-MEM containing 10% FBS and penicillin/streptomycin (100 U mL^{-1}; 0.1 mg mL^{-1}). For transport studies, the cells were seeded at a density of 1×10^5 cells per insert in Transwell polycarbonate culture well inserts (0.4 μm pore size, 12 mm diameter) as described previously (Walle & Walle 1999). The medium was renewed every other day and changed 24 h prior to initiation of the experiments. The cell monolayers were used for experiments at 21–24 days after seeding. The integrity of the monolayer was evaluated by measuring the transepithelial electrical resistance (TEER) values with a Millicell-ERS volt/ohmmeter. Cell inserts with resistance values > 350 Ω cm^2 were used for transport studies.

For cell uptake studies Caco-2 cells were seeded in six-well tissue culture dishes at a density of 1×10^5 cells well^{-1}. The cells were maintained in α-MEM containing 10% FBS and penicillin/streptomycin (100 U mL^{-1}; 0.1 mg mL^{-1}). The medium was renewed three times a week and the cells were used for experiments at 10–14 days after seeding. Fresh culture medium was added 24 h before experiments.

Transport studies

Prior to the start of the experiments the monolayers were washed twice for 30 min with warm HBSS containing 25 mM HEPES, pH 7.2 (HBSS/HEPES). ^{14}C-SAMe (0.075 μCi mL^{-1}; various concentrations) was dissolved in HBSS/HEPES buffer and added to either the apical (0.5 mL) or basolateral (1.5 mL) chamber. HBSS/HEPES buffer was added to the opposing compartment. Transport of ^{14}C-mannitol was monitored in parallel as a marker of paracellular transport. A known volume of the receiver compartment was collected and mixed with 10 mL Ecoscint A scintillation cocktail. The amount of radioactivity in each sample was determined by liquid scintillation spectroscopy using a Beckman 6000LSC scintillation counter. In transport studies designed to determine the effect of removal of Ca^{++} on transport, Ca^{++}-free HBSS was used for the 30 min washes and for the transport buffer. The following equation was used to calculate the apparent permeability coefficient (P_{app}):

$$P_{app} = (V/AC_0)(dC/dt) = \text{cm s}^{-1}$$

where V is the volume of the solution in the receiving compartment, A is the membrane surface area, C_0 is the initial concentration in the donor compartment and dC/dt is the change in drug concentration in the receiver solution over time (Artursson & Karlsson 1991; Lu et al 1996).

HPLC analysis of SAMe

Separation of SAMe from its major initial metabolite, S-adenosylhomocysteine, was achieved using an isocratic mobile phase system. Standards (1.0 mM) of SAMe and S-adenosylhomocysteine, either alone or as a mixture, were prepared in 0.1 N H_2SO_4 and a 20 μL aliquot was injected onto the HPLC column. SAMe and S-adenosylhomocysteine were separated using a Waters Novapak C8 reversed-phase column (3.9 × 150 mm). The compounds were eluted using an isocratic mobile phase of 4 mM heptanesulfonic acid + 50 mM KH_2PO_4, pH 2.4:methanol (88%:12%) at a flow rate of 1.0 mL min^{-1}. Using these conditions SAMe and S-adenosylhomocysteine eluted from the column after 22 and 25 min, respectively, with baseline separation.

For HPLC analysis of SAMe in Caco-2 cell transport studies, 1 mM SAMe (1.1 μCi mL^{-1}) was dissolved in HBSS/HEPES buffer and added to the apical chamber

of Caco-2 cell monolayers in Transwell inserts. One hour later the buffer from the basolateral chamber (1.3 mL) was removed and acidified with 13 μL of 5.0 N HCl. The samples were frozen in a dry ice/ethanol bath and stored at −80°C until analysis. The presence of SAMe was determined by HPLC analysis of 20 μL aliquots of the samples. The effluent off the column was monitored sequentially by UV absorption at 254 nm, followed by collection of fractions at 1 min intervals using a Gilson FC 203 fraction collector. Ecoscint A scintillation cocktail (0.5 mL) was added to each fraction, and the amount of radioactivity was determined by liquid scintillation spectroscopy as described above. The amount of radioactivity in each fraction was plotted over time and the profile was compared to the elution profile for SAMe and S-adenosylhomocysteine standards.

Caco-2 cell uptake studies

The cells were washed twice for 30 min with warm HBSS/HEPES prior to the start of the experiments. ^{14}C-SAMe (0.075 μCi mL^{-1}; various concentrations) was dissolved in HBSS/HEPES buffer and added to the cultured cells. At various times, the cells were washed four times for 30 s with ice-cold phosphate-buffered saline and solubilized overnight by the addition of 1.0 N NaOH (1.0 mL). A 50 μL aliquot of the solubilized cell suspension was collected for protein quantification according to the method of Smith et al (1985). An equal volume of 0.5 N HCl was added to the solubilized cell suspension and the amount of radioactivity in the suspension was determined by liquid scintillation spectroscopy.

Hepatocyte uptake studies

Adult male Long Evans rats were purchased from Harlan Lab Animals (Indianapolis, IN). They were housed in AAALAC-accredited animal facilities and maintained on a 12/12 h light/dark cycle with free access to food and water. They were used for hepatocyte isolation when they reached a weight of 150–250 g. Hepatocytes were isolated by collagenase perfusion as previously described (McMillan & Jollow 1992). Hepatocytes were purified from the liver cell suspension by centrifugation twice at 50 × g for 2 min at 4°C and resuspended in William's medium E containing 10% FBS, 2 mM glutamine, 34 μg mL^{-1} insulin and 0.1 mg mL^{-1} gentamicin sulfate. Viability was >80%, as determined by Trypan blue exclusion. Hepatocytes were plated in six-well collagen-coated culture dishes at confluency (6.25×10^5 cells well^{-1}) and allowed to attach for 2 h. The unattached cells and old medium were removed and replaced with fresh medium. The cells were used for uptake studies 24 h later. The cells were washed twice for 30 min with warm HBSS/HEPES prior to the start of the experiments. ^{14}C-SAMe (0.075 μCi mL^{-1}; various concentrations) was dissolved in HBSS/HEPES buffer and added to the cultured cells. At various times, the cells were washed four times for 30 s with ice-cold phosphate-buffered saline and solubilized overnight by the addition of 0.5 mL of 1.0 N NaOH. A 50 μL aliquot of the solubilized cell suspension was collected for protein quantification as described for Caco-2 cells. An equal volume of 0.5 N HCl was added to the solubilized cells and the amount of radioactivity in the suspension was determined by liquid scintillation spectroscopy.

Statistics

Statistical differences ($P < 0.05$) were determined by the Mann–Whitney U test. Results from a typical experiment are shown in the figures. Treatments were carried out in triplicate (n = 3) and each experiment was repeated at least twice.

Results

Transport of SAMe by Caco-2 cells

Using the Caco-2 cell culture model for enterocyte transport we determined whether or not transcellular transport of SAMe could be observed. When Caco-2 cells cultured on Transwell culture inserts were treated with ^{14}C-SAMe in the apical compartment, a time-dependent appearance of radioactivity on the basolateral side of the culture inserts was observed and was linear over 2 h (data not shown). The P_{app} for this transport was 0.7×10^{-6} cm s^{-1} (Figure 2A). Transport in the basolateral to apical direction occurred with a similar P_{app} of 0.6×10^{-6} cm s^{-1} (Figure 2B). These P_{app} values were both similar to the P_{app} values for mannitol transport (0.9×10^{-6} cm s^{-1}, apical to basolateral, and 0.6×10^{-6} cm s^{-1}, basolateral to apical), a compound known to be transported paracellularly. The similarity in P_{app} for SAMe and mannitol transport and the lack of direction-dependent transport suggested that SAMe may be transported paracellularly. Furthermore, the P_{app} values are much lower than those for lipid soluble compounds absorbed by passive diffusion (i.e. propranolol, P_{app} of 40×10^{-6} cm s^{-1}) (Artursson 1990).

If SAMe transport is paracellular then disruption of the tight junctions should increase the rate of flux across the Caco-2 cell monolayer. A well-accepted method for disrupting tight junctions in Caco-2 cell cultures is removal of Ca^{++} from the incubation medium (Artursson & Magnusson 1990; De Boer et al 1991). To disrupt tight junctions, transport was determined in Ca^{++}-free incubation medium. When Ca^{++} was removed, transcellular transport of SAMe was increased approximately four-fold in both the apical to basolateral (Figure 2A) and basolateral to apical (Figure 2B) directions. These increases were similar to the increases observed in mannitol flux upon removal of Ca^{++}. These data strongly suggest that SAMe is transported paracellularly across the Caco-2 cell monolayers.

To determine whether the radioactivity was present as intact SAMe or SAMe metabolites, 1.0 mM ^{14}C-SAMe was added to the apical compartment and buffer from the basolateral compartment was analysed by HPLC after a 1 h incubation period. The radioactivity in the collected buffer co-eluted with the peak for SAMe, as detected by UV absorption at 254 nm and radioactivity of the treatment

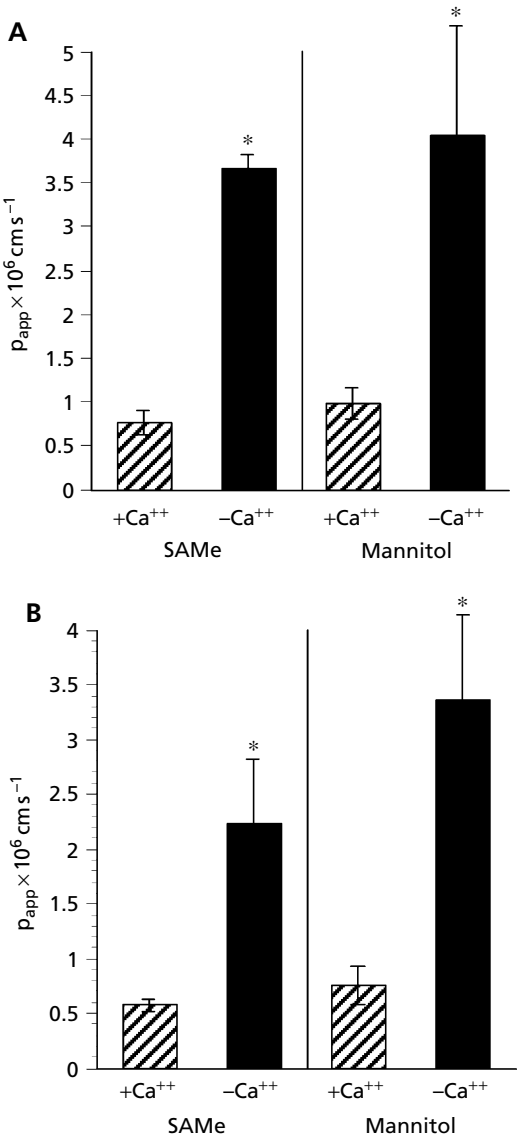

Figure 2 SAMe transport across Caco-2 cell monolayers. (A) Apical to basolateral transport. (B) Basolateral to apical transport. *Significantly different from value obtained in the presence of Ca^{++} (n = 3).

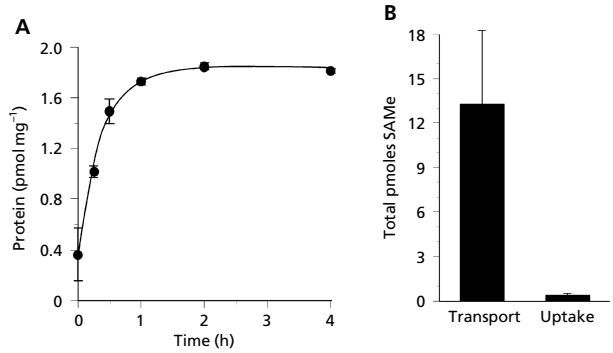

Figure 3 Uptake of SAMe by Caco-2 cells. (A) Time-dependent uptake of SAMe by Caco-2 cell cultures. (B) Comparison of SAMe transport across and uptake by Caco-2 cell monolayers grown on Transwell inserts (n = 3).

solution. No detectable radioactivity co-eluted with the S-adenosylhomocysteine peak, indicating that metabolism of SAMe and transport of its metabolites did not occur during this period of time.

Caco-2 cell uptake of SAMe

Although the fate of SAMe appeared to be paracellular transport, it was of interest to determine to what extent SAMe was taken up by Caco-2 cells. To address this question, ^{14}C-SAMe uptake by Caco-2 cells grown in six-well culture dishes was assessed (Caco-2 cells cultured on tissue culture plates have only the apical surface available for uptake). As shown in Figure 3A, time-dependent uptake of radioactivity was observed after addition of SAMe, suggesting apical absorption. Steady state uptake occurred approximately 1.0 h after addition of SAMe. At this time the concentration inside the cells was 1.7 pmol mg^{-1} protein. Assuming that the intracellular volume is 3.66 μL mg^{-1} protein (Blais et al 1987; Dantzig & Bergin 1990), this amount represented an intracellular concentration of 0.48 μM. This intracellular concentration is only one-half of the extracellular concentration of 1.0 μM.

The amount taken up (apical) by the cells was compared to the amount transported (apical to basolateral). For these studies, apical uptake and apical to basolateral transport were determined in Caco-2 cell monolayers grown on Transwell inserts during 1.0 h of incubation. At this time point uptake of SAMe by the cells had reached equilibrium (Figure 3A). The total amount of SAMe in the basolateral compartment was compared to the total amount of SAMe in the cells. As illustrated in Figure 3B, although uptake of SAMe by the Caco-2 cells occurred, the total amount was much less than the amount transported transcellularly.

Hepatocyte uptake of SAMe

SAMe that is absorbed into the mesenteric blood supply is carried first to the liver via the portal vein. Thus, we wished to determine the extent of SAMe uptake by hepatocytes and compare the uptake to that observed for the Caco-2 cells. Primary cultures of rat hepatocytes were used as the model system for hepatocyte uptake. As shown in Figure 4A, time-dependent uptake of ^{14}C-SAMe by cultured rat hepatocytes was observed and was very similar to that occurring with Caco-2 cells. Uptake of SAMe by hepatocytes reached steady state at approximately 2.0 h after SAMe addition. At this time the intracellular concentration was 2.0 pmol mg^{-1} protein. Assuming a cell volume of 11 000 μm^3 for a hepatocyte (Arias et al 1988) and a protein concentration of 1.0 mg protein 10^{-6} cells (personal observation), this amount represents an intracellular concentration of 0.19 μM. This amount is less than 20% of the extracellular concentration of 1.0 μM.

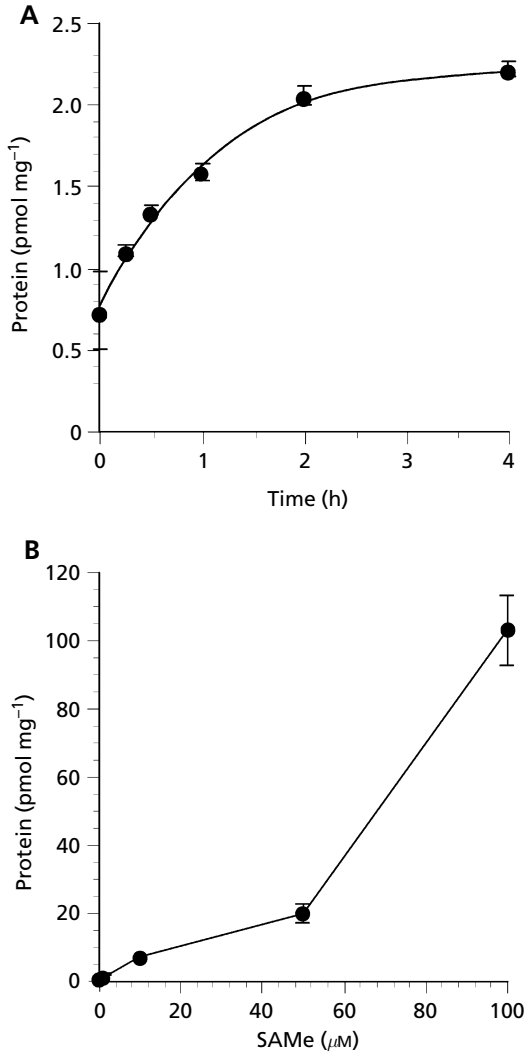

Figure 4 Hepatocyte uptake of SAMe. (A) Time-dependent uptake of SAMe. (B) Concentration-dependent uptake of SAMe (n = 3).

Concentration-dependent uptake of SAMe was determined at 30 min in the cultured hepatocytes (Figure 4B). Uptake was linear over the concentration range examined (0.01–100 μM). Saturation of uptake was not observed.

Discussion

Drug-induced liver injury is a persistent clinical problem and few specific agents are available for its treatment. Use of N-acetylcysteine in treatment of acetaminophen overdose is well known. However, its use as a treatment for other types of hepatotoxic injury, such as that associated with alcohol abuse, is variable. In contrast to N-acetylcysteine, SAMe has been shown to be effective in ameliorating hepatic injury from a variety of drugs and xenobiotics, including alcohol (Stramentinoli et al 1978; Friedel et al 1989; Lieber et al 1990; Bray et al 1992; Corrales et al 1992; Wu et al 1996). However, its therapeutic use appears limited by the low oral bioavailability of SAMe. In addition, the mechanisms of intestinal absorption and hepatic uptake of exogenously administered SAMe and the mechanism of its hepatoprotection remain unknown.

As a first step in determining how SAMe efficacy and bioavailability might be improved, we addressed the question of how SAMe is absorbed across the intestinal epithelial layer. We used the Caco-2 cell monolayer system as a model for human intestinal epithelial cells to determine the mechanism for transcellular transport of SAMe. Caco-2 cells grown on microporous membranes have been used successfully as an in-vitro cell culture model for the intestinal mucosa (Artursson 1990; Artursson & Karlsson 1991; Meunier et al 1995; Walle et al 2003). These cells, although derived from transformed human colonic adenocarcinoma cells, dramatically differentiate in culture to become similar to enterocytes. They are easily and reproducibly cultured as monolayers and, in addition, they express many of the transporters present in the normal human small intestine (Takanaga et al 1995; Tamai et al 1995; Brot-Laroche 1996; Matosin et al 1996; Wang et al 1997; Ward & Tse 1999; Herrera-Ruiz et al 2001; Walle et al 2003). Thus, they can be used to determine mechanisms of transport and the data can be used to predict what may occur in normal human intestine.

The data presented in the current studies demonstrate that SAMe is transported across the Caco-2 cell monolayer intact and strongly suggest that transport occurs by a paracellular mechanism without any involvement of membrane transporters. Thus, the transport is virtually identical to that of the paracellular transport marker mannitol and is dramatically increased by lowering Ca^{++}, a well-known tool for disrupting the tight junctions (Artursson & Magnusson 1990; De Boer et al 1991). This evidence was further supported by Caco-2 cell uptake studies. In these studies the amount of SAMe taken up by the Caco-2 cells was a very small fraction of that transported across the cell monolayer, i.e. again very similar to what is known for mannitol (the oral absorption of mannitol is only about 17%). For other drugs, absorption by only the paracellular route has limited their effectiveness when administered orally (Gan et al 1993; Nichols et al 1994; Samanen et al 1996; Smith & Lee 2001).

Our studies also demonstrated uptake of SAMe by cultured rat hepatocytes. However, uptake was low, i.e. did not result in cellular accumulation of SAMe. The intracellular amount in hepatocytes at equilibrium was only one-fifth that of the extracellular compartment.

Previous reports in the literature reached conflicting conclusions about uptake of SAMe by hepatocytes. Pezzoli et al (1978), using isolated hepatocyte suspensions, and Zappia et al (1978), using an isolated perfused liver system, reported uptake of radiolabelled SAMe by hepatocytes and liver cells, respectively. In contrast Hoffman et al (1980), using an isolated perfused liver system, and Van Phi & Soling (1982), using isolated hepatocyte suspensions, reported no evidence for uptake of radiolabelled SAMe into hepatocytes. Rather, they concluded that exogenous SAMe was

used in methylation reactions at the plasma membrane to form phosphatidylcholine from phosphatidylethanolamine. The SAMe used for our studies contained a ^{14}C-radiolabel on the carboxyl group of the methionine portion of the molecule; thus, use of SAMe in methylation reactions would not result in production of radiolabelled phosphatidylcholine. Although our studies do not preclude the possibility that SAMe is demethylated at the cell surface and enters the cell as S-adenosylhomocysteine, our studies do support the conclusion that SAMe is very inefficiently taken up into hepatocytes.

Conclusions

The low transport of SAMe through the paracellular route alone serves to limit its intestinal absorption and similarly limits the amount of SAMe both available to and taken up by hepatocytes. Thus, both of these factors together reduce its effectiveness as a therapeutic agent. Strategies to increase the lipid solubility of SAMe by development of a prodrug form may improve on both these properties.

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