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Effect of Phospholipase A_2 on Purified Gastric Vesicles

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The phospholipid and fatty acid composition and role of phospholipids in enzyme and transport function of gastric (H^++K^+)-ATPase vesicles was studied using phospholipase A_2 (bee venom). The composition (%) was phosphatidylcholine (PC) 33%; sphingomyelin (sph) 25%; phosphatidylethanolamine (PE) 22%; phosphatidylserine (PS) 11%; and phosphatidylinositol (PI) 8%. The fatty acid composition showed a high degree of unsaturation. In both fresh and lyophilized preparations, even with prolonged incubation, only 50% of phospholipids were hydrolyzed, but the amount of PE and PS disappearing was increased following lyophilization. There was a marked decrease in K^+-ATPase activity (75%) but essentially no loss of the associated K^+ p-nitrophenyl phosphatase was found. ATPase activity could be largely restored by various phospholipids (PE > PC > PS). There was also an increase in Mg^{2+}-ATPase activity, partially reversed in fresh preparations by the addition of phospholipids (PE > PS > PC). Proton transport activity of the preparation was rapidly inhibited, initially due to a large increase in the HCl permeability of the preparation. Associated with these enzymatic and functional changes, the ATP-induced conformational changes, as indicated by circular dichroism spectra were inhibited.

Key words: (H^++K^+)-ATPase, transport ATPase, proton transport, phospholipids, phospholipase A_2, CD spectrum, gastric ATPase

Recently, a K^+-activated adenosine triphosphatase (ATPase) has been isolated from gastric mucosa [1, 2] and further has been shown to transport both H^+ and K^+ [3, 4].

As isolated, this ATPase occurs in vesicles of uniform orientation as assessed by enzyme assay [2] and of highly restricted permeability to H^+, K^+, and Cl^-. The only class of peptides associated with these vesicles has a molecular weight of 105,000 and is phosphorylated by ATP, the phosphoenzyme having the kinetic characteristics of an intermediate in the overall reaction [5]. Evidence from tryptic hydrolysis [6] has been

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interpreted as indicating that possibly three peptides of mol wt 105,000 are involved in the ATPase activity and transport, only one of which is the catalytic subunit. Although in many of its properties, the enzyme is similar to the ($Na^+ + K^+$)-ATPase or Ca^{2+}-ATPase [7, 8], there are also many distinguishing features, not the least of which is the lack of antigenic cross-reactivity between antibody to this enzyme and the others.

Phospholipids have been shown to play an essential role in the enzymic activity of both the ($Na^+ + K^+$)-ATPase and the Ca^{2+}-ATPase [9, 10] and different phospholipids show different relative potency in terms of reconstitution of transport or enzyme activity [11, 12]. The role of phospholipids in another transporting vesicle, such as is readily available in the gastric (H^++K^+)-ATPase, has not previously been reported. Accordingly, by means of a purified fraction of hog gastric mucosa, the phospholipid composition and effect of phospholipase A_2 was examined.

MATERIALS AND METHODS

Phospholipase A_2 from bee venom was the highest-purity grade available commercially (Sigma Chemical Co.). By means of ^{14}C-labeled phosphatidylcholine, the enzyme was shown to hydrolyze the phospholipid at the 2 position.

Phosphatidylcholine from soybean (type II-S), phosphatidylethanolamine from bovine brain (type I), and phosphatidylserine (brain extract) were purchased from Sigma Chemical Co. Phosphatidylserine (bovine), phosphatidylcholine (bovine, egg, plant), and phosphatidylethanolamine (egg) were obtained from Supelco, Inc. and phosphatidylinositol (bovine) from Avanti Biochemical Inc. For thin-layer chromatography, standards were obtained from Sedary, Ontario.

All of the other chemicals were reagent grade.

Vesicle Preparation

A membrane fraction (GI), largely derived from the apical plasma membrane of parietal cells [13], was obtained from hog gastric mucosa by a combination of differential and zonal density gradient (Ficoll-sucrose) centrifugation by methods detailed previously [2]. This fraction is devoid of mitochondrial enzyme markers, RNA and (Na^++K^+)-ATPase, but is enriched by a factor of about 20 in the activities of K^+-ATPase and K^+-pNPPase (p-nitrophenyl phosphatase), which are copurified [2]. This fraction also has the highest H^+ and K^+ transport activity of the gastric membrane fractions [3, 4] as well as the highest ionophoretic stimulation of the K^+-ATPase activity.

For lyophilization, the fraction was diluted with water and spun down at 78,000g for 1 h. The pellet was homogenized in water, at a final protein concentration of 5 mg/ml and lyophilized. While this procedure does not affect the ATPase activity, more than 90% of proton transport activity, as well as 100% of the ionophoretic sensitivity, is abolished.

In the fresh GI vesicles, no latency of the ATPase or pNPPase was observed with NH_4^+ used as cation activator instead of K^+ [2], a finding interpreted as showing a uniform outside orientation of the ATP site of the enzyme. In lyophilized and resuspended GI vesicles, identical ATPase activity was obtained as in fresh vesicles in the presence of NH_4^+, indicating that if reorientation of ATP sites had occurred, ATP was freely permeable [2].

Electron Microscopy

Gastric vesicles, either fresh or lyophilized and resuspended as described above, at a protein concentration of approximately 1 mg/ml were fixed in 2% glutaraldehyde–1%

tannic acid in 0.1 M sodium cacodylate at pH 7.4 in the dark for 2 h at 4°C [14]. The samples were centrifuged for 30 min at 78,000g and the pellets were rinsed with 0.1 M sodium cacodylate. The final pellets were embedded in Epon and sectioned for electron microscopy.

Phospholipid Analysis

The phospholipid composition of fresh, lyopholized, and phospholipase A_2-treated membranes was analyzed. Lipid extraction was carried out as previously described [15]. The lipid extract was diluted with chloroform to a known volume and an aliquot was taken for phosphorus analysis [16]. Phospholipids were separated by thin-layer chromatography on silica gel HR plates (Merck Laboratory Chemicals) using a solvent system containing chloroform–methanol–acetic acid–water (50:20:6:2). Identification of the various phospholipids was made by R_f comparison with known phospholipid standards. After development, the plates were visualized with I_2 vapor. The spots were outlined, scraped, and processed for phosphorus determination [17]. Fatty acids composition of the various phospholipids were analyzed by gas-liquid chromatography [18], following separation by thin-layer chromatography (TLC) as described above and with 0.01% 2,7-dichlorofluorescein used for visualization.

Phospholipase A_2 Treatment

Using radioactive substrate, this enzyme was shown to be specific for the 2 position. No protease activity was detected since SDS gel patterns did not change at all following treatment. The final experimental conditions were chosen to obtain maximum loss of K^+-ATPase activity and maximal phospholipid hydrolysis. In pilot experiments incubation time and phospholipase A_2/membrane protein ratio were varied and maximal loss of K^+–ATPase activity and phospholipid hydrolysis was obtained using 130 IU of phospholipase A_2 (1 μg = 1.3 IU), 2 mg membrane protein, and an incubation time of 60 min at 22°C.

For fresh vesicles, the incubation was carried out with shaking in 1 ml solution containing 0.25 M sucrose, 2×10^{-3}M $CaCl_2$, 0.02 M Tris-Cl (pH 7.4). For the lyophilized vesicles, incubation was carried out under the above conditions, except for the omission of 0.25 M sucrose from the reaction medium.

The reaction was stopped by diluting the incubation mixture 30-fold with ice-cold fat-free 1% bovine serum albumin (BSA) in 0.02 M Tris-Cl, pH 7.4. The mixture was centrifuged at 78,000g for 40 min at 4°C and the pellet was washed twice with 20 ml ice-cold 0.02 M Tris-Cl buffer. Control studies with or without incubation, with or without phospholipase A_2, were run and showed no loss of ATPase activity. Hence, this procedure effectively stopped phospholipid hydrolysis. In absence of phospholipase A_2, no loss of ATPase activity was observed. It was also found that albumin washing was necessary to stop any further action of enzyme.

Reconstitution of Enzyme Activity

Reconstitution of enzyme activity was attempted in several ways. The most effective was found when phospholipid dispersions were mixed with the treated enzyme. Lipids at a concentration of 25 mg/ml were dispersed to clarity in 0.02 M Tris-Cl at pH 7.4 by sonication in a water-bath sonicator (Chemical Supplies Co.) under a nitrogen atmosphere at 22°C. The pH of the dispersion was checked and when necessary readjusted to 7.4. The phospholipid concentration in the dispersions was calculated from phosphorus determinations.

Reactivation of phospholipase-treated fresh or lyophilized membranes was carried out by mixing membrane protein and phospholipid dispersions and incubating for 15 min at 37°C. The ratio (w/w) of protein/phospholipid was varied between 1/1 and 1/50 for phosphatidylcholine and between 1/1 and 1/30 for phosphatidylserine and phosphatidylethanolamine. The recombinate was vigorously mixed, diluted with ice-cold water to give a final protein concentration of 0.1 mg/ml, and kept at 4°C.

Enzyme assays were carried out immediately.

Enzyme Assays

ATPase activity was measured in a medium containing 2×10^{-3}M $MgCl_2$, 2×10^{-3}M ATP (disodium salt), 0.04 M Tris-Cl pH 7.4 with or without 0.02 M KCl and 10 μg of protein in a final volume of 1 ml. After incubation at 37°C for 15 min, the inorganic phosphate released was assayed as previously described [2]. p-Nitrophenyl phosphate hydrolysis was measured in a similar medium, except that 6×10^{-3}M $MgCl_2$ and 6×10^{-3}M pNPP were used. The p-nitrophenol released was assayed as earlier reported [2]. To obtain the K^+-dependent ATPase and pNPPase activities, the "basal" Mg^{2+} activity was subtracted. In order to correct for a nonenzymatic hydrolysis of ATP or pNPP, all measurements were standardized with respect to time and compared with appropriate blanks.

Activities were measured on the same day as phospholipase A_2 treatment. Protein concentration was measured according to Lowry et al [19].

Proton Transport

The technique used has been previously described [20]. A fresh vesicle preparation (1 mg) was treated with 65 IU of phospholipase A_2 in a solution containing 0.25 M sucrose, 10^{-3}M $CaCl_2$, and 0.02 M Tris-Cl (pH 7.4) in 1 ml and incubated at 22°C for a period of time varying from 5 to 30 min. The digested membrane protein was then diluted to 3 ml in 0.15 M KCl, 2×10^{-3}M $MgCl_2$ glycyl-glycine buffer at pH 6.1. Proton transport was initiated with the addition of ATP to give a final concentration of 1.5×10^{-7}M and expressed as nmoles of H^+ uptake/mg of protein. Nigericin (1 μg/ml final concentration) was added in 10 μl methanol after the peak pH had been observed. The ionophore-dissipatable pH gradient corrects for any change of pH due to phospholipase A_2 action.

The initial rate of H^+ uptake is a function of H^+ pump activity and H^+ leak rate. The rate of dissipation of the H^+ gradient following ATP consumption is a measure of the H^+ leak rate alone. Comparing these parameters to ATPase activity measured in identical samples allows discrimination between an effect primarily on permeability and one primarily on pump activity.

In these experiments, control transport studies omitting phospholipase A_2 were carried out under identical conditions. It should be noted that phospholipase A_2 and by-products of the digestion were not removed in these experiments; therefore digestion was proceeding during the measurements of proton transport.

Circular Dichroism Measurements

The circular dichroism (CD) spectra were recorded at 22°C on a Cary 60 spectropolarimeter equipped with a model 6001 CD accessory. Protein concentrations were varied between 0.36 and 0.72 mg/ml and cells of 0.1 or 0.2 mm were used. Fresh and lyophilized membrane preparations before and after phospholipase A_2 treatment were resuspended in 0.02 mM Tris-Cl at pH 7.4 with additions as noted in figure legends and tables. The

spectrum of each sample was determined at least three times with two or more preparations and the results were averaged.

The CD spectra obtained were corrected [21] and the data were expressed as the mean residue ellipticity [Θ] ($deg \cdot cm^2 \cdot dmole^{-1}$) using 124 as the calculated mean residue weight of the protein. The pseudo reference state was achieved by adding 0.1% or 0.2% sodium dodecyl sulfate (SDS) and 10% or 20% trifluoroethanol (TFE) to the membrane suspensions and stirring for 30 min at 22°C.

RESULTS

Morphology of Gastric Vesicles

The tannic acid method outlines the orientation of proteins in biologic membranes [14]. In intact tissue, distinct asymmetry of the plasma membrane is observed, indicating that penetration of tannic acid is not a limiting factor in this procedure. A typical section of fresh vesicles shows that protein is oriented to the external face of the vesicles. A quantitative measure is that 90% of the vesicles show this orientation, and essentially all of the vesicles are intact. In contrast, following lyophilization and reconstitution, a number of the vesicles remain unsealed (37%) and also the protein reorients so now 62% have protein symmetrically distributed (Fig. 1).

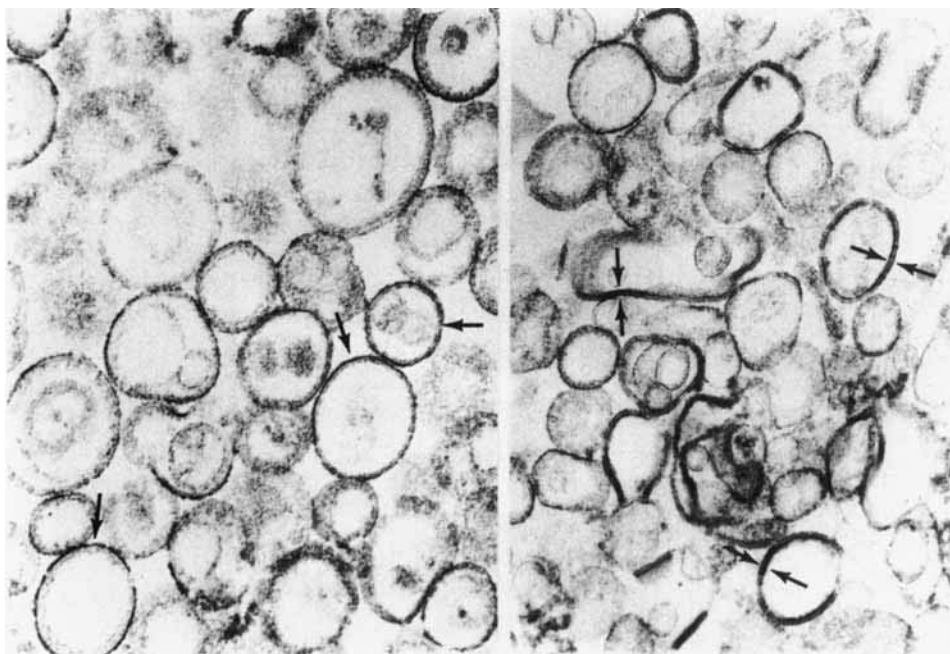


Fig. 1. Left: Electron micrograph of the fresh purified gastric vesicles fraction fixed in 2% glutaraldehyde and 1% tannic acid. This fraction consists almost exclusively of smooth vesicles. The outermost leaflet is almost always thicker than the inner ones (as indicated by the arrows). Some of the vesicles contain one or more smaller vesicles × 130,000. Right: Electron micrograph of the gastric vesicles preparation fixed in 2% glutaraldehyde and 1% tannic acid after lyophilization and resuspension. In contrast to the vesicles from the fresh material, those seen here are mostly surrounded by a symmetrical membrane (as indicated by the arrows) × 62,000.

Phospholipid Composition of the Gastric Membrane

As can be seen in Table I, gastric membrane preparations contain about 0.8 μmoles of phospholipid per milligram of protein. Since the protein has a molecular weight of 105,000, this corresponds to a molar ratio of 80. Thin-layer chromatography of the membrane lipid extract showed the main phospholipid components to be phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol in approximate ratio of 1:0.7:0.6:0.3:0.2. The fatty acid composition of the acyl groups of the various phosphoglycerides were substantially different, as shown in Table II. Unsaturated fatty acids accounted for approximately 50% of the acyl groups of phosphatidylcholine and 68–76% of the acyl groups of phosphatidylinositol, phosphatidylserine, and phosphatidylethanolamine. The phosphatidylcholine had equal amounts of 18:1 and 18:2; phosphatidylethanolamine and phosphatidylinositol contained 20:4, and only phosphatidylserine contained significant amounts of 22:6.

TABLE I. Phospholipid Composition of Control and Phospholipase A_2-Treated Gastric Membranes (μmoles/mg protein)*

			Phospholipase A_2-treated vesicles	
Phospholipid[a]	Control	% Total	Fresh	Lyophilized
Total	0.832 ± 0.02	100.0	0.445	0.406
Sphingomyelin	0.208 ± 0.01	25.0	0.201	0.213
Glycerol-phospholipids[a]	0.624 ± 0.01	74.4	0.244	0.193
PC	0.273 ± 0.02	32.8	0.082	0.087
PE	0.182 ± 0.02	21.9	0.078	0.040
PS	0.094 ± 0.01	11.3	0.048	0.028
PI	0.070 ± 0.01	8.4	0.035	0.037

*Phospholipid composition of control (intact vesicles and lyophilized) membranes is the mean of four different preparations (± SD). The values shown for phospholipase A_2-treated membranes are the average of two separate experiments.
[a]PC – phosphatidylcholine; PE – phosphatidylethanolamine; PS – phosphatidylserine; PI – phosphatidylinositol.

TABLE II. Fatty Acid Composition (weight %) of the Major Phospholipids* Isolated From Gastric Membranes

Fatty acids	PC	PE	PS	PI
16 : 0	39.5	13.8	2.9	9.8
18 : 0	11.0	14.9	21.1	21.7
18 : 1	24.2	40.8	59.5	25.0
18 : 2	25.3	16.3	–	17.5
20 : 4	–	14.2	–	26.0
22 : 6	–	–	16.5	–
% Unsaturated	49.5	71.4	76.0	68.5

The fatty acid composition of purified phosphoglycerides was analyzed and quantitated using a Hewlett-Packard 5830A gas chromatograph with a 1,500- × 2-mm (inside diameter) glass column packed with 10% SP-2340 on Chromosorb W AW. Column temperature was 180°C.
*Abbreviations: PC – phosphatidylcholine; PE – phosphatidylethanolamine; PS – phosphatidylserine; PI – phosphatidylinositol.

Phospholipase A_2 Treatment

Effect on phospholipids. The results of hydrolysis of the phospholipids of fresh and lyophilized vesicles are shown in Table I. Consonant with the small increased number of broken membranes seen in electron micrographs of the lyophilized fraction, there was a small increase of loss (8%) of hydrolyzable phospholipid in this fraction compared to the fresh membranes. The sphingolipids did not change, since no ester bond is present, and hence their contribution to the phospholipid composition rose from 25% in control to 49% in the treated fresh and 52% in the treated lyophilized membranes.

The level of total glycerol phospholipids fell by 60% in fresh and 70% in lyophilized membranes. Phosphatidylcholine and phosphatidylinositol levels were reduced by 70% and 50%, respectively, in both fresh and lyophilized membranes.

In contrast to the similar levels of hydrolysis of the above, the hydrolysis of both phosphatidylethanolamine and phosphatidylserine nearly doubled in lyophilized as compared to fresh membranes. This shows a difference in availability of these two phospholipids as a result of lyophilization.

Effect on enzyme activity. Although there are species variations in the ATPase to pNPPase activation ratios [22], in the case of hog stomach the two enzymes copurify in the fractionation procedure. ATP is a competitive inhibitor of pNPPase activity and pNPP is a competitive inhibitor of the ATPase [23]; hence although the binding sites for the two substrates are in all likelihood not identical, they clearly reside on the same group of 105,000-dalton peptides [6].

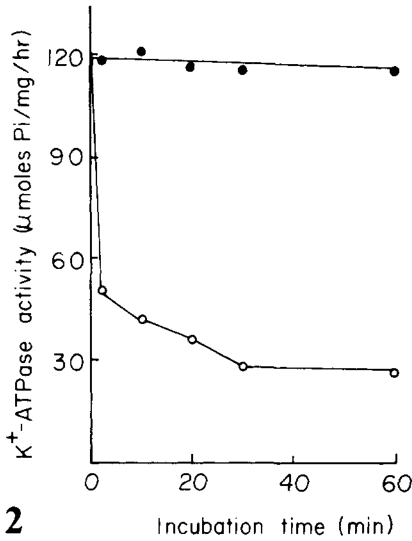
In the case of K^+-ATPase, at a level of 100 μg phospholipase A_2 there was a rapid loss of activity which leveled out at 30 min (Fig. 2). When the incubation time was fixed at 30 or 60 min, but the level of phospholipase A_2 was varied at a fixed membrane protein level, there was a phospholipase A_2 concentration-dependent loss of enzyme activity, reaching a maximum of 75% at the 60-min incubation time (Fig. 3). Omitting the 1% BSA wash resulted in partial (29%) protection of the ATPase activity (data not shown). No significant difference in rate or extent of inactivation was noted in the lyophilized preparation. No loss of activity occurred in the absence of added Ca^{2+}.

In contrast to the K^+-ATPase activity, K^+-pNPPase activity was not affected in fresh vesicles and only 15% was lost in lyophilized membranes under identical conditions. The omission of the BSA wash showed that, in contrast to the protection afforded the ATPase activity by the hydrolysis products, the products removed by BSA were inhibitory to the pNPPase, since 40% and 55% of pNPPase activity was inhibited (Fig. 4). Analysis of the BSA extract showed the presence of free fatty acids, hence their presence in the membrane without BSA washing is responsible for the pNPPase inhibition.

The protein recovery and the peptide pattern of the fresh or lyophilized membranes were identical before and after phospholipase A_2 treatment, showing that contamination by protease activity was not responsible for any of the inhibition observed. This was further borne out by the results of the reactivation experiments to be discussed below.

A surprising result, for which we have no explanation, was the stimulation of Mg^{2+}-ATPase activity as a result of phospholipase A_2 treatment, a 3.5-fold increase being noted in fresh membranes.

Reactivation studies. Preliminary experiments were carried out to determine the optimum conditions for restoration of ATPase activities to control levels by adding phospholipids. Figure 5 shows the results obtained at varying phospholipid-to-protein ratios for ATPase activity using a variety of phospholipids. There was a different optimum ratio for the various phospholipids studied. Using these optimum ratios, the effects of these



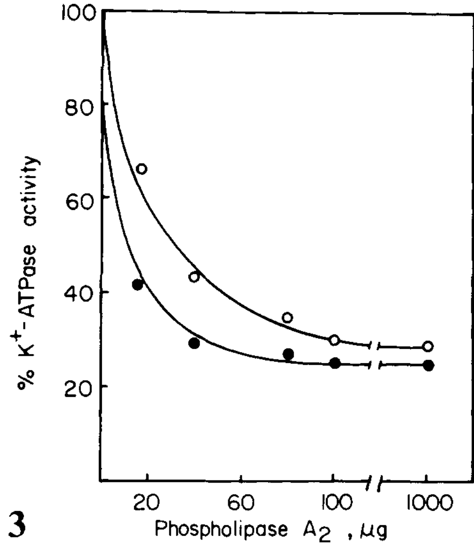


Fig. 2. Loss of K^+-ATPase activity (○—○) of gastric membrane vesicles as a function of the time of incubation with phospholipase A_2. Membrane protein (2 mg) was incubated with 100 μg of phospholipase A_2 at 22°C in one ml solution containing 0.25 M sucrose, 2×10^{-3}M $CaCl_2$, and 0.02 M Tris-Cl (pH 7.4) for different periods of time. Reactions were stopped by dilution of the incubation mixtures with ice-cold fat-free 1% BSA in 0.02 M Tris-Cl. After centrifugation the pellet was washed and spun down, and the final sediment was tested for K^+-ATPase activity (see Materials and Methods). Control experiments (●—●) were carried out under the above conditions in absence of phospholipase A_2.

Fig. 3. Effect of varying phospholipase A_2 concentration on gastric K^+-ATPase activity. Membrane protein (2 mg) was incubated at 22°C for 30 min (○—○) and 60 min (●—●) with different quantities of phospholipase A_2 in 1 ml medium containing 2×10^{-3}M $CaCl_2$ and 0.02 M Tris-Cl (pH 7.4). The reaction was terminated by dilution of the mixture with ice-cold 1% fat-free BSA in 0.02 M Tris-Cl. K^+-ATPase activity was assayed as described in Materials and Methods.

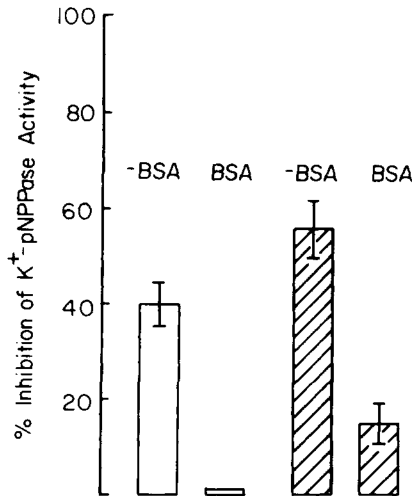


Fig. 4. Loss of K^+-pNPPase produced by phospholipase A_2 treatment of the intact vesicles (open columns) and lyophilized gastric membrane preparations (shaded columns). For each experiment 2 mg of membrane protein was digested with 130 IU of phospholipase A_2 at 22°C for 60 min in a medium containing 2×10^{-3}M $CaCl_2$ and 0.02 M Tris-Cl (pH 7.4), with or without 0.25 M sucrose. Reaction was terminated by dilution of the mixture with ice-cold 1% fat-free BSA in 0.02 M Tris-Cl (+BSA) or with ice-cold 0.02 M Tris-Cl (–BSA). K^+-pNPPase activity was assayed as indicated in Materials and Methods.

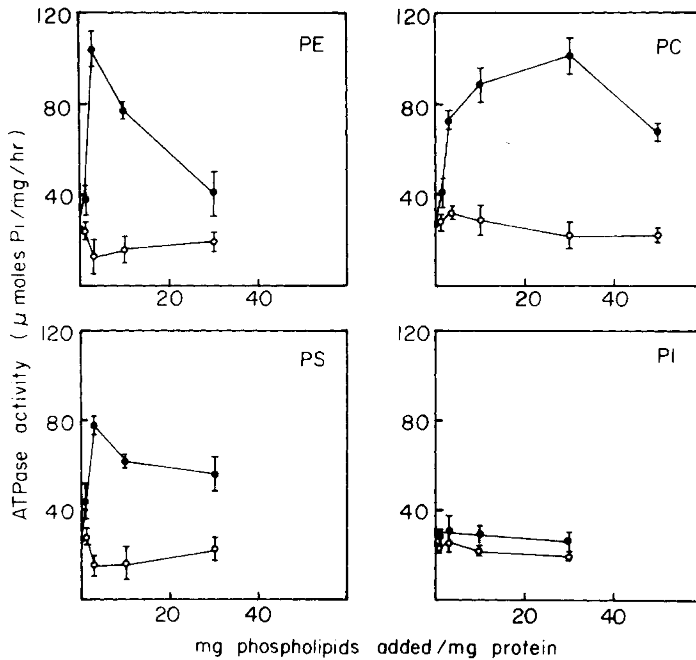


Fig. 5. Reconstitution of the Mg^{2+}-ATPase (o—o) and K^+-ATPase (•—•) activities in phospholipase A_2-treated gastric membranes by various phospholipids at varying phospholipid/protein ratio. ATPase was reconstituted with sonicated phospholipids according to the procedure of Materials and Methods. PE, Brain extract phosphatidylethanolamine; PC, soybean phosphatidylcholine; PS, bovine phosphatidylserine; PI, bovine phosphatidylinositol.

lipids on Mg^{2+} and K^+-ATPase activities were tested. As shown in Table III, varying degrees of reactivation were obtained, both as a function of the phospholipids and as a function of the state of the vesicles (fresh or lyophilized).

The most effective reactivation of K^+-ATPase both in lyophilized and fresh material was obtained with phosphatidylethanolamine, but the extent of reactivation was greater in the fresh membranes. Phosphatidylcholine, while as effective in the fresh material, was considerably less effective in the lyophilized membrane preparation where more of the phosphatidylethanolamine had been hydrolyzed. Phosphatidylserine was less effective than the above in fresh material, but as effective as phosphatidylcholine in the lyophilized material. Phosphatidylinositol partially inhibited the K^+-ATPase activity remaining after lipolytic digestion. Phospholipids from other sources, as noted in the methods section were less effective than those used in these experiments.

Considering the reversal of the Mg^{2+}-ATPase enhancement, there was partial reversal in fresh but not in lyophilized membranes. In this assay, as for the K^+-ATPase activity, phosphatidylethanolamine was the most effective in reducing the K^+-independent activity, followed by phosphatidylserine. Phosphatidylcholine and phosphatidylinositol were without effect.

Thus, phosphatidylethanolamine seemed to be the most effective phospholipid in restoring the original parameters of Mg^{2+} and K^+-ATPase activities in lipid-depleted membranes.

TABLE III. Effect of Phospholipid Addition on the Mg^{2+}-ATPase and K^+-ATPase Activities of Phospholipase A_2-Treated and BSA-Washed Gastric Membranes

Systems	Phospholipids added	Mg^{2+}-ATPase (μmoles Pi $mg^{-1}h^{-1}$)	K^+-ATPase[a] (μmoles Pi $mg^{-1}h^{-1}$)
Intact vesicles (untreated)	–	7.4 ± 1.0	119.6 ± 2.3
Intact vesicles (treated)	–	26.4 ± 1.7	29.9 ± 4.3
Intact vesicles	PE (brain extract)	13.4 ± 0.7	104.1 ± 11.9
Intact vesicles	PC (soybean)	23.5 ± 1.5	102.5 ± 10.4
Intact vesicles	PS (bovine)	15.1 ± 2.0	78.4 ± 1.5
Intact vesicles	PI (bovine)	25.3 ± 0.9	25.9 ± 3.1
Lyophilized vesicles (untreated)	–	7.5 ± 1.1	107.6 ± 5.4
Lyophilized vesicles (treated)	–	12.7 ± 4.7	26.2 ± 4.3
Lyophilized vesicles	PE (brain extract)	10.7 ± 2.1	68.6 ± 9.6
Lyophilized vesicles	PC (soybean)	14.2 ± 0.8	50.1 ± 0.3
Lyophilized vesicles	PS (bovine)	12.5 ± 1.7	47.8 ± 0.8
Lyophilized vesicles	PI (bovine)	13.1 ± 2.6	12.4 ± 1.4

Phospholipase A_2-treated membranes were incubated at 37°C for 15 min with phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylinositol (PI) at concentration ratios 3/1, 30/1, 3/1, 3/1, (w/w), respectively, and ATPase assay was carried out as described in Methods and Materials (mean of six experiments ± SD).

[a] Activity in presence of 0.02 M KCl minus the "basal" Mg^{2+}-ATPase rate. For intact vesicles (untreated) the K^+-ATPase activity was measured in presence of 10^{-5} M valinomycin.

H^+ Transport

Under control conditions, the addition of 1.5×10^{-7}M ATP resulted in an uptake of 33 nmoles H^+/mg protein in the first 6 sec and a maximum uptake of 54 nmoles H^+/mg protein, which was reached in 3 min. A slow decline in the H^+ gradient was noted after about 4 min and the residual gradient was dissipated by nigericin (curve A, Fig. 6).

Treatment of the gastric vesicles with phospholipase A_2 for 5 min, which resulted in only 10% reduction of K^+-ATPase activity, reduced the initial H^+ uptake by 45% and the maximal H^+ uptake by 16%. The most striking effect, however, was that the time of maintenance of the gradient was reduced, dissipation starting to occur at 90 sec following the addition of ATP. Again the residual gradient was abolished by nigericin (curve B, Fig. 6). Since ATPase activity was only slightly affected, the reduction in the initial rate, and the reduction in time of maintenance of the gradient, were due to an increased H^+ leak in the vesicle membrane.

At 15 min incubation, where 60% of the ATPase activity was abolished, and the H^+ uptake parameters were reduced (curve C, Fig. 6). These effects were therefore due to a combination of both H^+ pump inhibition and H^+ leak enhancement.

CD Measurements

Evidence that functional parameters of membrane-bound enzymes can be modified by changes in the structure and composition of the membrane suggest the possibility that lipids evoke a conformational change in the protein. In this regard, circular dichroism provides a method, although in part empirical, with which to demonstrate this role. Figures 7 and 8 show the CD spectra of the intact vesicles and lyophilized membrane suspensions

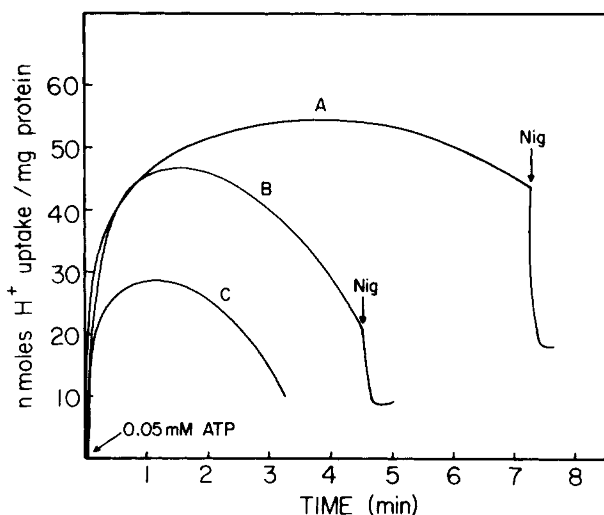


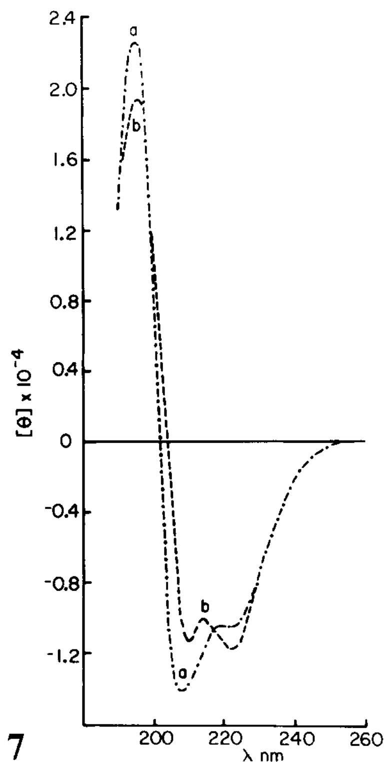
Fig. 6. Effect of phospholipase A_2 digestion on H^+ uptake. Intact vesicles (1 mg of protein) were in-incubated with 65 IU of phospholipase A_2 at 22°C in 1 ml solution containing 10^{-3}M $CaCl_2$, 0.02 M Tris-Cl (pH 7.4), and 0.25 M sucrose for 5 and 15 min. The treated membrane suspension was diluted to 3 ml in 0.15 M KCl, 2×10^{-3}M $MgCl_2$, and 5×10^{-3}M glycyl-glycine buffer at pH 6.1. Proton transport was initiated with the addition of 1.5×10^{-7}M ATP (final concentration). Proton transport is expressed as nmoles H^+ uptake/mg protein. A, Control experiment; B, 5 min phospholipase A_2 treatment of vesicles; C, 15 min phospholipase A_2 treatment of vesicles. At times indicated nigericin (Nig) was added.

before and after phospholipase A_2 treatment. The CD spectra of both untreated membrane systems were similar, indicating that the lyophilization process did not sensibly affect the structure of the enzyme and its activity. The shape of the curves were indicative of a protein structure with α-helix content. The percentage of α-helix structure calculated on the basis of the ellipticity values of the peak at 222 nm [24] was estimated to be approximately 25% for both systems.

The spectra were characterized by an intense negative peak centered at 208 nm and by a less pronounced negative peak at 222 nm. Associated with these peaks was a strong positive band at 195 nm and a cross-over at 202 nm. Phospholipase A_2 treatment resulted in a change of the CD spectra for both fresh and lyophilized membrane preparations.

A red shift of about 2 nm in the cross-over was found with a net decrease and a red shift, of the peak at 208 nm. No shift was found in the negative peak at 222 nm, which slightly increased in intensity in the treated fresh vesicles. The positive peak at 195 nm was lowered by about 15–25% and also red-shifted, indicating the presence of some protein-protein or stronger lipid-protein interaction.

The addition of ATP to the untreated membrane preparation produced a large decrease in the ellipticity in the 208-nm region (Saccomani et al, in preparation), resulting in a marked fall in the ratio $[\theta]_{208}/[\theta]_{222}$. This may be interpreted as due to an increase in the α-helix content [24]. Following phospholipase A_2 digestion, the ellipticity values were already shifted in the direction induced by ATP addition to the untreated material; hence, the addition of ATP produced no change in the CD spectra. Thus, phospholipase induced a conformational change in the protein and inhibited the ATP-induced structural change (Table IV).



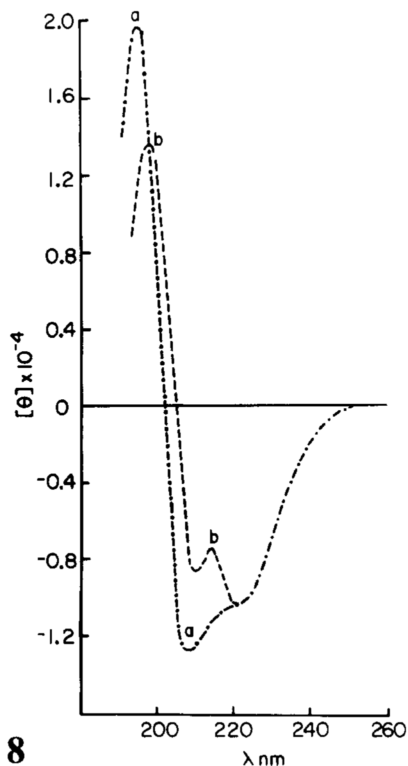


Fig. 7. Corrected circular dichroism spectra of the gastric membranes. Fresh vesicles (a) were used at concentration 0.57 mg/ml with a cell of path length 0.211 mm. The pseudoreference state was achieved by solubilizing the suspension in 0.1% sodium dodecyl sulfate (SDS) and 20% trifluoroethanol (TFE). Phospholipase A_2-treated vesicles (b) were used at concentration 0.72 mg/ml with a cell of path length 0.103 mm. For solubilization 0.2% SDS–10% TFE was used.

Fig. 8. Corrected circular dichroism spectra of the gastric membranes. Lyophilized membranes (a) at concentration 0.56 mg/ml were solubilized in 0.1% SDS–20% TFE and a cell of 0.211 mm path length was used. Phospholipase A_2-treated membrane (b) (0.75 mg/ml) was solubilized in 0.1% SDS–20% TFE and a cell path length of 0.103 mm was used.

TABLE IV. Ellipticities of Gastric Membranes Reported in $[\theta] \times 10^{-4}$M

	– ATP			+ ATP[a]		
Membrane systems[b]	$[\theta]_{208}$	$[\theta]_{222}$	$\frac{[\theta]_{208}}{[\theta]_{222}}$	$[\theta]_{208}$	$[\theta]_{222}$	$\frac{[\theta]_{208}}{[\theta]_{222}}$
Intact vesicles	– 1.40	– 1.03	1.36	– 0.83	– 1.22	0.69
Intact vesicles/PL-A_2	– 0.89	– 1.17	0.76	– 1.17	– 1.40	0.84
Lyophilized membrane	– 1.27	– 0.95	1.34	– 0.82	– 0.98	0.84
Lyophilized membrane/ PL-A_2	– 0.69	– 0.98	0.70	– 0.73	– 0.90	0.81

Ellipticities were calculated using 0.1% SDS–20% TFE as pseudoreference states.

[a] Samples at conc. 0.72 mg/ml were incubated for 10 min at 22°C in the presence of 2×10^{-3} M $MgCl_2$, 2×10^{-3}M ATP, 0.02 M KCl, and experiments were carried out with 0.1-mm path length cell.

[b] Abbreviation: PL-A_2 – phospholipase A_2.

DISCUSSION

In this work we have approached several of the phospholipid-related properties of the H^+ and K^+ transporting vesicles of gastric mucosa, which contain a K^+-ATPase activity. Thus, we have studied phospholipid and fatty acid composition, the effect of phospholipase A_2 on phospholipid composition in fresh and lyophilized membrane preparation, its effect on ATPase and pNPPase activities, the reactivation of the lost enzyme activity, and the effect of lipid hydrolysis on proton transport and protein structure as revealed by circular dichroism.

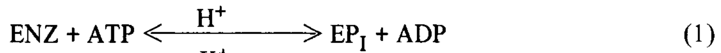
There seems to be nothing remarkable about the phospholipid composition of these vesicles, in that many other membranes exhibit the same ratio of phospholipids [25, 26]. The fatty acids show relatively high quantities of unsaturated fatty acids as compared, for example, to erythrocyte membranes [27]. This perhaps may have a teleologic explanation because gastric lumen is exposed to lower temperatures on occasion, but perhaps more importantly because the apical membranes of the gastric parietal cell (which account for the majority of membranes in the GI fraction) undergo reversible fusion-eversion as a function of secretory status [28].

The asymmetric distribution of membrane phospholipids [29–32] as well as the lipid dependence of membrane-bound enzymes [33–35] can be studied by selective degradation of different phospholipid pools using various phospholipases.

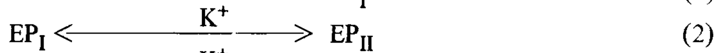
All of these studies have concluded that the lipid bilayer is asymmetric with respect to the distribution of at least some phospholipid species. The fact that such studies have been limited to relatively simple membrane systems emphasizes the formidable problems of obtaining membrane preparations that are free of contaminating lipids, sealed, and of uniform sidedness [36]. The procedure chosen in this work – the use of fresh, ion-tight membrane vesicles in terms of phospholipase A_2 (bee venom) hydrolysis – provides evidence of some degree of transbilayer asymmetry.

Considering the phospholipid hydrolyzed, as shown in Table III if we make the assumption, based on the loss of about 60% of the glycerol lipids due to hydrolysis by phospholipase A_2 and based on studies in the red cell [37], that only the outer leaflet of the bilayer is attacked, we can estimate that 70% of the phosphatidylcholine, 62% of the phosphatidylethanolamine, 40% of the phosphatidylserine, and 50% of the phosphatidylinositol reside in the outer half. Accordingly, the majority (77%) of the sphingolipids have to be on the inner half of the bilayer. Lyophilization results in only an 8% increase in loss of glycerol phospholipids, hence it follows, also on the basis of the electron microscopic data, that most of the vesicles reformed following lyophilization have to be closed and not permeated by phospholipase A_2; otherwise total hydrolysis of the glycerol phospholipids would be likely to occur. Since the pattern of hydrolysis of phosphatidylcholine and phosphatidylinositol does not change after lyophilization, no significant reorientation of these lipids has occurred. In contrast there is a doubling of hydrolysis of phosphatidylserine, and hydrolysis of phosphatidylethanolamine increases from 60% to almost 80%. Thus, lyophilization results in an increased access of phospholipase A_2 to these two substrates. An exchange of these phospholipids with sphingolipids in the outer leaflet of the bilayer during lyophilization can be suggested, as has been proposed for other systems [38].

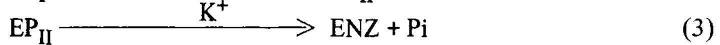
Inactivation of the ATPase with sparing of the pNPPase can readily be explained on the basis of the following model of enzyme activity, analogous to that established for the $(Na^+ + K^+)$-ATPase [7].



$$\mathrm{ENZ + ATP} \xleftrightarrow{H^+} \mathrm{EP_I + ADP} \quad (1)$$



$$\mathrm{EP_I} \xleftrightarrow{K^+} \mathrm{EP_{II}} \quad (2)$$



$$\mathrm{EP_{II}} \xrightarrow{K^+} \mathrm{ENZ + Pi} \quad (3)$$

Thus, loss of some of the phospholipids affects reactions (1) or (2) with no effect on reaction (3), which is reflected by the K^+-dependent pNPPase activity. The role of phospholipids therefore, in this system, is to maintain the active state of the enzyme for the initial stages of the reaction. This is in contrast with the Ca^{2+}-ATPase, where the dephosphorylation step is more sensitive to the phospholipid environment [39]. Without BSA washing to remove free fatty acids, pNPPase activity was significantly reduced following phospholipase A_2 digestion. Thus, it seems that free fatty acids can selectively inhibit the dephosphorylation step in the gastric K^+-ATPase. All these data indicate a significant but complex role of phospholipids in maintaining enzyme activity and the relative activity of the two reaction sites.

This is also illustrated by the reactivation of ATPase by added phospholipids. In an unknown fashion phospholipids also seem to regulate the K^+ requirement for ATPase activity since Mg^{2+}-ATPase activity increased. Referring to the reaction scheme, if the interconversion of EP_I and EP_{II} is rate-limiting and requires the presence of K^+ and the breakdown of EP_{II} requires the presence of K^+, then removal of phospholipid, especially phosphatidylethanolamine, has altered the reaction path, allowing ATP hydrolysis to proceed without the K^+-dependent steps. Alternatively, activation of a nonspecific ATPase may be suggested. Against this latter possibility, in more highly purified fractions, where the absence of nonspecific ATPase is established on the basis of either enzyme assay or antibody studies [40], similar data were obtained.

The finding that phospholipid hydrolysis rapidly affects the H^+ permeability of gastric vesicles is not surprising. These vesicles are remarkably ion-tight with a $t_{1/2}$ for H^+ of 5 min, and for Cl^- and K^+ of 40 min at 22°C [5]. Aging or trypsinization induce a Cl^- conductance [41], which is in part responsible for an increased HCl leak. In the present case, the H^+ leak induced by lipolytic digestion is greater, indicating that ion-tightness is more a function of the phospholipid than of the membrane protein.

Intact vesicles and lyophilized membrane preparations exhibit similar CD patterns with an α-helix content of about 25%.

Significant changes in the CD spectra as originally recorded were obtained when gastric membranes were treated with phospholipase A_2. The net decrease of the transition at 208 nm and concomitant decrease of the transition at 198 nm, as well as a red shift of the cross-over, could be explained by the fact that lipolytic digestion and consequent removal of the free fatty acids by BSA may induce a real change in the protein conformation owing to a stronger lipid-protein and/or protein-protein interaction, keeping the protein in a more hydrophobic and highly polarizable environment, as suggested by others [42].

Since ATP addition to untreated gastric membranes has also been found to induce conformational changes in the protein structure, we have also investigated the possibility of a structural modification of the phospholipase A_2-treated membrane protein upon addition of ATP. A useful way to detect relative changes in the enzyme structure is to use the ratios between the negative ellipticity values of the CD bands at 208 and 222 nm [43]. Table IV shows that for fresh vesicles and lyophilized membrane preparations the calculated $[\theta]_{208}/[\theta]_{222}$ are very similar and ATP addition to both systems caused a drop in these

values, suggesting that some conformational change had occurred with a possible increment in the α-helix content as indicated by the slight increase of the ellipticity values at 222 nm. More interesting, however, is the fact that the ellipticity ratios at 208 and 222 nm for the CD spectra of the phospholipase A_2-treated membranes decrease and remain approximately the same upon ATP addition. This could suggest that in the inactive state the ATP hydrolytic sites are not accessible to the substrate, indicating that perturbation of the protein structure and enzyme activity are closely associated.

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REFERENCES

1. Ganser AL, Forte JG: Biochim Biophys Acta 307:169, 1973.
2. Saccomani G, Stewart HB, Shaw D, Lewin M, Sachs G: Biochim Biophys Acta 465:311, 1977.
3. Sachs G, Chang HH, Rabon E, Schackmann R, Lewin M, Saccomani G: J. Biol Chem 251:7690, 1976.
4. Schackmann R, Schwartz A, Saccomani G, Sachs G: J Membr Biol 32:361, 1977.
5. Sachs G, Rabon E, Saccomani G: In Mukohata T, Packer L (eds): "Cation Flux Across Biomembranes." New York: Academic, 1979, p 53.
6. Saccomani G, Dailey DW, Sachs G: J Biol Chem 254:2821, 1979.
7. Albers RW: In Martonosi A (ed): "The Enzymes of Biological Membranes." New York: Plenum, 1976, vol 3, p 283.
8. MacLenan DH, Holland PC: In Martonosi A (ed): "The Enzymes of Biological Membranes." New York: Plenum, 1976, vol 3, p 221.
9. Fiehn W, Hasselbach W: Eur J Biochem 13:510, 1970.
10. Roelofsen B, van Deenen LLM: Eur J Biochem 40:245, 1973.
11. Kimelberg HK, Paphadjopoulos D: J Biol Chem 249:1071, 1974.
12. Coleman R, Bramley TA: Biochim Biophys Acta 382:565, 1975.
13. Saccomani G, Crago S, Mihas AA, Dailey D, Sachs G: Acta Physiol Scand. (Special Suppl), 1978, p 293.
14. Saito A, Wang CT, Fleischer S: J Cell Biol 79:601, 1978.
15. Spenney JG, Saccomani G, Spitzer HL, Tomana M, Sachs G: Arch Biochem Biophys 161:456, 1974.
16. Bartlett GR: J Biol Chem 234:466, 1959.
17. Skipski VP, Barclay M, Archibald WM, Terebus-Kekich O, Reichman ES, Good JJ: Life Sci 4:1673, 1965.
18. Balint JA, Beeler DA, Treble DH, Spitzer HL: J Lipid Res 8:486, 1967.
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265, 1951.
20. Chang H, Saccomani G, Rabon E, Schackmann R, Sachs G: Biochim Biophys Acta 464:313, 1977.
21. Urry DW: Biochim. Biophys Acta 265:115, 1972.
22. Saccomani G, Crago S, Mihas AA, Chang HH, Sachs G: J Clin Invest 64:627, 1979.
23. Sachs G: Rev Physiol Biochem Pharmacol 79:134, 1977.
24. Chen YH, Yang JT, Martinez HM: Biochemistry 11:4120, 1972.
25. Chap HJ, Zwaal RFA, van Deenen LLM: Biochim Biophys Acta 467:146, 1977.
26. De Pont JJHHM, van Prooijen-van Deenen A, Bonting SL: Biochim Biophys Acta 508:464, 1978.
27. van Deenen LLM, de Gier J: In Surgenor SM (ed): "The Red Blood Cell." New York: Academic, 1974, p 147.
28. Sedar AW: Fed Proc 24:1360, 1965.
29. Colley CM, Zwaal RFA, Roelofsen B, van Deenen LLM: Biochim Biophys Acta 307:74, 1973.
30. Verkleij AJ, Zwaal RFA, Roelofsen B, Comfurius P, Kastelijn D, van Deenen LLM: Biochim Biophys Acta 323:178, 1973.

31. Zwaal RFA, Roelofsen B, Comfurius P, van Deenen LLM: Biochim Biophys Acta 406:83, 1975.
32. Rothman JE, Tsai DK, Dawidowicz EA, Lenard J: Biochemsitry 15:2361, 1976.
33. Yu BP, DeMartinis FD, Masoro EJ: J Lipid Res 9:494, 1968.
34. Meissner G, Fleischer S: Biochim Biophys Acta 255:19, 1972.
35. Roelofsen B, Schatzmann HJ: Biochim Biophys Acta 464:17, 1977.
36. Rothman JE, Lenard J: Science 195:743, 1977.
37. Roelofsen B, Zwaal RFA, Comfurius P, Woodward CB, van Deenen LLM: Biochim Biophys Acta 241:925, 1971.
38. Roberts MF, Bothner-By AA, Dennis EA: Biochemistry 17:935, 1978.
39. Martonosi A, Donley JR, Pucell AG, Halpin RA: Arch Biochem Biophys 144:529, 1971.
40. Saccomani G, Helander HF, Crago S, Chang HH, Dailey DW, Sachs G: J Cell Biol (In press).
41. Rabon E, Kajdos I, Sachs G: Biochim Biophys Acta (In press).
42. Straus JH, Gordon AS, Wallach DFH: Eur J Biochem 11:201, 1969.
43. Cockle SA, Epan RM, Boggs JM, Moscarello MA: Biochemistry 17:624, 1978.