Biochimica et Biophysica Acta 862 (1986) 87–99
Elsevier

BBA 73331

Phospholipid topology and flip-flop in intestinal brush-border membrane

Leonid I. Barsukov [a], Lev D. Bergelson [a], Martin Spiess [b], Helmut Hauser [b] and Giorgio Semenza [b]

Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, Ul. Miklukho-Maklaya, 16/10, 117871 GSP Moscow, V-437 (U.S.S.R.) and Laboratorium für Biochemie, Eidgenössische Technische Hochschule Zürich, Zürich (Switzerland)

(Received 5 May 1986)

Key words: Phospholipid topology; Lipid topology; Membrane organization; Flip-flop; (Brush-border membrane)

The topological distribution of the two major phospholipids of brush-border membrane, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), has been investigated using brush-border membrane vesicles from rabbit small intestine. Bee venom phospholipase A_2 and phosphatidylcholine exchange protein from bovine liver were used as membrane probes. It is shown that the brush-border membrane retains its integrity under conditions of phospholipase hydrolysis and intermembrane phospholipid exchange. Kinetic analysis of the data of phospholipase hydrolysis and phospholipid exchange at temperatures under 10°C shows that both PC and PE occur in two pools: a minor (about 25%) more readily accessible pool and a major one (about 75%) less readily available. The rate of PC exchange between these two pools is relatively fast. The half-time derived under conditions of phospholipase hydrolysis is of the order of 20 min. Under conditions of phospholipid exchange the exchange rates may be even faster. The difference in exchange kinetics observed with the two methods of probing is probably due to changes in membrane properties such as the bilayer fluidity induced by the probing process itself. It is proposed that the two pools represent the transverse distribution of the phospholipids. The two major phospholipids of brush-border membranes, PC and PE, would be distributed mainly on the inner (cytoplasmic) side of the brush-border membrane. The phospholipid exchange between the brush-border vesicles and unilamellar phosphatidylcholine vesicles in the presence of phosphatidylcholine exchange protein reveals that significant quantities of phospholipid are taken up by brush-border membrane independently, i.e., in a separate process independent of the exchange protein-catalyzed phosphatidylcholine exchange.

Introduction

It is now generally accepted that biological membranes are organized asymmetrically. Experimental evidence exists that phospholipids are distributed asymmetrically between the two bilayer halves, but frequently serious problems are encountered in providing this evidence. The information available about the phospholipid distribution in biological membranes is sometimes puzzling and even contradictory [1–3]. One of the possible reasons is that phospholipid asymmetry may be coupled to rapid transmembrane migration (flip-flop) of phospholipid molecules. Determination of the transverse distribution of phospholipids under conditions of rapid flip-flop re-

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; SDS, sodium dodecyl sulphate.

Correspondence: Dr. H. Hauser, Laboratorium für Biochemie, E.T.H. Zürich, ETH-Zentrum, Universitätstrasse 16, CH-8092 Zürich, Switzerland.

quires special precautions. In studies of the topological distribution of phospholipids the proper choice of the system to be studied is very important. Obligatory requirements are not only the purity and homogeneity of the membrane preparation but also the integrity of the membrane and uniformity of the membrane orientation.

The brush-border membrane of the small intestine is a highly specialized part of the plasma membrane responsible for the digestive and absorptive functions of the enterocyte. Brush-border membrane is easily obtainable in relative large amounts in a sufficiently pure state [4]. The resulting brush-border vesicles are relatively homogeneous with respect to size [5] and retain the original membrane orientation present in the enterocyte to more than 90% [6]. Further, the orientation of the brush-border vesicle membrane appears to be stable and cannot be reversed even by ultrasound treatment. Nevertheless, the internal membrane surface can be made accessible to protein through mild detergent treatment [6].

The hydrolytic activity and transport functions of the brush-border membrane have been studied extensively; however, the molecular organization of this membrane is poorly understood. Information about the phospholipid topology of this membrane is still lacking. The present paper is adressed to the question of the organization of the major phospholipids (PC and PE) in this membrane.

Materials and Methods

Vesicles of rabbit small intestinal brush-border membrane were prepared according to Hauser et al. [7]. Their properties were similar to those described in this reference. Brush-border vesicles were dispersed in buffer 1: 300 mM D-mannitol/1 mM EDTA/10 mM Tris-KOH (pH 7.5). Protein was determined following the Lowry procedure as modified by Peterson [8] with bovine serum albumin as the reference. Lipid phosphorus was measured by the method of Chen et al. [9].

Treatment of brush-border vesicles with phospholipase A_2

Bee venom phospholipase A_2 (*Apis mellifica*, EC 3.1.1.4, specific activity 1300 units/mg) was isolated and purified according to Gritsuk et al. [11]. In a typical experiment, brush-border membranes (1.6 mg protein at 8 mg/ml) were incubated in buffer 1 (total volume 0.2 ml) containing 5 mM $CaCl_2$ and phospholipase A_2. The incubation was stopped by the addition of a 2-fold excess of EDTA over Ca^{2+}, immediately followed by lipid extraction according to Folch et al. [12]. The phospholipids were separated by two-dimensional chromatography on silica-gel microthin-layer plates (5 × 5 cm) according to Bergelson [13] (solvent systems: first dimension, chloroform/methanol/water, 65 : 25 : 4 (v/v); second dimension, chloroform/methanol/25% ammonium hydroxide, 14 : 6 : 1 (v/v)). Lipids were stained with iodine vapor, scraped off the plate and analyzed for phosphorus [10]. Each measurement was done in triplicate.

Phospholipid exchange between brush-border vesicles and sonicated phosphatidylcholine liposomes

PC exchange protein (specific activity 600 units/mg) was isolated from bovine liver according to Kamp et al. [14] except that the last purification step by gel-filtration on Sephadex G-50 was omitted. Donor liposomes were prepared from egg-yolk PC (Lipid Products, U.K.) containing [*N-methyl*-^{14}C]PC and traces of tri[9,10(n)-^{3}H] oleoylglycerol as a marker non-exchangeable by PC exchange protein (both from Amersham International, UK). PC dispersions in buffer 1 were made as described before [15] and sonicated using a Branson sonifier (with a microtip, in a pulsed mode, under N_2 at 4°C for 10 min). The liposomes were centrifuged for 30 min at 27000 × *g* before use. In a typical experiment brush-border vesicles (0.2 mg protein, 14.5 nmol PC) were incubated with an excess of liposomes (1.2 μmol PC) in the presence or absence of PC exchange protein in a total volume of 0.7 ml at the indicated temperature. Due to the about 80-fold excess of liposomes, the decrease in specific radioactivity of the liposomes during incubation was negligible. Incubation was stopped by cooling on ice and samples were centrifuged at 27000 × *g* for 30 min. The lipids were extracted from pelleted membrane vesicles according to Bligh and Dyer [16]. The ^{3}H radioactivity in this pellet is a measure of the non-specific adsorption of liposomal PC by brush-border vesicles, while the ^{14}C radioactivity

is the sum of two contributions, one due to specific PC exchange and the other due to non-specific PC adsorption. The difference between the ^{14}C and ^{3}H radioactivity is a measure of the protein-catalyzed PC exchange discussed in this paper.

Results

Characterization and stability of brush-border vesicles

Brush-border vesicles prepared as described under Materials and Methods were analyzed for total lipid, phospholipid, cholesterol and protein. The values obtained were in good agreement with published data [7]. Our brush-border vesicle preparation consisted of right-side-out vesicles for about 90% of sucrase-isomaltase was released by papain treatment. No latency of the sucrase activity was detected with 1% Triton X-100. The integrity of the brush-border vesicle membrane was checked by measuring the ability of the vesicles to accumulate and retain D-[^{3}H]glucose [4]. Furthermore, the inaccessibility of actin on the inner membrane surface to trypsin was taken as a criterion for membrane integrity (see below).

Treatment of brush-border vesicles with bee venom phospholipase A_2

It has been reported that small intestinal epithelium contains endogenous phospholipase A and B activity [18–21]. Part of this phospholipase activity of rat small intestine has been shown to be localized in the microvillus membrane [22]. Since endogenous phospholipase could interfere with the interpretation of the results of phospholipase probing, we first determined the phospholipase activity in our brush-border vesicle preparation. Prolonged incubation of brush-border membrane at 37°C was accompanied by significant phospholipid degradation. The lipid primarily affected was PE; 80% of the total PE was degraded after incubation at 37°C for 15 h. That this degradation did not lead to disruption of brush-border vesicles could be shown by repeated sedimentation and redispersion of brush-border vesicles after the incubation. Practically all the remaining phospholipid, cholesterol and membrane protein sedimented with brush-border vesicles. At shorter incubation times and lower temperatures the extent of degradation was significantly decreased, even in the presence of Ca^{2+}.

As can be seen from Table I, the PC and PE

TABLE I

EFFECT OF ENDOGENOUS PHOSPHOLIPASES ON INTACT AND PARTIALLY HYDROLYZED BRUSH-BORDER VESICLES

Brush-border vesicles (8 mg protein/ml; total sample volume 0.2 ml) were incubated at 30°C for various periods of time with and without added Ca^{2+} and phospholipase A_2 (PLase A_2) and the PC, PE, lyso-PC (LPC) and lyso-PE (LPE) content determined as described under Materials and Methods. All measurements were made in triplicate.

Conditions	Time of incubation (min)	Content (% of total phospholipid)					
		PC	LPC	PC + LPC	PE	LPE	PE + LPE
Brush-border vesicles (control)	0	31.5	3.9	35.4	38.9	4.1	43.0
	20	32.0	3.0	35.0	38.1	3.5	41.6
	45	31.5	3.0	34.5	39.4	3.9	43.3
Brush-border vesicles + Ca^{2+} (5 mM)	0	32.7	2.0	34.7	38.6	3.3	41.9
	20	32.2	2.0	34.1	32.9	6.6	39.5
	45	32.6	3.8	36.4	31.3	9.6	40.9
Brush-border vesicles + PLase A_2 (0.4 μg) + Ca^{2+} (5 mM)	0	32.2	2.2	34.4	38.3	4.6	42.9
	20	17.2	13.8	31.0	23.1	19.5	42.6
	45	16.8	15.6	32.4	15.2	26.3	41.5

content as well as the content of their lyso-derivatives did practically not change during incubation of brush-border vesicles at 30°C and pH 7.5 for 45 min. In the presence of 5 mM Ca^{2+} the content of PC and lyso-PC remained constant, whereas a small decrease in PE content and a concomitant increase in lyso-PE content were observed under these conditions. In contrast, in the presence of exogenous bee venom phospholipase A_2 and Ca^{2+} at pH 7.5 and 30°C, about 50% PC and 60% PE were hydrolyzed in 45 min. The reduction in the PC and PE content was accompanied by a corresponding increase in lysophospholipid content. The sum of PC and lyso-PC and the sum of PE and lyso-PE remained constant within the error of the measurement. Similar observations were made when brush-border vesicles were treated with phospholipase A_2 at 4°C.

The dependence of the extent of hydrolysis on the phospholipase A_2 concentration (Fig. 1A) and time (Fig. 1B) shows that PC and PE are hydrolyzed to approximately the same extent. In the presence of deoxycholate, that was shown to disrupt the brush-border vesicle membrane [6,17,23] the accessibility of phospholipids to phospholipase A_2 was significantly increased (Fig. 1B).

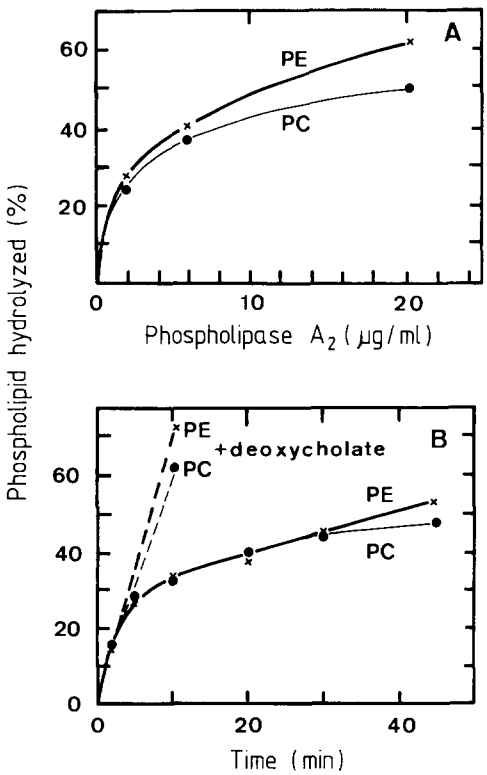

Fig. 1. (A) Hydrolysis of phospholipids in brush-border vesicles (8 mg protein/ml) as a function of increasing concentrations of phospholipase A_2 from bee venom. The hydrolysis of the two major phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), is expressed as a percentage of the initial PC and PE content, respectively. The hydrolysis was carried out in a sample volume of 0.2 ml for 10 min at 30°C. Each point is the mean of three measurements. (B) Kinetics of phospholipid hydrolysis in brush-border vesicles (8 mg protein/ml) treated with phospholipase A_2 (2 μg/ml) from bee venom at 30°C. Each point is the mean of three measurements, the standard deviation is given by the size of the symbols. For comparison, brush-border vesicles (8 mg protein/ml) were incubated with 0.2% deoxycholate and phospholipase A_2 (2 μg/ml) for 10 min at 0°C (no Ca^{2+} present, total sample volume 0.2 ml). Phospholipase A_2 was then activated by the addition of 7.5 mM $CaCl_2$ and raising the temperature to 30°C. At the same time deoxycholate was precipitated by Ca^{2+}. The hydrolysis was stopped after 10 min and the quantity of PC and PE that was hydrolyzed under these conditions was determined (+deoxycholate is the average of two measurements).

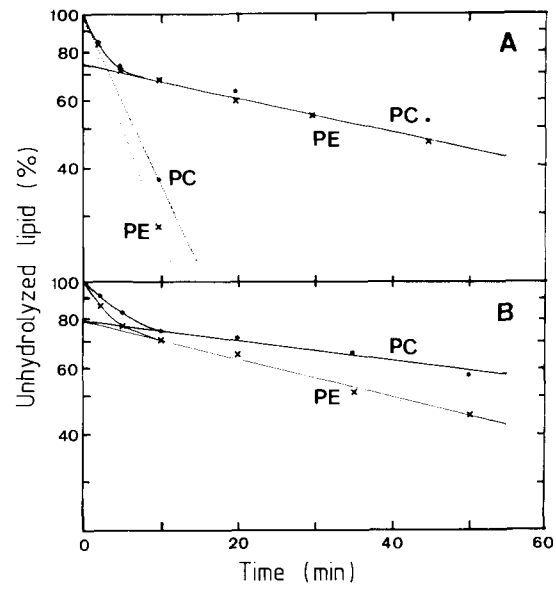

Fig. 2. (A) Kinetics of phospholipid (PC and PE) hydrolysis in brush-border vesicles (8 mg protein/ml) in the presence of phospholipase A_2 (2 μg/ml) from bee venom at 30°C. The data in Fig. 1B are plotted in a semi-logarithmic form of unhydrolyzed lipid (%) remaining in brush-border vesicles vs. time (min). (B) Hydrolysis of PC and PE in brush-border vesicles (8 mg protein/ml) in the presence of 6 μg/ml phospholipase A_2 (total sample volume 0.2 ml) at 4°C. Each data point in (A) and (B) is the average of two experiments.

The semi-logarithmic plots shown in Fig. 2 reveal the biphasic character of the hydrolysis process at 30 and 4°C, respectively. This indicates that both PC and PE are distributed (in the brush-border vesicle membrane) between two pools differing in their accessibility to phospholipase A_2. Extrapolation of the straight-line portion in Fig. 2 to zero time gives a rough estimate of the phospholipid fraction undergoing rapid hydrolysis. Approx. 20–25% of both PC and PE are cleaved rapidly by phospholipase A_2 whereas the rest is hydrolyzed at a considerably lower rate. It should be noted that as a result of deoxycholate treatment the less accessible pool was eliminated and the rate of hydrolysis became similar to that of the easily available phospholipid pool in intact brush-border vesicle membranes (dotted line, Fig. 2A).

Exchange of PC between brush-border vesicles and sonicated egg PC liposomes

It was shown previously [24] that the brush-border vesicle membrane is capable of incorporating significant amounts of [^{14}C] phosphatidylcholine from sonicated liposomes in the presence of PC exchange protein. In order to establish whether the incorporation resulted from the net transfer of PC from liposomes to brush-border vesicles or from molecular exchange of phospholipid molecules between donor and acceptor membranes, the following experiments were performed. Brush-border vesicles were incubated with an excess of sonicated labeled PC liposomes (liposomal PC/brush-border membrane PC, 80 : 1, mole ratio) in the presence and absence of PC exchange protein at 37°C for 2 h. The brush-border vesicles were then separated from the liposomes by centrifugation. The amount of liposomal PC transferred to brush-border vesicle membrane by PC exchange protein was obtained after correcting for the non-specific adsorption of PC. The PC exchange protein-catalyzed PC exchange was $98 \pm 4\%$ of the inital PC present in brush-border vesicles. Further and importantly, the specific radioactivity of PC in brush-border vesicles was $96 \pm 6\%$ of that present in liposomes. If the incorporation of PC into the brush-border vesicle membrane was due to net transfer, then the maximum specific radioactivity of the PC fraction of brush-border vesicles would have been one-half of the initial specific radioactivity in the liposomes due to dilution of the labelled PC molecules with non-labeled ones from the brush-border membrane. Since the final maximum specific radioactivity was $96 \pm 6\%$, no such dilution was observed; incorporation of labelled PC into brush-border vesicles must have proceeded with the removal of an equal amount of non-labelled PC molecules from brush-border vesicles. That the PC exchange protein-mediated exchange is specific for PC and is characterized by a 1 : 1 stoichiometry was also confirmed by phospholipid analysis of brush-border vesicles after exchange. It was found that the content of three major brush-border vesicle phospholipids, PC, PE and sphingomyelin, determined after PC exchange and after correction for non-specific adsorption of PC, was identical within the experimental error to that of untreated brush-border vesicles. Thus the data obtained show that protein catalyzed incorporation of [^{14}C]PC into the brush-border membrane results from a specific one-to-one exchange and not from net transfer of PC molecules. It is important to note that only PC was exchanged by the protein; other phospholipids of the brush-border vesicle membrane did not participate in the exchange process.

Kinetics of the PC exchange between brush-border vesicles and liposomes

The kinetics of the protein-mediated PC exchange are shown in Fig. 3. At 37°C and 27°C all PC in the brush-border vesicle membrane is readily accessible and rapidly exchanged at rates dependent on the PC exchange protein concentration. At 17°C the exchange rate is considerably reduced compared to 27°C, even though more than twice as much PC exchange protein was used. Decreasing the temperature further produced a marked decrease in the exchange rate. Similar to the data at 17 and 27°C, the data at 10 and 4°C can be readily fitted by a linear regression analysis. However, in contrast to the experiments at higher temperatures, the linear relationships at 10 and 4°C do not extrapolate to 100% at time zero but to about 75% (Fig. 3). This may be interpreted in terms of biphasic exchange kinetics observable only at temperatures under about 10°C. The results at low temperatures (no higher

than 10°C) would then be consistent with PC of the brush-border vesicle membrane being distributed between two pools with different accessibility for PC exchange protein. It is noteworthy that the pattern of the PC distribution is very close to that deduced from phospholipase A_2 probing: only about 25% of PC belongs to the easily available pool, whereas the major part (75%) is present in the less available pool (cf. Figs. 2 and 3). However, the finding that all PC is exchangeable with PC exchange protein at 17°C is at variance with the phospholipase A_2 results (cf. Figs. 2 and 3). This could be due to differences in experimental conditions. The hydrolysis by phospholipase A_2 requires Ca^{2+}, which was not present in the exchange experiment. Furthermore, incubation of brush-border vesicles with excess PC liposomes could lead to cholesterol depletion of the brush-border membrane [25]. Both Ca^{2+} and cholesterol are known to influence the phospholipid dynamics [26–28] and could possibly account for the observed difference. In order to shed light on this question, the phospholipid exchange was carried out under the same conditions as the phospholipase hydrolysis, i.e., in the presence of 5 mM Ca^{2+}. In addition, the exchange experiments were performed with an excess of liposomes containing PC and cholesterol in a ratio of 9 : 1 (by weight), corresponding to the total content of cholesterol in the brush-border vesicle membrane [7]. No difference in the exchange kinetics was observed in the presence or absence of 5 mM Ca^{2+} and when the exchange was carried out with either PC liposomes or PC/cholesterol liposomes. Hence, the presence of cholesterol in PC liposomes and of Ca^{2+} in the medium cannot account for the observed differences.

In another series of experiments, brush-border vesicles were treated first with phospholipase A_2 for 10 min at 30°C. As indicated in Fig. 4, this treatment resulted in the hydrolysis of about 30% of PC. The hydrolysis was stopped by adding 10 mM EDTA, and the partially hydrolyzed brush-border vesicle membrane was incubated with an excess of sonicated PC vesicles in the presence of PC exchange protein. Control experiments were carried out under identical conditions except that phospholipase A_2 treatment was omitted. Fig. 4 shows that the PC exchange rate after phospholipase A_2 treatment is markedly reduced compared

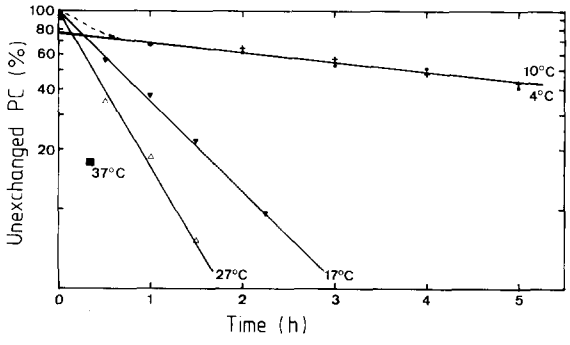

Fig. 3. Kinetics of PC exchange between brush-border vesicles and PC liposomes in the presence of phosphatidylcholine exchange protein. Brush-border vesicles (0.29 mg protein/ml; total sample volume 0.7 ml) were incubated with sonicated PC liposomes (1.7 mM) in the presence of 15 μg PC at 27°C and 37°C, 36 μg PC exchange protein at 17°C, 23 μg PC exchange protein at 10°C and 45 μg exchange protein at 4°C. The PC exchange was measured as a function of time as described under Materials and Methods. The data are corrected for non-specific adsorption by PC by brush-border vesicles.

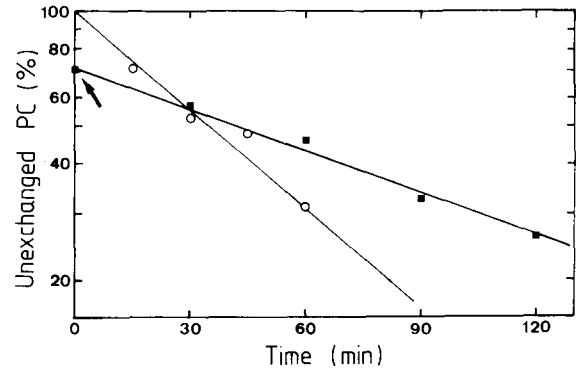

Fig. 4. PC exchange between PC liposomes and brush-border vesicles after partial hydrolysis of the brush border membrane phospholipids by phospholipase A_2. Brush-border vesicles (8 mg protein/ml, total sample volume 0.2 ml) were incubated with 2 μg phospholipase A_2 from bee venom and 5 mM $CaCl_2$ at 30°C for 10 min. This treatment (see arrow) resulted in the hydrolysis of about 30% each of PC and PE. After 10 min the hydrolysis was stopped by adding EDTA (10 mM). Aliquots of the reaction mixture (containing 0.2 mg protein) were incubated with excess liposomes (1.7 mM) in the presence of 15 μg PC exchange protein (total volume 0.7 ml). The PC-exchange was measured at 30°C as a function of time (■). In a control experiment (○) the PC exchange was measured between untreated brush-border vesicles and PC liposomes under otherwise identical conditions. Each data point is the average of two measurements.

to the control. A similar result was reported for rat liver microsomal membranes [33]. After phospholipase A_2 treatment, the rate of PC exchange between the microsomal and sonicated egg PC vesicles in the presence of PC exchange protein was significantly reduced.

Retention of the integrity of the brush-border vesicle membrane under the conditions of phospholipase A_2 hydrolysis and intermembrane phospholipid exchange

When elucidating the topological distribution of phospholipids it is important to know whether the membrane integrity and the permeability barrier of the membrane are maintained throughout the phospholipase treatment and phospholipid exchange. Since it is difficult to measure directly the diffusion of phospholipase A_2 across the brush-border membrane, we measured the permeability of brush-border vesicles to trypsin, the molecular weight of which ($M_r = 23\,800$) is close to that of the bee venom phospholipase A_2 ($M_r = 20\,000$; [29]). Trypsin was used earlier [6,23] to monitor the intactness of brush-border vesicles; this assay is based on the digestion of the cytoskeletal protein actin which in intact brush-border vesicles is not accessible to trypsin. Fig. 5 shows the effect of trypsin on brush-border vesicles treated with phospholipase A_2. The SDS-polyacrylamide gel electrophoresis of intact brush-border vesicles is shown as a control in lane A. Incubation of brush-border vesicles with trypsin had no visible effect on the protein pattern (lane B) while trypsin in the presence of Triton X-100 at concentrations known to disrupt the membrane, almost completely digested actin (lane F, see arrow). Phospholipase treatment of brush-border vesicles did not activate any endogenous proteolytic enzyme(s) (lane C) and the subsequent addition of trypsin did not reduce the intensity of the actin band (lane D). We conclude that even extensive hydrolysis of the phospholipids (up to 50%) in brush-border vesicles does not disrupt the membrane and that the inner membrane surface most probably remains inaccessible to phospholipase A_2. The effect of phospholipase A_2 treatment on the permeability of the brush-border vesicle membrane was also checked. For this purpose the ability of brush-border vesicles to accumulate and retain D-glucose in their inner space was used [4]. The membrane vesicles were equilibrated with D-[^{3}H]glucose at 37°C for 1 h and after treatment

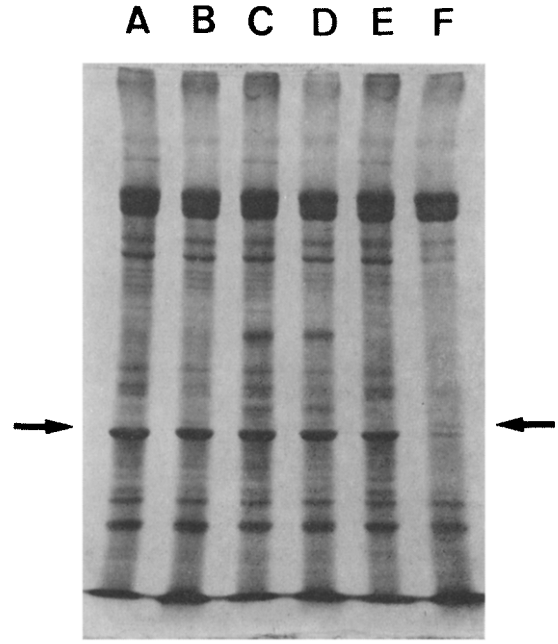

Fig. 5. Determination of membrane integrity of brush-border vesicles after phospholipase A_2 treatment. The membrane integrity was monitored by measuring the accessibility to trypsin of the cytoplsmic protein actin (arrows). Sample A (control): brush-border vesicles (8 mg protein/ml; total sample volume 0.2 ml) in buffer containing 5 mM $CaCl_2$ were incubated at 30°C for 15 min (lane A). Sample B: an aliquot of sample A (control) was cooled to room temperature and trypsin (0.15 mg/ml) was added. After 20 min incubation at room temperature, SDS (final concentration 2%, w/v) and mercaptoethanol (final concentration 5%, v/v) were added followed by boiling for 2 min (lane B). Samples C and D: aliquots of sample A were incubated with phosholipase A_2 (2 μg/ml) for 15 min; about half of the membrane PC and PE was hydrolyzed by this treatment. The hydrolysis was stopped by adding EDTA to a final concentration of 25 mM. Both samples were cooled to room temperature and to sample D trypsin was added (0.15 mg/ml) and the sample treated as sample B (lane D). Sample C is as sample D except for the trypsin treatment (lane C). Samples E and F were treated as samples C and D except that both E and F contained 0.4% Triton X-100. Sample E: brush-border vesicles treated with phospholipase A_2 in the presence of 0.4% Triton X-100 (lane E); sample F: brush-border vesicles treated with phospholipase A_2 and trypsin in the presence of 0.4% Triton X-100 (lane F). The SDS-polyacrylamide gel electrophoresis (gel 8.4×2.7) was carried out according to Ref. 30.

with phospholipase A_2 the D-[^{3}H]glucose contained within the brush-border vesicles was measured by rapid filtration. Table II gives the glucose retention of brush-border vesicles before and after phospholipase A_2 treatment. The amount of D-glucose retained is reduced by about 50% compared to untreated vesicles, indicating that the accumulation of the hydrolysis products (lysophospholipids and fatty acids) render the brush-border vesicle membrane more permeable to small molecules like glucose. To check the brush-border vesicle membrane integrity under the conditions of PC exchange the same assay was used. The retention of radioactive D-glucose by brush-border vesicles was measured before and after incubation with PC liposomes and PC exchange protein. The results are summarized in Table II. Neither liposomes nor PC exchange protein enhanced the permeability of brush-border vesicle membrane for D-[^{3}H]glucose. The content of radiolabelled glucose in the brush-border vesicles did not decrease during the simultaneous incubation with liposomes and PC exchange protein. During this incubation 90% of the PC of brush-border vesicles was exchanged. These experiments indicate that the integrity of the brush-border membrane was not affected during the intermembrane phospholipid exchange. Thus, the experiments described above demonstrate that under the conditions of phospholipid exchange and phospholipase hydrolysis, the brush-border vesicle membranes do not become leaky to proteins: the inner surface of the brush-border vesicle membrane appears to be inaccessible to both phospholipase A_2 and PC exchange protein.

TABLE II

D-GLUCOSE RETENTION BY BRUSH-BORDER VESICLES TREATED WITH PHOSPHOLIPASE A_2 AND AFTER EXTENSIVE EXCHANGE OF PC BETWEEN PC LIPOSOMES AND BRUSH-BORDER VESICLES

Incubation of brush-border vesicles – additions	D-[^{3}H] Glucose retention
None (control) [a]	100 ± 5
+ 5 mM Ca^{2+} [b]	107 ± 8
+ phospholipase A_2 and 5 mM Ca^{2+}	56 ± 6
+ PC liposomes [c]	117 ± 17
+ PC exchange protein	101 ± 3
+ PC liposomes and PC exchange protein	106 ± 21

[a] Control: Brush-border membrane vesicles were equilibrated with D-[^{3}H]glucose (1–2 mM) for 90 min in buffer 1 at 37°C.

[b] Control: Aliquots of 1.6 mg protein in 0.2 ml were incubated with 5 mM $CaCl_2$ in the absence and presence of phospholipase A_2 (0.4 μg) for 10 min at 30°C. This treatment resulted in the hydrolysis of 30–40% each of PC and PE. Aliquots of 50 μg protein were finally diluted with 3.5 vol. of cold 0.15 M NaCl and immediately filtered through cellulose nitrate filters (Sartorius, Göttingen, F.R.G.; 0.65 μm nominal pore diameter) as described by Kessler et al. [4]. The amount of radioactivity retained by brush-border vesicles on the filter was determined; the radioactivity associated with brush-border vesicles in the absence of Ca^{2+} and phospholipase A_2 was taken as 100%. Results are presented as the mean ± S.D. of three measurements.

[c] Aliquots containing 0.2 mg protein were incubated with liposomes (1.7 mM), or with PC exchange protein (8 μg) or both in a total volume of 0.7 ml. Immediately after mixing and after 1 h at 37°C, aliquots containing 50 μg protein were diluted with 3.5 vol. of cold 0.15 M NaCl and filtered as described above. The values are the average ± S.D. of six experiments.

Discussion

Probing with phospholipase A_2

The main conclusion from the phospholipase A_2 experiment is that PC and PE are distributed in the brush-border vesicle membrane between two pools differing by their accessibility to the probing reagents. The phospholipid hydrolysis by phospholipase A_2 may be described by a kinetic model of two pools between which exchange of phospholipids occurs (see Appendix, Fig. 6). Using this two-pool model the biphasic curves (Fig. 2) can be fitted by the sum of two exponentials:

$$(\%\ \text{unhydrolyzed lipid}) = K_1\, e^{-g_1 t} + K_2\, e^{-g_2 t}$$

referring in turn to the fast and the slow phases of the probing process. The size of the two phospholipid pools and the rate constants derived from the constants K_1, K_2, g_1, g_2 (Table III) as described by Bloj and Zilversmit [31] are summarized in Table IV. The following conclusions can be drawn: (i) PC and PE of the brush-border vesicle membrane are distributed in this membrane in a similar way. (ii) The major fraction of

TABLE III

KINETIC ANALYSIS OF PHOSPHOLIPID HYDROLYSIS INDUCED BY PHOSPHOLIPASE A_2 IN BRUSH-BORDER VESICLES

Values for K and g were derived from approximating the experimental curves (Fig. 2) by the sum of two exponentials.

Phospholipid hydrolyzed	Temp. (°C)	K_1	K_2	g_1 (h^{-1})	g_2 (h^{-1})
PC	30	0.26 ± 0.01	0.75 ± 0.01	25 ± 6	0.64 ± 0.04
PE	30	0.24 ± 0.02	0.76 ± 0.02	27 ± 6	0.66 ± 0.05
PC	4	0.18 ± 0.01	0.82 ± 0.03	15 ± 6	0.41 ± 0.05
PE	4	0.19 ± 0.01	0.81 ± 0.03	25 ± 10	0.71 ± 0.06

these phospholipids (about 75%) is located in the less accessible pool, whereas the remaining 25% are in the readily accessible pool. (iii) The pattern of phospholipid distribution is not particularly sensitive to temperatures between 4 and 30°C. (iv) The rate of exchange of PC between the two pools of the brush-border membrane is characterized by half-times of about 20 min. In this context it is interesting to note that Bloj and Zilversmit [25] demonstrated that cholesterol of brush-border membrane also exists in two pools: one of about 30% which is readily accessible to oxidation in the presence of cholesterol oxidase and a major one of about 70% less readily accessible to this enzyme. The values for the sizes of the two cholesterol pools are close to those derived for PC and PE (cf. Table IV). Whatever the assignment of these two pools may be, it is interesting that the distribution of cholesterol in brush-border membrane seems to follow that of the major phospholipids (PC and PE).

Assignment of the two phospholipid pools in brush-border membrane

Considering the origin of the two phospholipid pools the following observations are pertinent: (i) The pattern of the phospholipid distribution is not particularly sensitive to temperatures between 4 and 30°C. It was shown by differential scanning calorimetry that the lipids in brush-border membrane undergo a broad reversible phase transition in this temperature range. The assignment of the two phospholipid pools to different lipid phases can be ruled out. In this case the size of the pools is expected to be sensitive to temperatures between 4 and 30°C. (ii) The integrity of the brush-border membrane was maintained under the conditions of phospholipase hydrolysis and phospholipid exchange at least to the extent that the membrane does not become permeable to proteins. (iii) The less accessible pools of PC and PE became available to phospholipase A_2 after deoxycholate treatment of the brush-border mem-

TABLE IV

KINETIC PARAMETERS FOR THE HYDROLYSIS OF PC AND PE OF BRUSH-BORDER VESICLES IN THE PRESENCE OF PHOSPHOLIPASE A_2

Experiment	Phospholipid	Temp. (°C)	Pool size (%)		Rate constants (h^{-1})			Half-time ($t_{1/2}$)(min) [b]
			a [a]	b	k_0	k_{ab}	k_{ba}	
Phospholipid hydrolysis	PC	30	30 ± 2	70 ± 2	23 ± 6	0.69 ± 0.04	1.6 ± 0.2	18
	PC	4	23 ± 4	77 ± 4	13 ± 5	0.46 ± 0.05	1.6 ± 0.5	20
	PE	30	28 ± 3	72 ± 3	25 ± 6	0.72 ± 0.05	1.9 ± 0.4	16
	PE	4	24 ± 4	76 ± 3	23 ± 9	0.80 ± 0.05	2.6 ± 0.8	12

[a] a, readily accessible pool; b, less accessible pool.

[b] Half-time for phospholipid flip-flop between pool b and a. Half-times were calculated according to Bloj and Zilversmit [31].

brane. This treatment has been shown to render brush-border vesicles permeable to proteins without solubilizing the membrane [6,23]. (iv) The PC exchange at low temperatures (under 10°C) suggests that PC exchange protein senses two pools of PC in brush-border membrane. The size of the two pools derived from the PC exchange is in good agreement with values derived from phospholipase A_2 probing. This is remarkable considering that not only the two methods of probing are basically different but also their experimental conditions. Taking all the evidence discussed above together, it is tempting to assign the less accessible phospholipid pool to the inner (cytoplasmic) side of the membrane and the readily available pool to the external membrane surface, exposed in vivo to the lumen of the small intestine. Shielding of the less accessible phospholipid pool by other membrane components would be difficult to reconcile with the finding that all endogenous PC is accessible to PC exchange protein at temperatures of at least 17°C. However, despite all the blank experiments carried out, we still cannot rule out the possibility that intrinsic phospholipases are activated in the presence of externally added phospholipase. If such an activation takes place, relative hydrolytic rates would be difficult to predict. On the other hand, the Langmuir-type curves presented in Fig. 1 do not support the activation of intrinsic phospholipases by externally added phospholipase. In case of such an activation sigmoidal curves characteristic of cooperative phenomena are predicted. Certainly more experimental work is required; probably studying the topological distribution of the negatively charged phospholipids and glycolipids may be useful and would help in solving the problem. The assignment of the two pools to the two membrane halves would imply that the distribution of the major phospholipids, PC and PE, is highly asymmetric, with a predominance on the cytoplasmic side. This would be remarkable, because it is different from other plasma membranes, e.g., the erythrocyte membrane [32]. In this context, it is interesting to note that an asymmetric lipid distribution was proposed for pig brush-border membrane (Patton, J.S., personal communication). Based on molecular packing considerations it was concluded that the outer leaflet of brush-border membrane is poor in glycerophospholipids and enriched in glyco- and sphingolipids, whereas glycerolipids occur predominantly on the cytoplasmic side.

Probing with PC exchange protein

The PC exchange between sonicated PC liposomes and brush-border vesicles in the presence of PC exchange protein is difficult to interpret for several reasons. One major problem arises from the non-specific lipid adsorption by brush-border membrane. A measure of this is the amount of ^{3}H-labelled triolein associated with brush-border membrane. Evidence for non-specific adsorption is the fact that the ^{3}H radioactivity measured in brush-border vesicles was the same regardless of whether they were incubated with liposomes in the presence or absence of PC exchange protein. We conclude, therefore, that the triolein uptake represents the lipid adsorption which is not catalyzed by PC exchange protein. Furthermore, under conditions of extensive PC exchange, (i.e., in the presence of a large excess of PC liposomes, sufficient PC exchange protein and with long incubation times) the amount of PC exchanged approached a limiting value which was identical to the amount of endogenous PC. In contrast, the PC non-specifically adsorbed appeared to increase continuously with increasing ratio liposomal PC/brush-border vesicle PC (see discussion below). Thus, non-specific lipid adsorption and specific, PC exchange protein-catalyzed PC exchange appear to be independent processes which, however, may affect each other indirectly (see below).

The contribution from non-specific adsorption depends on the experimental conditions. It was found [36] that it increased with the liposome/brush-border vesicle ratio. If equal amounts of liposomes and brush-border vesicles (referred to the PC content of brush-border vesicles) were incubated, less than 10% of the brush-border vesicle PC originated from liposomes. In contrast, if brush-border vesicles were incubated with a 100-fold excess of liposomal PC for several hours, 50–60% of the brush-border membrane PC was of liposomal origin. Under the conditions of extensive PC exchange, the contribution from non-specific lipid adsorption is therefore hardly trivial and a correction for PC adsorbed by this mechanism becomes essential. It should be stressed that

with this correction the amount of PC exchanged in the presence of PC exchange protein could be determined sufficiently accurately even in unfavourable cases where the contribution from the non-specific lipid adsorption was of the order of 50%.

The mechanism of non-specific lipid adsorption remains unclear. What is, however, clear is that the lipid adsorbed is firmly associated with the brush-border membrane. It cannot be removed from the membrane by repeated sedimentation and redispersion of brush-border vesicles or by density gradient centrifugation [36]. In view of the amount of lipid adsorbed by this mechanism, the term non-specific seems inappropriate. On the other hand, the process may be of physiological importance and warrants a separate investigation and an examination as to its physiological relevance. The ability of brush-border vesicles to adsorb different lipids from aqueous dispersions has been reported earlier from different laboratories [34–36]. It is also clear that, although lipid adsorption and PC exchange protein-catalyzed PC exchange may be independent processes, incorporation of exogenous PC into the brush-border membrane will affect membrane properties such as the membrane fluidity [17,36], which in turn may affect the PC exchange. Therefore, the PC exchange data must be interpreted cautiously.

With the proposed assignment of the two phospholipid pools to different membrane surfaces, the exchange rate between these two pools becomes the membrane flip-flop rate. The values estimated from the phospholipase experiment are relatively high (with half-times of about 20 min) compared to other plasma membranes [37]. It has been pointed out that the exchange kinetics at 17°C are monophasic. Hence, under these conditions the PC flip-flop cannot be rate-limiting and is probably even much faster than under conditions of phospholipid hydrolysis. Control experiments showed that the difference in rate constants cannot be attributed to differences in the experimental conditions of probing with phospholipase A_2 and PC exchange protein. Experiments shown in Fig. 4 indicate that the exchange rate was reduced after partial phospholipid hydrolysis. This is probably due to changes in the lipid composition resulting from the partial hydrolysis of the membrane phospholipids. The question whether or not the observed exchange rates indeed represent flip-flop rates remains unanswered and awaits further experimentation. Still, even if the assignment of the two pools turns out to be correct, the rate constants derived from either probing methods can hardly be regarded as intrinsic in the light of the extensive modification of the bilayer properties brought about by these probing procedures. Furthermoe, any stable asymmetric distribution of the phospholipids would then have to be reconciled with supposedly high flip-flop rates.

In summary, the work presented here demonstrates the difficulties encountered in studying the topological distribution of phospholipid using phospholipase A_2 and PC exchange protein as membrane probes with such a highly specific and functionally active membrane as the brush-border membrane. Besselaar et al. [33] have reported similar difficulties, probing the distribution of PC in rat liver microsomal membranes using phospholipase A_2 and PC exchange protein. One difficulty arises from the presence of intrinsic lipases and hydrolases in general; their activity is subject to control mechanisms that are presently not understood. Secondly, the probing based on phospholipid exchange in the presence of PC exchange protein is hampered by the ability of brush-border membrane to take up phospholipids readily from exogenous sources. As a result the intrinsic bilayer properties may be modified. Furthermore, attempts to verify the possible asymmetric distribution of phospholipids in brush-border membrane by chemical labelling methods failed for reasons elaborated on previously [38]. It was shown that labels of low molecular weight (under 700) considered to be impermeant to other plasma membranes readily permeate across the brush-border membrane, interacting with actin, a protein located on the cytoplasmic side of brush-border vesicles. The explanation given for this behaviour was [38] that a large fraction of brush-border vesicles is unsealed or at least leaky to low molecular weight compounds. Future studies will have to be addressed to these problems before information on the topology and dynamics of lipids in brush-border membrane can be obtained.

Appendix

The kinetic analysis of the curves in Fig. 2 is based on the two-pool model shown in Fig. 6. The differential equations describing the changes in PC or PE concentration in the two pools are

$$dq_a/dt = k_{ab}q_b - k_{ba}q_a - k_0 q_a = k_{ab}q_b - k_{aa} \quad (1)$$

$$dq_b/dt = k_{ba}q_a - k_{ab}q_b \quad (2)$$

$$k_{aa} = k_0 + k_{ba}$$

where q_a and q_b are the PC (PE) concentrations in pool a and b, respectively, and k are rate constants are shown in Fig. 6. Integration of eqs. 1 and 2 is carried out by Laplace transformation [39]:

$$q_a/q_t = \frac{k_{ab} - (q_{a,0}/q_t)g_1}{g_2 - g_1} e^{-g_1 t} + \frac{k_{ab} - (q_{a,0}/q_t)g_2}{g_1 - g_2} e^{-g_2 t} \quad (3)$$

$$q_b/q_t = \frac{k_{ba} + (q_{b,0}/q_t)(k_0 - g_1)}{g_2 - g_1} e^{-g_1 t} + \frac{k_{ba} + (q_{b,0}/q_t)(k_0 - g_2)}{g_1 - g_2} e^{-g_2 t} \quad (4)$$

where $q_{a,0}$ and $q_{b,0}$ are PC (PE) concentrations at $t = 0$ in pool a and b, respectively, and $q_t = q_{a,0} + q_{b,0}$. Addition of Eqns. 3 and 4 yields the fraction $(q_a + q_b)/q_t$ of the total PC (PE) remaining in both pools (a + b) at time t. The fraction is determined experimentally as a function of time.

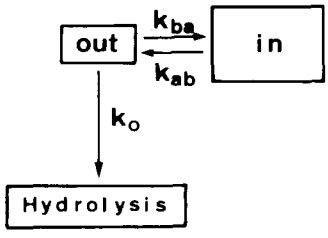

Fig. 6. Two-pool model for the hydrolysis of PC by phospholipase A_2: a = readily accessible pool (25%); b = less accessible pool (75%).

$$\frac{q_a + q_b}{q_t} = K_1 e^{-g_1 t} + K_2 e^{-g_2 t} \quad (5)$$

$$K_1 = \frac{k_{ab} + k_{ba} - g_1 + k_0(q_{b,0}/q_t)}{g_2 - g_1} = \frac{r_a k_0 - g_2}{g_1 - g_2} \quad (6)$$

$$K_2 = \frac{k_{ab} + k_{ba} - g_2 + k_0(q_{b,0}/q_t)}{g_1 - g_2} = \frac{g_1 - r_a k_0}{g_1 - g_2}$$

where

$$r_a = \frac{q_{a,0}}{q_t} = \frac{k_{ab}}{k_{ab} + k_{ba}} \quad (8)$$

$$g_1 + g_2 = k_0 + k_{ab} + k_{ba} \quad (9)$$

$$g_1 g_2 = k\, k_{ab} \quad (10)$$

References

1 Op den Kamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47–71
2 Etemadi, A.-H. (1980) Biochim. Biophys. Acta 604, 423–475
3 Krebs, J.J.R. (1982) J. Bioenerg. Biomembranes 14, 141–157
4 Kessler, M., Acuto, O., Storelli, C., Murer, H., Müller, M. and Semenza, G. (1978) Biochim. Biophys. Acta 506, 136–154
5 Perevucnik, G., Schurtenberger, P., Lasic, D.D. and Hauser, H. (1985) Biochim. Biophys. Acta 821, 169–173
6 Klip, A., Grinstein, S. and Semenza, G. (1979) FEBS Lett. 99, 91–96
7 Hauser, H., Howell, K., Dawson, R.M.C. and Bowyer, D.E. (1980) Biochim. Biophys. Acta 602, 567–577
8 Peterson, G.L. (1977) Anal. Biochem. 83, 346–356
9 Chen, P.S., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756–1758
10 Vaskovsky, V.E., Kostetsky, E.Y. and Vasendin, I.M. (1975) J. Chromatogr. 114, 129–141
11 Gritsuk, V.I., Mesheryakova, E.A., Ohanov, V.V., Efremiv, E.S. and Miroshnikov, A.I. (1979) Bioorg. Khim. 5, 1222–1232
12 Folch, J., Lees, M. and Stanley, G.H.S. (1957) J. Biol. Chem. 226, 497–509
13 Bergelson, L.D. (1980) Lipid Biochemical Preparations, Elsevier Science Publishers, Amsterdam
14 Kamp, H.H., Wirtz, K.W.A. and Van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 318, 313–325
15 Gains, N. and Hauser, H. (1983) Biochim. Biophys. Acta 731, 31–39
16 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917
17 Mütsch, B., Gains, N. and Hauser, H. (1983) Biochemistry 22, 6326–6333
18 Epstein, B. and Shapiro, B. (1959) Biochem. J. 71, 615–619
19 Ottolenghi, A. (1964) J. Lipid Res. 5, 532–537

20 Bonnefis, M.J., Thourenot, J.P. and Douste-Blazy, L. (1977) Biochimie (Paris) 59, 355–361
21 Takagi, R. and Sasaki, T. (1979) J. Biochem. (Tokyo) 85, 29–39
22 Subbaiah, P.V. and Ganguly, J. (1970) Biochem. J. 118, 233–239
23 Klip, A., Grinstein, S. and Semenza, G. (1979) J. Membrane Biol. 51, 47–73
24 Barsukov, L.I., Hauser, H., Hasselbach, H.-J. and Semenza, G. (1980) FEBS Lett. 115, 189–192
25 Bloj, B. and Zilversmit, D.B. (1982) J. Biol. Chem. 257, 7608–7614
26 Ohyashiki, T. and Mohri, T. (1982) J. Biochem. (Tokyo) 91, 1575–1581
27 Brasitus, T.A. and Schachter, D. (1982) Biochemistry 21, 2241–2246
28 Hauser, H., Gains, N., Semenza, G. and Spiess, M. (1982) Biochemistry 21, 5621–5628
29 Shipolini, R.A., Callewaert, G.L., Cottrell, R.C. and Vernon, C.A. (1974) Eur. J. Biochem. 48, 465–476
30 Brunner, J., Hauser, H., Braun, H., Wilson, K.J., Wacker, H., O'Neill, B. and Semenza, G. (1979) J. Biol. Chem. 254, 1821–1828
31 Bloj, B. and Zilversmit, D.B. (1976) Biochemistry 15, 1277–1283
32 Rothman, J.E. and Lenard, J. (1977) Science 195, 743–753
33 Van den Besselaar, A.M.H.P., De Kruijff, B., Van den Bosch, H. and Van Deenen, L.L.M. (1978) Biochim. Biophys. Acta 510, 242–255
34 Proulx, P., McNeil, J., Brglez, I. and Williamson, D.G. (1982) Can. J. Biochem. 60, 904–909
35 Proulx, P., Aubry, H., Brglez, I. and Williamson, D.G. (1984) Biochim. Biophys. Acta 775, 341–346
36 Mütsch, B., Gains, N. and Hauser, H. (1986) Biochemistry 25, 2134–2140
37 Housley, M.D. and Stanley, K.K. (1983) Dynamics of biological Membranes, pp. 43–48, John Wiley, Chichester
38 Gains, N. and Hauser, H. (1984) Biochim. Biophys. Acta 772, 161–166
39 Shipley, R.A. and Clark, R.E. (1972) Tracer Methods for in vitro kinetics – Theory and Applications, Academic Press, New York