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Oleic acid uptake into rat and rabbit jejunal brush border membrane

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Abstract

Oleic acid uptake was studied using adult rabbit and rat jejunal brush border membrane vesicles. There was a reduction of oleic acid uptake following trypsin-treatment. Opposing Na^+/H^+ gradients (inward Na^+ and outward H^+ gradients) increased oleic acid uptake by about 40%, as compared with only an inward Na^+ gradient, only an outward H^+ gradient, or the absence of either Na^+ or H^+ gradients. The addition of mucin further increased the enhanced uptake of oleic acid observed in the presence of opposing Na^+/H^+ gradients. Amiloride, an inhibitor of the Na^+/H^+ exchanger, reduced by about 40% the uptake of oleic acid into sheets of rat jejunum, and this inhibitory effect was observed over a range of rates of stirring of the bulk phase. In rabbit jejunal brush border membrane vesicles, amiloride reduced oleic acid uptake in the presence but not in the absence of opposing Na^+/H^+ gradients, with a K_i of approx. 36 μM. Thus, oleic acid uptake occurs largely by partitioning of the lipid into the brush border membrane, influenced by a process which involves the activation of the brush border membrane Na^+/H^+ exchanger.

Keywords: Oleic acid; Brush-border membrane; Sodium ion–proton exchanger; (Jejunum); (Rat); (Rabbit)

1. Introduction

It is widely accepted that lipids cross the intestinal brush border membrane (BBM) by a process of passive diffusion [1–3]. Evidence for passive absorption includes: (1) in most studies, rates of lipid absorption are a linear function of concentration; (2) competition between lipids for absorption has not been unequivocally demonstrated; and (3) for any given membrane, the permeability coefficient for a homologous series of lipid probes predictably increases with hydrophobicity). For example, there is a linear relationship between the number of CH_2 groups and a function of the rate of uptake of lipid [2,3]. Molina and coworkers [4] suggested that at low concentrations lipid uptake occurs by facilitated diffusion, while at higher lipid concentrations uptake occurs primarily by passive diffusion. Evidence for a protein-mediated component includes studies of the use of polyclonal antibody against the plasma membrane fatty acid-binding protein ($FABP_{PM}$), with decreasing fatty acid and cholesterol uptake when this antibody (anti $FABP_{PM}$-Ab) is added to brush border-membrane vesicles, perfused intestinal segments, or to isolated enterocytes [5].

The intestinal microenvironment is important for the absorption of weak organic acid, such as fatty acids, folic acid and dipeptides [6]. An increase in the microenvironment pH decreases the absorption of oleic acid into intestinal everted sacs [7,8]. In order to maintain this acidic microenvironment, a barrier must be present between the brush border membrane (BBM) and the bulk phase in the intestinal lumen [10,9,11]. The existence of an unstirred water layer and a coat of mucus adjacent to the BBM has been demonstrated in animals and in humans [11,12]. The lower pH in the acid microclimate may influence fatty acid uptake in several ways, including: (1) increasing the critical micellar concentration of bile acids for lipids, thereby enhancing their dissociation from the micelle and increasing their availability for BBM uptake; (2) increasing the proportion of protonated to non-protonated fatty acids,

Abbreviations: BBM, brush border membrane; BBMV, brush border membrane vesicles; CMC, critical micellar concentration of bile acid micelles; FA^-, non-protonated (ionized) fatty acid; FAH, protonated fatty acid; $FABP_{PM}$, plasma membrane fatty acid-binding protein; NHE-3, Na^+/H^+ exchanger or antiporter in the BBM oleic acid; TC, taurochloric acid, sodium salt; UWL, unstirred water layer.

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with greater lipophilicity of the protonated form and therefore greater ability to partition into the BBM; and (3) faster equilibration of the protonated fatty acids between the outer and the inner leaflets of the BBM [11,13].

The electrolyte composition and pH of the mucus coat appear to be fairly uniform [6]. One interesting property of intestinal mucus is its ability to decrease the lateral diffusion of hydrogen (H^+) ions, thereby acting as a 'trap' for H^+. Moreover, the interaction of free fatty acid with mucus produces a further decrease in the lateral diffusion of H^+ [14]. The presence of an acidic mucin layer facilitates micelle dissociation and fatty acid diffusion and the acidic microenvironment plays an important role in the intestinal uptake of oleic acid [11]. How is this acidic microenvironment produced? It may be maintained by the activity of a Na^+/H^+ exchanger or antiporter [15]. These antiporters have been described in the BBM and in the basolateral membrane of the enterocyte [16,17]. The importance of luminal Na^+ in the uptake of lipids is uncertain, and although fatty acids may influence the uptake of Na^+, the role of the exchanger in the BBM (NHE-3) in fatty acid uptake is unknown.

Accordingly, this study was undertaken with adult rabbit and rat jejunal brush border membrane vesicles and with sheets of jejunum to test the hypothesis that the uptake of oleic acid is passive and is assisted by an acid microclimate maintained by the amiloride-inhibitable Na^+/H^+ exchanger in the brush border membrane (NHE-3).

2. Materials and methods

2.1. Reagents

D-[^{3}H]Glucose, [^{3}H]inulin and [^{14}C]- or [^{3}H]oleic acid (18:1) were purchased from Amersham (Canada). All other chemicals were purchased from Sigma (Canada) or Fisher (Canada), and were of the highest quality available.

2.2. Rabbit brush border membrane vesicle studies

The guiding principles in the care and use of laboratory animals, approved by the Canadian Federation of Biological Societies, were observed in the conduct of this study. Adult male New Zealand white rabbits weighing 2.5–3.0 kg were purchased from Van der Meer Rabbitry (Edmonton). The animals were sacrificed by anesthetic overdose (sodium pentobarbital, 800 mg/kg body weight). Approx. 80 cm of jejunum distal to the ligament of Treitz was quickly removed, and flushed three times each with 60 ml of ice-cold saline to remove visible mucus and debris.

Brush border membrane vesicles (BBMV) were prepared from rabbits at 4° C by a modification of previously published techniques [18,19], using the buffers described by Dudeja and coworkers [20]. The intestine was removed and placed on a pre-chilled glass plate moistened with saline, and the mesenteric fat was removed. The mucosa was exposed by cutting along the mesenteric border, and then gently blotting it with lint-free tissue to remove excess moisture. The mucosa was removed by gently scraping with two glass slides, and placed into pre-weighed vials containing 5 ml Isolation Buffer (300 mM D-mannitol, 5 mM Tris, pH 7.4) so that the wet weight of the mucosal scrapings could be determined. Mucosal scrapings were homogenized in 115 ml Isolation Buffer using a Brinkman Polytron® at setting '9' for 30 s. The homogenate was centrifuged at $500 \times g$ for 15 min (Beckman J2-21 high speed centrifuge with JA-14 rotor, Beckman Instruments, Palo Alto, CA). The supernatant was collected, sufficient 1 M $CaCl_2$ was added to yield a final concentration of 10 mM, and the solution was stirred on ice for 10 min. The supernatant was then centrifuged at $7500 \times g$ for 20 min using a JA-14 rotor. The resulting supernatant was centrifuged at $28\,000 \times g$ for 20 min using a JA-20 rotor. The pellet was resuspended in Resuspension Buffer at pH 5.5 (145 mM KCl, 25 mM Mes, 4.6 mM Tris, 2 mM NaCl) or at pH 7.4 (144.5 mM KCl, 5 mM Mes, 13 mM Tris, 13 mM Hepes, 2 mM NaCl) according to the desired final pH, and the pellet was homogenized with the Polytron® at setting '8' for 30 s. The homogenate was centrifuged at $30\,000 \times g$ for 20 min. The final pellets were resuspended in Resuspension Buffer at the same pH as before, and homogenized with the Polytron® at setting '6' for 15 s. The presence of 2 mM NaCl in the Resuspension Buffer was necessary to equilibrate the Na^+ gradient caused by the unavoidable presence of the bile acid solubilizer (taurocholic acid, sodium salt (TC)) in the Lipid Transport Buffer. Although this was below the critical micellar concentration of TC, the 2 mM concentration was chosen since this did not cause measurable damage to the BBMV, as demonstrated by glucose time-course uptake studies performed in the presence of increasing concentrations of TC [21].

BBMV were freshly prepared on the day of the uptake experiments. The protein concentrations of the vesicle preparations were determined by the Hartree modification [22] of the method of Lowry [23], using bovine serum album in as a standard. Sucrase and alkaline phosphatase activities [24,25] of the final BBMV preparation were enriched at least 8-fold over their activities in the initial homogenates [21]. The BBMV preparations were previously shown to be relatively free of other subcellular membranes, as assessed by the lack of activity of Na^+/K^+-ATPase, glucose-6-phosphatase, β-glucuronidase, and by the lack of DNA contamination. The functional integrity of the BBMV was validated by the study of Na^+-dependent glucose uptake, as described below.

To determine if there was a protein-mediated step in oleic acid uptake, rabbit BBMV were treated with trypsin. In these studies, the pellet resulting from the centrifugation at $28\,000 \times g$ was resuspended in Resuspension Buffer pH

7.4, homogenized with the Polytron® at setting '8' for 15 s, divided into two tubes, and centrifuged again at 28 000 $\times g$ for 20 min. The resulting pellets were resuspended in either Resuspension Buffer pH 7.4 containing 0.025 g% of trypsin and 0.5 g% of chymotrypsin (trypsin-treated), or in Resuspension Buffer 7.4 (control). Each resuspended pellet was incubated for 5 min at room temperature. The BBMV were then diluted with 50 ml of Resuspension Buffer 7.4, and then centrifuged at 28 000 $\times g$ for 20 min. The pellets were resuspended in Resuspension Buffer at pH values ranging from 5.5 to 7.4 (different pH values were obtained by mixing proportional amounts of Resuspension Buffer 5.5 and 7.4). Resuspensions were then homogenized with Polytron® at setting '8' for 15 s and centrifuged at 28 000 $\times g$ for 20 min. From this point on, the pH of the Resuspension Buffer was kept constant. The resulting pellets were resuspended and homogenized with the Polytron® at setting '8' for 30 s. The homogenate was centrifuged at 30 000 $\times g$ for 20 min. The final pellets were resuspended in Resuspension Buffer, and homogenized with the Polytron® at setting '6' for 15 s. The final BBMV suspensions were allowed to equilibrate for 90 min at 4° C and then for 20 min at room temperature (20° C) prior to performing the uptake studies.

To determine the role of Na^+ gradients, H^+ gradients, or of opposing Na^+/H^+ gradients (inward Na^+ gradient and outward H^+ gradient) on oleic acid uptake into rabbit BBMV, the concentrations of Na^+ and/or H^+ were varied inside and outside of the vesicles, by varying the pH of the Resuspension Buffer (H^+ concentration inside BBMV) or by varying the Na^+ concentration in the Lipid Transport Buffer (Na^+ concentration outside BBMV).

To determine the effect of mucin on the rabbit BBMV uptake of oleic acid, mucin was added to the external buffer. A Mucin Stock Solution was prepared by homogenizing mucin (Sigma) in Resuspension Buffer at the desired pH with the Polytron® at setting '15' for 1 min. The addition of mucin to the Resuspension Buffer did not alter its pH. Following the 90 min equilibration period, the BBMV were mixed with the Mucin Stock Solution in different proportions to obtain various concentrations of mucin exterior to the BBMV. Both mucin and BBMV were at the same pH, and an additional 30 min equilibration was used before the uptake experiments were performed.

To determine the effect of amiloride (an inhibitor of the Na^+/H^+ exchanger) on the uptake of oleic acid, studies were performed either with sheets of rat intestine and with rabbit BBMV. To determine the concentration of amiloride (Sigma) which could produce 50% inhibition of oleic acid uptake (K_i), the effect of six different concentrations of amiloride on the uptake of 30 μM oleic acid was examined using rabbit BBMV. For the amiloride inhibition experiments, stock solutions with different concentrations of amiloride were made by solubilizing appropriated amounts of amiloride with Resuspension Buffer pH 7.4 or 5.5, using stirring and gentle heating. After allowing 90 min for equilibration, BBMV were mixed with amiloride solutions in a proportion of 1 part of amiloride to 4 parts of BBMV. An additional 30 min equilibration was allowed before the uptake experiments were performed. Studies performed to determine the influence of amiloride on oleic acid uptake into rabbit BBMV were conducted in the presence and in the absence of opposing Na^+/H^+ gradients.

2.3. Rat brush border membrane vesicle studies

Male Sprague-Dawley rats weighing 350–400 g were killed by the intraperitoneal injection of sodium pentobarbitol (800 mg/kg body weight). Approx. 60 cm of the proximal small intestine (jejunum) was removed and irrigated with 60 ml ice-cold saline (0.9% NaCl). Six rats were used for each experiment. Each intestine was cut into seven 6 cm length segments. Segments were inverted onto plastic pegs which were placed into 600 ml of ice-cold oxygenated 2.5 mM EDTA buffer (2.5 mM EDTA, 300 mM Mannitol, 10 mM Tris-HCl, 5 mM Tris, pH 7.4), and were fitted into a plexiglass pegboard. Intestinal villus cells were released by mechanical vibration of the apparatus at 1600 rpm for 30 min [26]. The cell suspension was centrifuged at 15 300 $\times g$ for 15 min, and the resultant pellet of cellular components was resuspended in ice-cold Isolation Buffer, homogenized using a Polytron® at setting '9' for 15 s, and then centrifuged for 20 min at 600 $\times g$. Sufficient 1 M $CaCl_2$ was added to the supernatant to yield a 10 mM concentration. The supernatant was stirred on ice for 10 min to aggregate the intracellular contaminants, and was centrifuged at 3000 $\times g$ for 15 min. The supernatant was centrifuged for 20 min at 29 800 $\times g$. The pellet was resuspended in Resuspension Buffer pH 7.4, homogenized in the Polytron® at setting '9' for 30 s, and was centrifuged at 19 500 $\times g$ for 20 min. The final pellet was resuspended in the appropriate amount of Resuspension Buffer at pH 7.4.

2.4. Probe preparation

Glucose

To demonstrate that the BBMV were sealed and functionally intact, validation studies were performed with D-[^{3}H]glucose. Glucose Stock Solution (198 μM) was prepared by dissolving unlabelled D-glucose in Glucose Transport Buffer (1 mM $MgCl_2$, 2 mM $CaCl_2$, 141 mM NaCl, 5 mM Mes, 13 mM Tris, 13 mM Hepes, pH 7.4). D-[^{3}H]Glucose was dried down under nitrogen, reconstituted in Glucose Transport Buffer, and mixed with Glucose Stock Solution. The final specific activity of the probe solution was 55 μCi/mmol.

These validation studies demonstrated the expected Na^+-dependent D-glucose overshoot, with peak values in rabbit BBMV of about 800 pmol/mg protein at 1 min for

a glucose concentration of 100 μM, with a rapid initial rate and a decline to equilibrium values of approx. 200 nmol/mg protein at 15 min incubation (Fig. 1). Similar time-course studies were performed with 100 μM D-glucose with the BBMV H^+ loaded with 2% or 3% mucin on the exterior of the BBMV, or with 125 μM amiloride on the exterior of the BBMV. These treatments had no effect on the uptake of glucose (data not shown). The glucose overshoot was not influenced by 2 mM TC, although further increases in the concentration of TC reduced the Na^+-dependent glucose overshoot [21]. Trypsin treatment of the BBMV inhibited ($P < 0.05$) the uptake of glucose, with an approx. 40% decline in the uptake of 100 μM D-glucose at 1 min incubation (data not shown).

Oleic acid

A 600 μM oleic acid Stock Solution was prepared by dissolving unlabelled oleic acid in 25 ml of chloroform. Aliquots of oleic acid Stock Solution were dried down under nitrogen and stored at $-80°$ C until required. The ^{14}C-labelled oleic acid was added to reconstituted oleic acid Stock Solution and dried down together under nitrogen before reconstitution in Lipid Transport Buffer with sodium (1 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM NaCl, 115 mM KCl, 5 mM Mes, 13 mM Tris, 13 mM Hepes, 2 mM TC, pH 7.4), or Lipid Transport Buffer without sodium (1 mM $MgCl_2$, 2 mM $CaCl_2$, 140 mM KCl, 5 mM Mes, 13 mM Tris, 13 mM Hepes, 2 mM TC, pH 7.4). The probe solution was then sonicated until clear. The final specific activity was 7 μCi/mmol.

2.5. Brush border membrane vesicle (BBMV) uptake studies

Rabbit and rat BBMV uptake of substrate was performed at room temperature (20° C) using the rapid filtration method described by Hopfer and coworkers [27] and by Maenz and Cheeseman [28], and using the buffers described by Dudeja and coworkers [20]. For each incubation, 80 μl probe and 20 μl BBMV were pipetted into a 16 × 100 mm polystyrene test tube, ensuring that the two drops did not mix. To start the uptake incubation, the tube was placed in a vortex for 2 s, and was then allowed to stand for the duration of the incubation time. For the oleic acid uptake studies, the reaction was stopped at preset times using 1.25 ml ice-cold Lipid Stop Buffer (155 mM KCl, 5 mM Tris, 5 mM Hepes, 0.2 mM phloretin (prepared fresh on day of experiment)). For the studies with albumin, 0.05% albumin was added to the Lipid Stop Buffer. For the water-lysis experiments, ice-cold deionized water was substituted for Stop Buffer. For the glucose uptake studies, the reaction was stopped with 1.25 ml of Glucose Stop Buffer (155 mM NaCl, 5 mM Tris, 5 mM Hepes, 0.8 mM phlorizin (prepared fresh on day of experiment)). 1.0 ml of the stopped incubation mixture was rapidly filtered through 0.45 μ pre-saturated cellulose

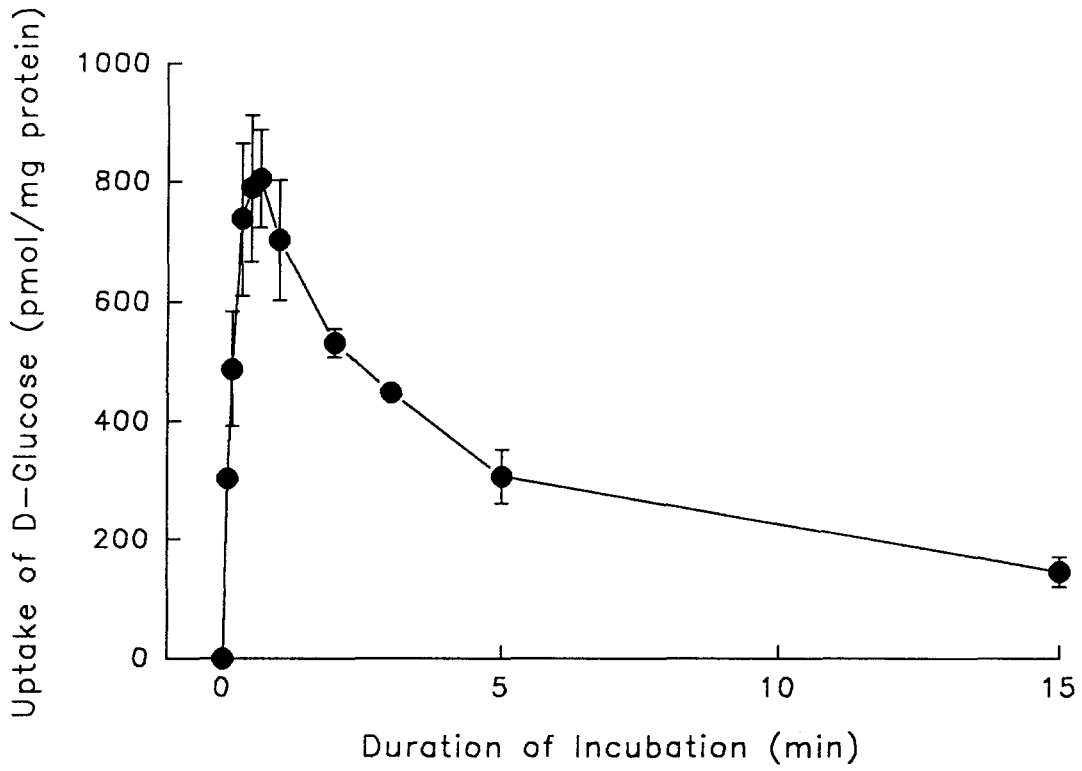

Fig. 1. Time-course of Na^+-dependent D-glucose uptake into rabbit jejunal brush border membrane vesicles (BBMV) in the presence of an inwardly-directed 140 mM Na^+ gradient. Results are presented as mean values ± S.E.

acetate filters (Millipore® Micron Sep, Fisher) under vacuum on an Amicon Filtration Manifold VFMI, rinsed twice with 3 ml of ice-cold Stop Buffer, and transferred to scintillation vials. Pre-saturation of the filters (necessary to reduce nonspecific binding of labelled substrate to the filters) was performed by immersing the filters for at least 20 min in 1.0 mM unlabelled glucose in Glucose Transport Buffer (for glucose uptake studies) or in 500 μM unlabelled oleic acid in Lipid Transport Buffer (for oleic acid uptake studies).

After drying the filters for 20 min at 55° C, HP Ready-Solv Scintillant® (Beckman) was added, the vials were vortexed and then counted on a Beckman LS 9800 liquid scintillation counter (Beckman, Palo Alto, CA). Blanks were prepared in the same way as the samples, except that the BBMV preparation was substituted with Resuspension Buffer.

To determine the role of Na^+ gradients, H^+ gradients, or of opposing Na^+/H^+ gradients (inward Na^+ gradient and outward H^+ gradient) on oleic acid uptake into rabbit BBMV, the concentrations of Na^+ and/or H^+ were varied inside and outside of the vesicles, by varying the pH of the Resuspension Buffer (H^+ concentration inside BBMV) or by varying the Na^+ concentration in the Lipid Transport Buffer (Na^+ concentration outside BBMV).

2.6. *In vitro uptake of oleic acid using sheets of rat jejunum*

Male Sprague-Dawley rats weighing 400–500 g were killed by the intraperitoneal injection of sodium pentobarbitol (800 mg/kg body weight). Approx. 40 cm of jejunum was removed rapidly through a mid line incision, and was gently washed with 50 ml of ice-cold normal saline (0.9% NaCl). The jejunum was placed on an ice-chilled tray with saline-soleic acidked filter paper, and the mesenteric fat was gently removed. The jejunum was opened along the mesenteric border, was cut into approx. 20×1 cm^2 sections starting at the proximal end of the jejunum, and was quickly placed in oxygenated ice-cold saline. The jejunal sections were then carefully mounted into the transport chambers [2]. These transport chambers were incubated for 15 min in the oxygenated Pre-Incubation Buffer (1 mM $MgCl_2$, 125 mM NaCl, 20 mM D-glucose, 20 mM KCl, 5 mM Mes, 13 mM Tris, 13 mM Hepes, pH 7.4 at 37° C). In order to vary the effective resistance of the intestinal unstirred water layer, the bulk phase was stirred at 100, 200, 400 or 600 rpm [2]. The chambers were then transferred to beakers containing the stirred Incubation Buffer, and were incubated for 6 min at 37° C. The Incubation Buffer was prepared by mixing appropriate amounts of both unlabelled and ^{14}C-labelled oleic acid in modified Lipid Transport Buffer pH 7.4 (1 mM $MgCl_2$, 2 mM $CaCl_2$, 100 mM NaCl, 20 mM D-glucose, 20 mM sodium taurocholate, 5 mM Mes, 13 mM Tris, 13 mM Hepes pH 7.4) to yield a final concentration of 100 μM oleic acid. The solution was gassed with 100% oxygen for 2 h at 37° C, and if necessary the pH was readjusted to 7.4. [^{3}H]Inulin (used as a nonabsorbable volume marker) was then added to the solution to correct for adherent mucosal fluid. The uptake chamber was removed from the Incubation Buffer and immersed in ice-cold saline for 10 s. The area of the intestine exposed to the uptake buffer was cut away from the aperture in the transport chamber, placed into preweighed scintillation vials, and dried overnight in a 55° C oven. The tissues were then saponified in NaOH, HP ReadySolv Scintillant® (Beckman) was added, and tissue radioleic acid activity was counted in a Beckman LS 9800 liquid scintillation counter (Beckman, Palo Alto, CA).

To determine the effect of amiloride on the uptake of oleic acid in rat, studies were performed with a fixed concentration of amiloride (1 mM) and a fixed concentration of oleic acid (100 μM), using sheets of rat intestine in vitro, in which the amiloride was added to either the Pre-Incubation Buffer, Incubation Buffer, or both.

2.7. *Data analysis and expression of results*

The data represent at least three experimental days for each figure. On each experimental day, every uptake point was measured in triplicate. Uptake was the net radioactivity after subtraction of non-specific radioactivity bound to the filters in the absence of BBMV (blanks). Uptake was expressed in pmol/mg protein for glucose and in nmol/mg protein for oleic acid. A time-course (rate of uptake versus time) was used to determine the linear range of the initial rate of uptake and the time required to reach equilibrium.

Only fatty acid monomers contribute to absorption [3]. However, for a constant bile acid concentration, the monomeric activity of the fatty acid is directly proportional to the total concentration of fatty acids in the medium [29]. For this reason, and for greater convenience, the fatty acid concentrations are expressed as total concentrations and not as monomeric concentrations.

In most studies, a paired t-test was used to test the statistical significance of differences between the means of two groups. A value of $P < 0.05$ was accepted as statistical significance. For the time-course experiments, the area under the curve was calculated by the mathematical integration of the formula $F = (A \cdot B)/(A + B)$, where F is the function, A is the mean value of uptake at the plateau, and B is the time required to reach one half of the plateau [30]. Where error bars do not appear in the figures, the size of the data symbol exceeds the value of the means ± S.E.

3. Results

3.1. *Effect of trypsin*

The rabbit jejunal brush border membrane vesicle (BBMV) uptake of 25 μM oleic acid solubilized in 2 mM taurocholic acid (TC) was studied as a function of time.

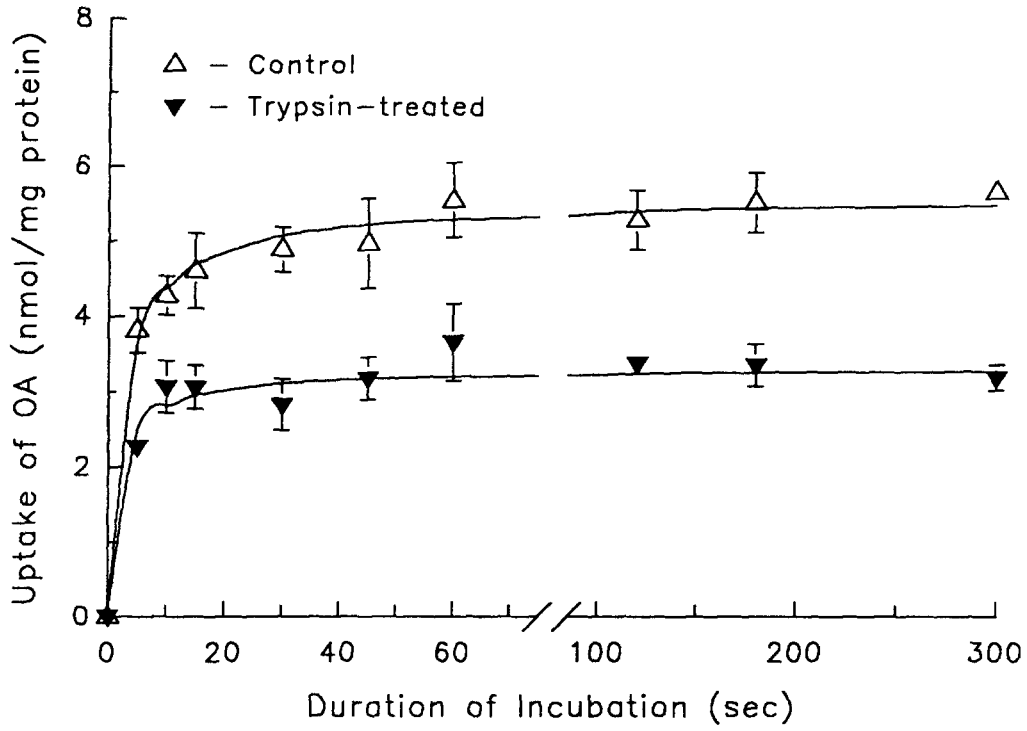

Fig. 2. Effect of trypsin on the time course of oleic acid uptake into rabbit jejunal brush border membrane vesicles (BBMV).

There was an initial rapid rate of uptake of oleic acid, which then reached a plateau at approx. 30 s incubation, with no overshoot observed (Fig. 2). Treatment of the BBMV with trypsin resulted in an approx. 40% reduction ($P < 0.05$) in the uptake of oleic acid when studied at varying durations of incubation (Fig. 2), and at varying concentrations of oleic acid incubated for 5 s (data not shown).

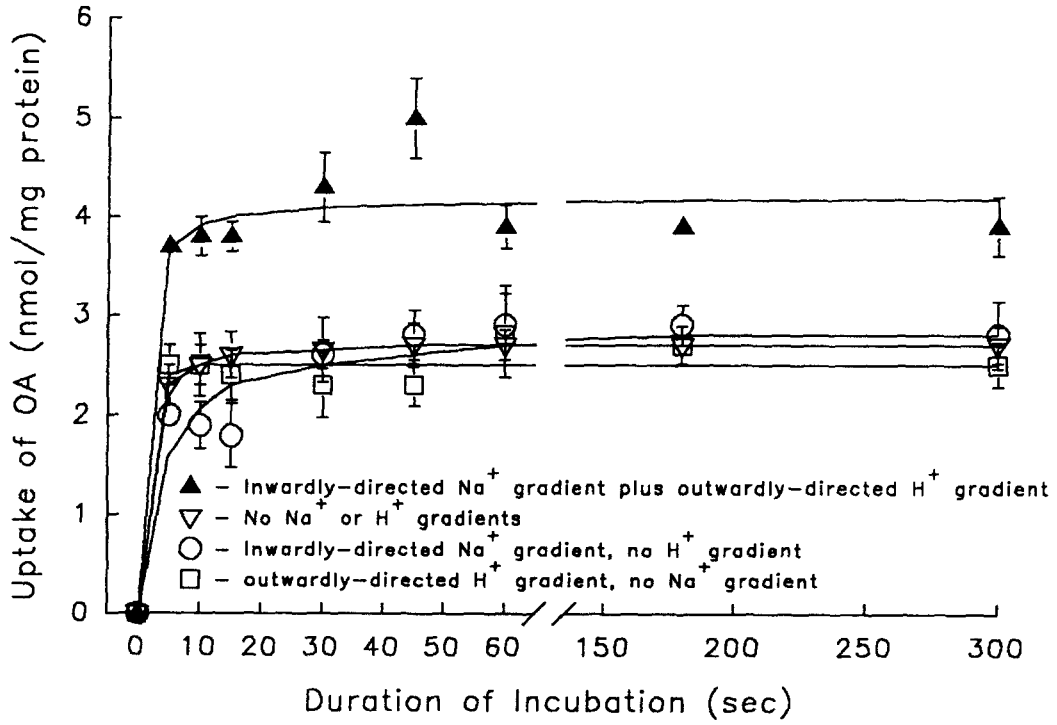

Fig. 3. Effect of Na^+ and/or H^+ gradients on the time-course of oleic acid oleic acid uptake into rabbit jejunal brush border membrane vesicles (BBMV).

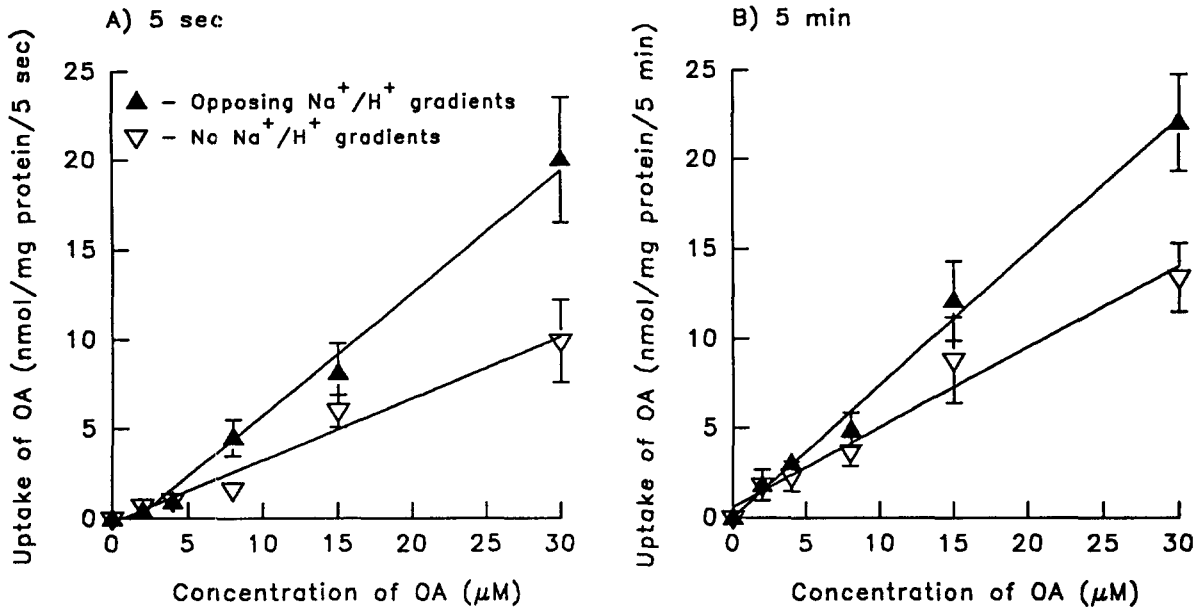

Fig. 4. Effect of opposing Na^+ and H^+ gradients on rabbit jejunal brush border membrane vesicles (BBMV) uptake of increasing concentrations of oleic acid.

3.2. Time-course

The mixture of 80 μl Transport Buffer pH 7.4 with 20 μl equilibrated BBMV pH 5.5 resulted in an external pH of 7.2, thereby creating an outwardly-directed H^+ gradient and inwardly-directed Na^+ gradient. There was an initial rapid rate of uptake of 15 μM oleic acid, which then reached a plateau (Fig. 3). The rate of uptake of oleic acid was approx. 40% higher ($P < 0.05$) in the presence of opposing Na^+/H^+ gradients (inwardly-directed Na^+ gra-

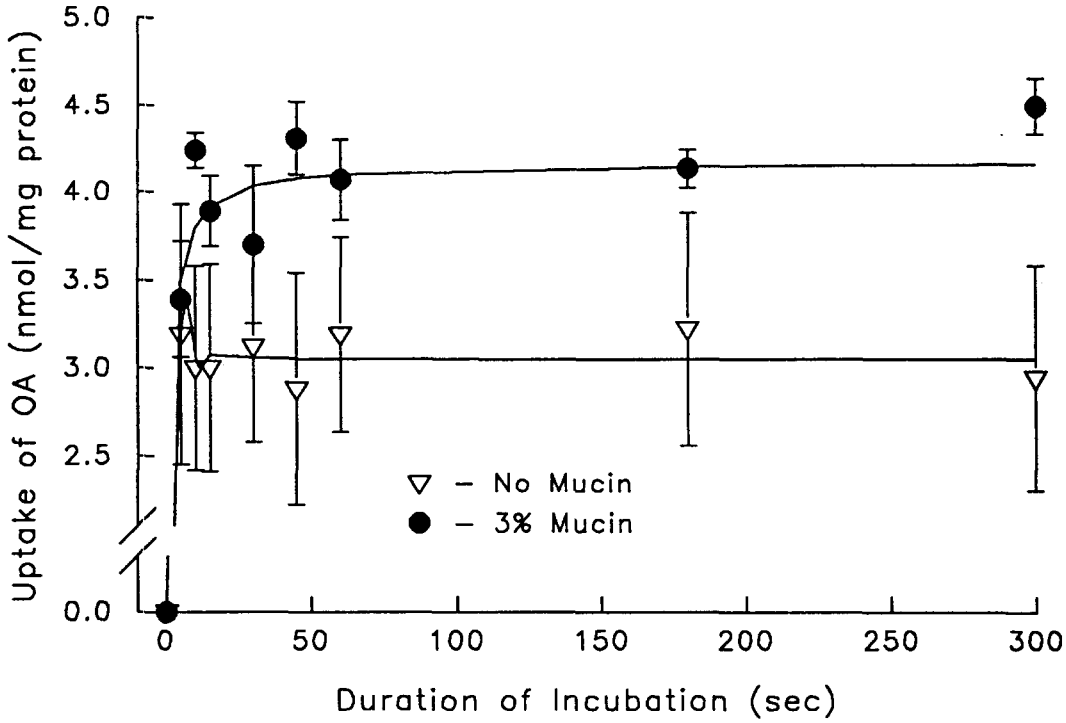

Fig. 5. Effect of mucin on the time-course of oleic acid uptake into rabbit jejunal brush border membrane vesicles (BBMV) in the presence of opposing Na^+ and H^+ gradients. BBMV were preloleic acidded with pH 5.5 Resuspension Buffer and then either no mucin or 3% mucin added prior to 5–300 s incubation at room temperature with 15 μM oleic acid in Lipid Transport Buffer with sodium. Uptake was stopped by the addition of Lipid Stop Buffer.

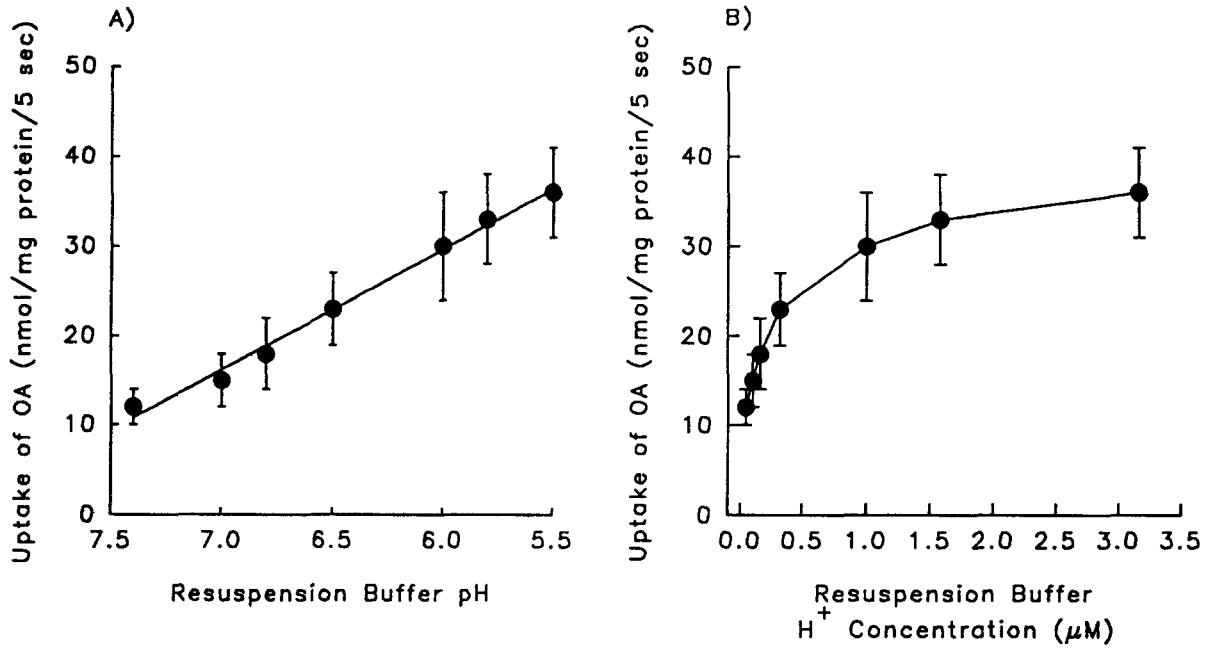

Fig. 6. Effect of Resuspension Buffer pH (A) and H^+ concentration (B) on the uptake of oleic acid into rabbit jejunal brush border membrane vesicles (BBMV) in the presence of a 20 mM inwardly-directed Na^+ gradient.

dient plus an outwardly-directed H^+ gradient), as compared with the three other test conditions. No difference in oleic acid uptake was observed when there was no Na^+ or H^+ gradient, no H^+ gradient in the presence of an inwardly-directed Na^+ gradient, or no Na^+ gradient in the presence of an outwardly-directed H^+ gradient. The up-

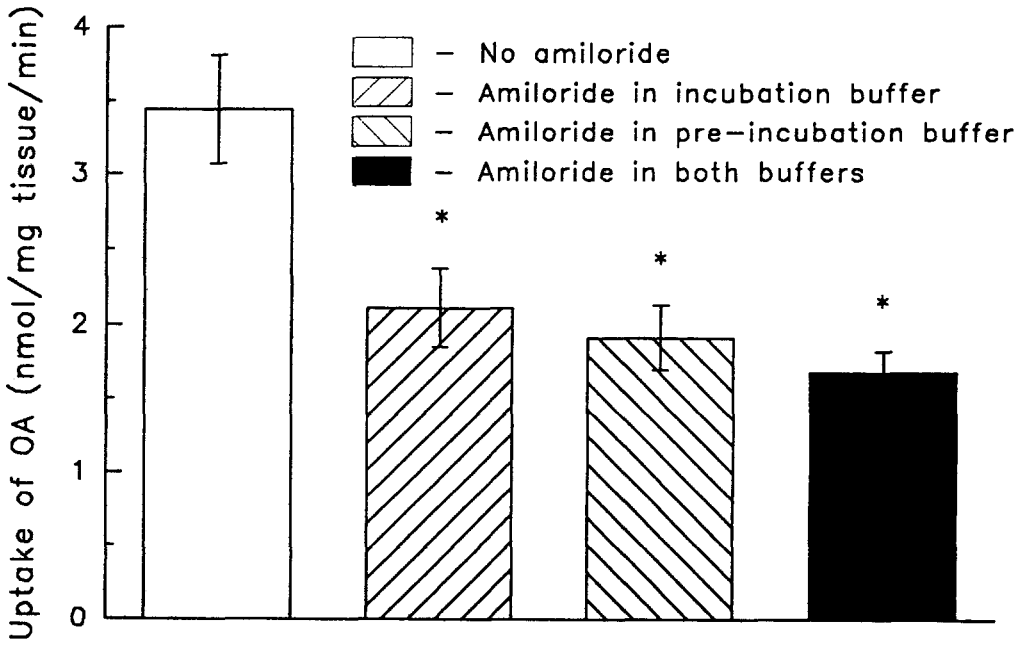

Fig. 7. Effect of amiloride on the in vitro uptake of oleic acid into sheets of rat jejunum. Rat jejunal sheets were pre-incubated 15 min in the absence or presence of 1 mM amiloride in oxygenated Pre-incubation Buffer prior to 6 min incubation with 100 μM oleic acid in the absence or presence of 1 mM amiloride 1 mM amiloride in oxygenated Incubation Buffer. The bulk phase of both Pre-Incubation and Incubation Buffers was stirred at 100 rpm. Uptake was terminated by immersion of the jejunal sheets in ice cold saline. An asterisk (*) indicates that the difference in oleic acid uptake of amiloride-treated versus control (no amiloride) was statistically significant ($P < 0.05$).

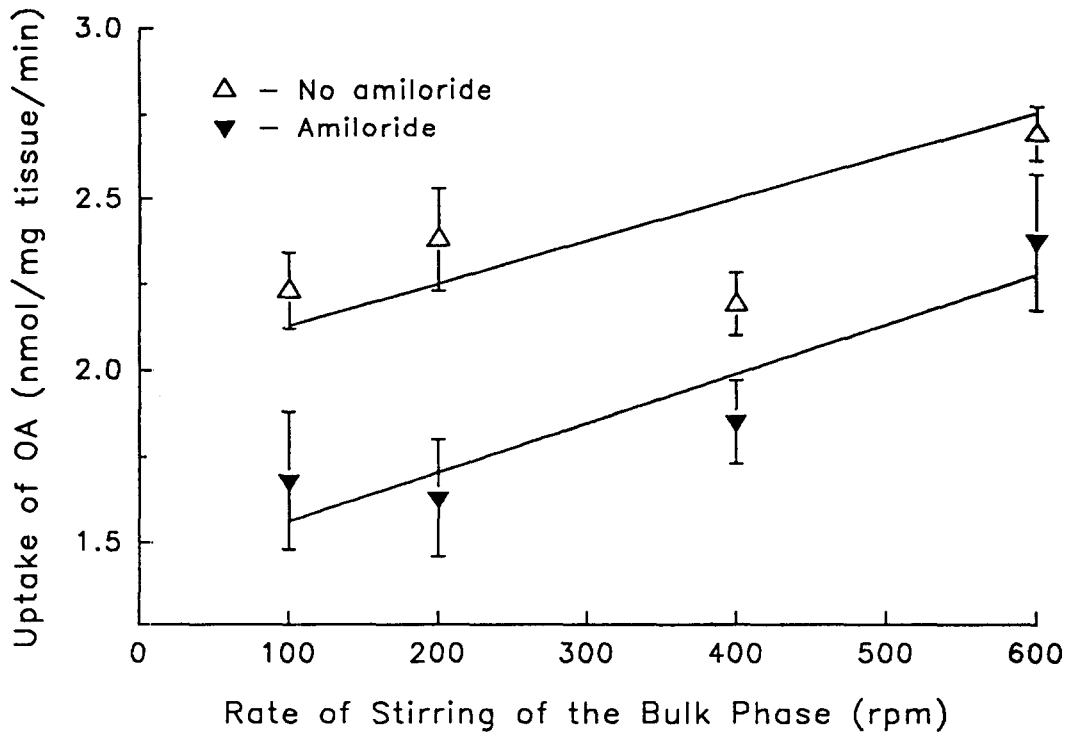

Fig. 8. Effect of amiloride on the in vitro uptake of oleic acid into sheets of rat jejunum with increased rates of stirring of the bulk phase. Rat jejunal sheets were pre-incubated 15 min in oxygenated Pre-Incubation Buffer at 37° C prior to 6 min incubation with 100 μM oleic acid in oxygenated Incubation Buffer at 37° C. Amiloride-treated rat jejunal sheets were exposed to 1 mM amiloride in both Pre-Incubation and Incubation Buffers, while control rat jejunal sheets were not. The bulk phase of both Pre-Incubation and Incubation Buffers was stirred at 100, 200, 400 or 600 rpm. Uptake was terminated by immersion of the jejunal sheets in ice cold saline. The linear regression equations for the lines were: for the control, $y = 2.1 + 0.0007x$ ($r = 0.694$); and for the amiloride-treated group, $y = 1.4 + 0.0014x$ ($r = 0.927$). The y-intercepts of the amiloride-treated versus control oleic acid uptake were significantly different ($P = 0.010$), but the slopes were not significantly different from each other ($P > 0.05$).

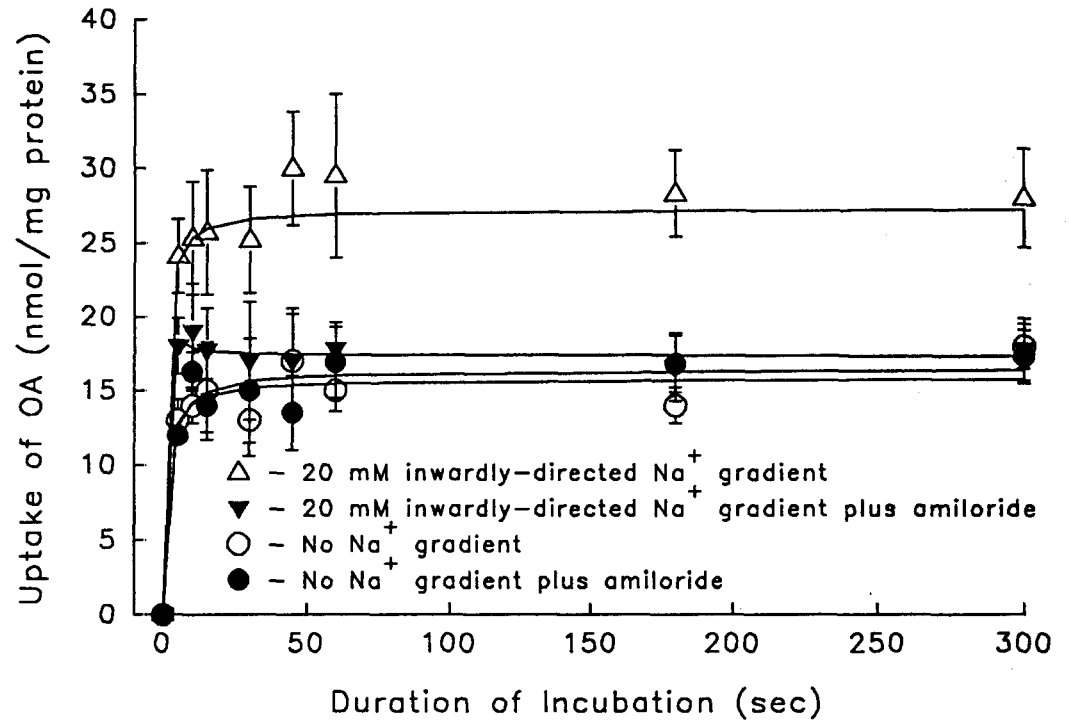

Fig. 9. Effect of amiloride on the time-course of oleic acid uptake into rabbit jejunal brush border membrane vesicles (BBMV) in the presence or absence of an inwardly-directed 20 mM Na^+ gradient.

take of the fatty acids 8:0, 10:0, 12:0, 14:0, 16:0 was also greater in the presence than in the absence of opposing Na^+/H^+ gradients (data not shown).

3.3. Effect of opposing Na^+/H^+ gradients

Oleic acid uptake was greater in the presence than in the absence of opposing Na^+/H^+ gradients over a range of concentrations of oleic acid, and both at 5 s (Fig. 4A) and at 5 min incubations (Fig. 4B).

3.4. Effect of mucin

The addition of 3% mucin to the solution bathing the exterior of the BBMV had no effect on the uptake of 15 μM oleic acid when there was no Na^+ gradient (data not shown). In contrast, in the presence of opposing Na^+ and H^+ gradients, adding 3% mucin increased oleic acid uptake ($P < 0.05$) from 5–300 s incubations (Fig. 5). A smaller increase in oleic acid uptake in the presence of a Na^+ gradient was observed with 2% mucin (data not shown).

3.5. Influence of resuspension solution pH

Uptake of 30 μM oleic acid into rabbit BBMV at 5 s was examined with seven values of resuspension solution pH in the presence of a 20 mM inwardly-directed Na^+ gradient. There was a linear relationship between decreasing values of resuspension pH and oleic acid uptake (Fig. 6A), and an increasing curvilinear relationship was noted between resuspension H^+ concentration and oleic acid uptake (Fig. 6B).

3.6. Effect of amiloride

The effect of 1 mM amiloride on the in vitro uptake of oleic acid was examined with sheets of rat jejunum, with the bulk phase stirred at 100 rpm. An approx. 40% reduction in the uptake of 100 μM oleic acid was observed when the amiloride was present in the Incubation Buffer, in the Pre-Incubation Buffer, or in both Buffers, when compared with oleic acid uptake in the absence of amiloride ($P < 0.001$) (Fig. 7). This inhibitory effect of 1 mM amiloride on the in vitro uptake of oleic acid into sheets of rat jejunum was also present when the rate of stirring of the bulk phase was varied (Fig. 8) to alter the effective resistance of the intestinal unstirred layer [2]. The values of the slopes of the lines was similar in the control and in the amiloride-treated groups, but the value of the y-axis intercept was significantly lower ($P < 0.01$) in the amiloride-treated rats. Thus, amiloride reduces the uptake of oleic acid when the effective resistance of the unstirred layer is low (600 rpm) or high (100 rpm).

Next, the effect of 100 μM amiloride on 30 μM oleic acid uptake was examined in rabbit jejunal BBMV. Over the 5–300 s incubation interval, amiloride reduced oleic acid uptake in the presence but not in the absence of

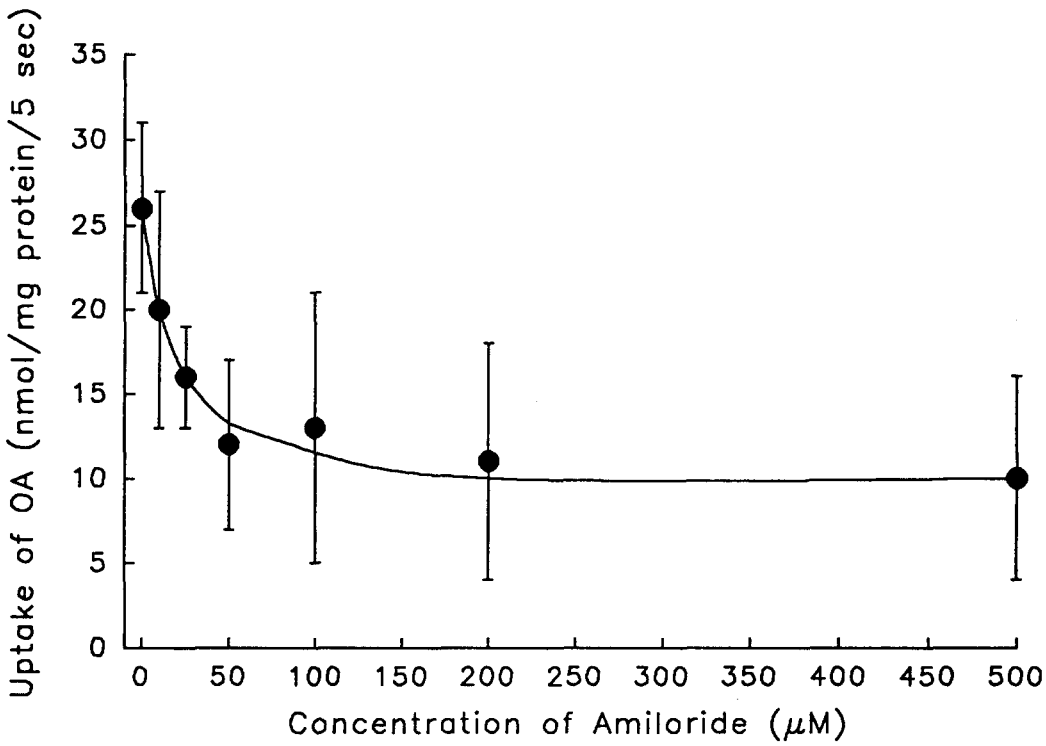

Fig. 10. Effect of increasing concentrations of amiloride on oleic acid uptake into H^+-loleic acidded rabbit jejunal brush border membrane vesicles (BBMV) in the presence of an inwardly-directed 20 mM Na^+ gradient.

opposing Na^+/H^+ gradients (Fig. 9). When increasing concentrations of amiloride were added to rabbit BBMV in the presence of opposing Na^+/H^+ gradients, there was a progressive decline in the uptake of 30 μM oleic acid (Fig. 10), with a K_i of 36 ± 9 μM. Amiloride had no effect on the uptake of glucose into rabbit jejunal BBMV (data not shown).

4. Discussion

Fatty acid uptake across the intestinal brush border membrane (BBM) is thought to be by a process of passive diffusion [1–3,31]. New evidence suggests a role for a specific protein in the BBM capable of mediating fatty acid uptake [5]. Passive permeation and protein-mediated transport are not necessarily mutually exclusive processes. Instead, both may occur concurrently, and the relative importance of each may vary under different physiological conditions. The physical properties of lipids influence their solubility in the bile acid micelle and in the luminal aqueous phase, their partitioning into the outer leaflet of the BBM, their equilibration with the inner leaflet of the BBM, and their release from the inner leaflet of the BBM and subsequent transfer to the cytoplasm. The physical properties of lipids may be modulated by the different values of pH in the intestinal lumen, in the intestinal acidic microclimate, and in the enterocyte cytoplasm [6,13,32]. This in turn will modify lipid uptake.

The pH in the intestinal lumen is important for lipid uptake. The pH may alter lipid uptake both by altering the bile acid critical micellar concentration (CMC), as well as by altering the relationship between the non-protonated and protonated fatty acids (FA^- and FAH, respectively). The presence of an acidic microclimate exterior to the BBM has been demonstrated [6,8,33]. The pH of the acidic microclimate is maintained by the activity of the BBM Na^+/H^+ exchanger (NHE-3), which exchanges H^+ metabolically generated in the cytoplasm for luminal Na^+ [15]. The pH in the microclimate is about 5.5 in the fasting state [6,8]. Changes in the microclimate pH are positively correlated to fat malabsorption in patients with tropical sprue [7]. Furthermore, the more acidic microclimate seen in diabetic rats and in rats with short bowel syndrome is associated with an increase in Na^+/H^+ exchanger activity [23,40]. Epidermal growth-factor increases the NHE-3 activity in the intestine, and decreases the pH in the microclimate [34]. NHE-3 may be involved in the ileal absorption of NaCl [35]. It is interesting to note that the intestinal uptake of fatty acid under these conditions is also increased. The generation of an acidic microclimate requires the means by which the secreted H^+ may remain concentrated near the BBM [10]. Intestinal mucus may act as a trap for H^+ extruded by the NHE-3 [14,36]. In brush border membrane vesicles (BBMV), the presence of an outwardly-directed H^+ gradient and an inwardly-directed Na^+ gradient has been observed to activate the exchanger [20,37,38]. The activation of NHE-3 may indirectly affect lipid uptake into the BBMV. In the present study, the presence of an inwardly-directed Na^+ gradient alters lipid uptake into BBMV (Figs. 3 and 4), and this effect was directly related to changes in the intravesicular H^+ concentration (Fig. 6). These data therefore support the hypothesis that the extrusion of H^+ in exchange for Na^+ is an important step for oleic acid uptake.

Na^+-coupled co-transport is the major driving force for glucose and amino acid absorption in the intestine [39]. Intestinal Na^+-dependent fatty acid absorption has been demonstrated using in vivo perfusion, and with the in vitro use of enterocytes and intestinal sheets, but not with BBMV [5,21,40]. This dependence on Na^+ is not known to influence the CMC or the partitioning of fatty acid into lipid membranes. The failure of some previous studies to demonstrate an effect of Na^+ in the intestinal lumen on fatty acid uptake may be explained by the observation in our study that Na^+ dependence was only demonstrable under the special condition of simultaneously opposing Na^+/H^+ gradients (Fig. 3). Under opposing Na^+ an H^+ gradient conditions as well as under no-gradient conditions, a linear relationship was observed with increasing concentration of fatty acid (Fig. 4).

The effect of amiloride, a known inhibitor of the Na^+/H^+ exchangers, was tested in rat jejunal sheets and in rabbit BBMV. The presence of 1 mM of amiloride in the incubation buffer, in the pre-incubation buffer, or in both the incubation and pre-incubation buffers, reduces oleic acid uptake into rat jejunal sheets (Figs. 7 and 8). In rabbit BBMV, lower concentrations of amiloride reduced the uptake of oleic acid only in the presence of opposing Na^+/H^+ gradients (Fig. 9), and the addition of increasing concentrations of amiloride to the outside of the BBMV inhibits oleic acid uptake in a dose-dependent relationship (Fig. 10). The K_i for amiloride inhibition of oleic acid uptake into rabbit BBMV was 36 μM, a lower value than that reported for inhibition of the exchanger in the rat intestine [37]. This difference may be due to a species-related sensitivity to amiloride, or due to variations in the intestinal preparations used to examine uptake. This inhibitory effect of amiloride could only be demonstrated under opposing Na^+/H^+ gradients (Fig. 9). Tse and coworkers cloned three rabbit ileal Na^+/H^+ exchangers, and NHE-3 is thought to be in the BBM [41,42]. We propose that this simultaneously opposing Na^+/H^+ gradient effect on lipid uptake is due to an action of the BBM NHE-3, because: (1) oleic acid uptake into rat intestinal sheets and rabbit BBMV is inhibited by amiloride in a dose-dependent manner over a range of concentrations; (2) amiloride affects oleic acid uptake into rabbit BBMV only in the presence of simultaneous Na^+/H^+ gradients; (3) the half-inhibitory (K_i) concentration of amiloride observed in this study (36 μM) is comparable to previously reported values for the reduction of Na^+ uptake in the presence of an

outwardly directed H^+ gradient; (4) when the Na^+ concentration is kept constant (20 mM), a curvilinear relationship was observed between the intravesicular H^+ concentration and oleic acid uptake (Fig. 6); suggesting saturation of oleic acid transport depending upon the intravesicular concentration of H^+, and also suggests that the intravesicular concentration of H^+ may be the rate-limiting step in passive lipid permeation through BBM; and (5) the presence of amiloride exterior to the BBMV affected oleic acid uptake, but did not decrease the Na^+-dependent D-glucose overshoot (data not shown), suggesting that the presence of amiloride exterior to the BBMV exerts its effect on lipid uptake by blocking NHE-3 and not by damaging the integrity of the BBMV. Thus, we propose that the influence of simultaneously opposing Na^+/H^+ gradients on oleic acid uptake into sheets of rat jejunum and into rabbit BBMV is due to an effect on the BBM Na^+/H^+ exchanger, NHE-3.

The presence of a mucus layer exterior to the BBMV also appears to be important for oleic acid uptake into BBMV, since the effect of opposing Na^+/H^+ gradients on uptake was more pronounced in the presence than in the absence of mucin (Fig. 5). We did not determine the pH in the mucin layer [14], so it is possible that the pH in regions adjacent to the NHE-3 reaches values even lower than the average pH in the microclimate, thereby further facilitating permeation of oleic acid across the BBM. The mucin was mixed in the buffer, so that the mucin was present not just in the aqueous layer adjacent to the brush border membrane vesicles. The mucus would be expected to impair the diffusion of the oleic acid through the unstirred water layer, and thereby reduce uptake. This did not occur. The enhancement rather than a reduction in uptake of oleic acid in the presence of mucin supports the proposal for the presence of an H^+ trap. Further support for this suggestion, rather than the effect of mucin being non-specific, comes from the finding that this increase in oleic acid uptake was only seen in the presence of inward Na^+ and outward H^+ gradients. Therefore, we propose that the mucus acts primarily as a trap for H^+ which has been extruded by NHE-3.

Increasing the rate of stirring of the bulk phase reduces the effective resistance of the intestinal unstirred water layer [2,40]. The persistence of the inhibitory effect of amiloride on oleic acid uptake into sheets of rat jejunum accomplished by varying the rate of stirring the bulk phase (Fig. 8) indicates that the effect of mucus is not dissipated by changing the effective resistance of the unstirred water layer, and that the effect of amiloride is independent of an effect of stirring. It is likely that the acid microclimate adjacent to the brush border membrane is not removed by gently stirring the bulk phase, and is relatively independent of unstirred layer resistance.

The activation of the Na^+/H^+ exchanger mediates net Na^+ and H^+ transport [43]. When the transmembrane concentration gradient for Na^+ is thermodynamically balanced by an equivalent but oppositive H^+ gradient (i.e., $[Na^+]_0/[Na^+]_i = [H^+]_0/[H^+]_i$), no net movement of these ions occurs [44]. The only driving force is the combined gradients of Na^+ and H^+ in opposite directions, without intervention of energy from metabolic reactions [43]. Normally, during the absorptive process the concentration of Na^+ is higher in the intestinal lumen than in the enterocytes ($[Na^+]_0 > [Na^+]_i$). The normal intracellular pH of the enterocytes is around 7 [45]. Under these conditions, the exchangers would have little effect of the electroneutral absorption of Na^+, since $[H^+]_0 > [H^+]_i$ (71). Fatty acid absorption may counterbalance that situation by permeating H^+ across the BBM by way of the preferred transport of the protonated fatty acids, thereby creating a region with high concentration of H^+ on the cytoplasmic side of the BBM. This would result in a favourable condition for the electroneutral absorption of Na^+ [46]. The 'recycling' of the H^+ transport during oleic acid absorption, in exchange for Na^+, could explain the indirect dependence of uptake on Na^+: withdrawal of Na^+ from the bath solution, substitution of Na^+ by Li^+, or the use of Na^+/K^+-ATPase blockers, such as ouabain, decrease lipid uptake. Situations which create an accumulation of $[Na^+]_i$ (such as inhibitors of the Na^+/K^+-ATPase), or which block NHE-3, or which substitute Na^+ in the BBM NHE-3 but not in the basolateral membrane Na^+/K^+-ATPase, will potentially further reduce lipid uptake [39]. Na^+-dependent lipid uptake would not be expected under this special condition, since the outwardly-directed H^+ gradient would not exist (unless created by experimental conditions).

On the basis of these findings, a working model for intestinal fatty acid uptake is proposed (Fig. 11). Lipids are solubilized in mixed micelles in the intestinal lumen. The mixed micelle diffuses across the intestinal unstirred water

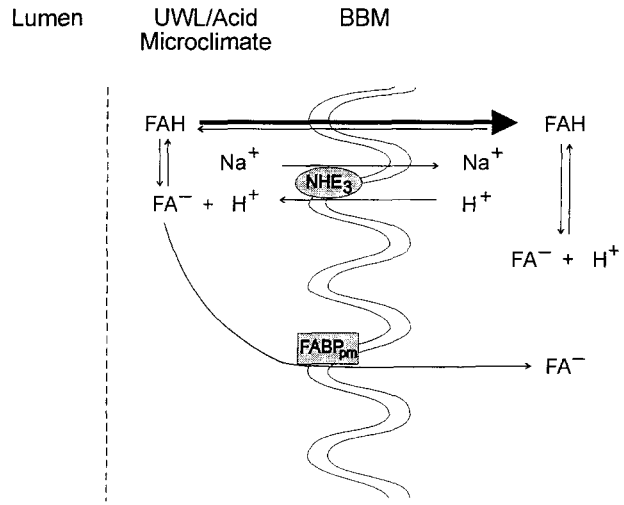

Fig. 11. A working model for intestinal fatty acid uptake is proposed. Please see the Discussion for details.

layer (UWL). Fatty acid partitions from the mixed micelle into an aqueous phase [3], although under some circumstances (determined by the composition of the mixed micelle), they may partition directly from the mixed micelle into the BBM [47,48]. Because the pK of fatty acids in water is about 4.8, in the bulk phase of the intestinal lumen where the pH is about 7 most fatty acids will be in the nonprotonated form. Once the fatty acids are solubilized in the mixed micelle, there is a shift in the pK of their carboxyl group so that the fatty acids will present as both protonated (FAH) and non-protonated ionized (FA^-) forms. When the mixed micelles enter the acidic microclimate exterior to the BBM, where the pH is about 5.5, a greater proportion of the fatty acids in the mixed micelles become protonated and the mixed micelles become less stable, resulting in an increase in the critical micellar concentration (CMC) of the bile acid micelles and breakdown of the micelles [13]. In the aqueous phase of the acidic microclimate at pH 5.5, most of the fatty acids become protonated (FAH). FAH (which is relatively less hydrophobic and more lipophilic than FA^-) partitions into the outer leaflet of the BBM, and equilibrates more rapidly ('flips') into the inner leaflet of the membrane. An acidic microclimate would have less if any effect on fatty acid uptake under conditions where there is direct partitioning of the mixed micelle into the BBM. Increased lipid uptake would result from a more acidic microclimate or from a more lipophilic BBM. The presence of the acidic microclimate would result from the trapping of H^+ in the intestinal mucus and in the UWL. The acidic microclimate is produced and maintained from H^+ secretion by the Na^+/H^+ exchanger in the BBM, NHE-3. Increased activity of the NHE-3 would enhance oleic acid uptake by increasing the acidity in the microclimate. An out-to-in Na^+ gradient can drive net uptake of oleic acid against a concentration gradient (measured as total concentration of protonated and unprotonated forms). Thus, the Na^+ gradient across BBM of the enterocyte is much greater than the opposing proton gradient, and may pump out protons that would otherwise accumulate in the cytosol. By raising the intracellular pH, an out-to-in Na^+ gradient will favour net uptake of fatty acids by 'trapping' them as the fatty acid an ion, thus reducing efflux back into the extracellular space. Therefore, the majority of the lipid uptake by the BBM, especially at higher concentrations, is usually by partitioning of the lipid into the brush border membrane, facilitated by the presence of the acidic microclimate resulting from the activity of NHE-3 and the trapping of H^+ by the intestinal mucus and intestinal UWL. It is possible, but not directly studied in these experiments, that under special conditions a portion of lipid uptake may be mediated by a fatty acid binding protein ($FABP_{PM}$) in the brush border membrane [5]. This model does not exclude the possibility that there may be facilitated uptake of fatty acids especially at low substrate concentrations [4], does not exclude the possibility of some paracellular transport, and does not exclude the possibility that NHE-3 may serve as the $FABP_{PM}$.

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