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Effects of exogenous fatty acids on calcium uptake by brush-border membrane vesicles from rabbit small intestine

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The uptake of a variety of fatty acids by isolated brush-border membranes from rabbit small intestine was studied. This uptake increased with acyl chain-length and was not diminished by washing of the lipid-treated membranes with 0.25 M CsBr. The binding of fatty acid was not accompanied by a decrease in endogenous acyl groups or of cholesterol and therefore corresponded to a net uptake accountable qualitatively and quantitatively by the fatty acid added to the membranes. The uptake of Ca^{2+} was stimulated by treatment of the membranes with low concentrations of unsaturated fatty acids (0.05 mM) as well as with various concentrations of caprylic acid (0.10–3.00 mM) and inhibited by treatment with higher concentrations of unsaturated fatty acids (0.20–0.60 mM). Saturated fatty acids had no marked effects on Ca^{2+} uptake. The stimulatory concentrations of unsaturated fatty acids did not change the Ca^{2+}-binding characteristics of the membranes, whereas the higher concentrations decreased equilibrium binding of Ca^{2+} and very probably the number of high-affinity binding sites. The results of this study are assessed in terms of the effects of normal fatty acids found in the diet on the absorptive properties of the brush-border membranes.

Introduction

Cells lining the lumen of the small intestine are constantly exposed to exogenous lipids derived from the diet. These lipids taken up by the luminal membrane modify its composition. Such compositional changes are likely to alter structure-function relationships in these membranes and consequently affect the absorption of other nutrients occurring concomitantly with the lipid. These effects remain largely unknown.

To probe this particular problem, we have initiated a series of studies in which the lipid composition of the brush-border membrane, isolated from rabbit small intestine, is modified by exposure to exogenous lipids and the subsequent effects on biological function are measured. To date we have shown that these membranes can incorporate a variety of lipids in vitro, including phosphoglycerides, cholesterol and fatty acids [1–4] under a variety of conditions. In the present report we describe in greater detail the incorporation of fatty acids into brush-border membranes and the effect of such uptake on their ability to take up Ca^{2+}.

Materials and Methods

(1-^{14}C)-labelled fatty acids were purchased from New England Nuclear Corp. (Boston) and diluted to the required specific activity with unlabelled analogues. The ionophore A23187 and unlabelled

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fatty acids were obtained from Sigma Chemical Co. (St. Louis).

Preparation of brush-border membranes

Brush-border membranes were routinely isolated from small intestine of female New Zealand white rabbits by the method of Selhub and Rosenberg [5] which makes use of Ca^{2+} precipitation. The rabbits were weaned at 4–6 weeks, after which time they received standard Purina rabbit chow. Methods substituting precipitation of contaminating membranes with Mg^{2+} or Mg^{2+} [6] and EGTA [7] for Ca^{2+} precipitation were also used for certain experiments. The purity of the membranes was checked by electron microscopy and by marker enzyme assays as described in previous reports [1–3]. The membranes appeared as closed vesicle structures and displayed Na^+ gradient-dependent glucose uptake with the usual overshoot phenomenon [8].

Uptake of exogenous fatty acids

The terms uptake, incorporation and binding are used synonymously throughout the present report and, in the case of fatty acids, are meant to signify mainly an intercalation of lipid within the core of the membrane, versus an adsorption at its surface. Evidence supporting this is presented in the the next section and in a previous report [4], although the possibility of some adsorption could not be precluded completely.

Unlabelled or (1-^{14}C)-labelled fatty acids were dissolved together with sodium taurocholate in 2 ml of chloroform/ethanol (1 : 1, v/v). The solvents were removed by thorough evaporation in vacuo and the residue was suspended in a 10 mM Hepes-Tris buffer (pH 7.5)/100 mM mannitol. The suspension was shaken at room temperature for 15 min and then sonicated twice for 2 min with the small probe of a Ultrasonics sonicator at a setting of 5. Solutions that were not clear after sonification were further clarified by centrifugation at $1 \cdot 10^7$ $g \cdot$ min. Brush-border membranes (300 μg protein) and various concentrations of the micellized fatty acid were incubated 10 min at 25°C in 1 ml of 100 mm mannitol 10 mM Hepes-Tris buffer (pH 7.5). For those fatty acid concentrations used, the final concentration of bile salt required to obtain clear micellar suspensions was 7.2 mM, except when palmitate was tested, which required 10 mM taurocholate. After incubation, the membranes were collected and washed with the same mannitol-containing buffer by centrifugation at $40000 \times g$ for 30 min. The pellets were either suspended in a small volume of water for counting [3] or for extraction of lipids [9], or were suspended in the usual mannitol/Hepes-Tris buffer for Ca^{2+}-uptake studies.

Ca^{2+} uptake

The procedure followed was essentially that of Miller and Bronner [10]. Calcium uptake was initiated by the admixture of 10 μl of brush-border membrane vesicles (30 μg protein) and 25 μl of $^{45}CaCl_2$, 0.36 mM final concentration. Incubations for 5 min, usually at 25°C, were terminated by the addition of 40 vol. of ice-cold stop solution comprising 100 mM mannitol/10 mM Hepes-Tris buffer (pH 7.5)/5 mM EDTA/20 μM $LaCl_3$ (pH 7.5). The suspension was then filtered through 0.45 μm nitrocellulose filters under low vacuum and the trapped membranes were washed twice with 2.5 ml of ice-cold stop solution. The filters were allowed to air-dry and were transferred to counting vials containing PCS (Amersham Radiochemicals)/toluene (1 : 1, v/v) and counted. Membrane preparations whose uptakes of Ca^{2+}, using these incubation and processing procedures, were found to be linear with protein concentration and time up to 5 min or longer were used for these experiments. However, on occasion, some membrane preparations displayed linearity of uptake for shorter periods under these conditions for reasons that are unclear.

Analytical procedures

Fatty acid methyl esters were prepared by treatment of lipid extracts with dry, 2.5% methanolic HCl at 70°C for 1 h under nitrogen. The methyl derivatives were analyzed with a varian 6000 gas chromatograph equipped with a flame ionization detector employing a 1.83 m × 2 mm packed glass column (10% SP-2330 on Chromosorb W, AW 100/20 mesh). The injector temperature, the ion temperature, N_2 flow rate, column pressure and temperature program were: 260°C, 300°C, 7.5 ml/min, 10 lb/in^2 and 160°C for 20 min followed by 8 Cdeg/min to 200°C, respectively. Hepta-

decanoic acid was used as internal standard. Cholesterol was determined on lipid extracts of the brush-border membranes as described by Bowyer and King [11]. Radioactive samples were counted in PCS/toluene (1 : 1, v/v) with a Beckman LS133 spectrometer. Proteins were determined by the method of Lowry et al. [12].

Variations in the results

All values were averages ± S.E. obtained from 6–10 determinations from at least three or four animals unless otherwise specified. Addition of taurocholate and fatty acid in the incubation mixture caused lysis of membranes ranging from 5% to 15% except when higher concentrations of oleate (0.2 mM) were used. All values of uptake were calculated on the basis of protein recovered.

Results

Results summarized in Table I reveal the extent of binding of various ^{14}C-labelled fatty acids to brush-border membranes as assessed by the amount of radioactivity recovered in the membranes after incubation with labelled analogues and washing with buffer of low ionic strength. The extent of fatty acid uptake was found to increase with acyl chain length, the octanoic acid analogue being retained very poorly by the membranes. Results summarized in Table II also indicate that washing the brush-border membranes with 0.25 M CsBr to dissociate any membrane surface, ionically-bound lipid [13] had no significant effect on the uptake values when compared to membranes washed with buffer of low ionic strength.

Results shown in Table III indicate that as the oleic acid concentration in the medium increased, the proportion of oleic acid in the total membrane lipids increased in concert with a decrease in the proportion of all the other fatty acids when these were quantified by GLC. After exposure of the membranes to 0.2 mM oleic and palmitic acids, the 18 : 1 and 16 : 0 components accounted for 62 and 36% of the total fatty acids, respectively. On a mg membrane protein basis, there occurred an increase in the amount of total fatty acid which was entirely accountable by the fatty acid added to the medium. These uptakes were similar to those calculated from the radioactivity incorporated when ^{14}C-labelled fatty acids were used. The re-

TABLE I

THE EFFECTS OF EXOGENOUS FATTY ACID ON Ca^{2+} UPTAKE BY BRUSH-BORDER MEMBRANE VESICLES

Fatty acid uptake was estimated from the radioactivity recovered in the membrane pellets incubated with labelled fatty acids. Ca^{2+} uptake was measured with membranes that had been treated with similar amounts of unlabelled fatty acid.

Fatty acid added	Concn. (mM)	Fatty acid uptake (nmol/mg protein)	Rate of Ca^{2+} uptake (percent of control) [a]
Nones	–	100	
Caprylic	0.100	3.4 ± 0.1	113 ± 4
	1.000	13.0 ± 0.8	127 ± 3
	3.000	37.3 ± 3.1	161 ± 9
Lauric	0.10	9.6 ± 0.2	102 ± 5
	1.00	270.7 ± 11.2	106 ± 7
Palmitic	0.06	58.6 ± 3.0	85 ± 8
	0.20	153.4 ± 2.9	85 ± 1
	0.38	274.5 ± 4.7	84 ± 7
Oleic	0.010	18.2 ± 0.8	113 ± 10
	0.025	47.8 ± 0.3	117 ± 7
	0.050	88.8 ± 3.7	137 ± 11
	0.100	147.9 ± 3.3	65 ± 8
	0.200	341.8 ± 16.6	48 ± 8
Linoleic	0.050	50.2 ± 3.2	143 ± 7
	0.600	1075.1 ± 36.6	6 ± 1

[a] The control uptake ranged from 6–8 nmol/mg protein per 5 min.

TABLE II

EFFECT OF WASHING WITH CsBr ON THE UPTAKE OF OLEIC ACID BY BRUSH-BORDER MEMBRANE VESICLES

Where indicated, 0.25 M CsBr was present in the wash buffer together with the other usual constituents, 100 mM mannitol and 10 mM Hepes-Tris (pH 7.5).

Oleic acid concentration (mM)	Wash solution	Fatty acid uptake (nmol/mg protein)
0.05	buffer	93 ± 7
0.05	buffer + CsBr	104 ± 4
0.20	buffer	273 ± 21
0.20	buffer + CsBr	293 ± 23

sults clearly indicate a net incorporation of fatty acids and a lack of readily measurable exchange with any major acyl group-containing fraction. Results shown in Table IV also indicate an absence of exchange between exogenous fatty acid and endogenous cholesterol. Any loss of this sterol during the fatty acid uptake process was entirely accountable by lysis of membrane. Membrane lysis was concluded on the basis of comparable loss of pelletable protein and turbidity of suspensions of vesicles treated with 0.2 mM oleic acid.

The effects of fatty acid uptake on function were tested specifically by assessing changes in the Ca^{2+} transport ability of the membrane vesicles. Since it was reported that the properties of brush-border membranes may be affected by the choice of divalent cation used to precipitate contaminating membranes during the isolation procedures, Ca^{2+} being an activator of phospholipase A of the microvilli membranes and causing breakdown of endogenous lipids [7], we decided to check the uptake of $^{45}Ca^{2+}$ with membranes isolated by different methods. Results illustrated in Fig. 1 indicate that the rates of uptake and the equilibrium uptake values for Ca^{2+} were not significantly affected by the type of divalent cation used for preparing the membranes. When prepared by precipitation with EGTA + Mg^{2+}, the membranes displayed higher initial rates of uptake and elevated equilibrium uptakes, probably because of chelation of Ca^{2+} by EGTA that had become trapped during vesiculization of the membranes (results not shown). On the basis of these results, we decided to retain our usual procedure for preparing brush-border membranes, which makes use of Ca^{2+} precipitation.

Interestingly, the uptake of unsaturated and shorter-chain fatty acids results in an altered ability of brush-border membranes to take up Ca^{2+}. Binding of low amounts of oleic acid and linoleic acid (50–90 nmol/mg protein) always resulted in a significant increase in the rate of Ca^{2+} uptake, whereas binding of larger amounts (over 150 nmol/mg protein) resulted in a very substantial

TABLE III

FATTY ACID COMPOSITION OF BRUSH-BORDER MEMBRANES BEFORE AND AFTER EXOGENOUS FATTY ACID UPTAKE

The values are averages of duplicate determinations for a single rabbit and are representative of several experiments yielding similar results. Membranes from a different rabbit were used for each oleic acid treatment and compared with untreated membranes of the same rabbit.

Fatty acid	Concn. (mM) acid added	Fatty acid composition (percent of total)							Increase in oleate (nmol/mg protein)
		16:0	18:0	18:1	18:2	18:3	20:3+20:4	others	
None		12.6	26.3	11.5	34.8	4.0	1.0	9.0	–
Oleic	0.01	11.9	23.5	17.6	30.4	4.0	1.9	10.7	18.3
	0.05	8.3	17.9	36.0	25.6	3.6	1.5	7.1	144.3
	0.20	5.2	11.5	62.0	15.9	2.4	0.8	2.2	404.6
									Increase in palmitate (nmol/mg protein)
Palmitic	0.20	36.0	17.3	9.8	24.2	2.9	1.2	8.6	137.9

TABLE IV

EFFECT OF FATTY ACID UPTAKE ON CHOLESTEROL CONTENT OF BRUSH-BORDER VESICLES

The values given are representative of several experiments yielding similar results. In the case of cholesterol content, the data are averages ± S.D. of four determinations performed on membranes from a single rabbit and are corrected for protein losses. Values indicating protein and turbidity losses are averages of two or more determinations performed on membranes from a single rabbit.

Fatty acid added	Concn. (mM)	Protein loss (% of control)	Decrease in turbidity of vesicles (% of control)	Cholesterol content (μg/mg prtoein)
None (control)		–	–	76 ± 6
Oleic	0.05	10		78 ± 6
	0.20	26	25	86 ± 2
Palmitic	0.38	0	–	76 ± 4
Caprylic	3.00	2	–	78 ± 6

decrease in the rate of Ca^{2+} uptake (Table I, Fig. 2.)

These values represent rates, since usually the membrane preparations used for these experiments displayed linear uptake of Ca^{2+} with time for at least 5 min. In fact, the stimulatory and inhibitory effects of low and high concentrations of oleic acid were seen with 1 min incubation times as well (results not shown).

It can be seen from the Scatchard plot of Fig. 3 that concentrations which enhanced the rate did not affect the equilibrium uptake, whereas concentrations which inhibited the rate also very substantially decreased the equilibrium uptakes. Caprylic acid which did not incorporate very readily into the brush-border membranes (under 40 nmol/mg protein) displayed only an enhancing effect under the conditions tested. Lauric acid had no significant effect on the rate of Ca^{2+} uptake when incorporations of this fatty acid varied from 10 to 270 nmol/mg protein, whereas palmitic acid had a slight inhibitory effect which did not increase when incorporations exceeded 60 nmol/mg protein.

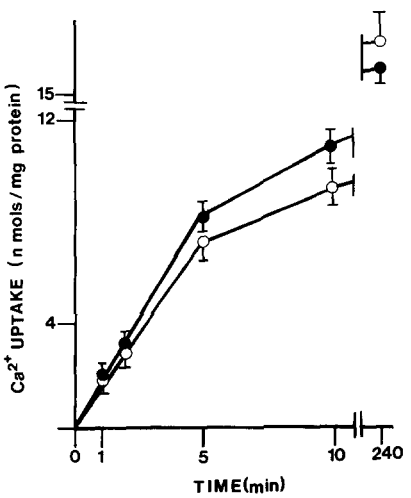

Fig. 1. Uptake of $^{45}Ca^{2+}$ by brush-border membrane vesicles. Isolation of membranes using (●) Ca^{2+} precipitation, (○) Mg^{2+} precipitation. The data are the average values for four determinations performed with membranes from a single rabbit and varied within 7% of the mean.

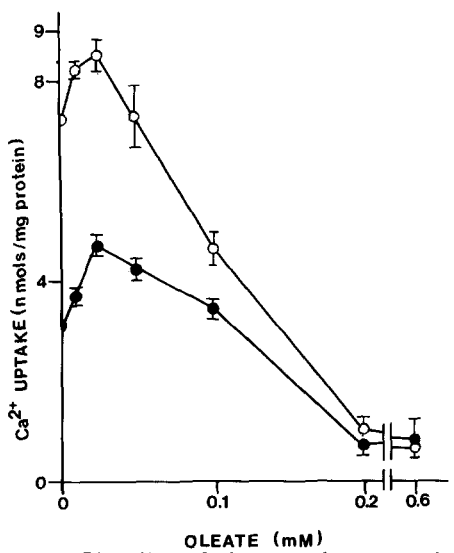

Fig. 2. The effect of oleate on the rate of Ca^{2+} uptake by brush-border membrane vesicles (○) with 15 μM A23187 and (●) without ionophore. The uptake data are for 2-min incubations.

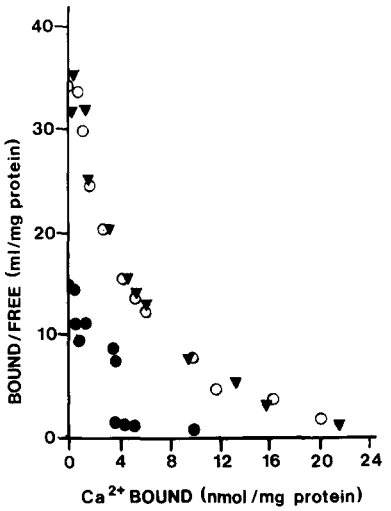

Fig. 3. Scatchard plot for Ca^{2+} binding obtained with oleate-treated membranes and with control membranes (○) no oleate; (▼) 0.05 mM oleate; (●) 0.20 mM oleate. Uptakes were measured after 150 min of incubation to obtain equilibrium values. The results are expressed as the average of four determinations with membranes from a single rabbit and are representative of several experiments yielding similar data.

The enhancing effect of oleic acid did not appear to be due to a change in the binding characteristics of the membrane, since a Scatchard plot of the data obtained with membranes treated with 0.05 mM oleate was identical to that obtained with control membranes (Fig. 2). On the other hand, exposure of the membranes to inhibitory concentrations of oleate (0.2 mM) resulted in a substantial change in the Ca^{2+}-binding characteristics. Although with inhibitory concentrations the binding data fitted neither a straight line nor a curved line very well, the results nevertheless suggested, when compared to those of other investigators [10,14,15], that high-affinity sites were decreased, whereas no conclusions could be made about the low-affinity sites. It may be said, however, that the affinity sites defined in the literature, principally on the basis of Scatchard plot analyses [10,14,15], have not yet been assigned any definite molecular basis and, furthermore, it is known that serious errors associated with analyses of such plots can easily arise [16,17]. Consequently, no attempt was made to calculate on this basis any definite binding parameters.

Data illustrated in Fig. 2 indicate that higher concentrations of oleic acid inhibit not only the intrinsic Ca^{2+} uptake mechanisms of the brush-border membrane, but the ionophore A23187-catalyzed system as well. This result can best be explained on the basis of a decrease in binding sites produced at higher oleate concentrations. The lower concentrations of oleic produce Ca^{2+} uptake enhancement which is additive to that produced by ionophore A23187, a result which is compatible with the possible ionophoric properties of this fatty acid.

Discussion

The membranes used throughout most of the present studies were prepared by a procedure involving Ca^{2+} precipitation [5], since procedures making use of Mg^{2+} precipitation have been reported to give membranes that are heterogeneous [18] and perhaps less pure [19] than membranes prepared by Ca^{2+} precipitation. A disadvantage with our choice of preparation method could be conceived from suggestions that the use of Ca^{2+} activates a membrane phospholipase A which allegedly causes extensive breakdown of endogenous lipids [7]. The accumulation of lyso-derivatives or perhaps free fatty acids in the membrane could affect various functions including Ca^{2+} transport. However, it is difficult to reconcile this possibility with recent reports describing the phospholipase A of intestinal mucosa as acting mainly on phosphatidylglycerol, originating from plants and/or bacteria [20,21]. Also, the phospholipase A activity in our preparations would probably be low, since these are prepared from whole small intestine comprising mainly the duodenum and jejunum segments, whereas most of the phospholipase A has been found to be present in the ileum [20]. It is possible that extensive degradation of endogenous lipids, when it occurs, results from contaminating luminal phospholipases or perhaps from freezing and thawing steps [7] that could activate a membrane phospholipase A of more general specificity. This point requires further investigation. A preliminary examination of the free fatty acid content of our brush-border membranes prepared by either of the precipitation methods did not indicate significant enhancement of phospholipid breakdown due to the presence of Ca^{2+}. To clarify this point

further, a detailed analysis of the lipid composition and phospholipase A activity of the membranes prepared by each precipitation method has been undertaken and will be the subject of a separate report. The almost identical uptake of $^{45}Ca^{2+}$ in the membranes prepared by Ca^{2+} or Mg^{2+} precipitation attests to their functional and, possibly, structural similarities. This point would require further investigation.

In a previous study, performed in the presence of Ca^{2+}, the uptake of fatty acid by isolated brush-border membranes was also found to increase with acyl chain length, but in this case the binding of oleic acid was reported to be less than that of palmitic acid [4]. The reason for this difference due to Ca^{2+} was not investigated further. That the uptake of fatty acid involves intercalation within the hydrophobic core (lipid bilayer) structure of the membrane can be inferred on the basis of increased binding with increase in acyl chain length and from the fact that bound fatty acid is not dissociated by increasing the ionic strength of the wash medium. Since the inulin space in the pelleted membranes is less than 2 μl/mg protein [4], less than 2% of the bound lipid would represent bulk phase contamination.

Since the rate of Ca^{2+} uptake and not the equilibrium values appeared to be affected by low concentrations of oleic acid (Table I, Figs. 2 and 3), one can suggest that unsaturated fatty acids may act as Ca^{2+} ionophores, at least when their uptakes do not exceed 90 nmol/mg protein. Ca^{2+} ionophore activity of ricinoleic acid has been proposed on the basis of similar results obtained with pig jejunal brush-border membrane exposed to low and moderate concentrations of this hydroxy unsaturated fatty acid [22]. Interestingly, higher concentrations of ricinoleic acid had an inhibitory effect on the uptake of Ca^{2+} similar to the one noted in the present study. These opposing effects of low and high concentrations of unsaturated fatty acids have also been seen in the case of Ca^{2+}-ATPase activity of human erythrocyte membranes [23].

Absorption of large amounts of unsaturated fatty acid destabilizes the membrane structure, as is evidenced by the lysis (above 20%) that occurs when membranes are exposed to concentrations of unsaturated fatty acids greater than 0.2 mM. Certain protein-mediated processes may be sensitive to these changes such as those believed to be involved in Ca^{2+} uptake [24]. There may result in such systems changes in the conformation of the ion-binding protein or in the structure of the ion channels involved. Whether the effect is stimulatory or not could depend on the extent of fluidization. Selective release of protein (e.g., Ca^{2+} binding protein) from the membrane as an explanation of the effect of higher concentrations of oleate is another possibility that has not been precluded completely, although protein losses were found to parallel closely the extent of lysis when an inhibitory concentration of oleic acid was used.

Interestingly, in preliminary experiments performed with lipid-treated membranes, D-glucose and D-fructose uptake were found not to be influenced by oleic acid incorporation into the membrane occurring at concentrations which inhibit Ca^{2+} uptake. Similar negative results were obtained by Fontaine et al. [24] studying glucose transport. It appears, therefore, that the effects of unsaturated fatty acids on membrane function are selective, but this specificity could merely reflect individual susceptibility of proteins to rather general changes on the fluidity of the membrane. This point would require further study.

Part of our results agree with those of previous studies showing the stimulatory effect of unsaturated methyl esters of vaccenic acid on Ca^{2+} transport in isolated chick brush-border membranes [24,25]. However, there is some disagreement in these reports as to whether it is the *cis* or the *trans* isomer that is most effective. It must be pointed out also that no attempt was made to establish the amount of vaccenic acid ester that entered the membrane, and whether essential components such as cholesterol and other lipids were lost during the study. Also, the effects of higher concentrations were not assessed. Kreutter et al. [26] also reported recently a simulatory effect of oleoyl CoA and linoleoyl CoA on Ca^{2+} transport of isolated chick brush-border membranes. Palmitoyl CoA, stearoyl CoA and arachidonoyl CoA had no effect. Our own study with free fatty acids extends such findings to rabbit-derived membranes, over a wider range of concentrations, defines the binding process more precisely in terms of whether or not a net uptake is concerned and

relates effects of Ca^{2+} transport not only to the type of fatty acid taken up but the amount as well.

Whether or not, under physiological conditions in vivo, the unsaturated fatty acid content of brush-border membranes can vary as extensively as in the present study remains to be established. Analyses of brush-border membrane lipids performed in our laboratory and elsewhere [7] have shown that the content of free fatty acids is high. Such a large pool might well be derived in part from the absorption of digestion products and consequently would have a composition fluctuating rapidly in conjunction with the fatty acid composition of the diet. It is conceivable therefore that the absorption of minerals such as Ca^{2+} would be controlled to a significant extent by the amount and the types of fatty acids in the diet through events occurring at the level of the brush-border membrane.

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