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# Characteristics of the Isolated Apical Plasmalemma and Intracellular Tubulovesicles of the Gastric Acid Secreting Cells: Demonstration of Secretagogue-Induced Membrane Mobilization†

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ABSTRACT: Separation of the gradient-purified gastric microsome into two membrane subfractions of distinct enzymatic and phospholipid composition has been achieved by mild SDS (0.033% w/v) treatment followed by sucrose gradient centrifugation of the pig and rabbit gastric microsomes. While the high-density membranes had all of the $(H^+,K^+)$-ATPase and $K^+$-pNPPase activities and revealed a single major 100-kDa band on SDS-PAGE, the low-density membranes contained all of the 5′-nucleotidase and nearly all of the $Mg^{2+}$-ATPase. In the present study, the low-density subfraction has been characterized to be derived from the apical membranes and the high-density one from the intracellular tubulovesicular membranes of the parietal cells. Such characterization was based primarily on sole dependency of the apical plasma membranes on the endogenous activator for $(H^+,K^+)$-ATPase activity, differential sensitivity of the activator (AF)-dependent and -independent $(H^+,K^+)$-ATPase on micromolar vanadate and $Ca^{2+}$, specific vitamin $B_{12}$ binding ability of the apical plasmalemma, phospholipid and protein profiles of the two membrane subfractions, and other parameters. The AF, mentioned previously, has recently been implicated as a cytosolic regulator of the gastric $(H^+,K^+)$-ATPase [Bandopadhyay et al. (1987) *J. Biol. Chem. 262*, 5664-5670]. Two different forms (i.e., AF-dependent and -independent forms) of the $(H^+,K^+)$-ATPase are suggested to be present in the tubulovesicles on the basis of differential vanadate sensitivity while the AF-dependent form alone is present in the apical membranes. The data have been discussed in terms of stimulation-induced membrane transformation characteristic of the $H^+$-secreting epithelia including the acid-secreting cells of the stomach.

Gradient-purified gastric microsomal vesicles have recently been proved to be a unique model system for studies on various aspects of the proton transport process (Ray & Fromm, 1981; Sen et al., 1980; Nandi & Ray, 1982; Ray et al., 1982, 1983; Nandi et al., 1983a-c) including molecular insights into the gastric $(H^+,K^+)$-ATPase system (Ray & Forte, 1976; Ray & Nandi, 1983; Nandi & Ray, 1987). The purified microsomal vesicles, highly enriched in gastric $(H^+,K^+)$-transporting ATPase activity, consist of a mixed membrane population derived primarily from the secretory surface (apical plasmalemma) and intracellular tubulovesicular membranes, the latter believed to act as an intracellular reserve for the former. Recycling of the secretory membranes is suggested to occur following cessation of secretion (Sedar & Friedman, 1961; Forte et al., 1977; Zalewsky & Moody, 1977; Ito & Schofield, 1974; Helander & Hirschwitz, 1974).

Previous efforts toward positive identification and biochemical characterization of the secretory plasma membranes and intracellular tubulovesicles were not successful due to their close functional similarities as well as lack of appropriate specific markers which could discriminate between these two membrane types. In a recent report (Nandi et al., 1987), we have demonstrated that treatment of purified gastric microsomes with low (0.033%) SDS under appropriate conditions followed by equilibrium sucrose density gradient centrifugation generates two membrane subfractions having different chemical and enzymatic profiles. In the present study, the use of a pure preparation of cytosolic activator protein (Bandopa-

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dhyay & Ray, 1986; Bandopadhyay et al., 1987) capable of uncovering a ($H^+$,$K^+$)-ATPase activity solely dependent on the activator, as well as specific association of the newly discovered phenomenon of intrinsic factor like activity with the secretory plasmalemma, allowed us to make differential identification of the low- and high-density membrane subfractions, as derived from the apical plasmalemma and the intracellular tubulovesicles, respectively. Additional features such as protein profile, morphological characteristics, ratios of phosphatidylcholine to phosphatidylethanolamine and of phosphatidylcholine to sphingomyelin, vanadate sensitivity, and $Ca^{2+}$ sensitivity of the ($H^+$,$K^+$)-ATPase in the presence and absence of the activator protein helped to differentiate between the high- and low-density membrane subfraction and thus aid in their subcellular identification.

The present data demonstrate fundamental differences as well as similarities between the apical plasmalemma and the intracellular tubulovesicular membranes. The data also suggest that appropriate modification of the tubulovesicles takes place prior to their incorporation into the cell surface membrane during the secretion-associated membrane transformation events. In addition, it was demonstrated that the tubulovesicles are mobilized with concomitant increase in the population of apical membranes during secretagogue stimulation (in vivo) of the rabbit stomach.

## Materials and Methods

*Preparation of Purified Gastric Microsomes.* Gradient-purified pig and rabbit gastric microsomes highly enriched in ($H^+$,$K^+$)-ATPase and $K^+$-pNPPase activities were isolated from the fundic mucosa following the procedure described previously (Ray, 1978; Ray & Nandi, 1986). No ouabain-sensitive ($Na^+$,$K^+$)-ATPase activity is detected in these membranes, suggesting negligible contamination by basolateral membranes. The preparation is also free from the mitochondrial marker enzymes such as succinic dehydrogenase and $HCO_3^-$-stimulated ATPase. The microsomes are derived primarily from the apical and tubulovesicular membranes of the parietal cells (Sachs et al., 1977).

*Preparation of the Activator Protein (AF).* The activator protein from pig and dog fundic cells was purified either following our recent procedure (Bandopadhyay & Ray, 1986; Bandopadhyay et al., 1987) or following a slight modification. The DTT (0.2 mM) used previously was omitted from all media without affecting the yield and purity of the AF. Briefly, the cytosolic fraction from the pig stomach was harvested by the procedure of Ray (1978). The soluble supernatant from the sucrose gradient (about 130 mL; 400–700 mg of protein) was concentrated while dialyzing at 0–4 °C (using a Micron-ProDicon, 60-kDa cutoff membrane) for 24 h against 2.5 L of 2 mM Pipes buffer, pH 7.4, containing 0.2 mM EDTA. The concentrated supernatant was carefully titrated at 4 °C with 0.1 M acetic acid to pH 4.8. The precipitate was pelleted at 10000*g* for 10 min at 0 °C. Following decantation of the supernatant, the pH precipitate was dissolved in an appropriate volume of 50 mM Tris, 0.25 M sucrose, and 0.2 mM EDTA, pH 7.4. Further purification of the pH precipitate was achieved by chromatography on Sephacryl S-200 columns and elution by 50 mM Tris (pH 7.4) containing 0.2 mM EDTA, as described recently (Bandopadhyay & Ray, 1986; Bandopadhyay et al., 1987).

*Isolation of Membrane Subfractions.* The procedure for the subfractionation of gradient-purified gastric microsomes has recently been reported (Nandi et al., 1987). In brief, pig gastric microsomes were extracted with 0.033% (w/v) SDS in 5 mL of a medium containing 250 mM sucrose, 2 mM ATP, 0.5 mM dithiothreitol (DTT), and 100 mM Tris–glycine buffer (pH 9.0). The extraction consisted of 6 min of sonication (Heat Systems Ultrasonics, Model W-225 R; using a cup horn with output control setting at 10) in a thick-wall glass tube (i.d. = 1.5 cm) at room temperature for 6 min followed by a period of 30-min incubation at 21 °C. It may be noted that sonication at 4 °C for about 15 min produces membrane subfractions similar to those obtained following room temperature sonication in about 60% of the cases. However, room temperature sonication consistently produces reproducible results. In addition, increasing the time of sonication up to 10 min at 21 °C does not alter either the relative yield of the subfraction or the response of the subfractions to the activator. After the extraction, the incubation mixture was diluted 1:2 with ice-cold 5 mM Pipes buffer (pH 6.8) and layered over linear (20–40%) continuous sucrose gradients. The materials were then spun for 16 h at 130000 rpm in an SW rotor (Beckman) at 4 °C. Following centrifugation, 1.5-mL fractions were collected with an LKB fractional collector and assayed for protein and enzyme activities. Band 1 (fractions 14–18) and band 2 (fractions 5–9) were separately pooled, diluted with homogenizing medium, and pelleted by high-speed centrifugation. The pellets were suspended in the homogenizing buffer at a protein concentration of 0.5 mg/mL and used for the study. Our recent experiments revealed that omission of DTT from the final suspending medium does not alter the ATPase activity.

*Enzyme Assays.* The ($H^+$,$K^+$)-ATPase, $Mg^{2+}$-ATPase, $K^+$-pNPPase, and 5′-nucleotidase were assayed as previously described (Nandi & Ray, 1984; Nandi et al., 1987). For the activator activation of the ($H^+$,$K^+$)-ATPase, the membranes were first preincubated for 10 min at 37 °C without (control) and with the desired concentration of the activator protein. Aliquots of the pretreated membranes were used as a source of the enzyme. Briefly, the incubation system for ATPase contained, in a total volume of 0.95 mL, 50 μmol of Pipes (pH 6.8), 5 μmol of $MgCl_2$, and 5 μg of the pretreated membrane with and without 20 μmol of KCl. Following 10-min preincubation at 37 °C, the reactions were started with 0.05 mL of 40 mM ATP and stopped after 10 more min with ice-cold TCA. The $P_i$ was assayed by the procedure of Sanui (1974). The assay conditions for $K^+$-*p*-nitrophenylphosphatase were the same as those for ATPase except the pH of incubation was 7.5 (50 μmol of Tris-HCl) and the substrate used was 5 mM *p*-nitrophenyl phosphate (Ray, 1978).

*Binding of Vitamin $B_{12}$.* The assay for vitamin $B_{12}$ (cyanocobalamin) binding was essentially the same according to Kolhouse et al. (1978) and consisted of, in a total volume of 0.2 mL, 20 mM Hepes/Tris buffer (pH 7.4), 1 mg of bovine serum albumin (BSA), 40–80 μg of the membrane proteins from various sucrose density gradient fractions, and 0.25 ng of radiolabeled cyanocobalamin ($^{57}Co$; Amersham Corp., specific activity 0.33 Ci/ng). The mixture was incubated for 10 min at room temperature and terminated by 20-fold dilution with ice-cold 20 mM Hepes/Tris buffer (pH 7.4). For the blanks, the membranes were preincubated for 5 min at room temperature with 25 ng of unlabeled vitamin $B_{12}$ prior to the addition of radiolabeled vitamin and processed in parallel. Following termination, 2 mL of a suspension of albumin-coated charcoal in Hepes/Tris buffer (pH 7.5) was added. The mixture was allowed to stand at room temperature for 15 min with continuous gentle shaking and then centrifuged for 10 min at 2000*g*. An aliquot (1.0 mL) of the supernatant was counted in the γ counter. It should be noted that in some preliminary experiments we found that the binding of vitamin

Table I: Recovery of Protein and ATPase Activities during Stimulation of Gastric Microsomes[a]

| fractions | sp act. [μmol/(mg·h)] | | | total act. (μmol/h) | |
|---|---|---|---|---|---|
| | total protein (mg) | $Mg^{2+}$-ATPase | $(H^+,K^+)$-ATPase | $Mg^{2+}$-ATPase | $(H^+,K^+)$-ATPase |
| gastric microsome | 50 | 10.1 | 62.4 | 505 | 3120 |
| SDS-soluble fraction | 20.6 | 0 | 0 | 0 | 0 |
| low-density subfraction | 9 | 50 | 0.3 | 450 | 2.7 |
| intermediate zone | 8 | 5 | 1.0 | 40 | 8 |
| high-density subfraction | 10 | 0.5 | 310 | 5 | 3100 |

[a] Following treatment of gastric microsomes with 0.033% SDS, the membrane suspension was layered over six continuous sucrose gradients and centrifuged in a Beckman SW25.1 rotor as detailed under Materials and Methods. The total number of gradient fractions collected (see Figure 1) was divided into the designated groups and pooled. The pooled fractions for the high-density subfraction included 5–9; intermediate zone, 10–13; low-density subfraction, 14–18; and soluble fraction, 19–24. The details for protein and enzyme assays are given under Materials and Methods.

$B_{12}$ was nearly complete within 1 min of incubation and the binding was linear with increasing low-density membrane protein. The presence of a 20-fold excess of unlabeled cobinamide dicyamide, a vitamin $B_{12}$ analogue, reduced the binding of radiolabeled vitamin $B_{12}$ to the extent of 50% in the low-density membrane subfraction.

*Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis.* SDS–PAGE was performed according to either Fairbanks et al. (1971) as previously described (Nandi & Ray, 1985) or by the Laemmli (1970) procedure.

*Electron Microscopy.* The membranes were prepared for electron microscopy by using standard techniques as previously described (Ray et al., 1983).

*Assay of Lipids. (A) Extraction of Lipids.* The lipids were extracted from the control microsomes and the light and heavy membrane subfractions by the method of Bligh and Dyer (1959). A nitrogen atmosphere was maintained during extraction to prevent aerial oxidation. Lipid phosphorus was assayed by the Bartlett (1959) procedure.

*(B) Separation, Identification, and Quantitation of Phospholipids.* The phospholipids were separated by two-dimensional TLC (silica gel H, Sigma) according to the procedure of Rouser et al. (1967) as described previously (Sen & Ray, 1980; Nandi & Ray, 1985). The solvent systems used were as follows: first dimension, chloroform/methanol/concentrated ammonia (65:35:5 v/v); second dimension, chloroform/methanol/acetone/acetic acid/water (50:10:20:10:5 v/v). The two-dimensional TLC plates were charred following the procedure of Rouser et al. (1967). The spots were marked, scraped off, transferred into Microkjeldahl flasks, and digested with 0.9 mL of perchloric acid. Appropriate blank spots were run in parallel. After digestion, the inorganic phosphate was estimated colorimetrically (Nandi et al., 1983c).

*Assay of Fatty Acids.* Fatty acids were obtained from individual phospholipids after mild alkaline hydrolysis followed by acidification with 1 N acetic acid (Nandi & Ray, 1985). The fatty acids thus obtained were esterified with diazomethane (Nandi & Ray, 1985). The fatty acid methyl esters were extracted with 3–4 volumes of low-boiling petroleum ether. After evaporation of solvent, the fatty acid composition was determined by gas chromatography.

## RESULTS

*Enzymatic Characterization and Identification of Various Sucrose Gradient Subfractions of Gastric Microsomes.* Equilibrium sucrose density gradient fractionation of pig gastric microsomes following treatment with low concentrations of SDS (0.033% w/v) is shown in Figure 1. Similar to the dog gastric microsomes described recently (Nandi et al., 1987), the gradient profile for pig and rabbit (data not shown) microsomes also revealed two major peaks and an intermediate zone, each containing about 20% of the total gastric microsomal proteins. The rest of the proteins (about 40%) are

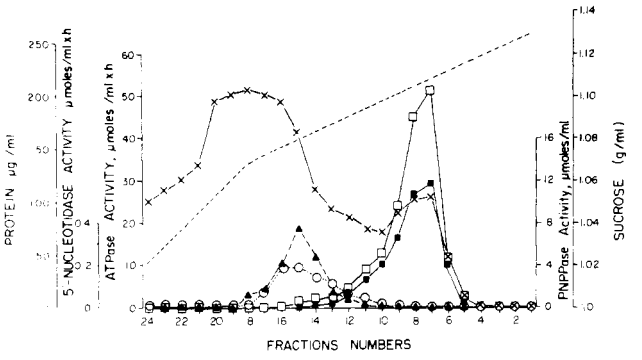


FIGURE 1: Fractionation of SDS (0.033% w/v) treated purified pig gastric microsomes on a linear sucrose density gradient. Details of the fractionation procedures are given under Materials and Methods. Both pig and rabbit (not shown) gastric microsomes give nearly identical profiles. Note that the $(H^+,K^+)$-ATPase and $K^+$-pNPPase activities associated with the heavy subfraction (buoyant density = 1.115 g/mL) are completely separated from the $Mg^{2+}/Ca^{2+}$-ATPase and 5′-nucleotidase activities associated with the low-density subfraction (buoyant density = 1.08 g/mL). A large part (about 40%) of the total gastric microsome-associated proteins remaining soluble following SDS treatment did not penetrate the gradient and did not have any detectable enzymatic activities. Notations are the following: sucrose gradient (---); protein (×); $(H^+,K^+)$-ATPase (□); $Mg^{2+}$- or $Ca^{2+}$-ATPase (○); $K^+$-pNPPase (■); 5′-nucleotidase (▲). The data are typical of several sucrose gradient runs. The details of enzyme assays are given under Materials and Methods.

solubilized during SDS treatment and remain at the upper part of the sucrose gradient. The buoyant densities of the two distinct gradient bands are 1.08 g/mL (low-density subfraction) and 1.115 g/mL (high-density subfraction), respectively. Recovery of the protein and ATPase activities in the major pooled fractions from the sucrose gradient are given in Table I. The origin of the membranes of intermediate density having poor ATPase activity remains unclear at present. While the high-density subfraction contained all of the $(H^+,K^+)$-ATPase and $K^+$-pNPPase activities, the low-density one contained all of the $Mg^{2+}$-ATPase and 5′-nucleotidase activities. It is noteworthy in this connection that cytochemical staining of the $Mg^{2+}$-ATPase in frozen sections of chicken gastric glands recently revealed (Koenig et al., 1987) that the $Mg^{2+}$-ATPase reaction occurs almost exclusively in the secretory pole of the oxyntic cells. Following histamine stimulation, a drastic increase in $Mg^{2+}$-ATPase occurs showing heavy stains at the secretory pole of the oxyntic cells (Koenig et al., 1987), thus consistent with the apical origin of such $Mg^{2+}$-ATPase.

The presence of the 5′-nucleotidase activity, a plasma membrane marker (Ray, 1971; Lauter et al., 1972) in the low-density subfraction, also suggests these membranes to be derived from the cell surface. In addition, similar to the high-density membranes showing a 100-kDa major peptide characteristic of the $(H^+,K^+)$-ATPase, the low-density membranes also contained the 100-kDa peptide together with several other low molecular weight peptides. The latter ob-

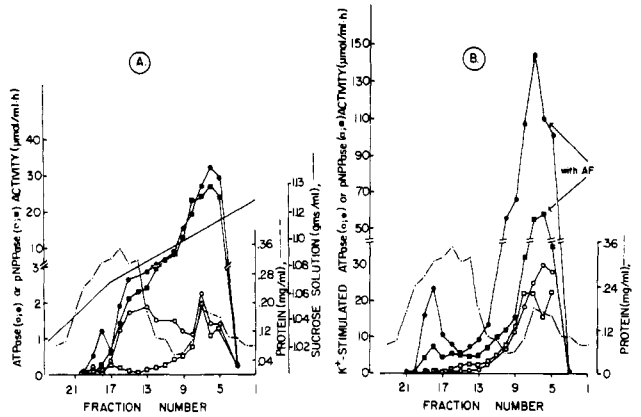


FIGURE 2: Effects of the pure endogenous activator on the $K^+$-stimulated ATPase and pNPPase activities of the various sucrose gradient fractions. The notations are as follows: sucrose density (—), protein (-·-), $Mg^{2+}$-ATPase (○), ($H^+$,$K^+$)-ATPase (●), $K^+$-pNPPase (□) activities in the absence (A) and presence (B) of the endogenous activator (AF). It may be noted that the AF causes remarkable activation (about 25-fold) of the ($H^+$,$K^+$)-ATPase in the low-density subfraction and also causes a modest (about 5-fold) stimulation of the highly active ($H^+$,$K^+$)-ATPase associated with the high-density subfraction. The details of the assay for each of the enzymes are given under Materials and Methods. The data are typical of three separate studies.

servation in conjunction with other preceding evidence implicated that these low-density membranes may have been derived from the secretory surface of the parietal cells. This possibility was further tested as follows.

An endogenous cytosolic regulator protein for the gastric ($H^+$,$K^+$)-ATPase has recently been characterized (Ray, 1978; Bandopadhyay & Ray, 1986; Bandopadhyay et al., 1987). It was reasoned that since the low-density membrane has the characteristic 100-kDa peptide but no significant ($H^+$,$K^+$)-ATPase activity, the lack of the endogenous activator may be the result of such an aberration. The data (Figure 2A,B) demonstrate that this was indeed the case. The low-density subfraction revealed a significant (about 25-fold) ($H^+$,$K^+$)-ATPase activity when assayed in the presence of the pure activator protein. The high-density subfraction containing a pure and highly active ($H^+$,$K^+$)-ATPase was also stimulated about 5-fold by the activator protein. The data suggested the low-density membranes to be derived primarily from the apical plasmalemma.

We found an additional specific marker for the apical plasmalemma of the parietal cells to be associated almost exclusively with the low-density membranes. It is well-known that in addition to acid secretion the parietal cells of most species (Hoedemaeker et al., 1964, 1966; Sahli & Hansen, 1969) studied, including man (Fisher & Taylor, 1969; Jacob & Glass, 1971), with the exception of rat (Sahli & Hansen, 1969; Hoedemaeker, 1964, 1965) possess another unique function such as secretion of the intrinsic factor, which is essential for vitamin $B_{12}$ absorption. However, the mechanism of transport of the intrinsic factor across the secretory plasma membrane remains unknown. There are at least two primary modes by which the intrinsic factor could be visualized to be transported out; an exocytotic release of prepackaged vesicles and a sequential transport of the intracellular intrinsic factor across the apical plasmalemma by some unknown mechanism. In the latter case, an obligatory association of the intrinsic factor with some specialized domain of the apical plasmalemma is expected. Several recent reports (Fisher & Taylor, 1969; Jacobs & Glass, 1971; De-Aizpurua et al., 1983a,b) testify to the association of intrinsic factor with the plasma membranes of parietal cells. Studies on the binding of radiolabeled vitamin $B_{12}$ to the various sucrose gradient fractions demonstrate that a specific binding of $B_{12}$ occurs predominantly in the low-density membrane subfractions (Figure 3). Studies on $B_{12}$ binding using the pooled and washed (see Materials and Methods for details) low- and high-density subfractions demonstrated the level of binding (femtomoles per milligram of protein ± SD, $n$ = 3) to be 350 ± 40 and 38 ± 10, respectively, thus showing the preferential nature of $B_{12}$ binding to low-density membranes. It is noteworthy in this connection that even though the antibody to the intrinsic factor is absent in many (about 40%) cases of pernicious anemia (Goldstone et al., 1973) antibody to the apical membranes is reported to be present (Samloff & Barnett, 1965; Samloff et al., 1967) in about 90% of the cases. Such differential development of an immune response may be due to some unique protein of the apical plasmalemma, presumably the putative intrinsic factor transporter. Hence, we concluded that the origin of the low-density membranes is primarily the secretory surface membrane (apical plasmalemma) of the parietal cells.

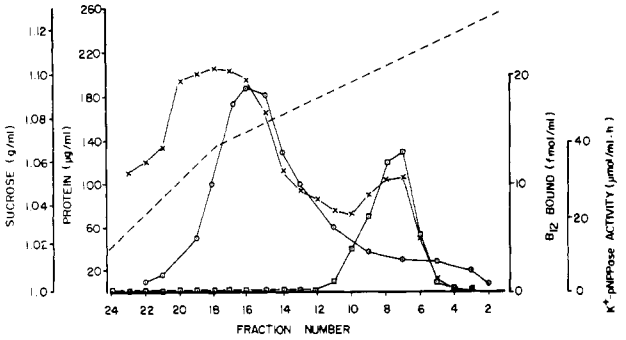


FIGURE 3: Binding of vitamin $B_{12}$ to various sucrose density gradient fractions of gastric microsomes. Notations are the following: protein (×); $K^+$-pNPPase activity (□); vitamin $B_{12}$ ([$^{57}$Co]cyanocobalamin) bound (○). The details of the vitamin $B_{12}$ binding assay and other assays are given under Materials and Methods. Alternate tubes from the continuous sucrose density gradient were assayed for $B_{12}$ binding. Fraction 16 was found to have a nearly identical level of binding as that of fraction 15. The data are typical of three separate studies.

The high-density membranes containing pure ($H^+$,$K^+$)-ATPase, on the other hand, were characterized to be derived from the intracellular tubulovesicles on the basis of the following analysis. Since the low-density (apical) membranes were separated from the mixture of apical and tubulovesicular membranes by using our current technique, it is only logical that the high-density membranes will be predominantly of tubulovesicular origin. The presence of high ($H^+$,$K^+$)-ATPase activity as well as enrichment of the characteristic 100-kDa catalytic subunit (Nandi et al., 1987) will be consistent with that idea. Also, consistent with the known phospholipid profile of the intracellular and surface membranes, the high-density membranes had lower phospholipid content, lower content of both phosphatidylcholine and sphingomyelin, and higher content of phosphatidylethanolamine compared to the low-density membranes (see below). In addition, the ratio of the low- and high-density membranes increased appreciably following histamine stimulation of acid secretion in rabbits (see Flow of Membranes in the Rabbit Stomach during Histamine-Stimulated Gastric Acid Secretion), an observation consistent with the known depletion of intracellular tubulovesicles and the concomitant increase in the apical plasmalemma under such conditions. The preceding information and other data (see Protein Profile and Morphology of the Low- and High-Density Membrane Subfractions and also see Lipid Profile of the Purified Apical and Tubulovesicular Membrane Subfractions Compared to the Unfractionated Microsomes)

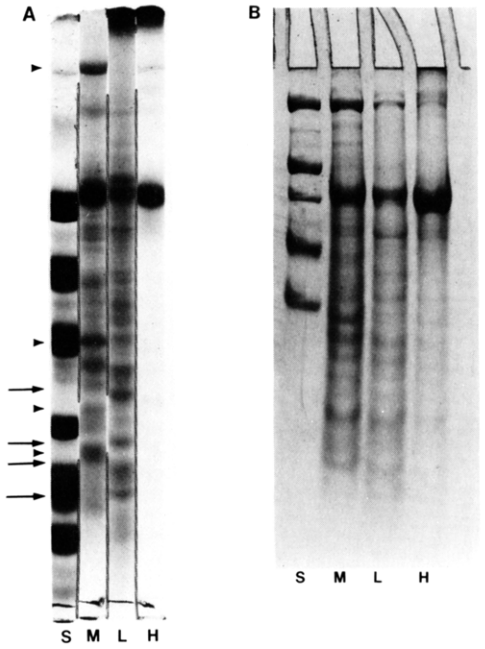


FIGURE 4: (A) SDS–PAGE of total gastric microsomes and the low- and high-density membrane subfractions from pig. S stands for molecular weight standards, M for the total (unfractionated) gastric microsomes, and L for the low-density and H for the high-density microsomal subfractions, respectively. The molecular weight standards are, from the top, 92.5K, 66.2K, 45K, 31K, 21.5K, and 14.4K, respectively. Note that contrary to the low-density membranes, the high-density one shows a single major 100-kDa band. Although both M and L have numerous peptide bands, there are some noteworthy differences. Thus, close comparison of M and L reveals differential depletion of some peptides (180, 45, 32, and 25.7 kDa shown by arrowheads) and enrichment of others (33.4, 26.6, 22.3, and 20.4 kDa shown by arrows) in the low-density (L) membrane subfraction. (B) SDS–PAGE of low- and high-density subfractions of rabbit gastric microsomes. L stands for low-density membranes, H stands for high-density membranes, and S is for standards.

are consistent with the apical plasmalemmal and tubulovesicular origin of the low- and high-density membranes, respectively.

*Protein Profile and Morphology of the Low- and High-Density Membrane Subfractions.* Figure 4A shows SDS–PAGE of the apical plasmalemmal (low-density) and tubulovesicular (high-density) membranes compared to the unfractionated gastric microsomes. It is clear that while the tubulovesicular membrane fraction contains a nearly pure $(H^+,K^+)$-ATPase as revealed by the single major 100-kDa catalytic subunit, the apical plasmalemma contains in addition to the 100-kDa band several other low molecular weight peptides. It is noteworthy that the high molecular weight (about 180K) protein along with three other major bands (45, 32, and 26 kDa, respectively) is lacking in the purified apical plasmalemmal fraction when compared to the unfractionated microsomes. Such peptides seem to have originated from soluble contamination during isolation of gastric microsomes since peptides of identical mobility on SDS–PAGE were present (data not shown) in the soluble fraction during subfractionation of microsomes following low (0.033%) SDS treatment. Several peptides of low molecular weight such as 33.4 K, 26.6 K, 22.3 K, and 20.4K, respectively, were found to be enriched in the apical plasma membranes.

The vesicular nature of the isolated apical membrane and portions of the tubulovesicular membranes is apparent in the electron micrographs (Figure 5B,C). The vesicles were found to be leaky as revealed by their lack of ability to hold a $\Delta$pH. The dimensions of the two types of vesicles were found to be strikingly different. Thus, the control microsomes (Figure 5A) were quite heterogeneous with respect to the vesicular size. Contrary to the control microsomes, the purified apical plasmalemma (Figure 5B) and the tubulovesicular fractions (Figure 5C) were largely homogeneous. Also, the apical plasmalemmal vesicles were considerably smaller compared to the tubulovesicles (compare panels B and C of Figure 5). The observed homogeneity in vesicular size in the two membrane subfractions may well be due to some characteristic features intrinsic to these membranes. However, considering the extensive sonication needed for their preparation, it is possible that sonication may contribute, at least in part, to such homogeneity.

*Lipid Profile of the Purified Apical and Tubulovesicular Membrane Fractions Compared to the Unfractionated Microsomes.* Lipid compositions of the various gastric microsomal fractions are shown in Table I. The lipid content of the low- and high-density subfractions and of the untreated control microsomes would be consistent with their buoyant densities (Figures 1 and 2). Thus, the untreated microsomes (buoyant density = 1.118 g/mL) had the least and the low-density subfractions (buoyant density = 1.08 g/mL) the most lipids on a milligram membrane protein basis, while the high-density subfraction (buoyant density = 1.115) shows an intermediate lipid value (Table I). It is noteworthy that even though the lipid contents of the low- and high-density subfractions are different, the cholesterol to phospholipid molar ratio is nearly identical for the two subfractions.

Phospholipid data (Table I) show that phosphatidylethanolamine (PE), phosphatidylcholine (PC), and sphingomyelin (Sph) are the major phospholipids in the untreated microsomes, as well as in the low- and high-density subfractions. The individual phospholipid values were significantly different in the three membrane fractions. The relative proportion of various phospholipids, which serves as an index of their relative distribution, illustrates this aspect. Thus, the relative distribution parameters differentiating the surface membranes from the intracellular ones, such as the ratios of PC/PE and PC/Sph, were remarkably different for the low- and high-density membrane subfractions. The data are consistent with the distribution of enzyme and other markers discussed previously [see Enzymatic Characterization and Identification of Various Sucrose Gradient Subfractions of Gastric Microsomes and also see Protein Profile and Morphology of the Low- and High-Density Membrane Subfractions), suggesting the apical plasmalemmal and intracellular tubulovesicular origin of the low- and high-density membrane subfractions, respectively.

Fatty acid compositions of various phospholipids derived from the three membrane fractions also show significant differences. Thus, the percentages of 16:0, 18:0, 18:2, and 20:4 in the PE from different membrane fractions were appreciably different. Phosphatidylcholine showed appreciable differences in 16:0, 18:1, 18:2, and 20:4 in various membrane subfractions. The fatty acid composition of PI from the three membranes exhibited significant differences in 18:1 and 20:4. It is clear that the fatty acid compositions of the various phospholipids, associated with the apical plasmalemma, such as PC, PE, and PI, are widely different from those associated with the intracellular tubulovesicles.

*Other Characteristics of the Apical and Tubulovesicular Membranes.* Vanadate (monosodium orthovanadate) showed some interesting differential inhibition of the activator-independent and activator-dependent $(H^+,K^+)$-ATPase activities (Figure 6A,B). Thus, while the endogenous activator-inde-

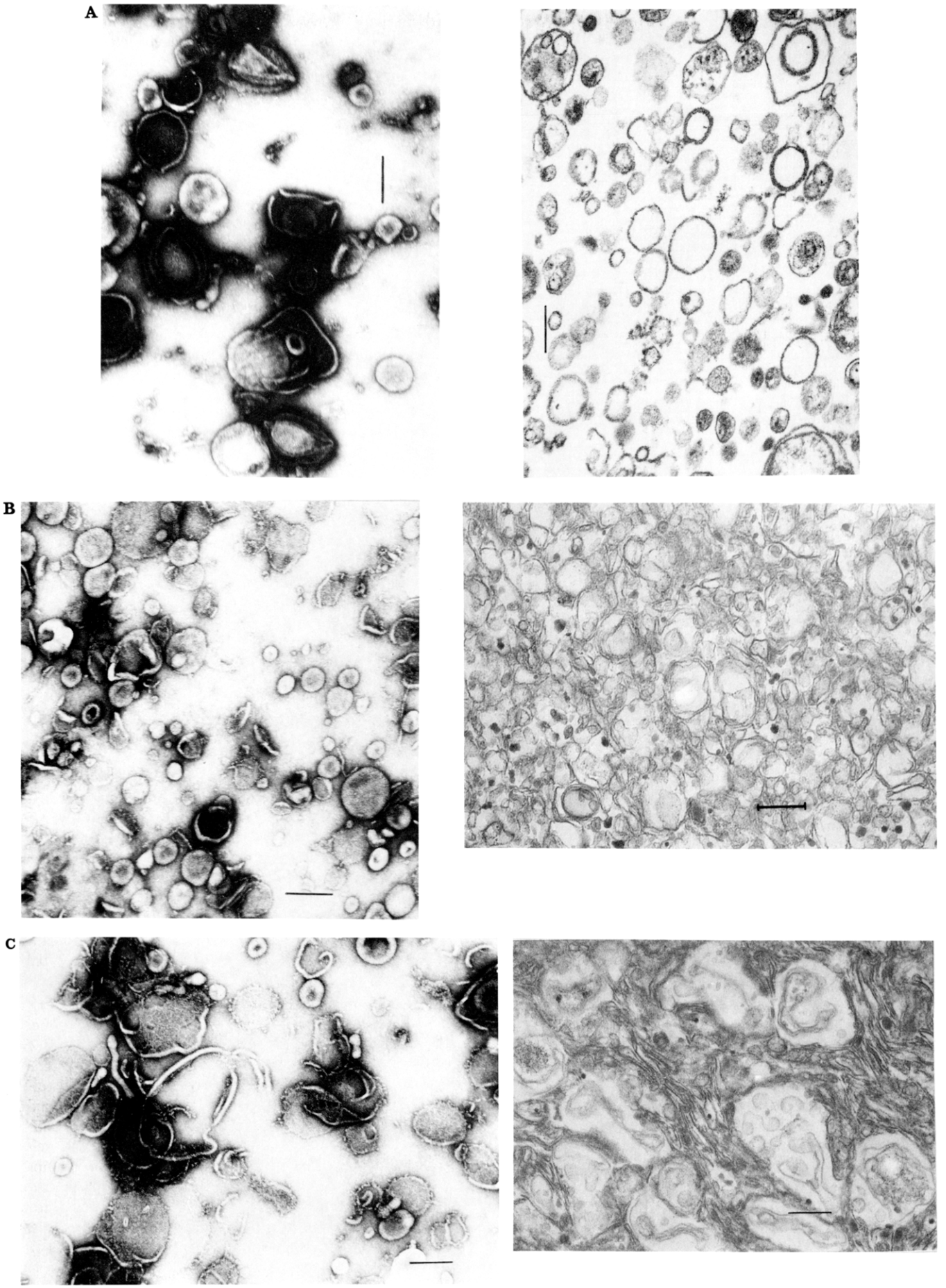


FIGURE 5: Electron micrographs of the control microsomes and of the low- and high-density membrane subfractions. Negatively stained (left panels) and thin-sectioned (right panels) preparations of control microsomes (A) and of the low-density (B) and high-density (C) membrane subfractions, respectively. Bars are 0.2 $\mu$m.

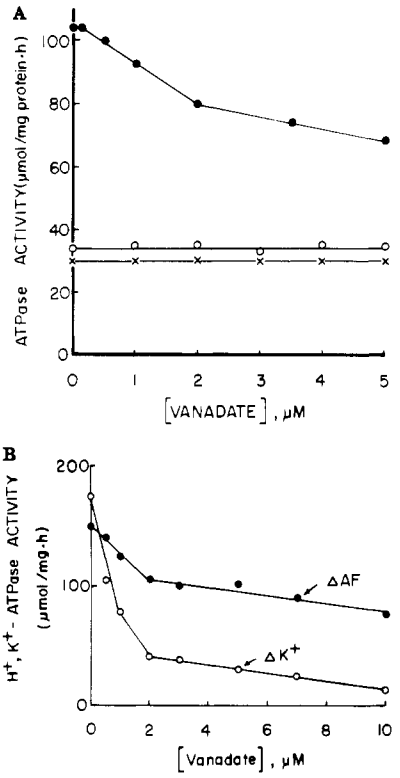


FIGURE 6: Effect of vanadate concentration on the ATPase activities associated with the low- and high-density gastric microsomal subfractions in the absence and presence of the pure endogenous activator protein (AF). Figure 6A represents the vanadate effects on the apical plasmalemmal (low-density subfraction) ATPase in the absence of the AF without (×) and with (○) $K^+$ and in the presence of the AF and $K^+$ (●). The data are typical of five separate studies. It is noteworthy that the AF used in this study was prepared in the DTT-free buffer medium (see Materials and Methods) and the membranes were washed free of DTT. The pattern of vanadate inhibition observed in the absence of DTT is nearly identical with that observed (not shown) with a low concentration of DTT present in the assay medium. Figure 6B shows the vanadate effect on high-density membrane-associated $K^+$-stimulated ATPase without AF (○) and the AF-dependent ($H^+$,$K^+$)-ATPase activity (●). The $K^+$-stimulated ATPase ($\Delta K^+$) was the rate in the presence of 20 mM $K^+$ minus the basal (with $Mg^{2+}$ as the only cation) rate. The basal rate was about 2 μmol/(mg·h), which was not affected by vanadate to any appreciable extent. The AF-dependent ($H^+$,$K^+$)-ATPase activity ($\Delta AF$) was calculated by subtracting the activity in the absence of the AF from those measured in presence of the AF, both containing $K^+$ in the assay. About 5 μg of the low- and high-density membrane subfractions and 5 μg of the pure AF were used for each assay. The details for the ATPase assay are given under Materials and Methods. The data are typical of studies from three different preparations.

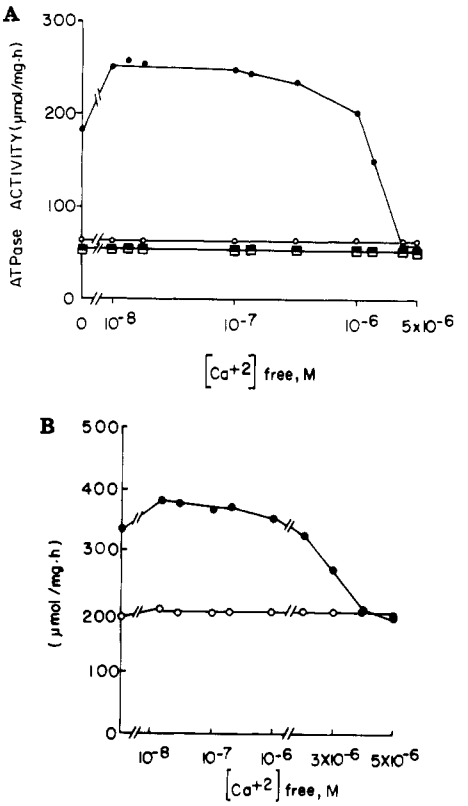


FIGURE 7: (A) Effects of calcium concentration on the AF-dependent ($H^+$,$K^+$)-ATPase activity associated with the low-density membrane subfraction. Notations are as follows: without AF without $K^+$ (□); without AF with $K^+$ (■); with AF without $K^+$ (○); with AF with $K^+$ (●). The concentration of free $Ca^{2+}$ in the assay was regulated by 0.5 mM EGTA (Nandi & Ray, 1984). Note that significant activation of the AF-dependent ($H^+$,$K^+$)-ATPase occurs between 0.01 and 0.5 μM $Ca^{2+}$ but a dramatic inhibition is evident between 2 and 4 μM $Ca^{2+}$. It should be pointed out that there is a small component of $K^+$-stimulated ATP hydrolysis [about 3 μmol/(mg·h)] in the absence of the activator, even though it is not clearly evident from the figure. The extent of activator stimulation was therefore about 40-fold. The data are typical of three separate studies. (B) Effects of calcium concentration on the high-density membrane-associated ($H^+$,$K^+$)-ATPase activity in the absence (○) and presence (●) of the AF. Note that similar to the low-density membrane-associated AF-dependent ($H^+$,$K^+$)-ATPase, a significant stimulation at low (below 1 μM) and a dramatic inhibition at high (2–4 μM) $Ca^{2+}$ concentrations are observed. The data are typical of three separate studies. The details of the assay are given under Materials and Methods.

pendent ($H^+$,$K^+$)-ATPase activity associated with the tubulovesicles (high-density subfraction) is highly sensitive to vanadate inhibition, the activator-dependent activity is poorly sensitive. It is noteworthy that the activator-dependent ($H^+$,$K^+$)-ATPase activity associated with the low-density (apical plasmalemma) subfraction also shows a nearly identical low vanadate inhibitory pattern (Figure 6A) as that of the observed activator-dependent ($H^+$,$K^+$)-ATPase of the tubulovesicles. The presence or absence of DTT in the enzyme and/or the activator preparation did not alter either the activity or the pattern of vanadate sensitivity (Figure 6A). The latter observation eliminated the possibility that DTT reduction of vanadate to lower valency states might have caused such low vanadate sensitivity of the activator-dependent ($H^+$,$K^+$)-ATPase activity. The observation will be consistent with the idea of the apical plasmalemmal $Mg^{2+}$-ATPase activity to be a modified form of the ($H^+$,$K^+$)-ATPase system. A recent report (Koenig et al., 1987) came up with a similar conclusion based on differential cytochemical distribution of the $Mg^{2+}$-ATPase of acid-secreting cells under resting and histamine-stimulated conditions. Differences in the activator-dependent and -independent ($H^+$,$K^+$)-ATPase activities were also revealed in the presence of low concentrations of $Ca^{2+}$ (Figure 7A,B). Low (micromolar) concentrations of $Ca^{2+}$ showed both activation and inhibition of the activator-dependent ($H^+$,$K^+$)-ATPase activity depending upon the concentration. Thus, below 1 μM concentration, $Ca^{2+}$ exhibits appreciable stimulation while obliterating the activator-dependent ($H^+$,$K^+$)-ATPase activity within a narrow range of 2–4 μM. Such $Ca^{2+}$ (up to 4 μM), however, does not have any effect on the activator-independent ($H^+$,$K^+$)-ATPase activity. Cyclic AMP, on the other hand, did not have any effect on either the activator-independent or the activator-dependent ($H^+$,$K^+$)-ATPase at any of the concentrations ($10^{-9}$–$10^{-4}$ M) tested (unpublished data).

It is noteworthy in this connection that the extent of activator stimulation of the ($H^+$,$K^+$)-ATPase has been found to be quite variable in the present as well as in previous studies (Bandopadhyay et al., 1987; Ray et al., 1987). The degree

Table II: Lipid Composition of Gastric Microsomes and Its Subfractions[a]

| | microsomes | low density | high density |
|---|---|---|---|
| lipid protein (mg/mg) | 0.98 | 2.08 | 1.47 |
| cholesterol/phospholipid (mol/mol) | 0.43 | 0.62 | 0.63 |
| phospholipid (μmol/mg of protein) | | | |
| total | 100 ± 1.8 | 218.4 ± 7.2 | 140.0 ± 10.5 |
| PE | 28.6 ± 0.4 | 48.4 ± 1.7 | 38.1 ± 2.6 |
| PC | 33.2 ± 1.5 | 67.0 ± 2.7 | 33.3 ± 1.4 |
| Sph | 30.2 ± 0.7 | 65.8 ± 2.1 | 58.5 ± 4.2 |
| PI | 6.8 ± 0.6 | 23.6 ± 2.1 | 13.1 ± 1.0 |
| PS | 1.5 ± 0.3 | 8.1 ± 0.5 | 3.5 ± 0.5 |
| cardiolipin | 0 | 0 | 0 |
| PC/PE | 1.16 | 1.38 | 0.87 |
| PC/Sph | 1.09 | 1.02 | 0.57 |

[a] Data are mean ± SD ($n$ = 3). Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; Sph, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine.

of activator stimulation of the tubulovesicular ($H^+$,$K^+$)-ATPase varies between 2- and 6-fold, whereas that from the apical plasmalemma varies from 25- to over 300-fold. The extent of stimulation in the latter membranes appears to bear some relationship with the secretory state of the parietal cells from which the membranes were harvested (see below).

*Flow of Membranes in the Rabbit Stomach during Histamine-Stimulated Gastric Acid Secretion.* A dramatic transformation in the secretory conformation of the parietal cells is known to occur following secretagogue stimulation (Sedar & Friedman, 1961; Forte et al., 1977). The intracellular tubulovesicles are greatly reduced with concurrent proliferation of the secretory plasma membranes, suggesting interconversion of the former into the latter during secretagogue-stimulated acid secretion. We studied this aspect of membrane migration using resting (cimetidine-injected) and histamine-stimulated rabbits followed by isolation of the apical and tubulovesicular membranes using the present technique. Besides the differences in relative yield and redistribution of ATPase activities, no other differences in properties of the harvested apical and tubulovesicular membranes from the cimetidine- or histamine-treated rabbits could be detected. A typical SDS–PAGE profile of the apical and tubulovesicular membranes harvested from rabbit fundic cells is shown in Figure 4B. Consistent with the observations of others (Hirst & Forte, 1985; Im et al., 1984), the histamine-stimulated (S) microsomal membranes (unpublished data) showed an increase in the basal ATPase activity and a concomitant decrease in the $K^+$-stimulated ATPase activity compared to the cimetidine-treated, resting (R) preparation. The differences between R and S microsomes were further revealed when the distribution of ATPase activities was studied in the apical and tubulovesicular membrane subfractions. Thus, in the apical plasmalemma (APM) from R and S, the basal and net ($\Delta K^+$) $K^+$-stimulated ATPase activities (in micromoles per milligram per hour) were the following: APM (R), 46 and 6.1; APM (S), 70 and 0.2, respectively. In the presence of the activator, the apical plasmalemma $\Delta K^+$ activities reached the same level (about 74) both in R and in S, thus showing activator activation of about 12- and 350-fold, respectively. In contrast, the tubulovesicles from both R and S showed about 3-fold stimulation of the $K^+$-stimulated ATPase by the activator.

The data (Table III) demonstrate that a definite increase in the population of the apical plasmalemma occurs following histamine-stimulation compared to the non-stimulated control animals, thus consistent with the membrane-flow hypothesis.

## Discussion

*Identification and Characterization of the Apical and the Tubulovesicular Membranes.* The data presented in this paper reveal that under appropriate conditions, SDS (0.033% w/v) removes about 40% of the total microsomal proteins in a soluble form with consequent generation of two membrane subfractions of low (buoyant density = 1.08 g/mL) and high (buoyant density = 1.115 g/mL) density, respectively. The soluble proteins are likely to be primarily derived from the cytoskeletal material entrapped within some of the vesicles and, in part, some soluble proteins absorbed to the membrane surface during the course of isolation. Frequent occurrence of some fuzzy materials almost exclusively within the small vesicular population (Figure 5A, thin section) of the unfractionated (control) microsomes but virtually absent in the SDS-fractionated ones (Figure 5B,C) would be consistent with such an idea. Hence, the SDS extraction procedure appears to solubilize and primarily release the entrapped cytoskeletal materials from the vesicle interior of the isolated apical plasma membranes by making them leaky, thereby regenerating their normal low buoyant density. It is noteworthy in this connection that the two types of membrane vesicles, besides having different buoyant densities, may also have differential surface charge densities. Thus, using free flow electrophoresis, Saccomani et al. (1977) reported the separation of a vesicle population enriched in ($H^+$,$K^+$)-ATPase from those containing the $Mg^{2+}$-ATPase. Subsequently, Schrijen et al. (1983) reported similar enrichment of ($H^+$,$K^+$)-ATPase using zonal electrophoresis. Although the precise origin of the electrophoretically separated membrane subfractions was not spec-

Table III: Fatty Acid Composition of Various Phospholipids Associated with Control Gastric Microsomes and Low- and High-Density Membrane Subfractions

| | fatty acids (% of total) | | | | | |
|---|---|---|---|---|---|---|
| lipids | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 | 20:4 |
| microsome | | | | | | |
| PE | 16.6 ± 0.5 | 13.2 ± 0.3 | 24.3 ± 0.6 | 20.5 ± 0.4 | trace | 21.2 ± 1.2 |
| PC | 32.1 ± 0.3 | 12.6 ± 0.8 | 17.4 ± 0.2 | 25.1 ± 0.2 | trace | 8.9 ± 0.3 |
| PI | 22.6 ± 0.2 | 23.3 ± 0.2 | 33.9 ± 0.3 | 14.4 ± 0.1 | trace | trace |
| Sph[a] | 16.1 ± 1.5 | 28.8 ± 1.4 | 9.1 ± 2.2 | trace | trace | trace |
| low-density subfraction | | | | | | |
| PE | 11.7 ± 0.3 | 18.9 ± 1.0 | 22.0 ± 1.7 | 9.1 ± 0.3 | 9.4 ± 0.3 | 10.2 ± 1.2 |
| PC | 34.6 ± 1.4 | 13.3 ± 0.5 | 18.8 ± 2.4 | 16.2 ± 0.2 | 1.8 ± 0.3 | 1.1 ± 0.05 |
| PI | 19.4 ± 1.6 | 25.3 ± 2.1 | 27.4 ± 1.6 | 6.4 ± 0.9 | 5.5 ± 0.7 | 4.6 ± 1.2 |
| high-density subfraction | | | | | | |
| PE | 31.7 ± 3.7 | 21.1 ± 0.6 | 22.0 ± 0.8 | 11.0 ± 0.7 | 4.4 ± 0.5 | 4.0 ± 1.0 |
| PC | 29.8 ± 1.1 | 11.6 ± 1.5 | 15.4 ± 0.4 | 11.0 ± 0.5 | 6.0 ± 0.4 | 5.2 ± 0.7 |
| PI | 24.3 ± 1.4 | 14.0 ± 2.1 | 17.7 ± 2.7 | 12.3 ± 2.2 | 2.0 ± 0.2 | 12.1 ± 1.3 |

[a] Sph contained 14:0 in a rather high amount (35.7 ± 3.2). The 14:0 content of other phospholipids was within 2%. Fatty acid composition of Sph from low- and high-density subfractions was not determined. Abbreviations have been explained in Table II. Data are mean ± SD ($n$ = 3).

Table IV: Distribution of Isolated Apical and Tubulovesicular Membranes under Resting and Stimulated Conditions[a]

| | ratio of apical plasmalemma to tubulovesicles (mg/mg) | |
|---|---|---|
| expt | resting stomach | secreting stomach |
| 1 | 0.5 | 1.30 |
| 2 | 0.76 | 1.84 |
| 3 | 0.9 | 1.85 |

[a] Six rabbits (three each for resting and stimulated) were used in each experimental group. The injection protocol used for the study was from Hirst and Forte (1985). For the resting stomach, the rabbits were injected twice with 0.3 mL (150 mg/mL) of cimetidine at an interval of 30 min. For the stimulated stomachs, 0.2 mL of histamine (100 mM) was injected twice at an interval of 10 min, the first two being subcutaneous and the last intravenous. Following the last injection, the animals were sacrificed after 10 min. Gastric microsomes from the two groups of animals were separately isolated following our conventional procedure (Ray & Nandi, 1986). For isolation of the apical (low-density subfraction) and tubulovesicular (high-density subfraction) membranes from gastric microsomes, the step gradient method for bulk purification (Nandi et al., 1987) was used. The membranes from the step gradient were washed once by dilution in 250 mM sucrose, 0.2 mM EDTA, and 0.2 mM Pipes buffer (pH 6.8) followed by centrifugation at 100000*g* for 90 min. The low- and high-density membranes harvested from the step gradient have been demonstrated to be of similar purity as those from the continuous gradient (Nandi et al., 1987).

ified (Saccomani et al., 1977; Schrijen et al., 1983), in view of the present study it appears likely that electrophoresis may be a good way to separate the apical from the tubulovesicular membranes. The feasibility of the electrophoretic approach in separating the functional membranes has recently been documented (Youmans & Brodsky, 1987) with turtle bladder cells.

Our data revealed that the endogenous activator (Bandopadhyay & Ray, 1986; Bandopadhyay et al., 1987) can induce appreciable ($H^+$,$K^+$)-ATPase activity in the low-density membranes, such activity being originally absent from these membranes. Studies on vanadate inhibition of the activator-stimulated ($H^+$,$K^+$)-ATPase associated with both the low- and high-density membrane subfractions revealed an identical nature of the activator-stimulated activities. The data, besides pointing out the differences in the nature of the ($H^+$,$K^+$)-ATPase associated with the low-density apical plasmalemma and the high-density tubulovesicles, emphasize the importance of the endogenous activator in the functional interconversion between these two membranes during gastric $H^+$ transport. The intrinsic factor like activity as demonstrated by specific vitamin $B_{12}$ binding was found to be associated almost entirely with the apical plasmalemma, thus providing another new and reliable marker for the secretory surface membranes. It is possible that the vitamin $B_{12}$ binding component may be either true intrinsic factor or similar protein factors called "R-proteins" (Kolhouse et al., 1978) associated with these membranes. Whatever may be the reasons, such specific binding of vitamin $B_{12}$ provides an excellent marker for the apical plasma membranes of the parietal cells.

Studies on phospholipid composition revealed characteristic differences consistent with the cell surface and intracellular nature of the low- and high-density membrane subfractions, respectively. Thus, both the phosphatidylcholine (micromoles per milligram of protein) content and the ratio of phosphatidylcholine to phosphatidylenthanolamine were appreciably higher in the low-density (apical plasmalemma) compared to the high-density (tubulovesicles) subfraction and were 67, 33 and 1.38, 0.87, respectively.

*Similarities between the Apical Plasmalemma and the Tubulovesicles.* Besides the differences, mentioned above, there are some notable similarities between the apical and tubulovesicular membranes. Thus, the cholesterol to phospholipid molar ratios are nearly identical in the two membranes. Also, both the apical and tubulovesicular membranes are rich in a 100-kDa peptide and a glycopeptide of about 85-kDa mass. In addition, both membranes demonstrate endogenous activator-stimulated ($H^+$,$K^+$)-ATPase, identified as the proton pump. Hence, even though the tubulovesicular membranes are of cytosolic origin, they share some important features with the apical plasmalemma. These similarities are, however, totally consistent with the idea of the tubulovesicles to be acting as a reserve for the apical plasmalemma (Forte et al., 1977).

*Two Forms of the Gastric ($H^+$,$K^+$)-ATPase.* Vanadate show appreciable differences in the inhibition of the activator-dependent and -independent ($H^+$,$K^+$)-ATPase activities associated with the two membrane subfractions, the latter being far more sensitive to vanadate than the former. Contrary to the apical plasmalemma, the tubulovesicular membranes contain both types of vanadate-sensitive activities, suggesting that there are two forms of the ($H^+$,$K^+$)-ATPase in the latter membrane. Since vanadate is believed (Beauge, 1979; Csermely et al., 1985) to serve as an analogue of $P_i$, and interacts with the enzyme in the $E_2$ conformation at the cytosolic $P_i$ binding site thus stabilizing the $E_2$ form of the enzyme, it is likely that the two forms (i.e., activator dependent and activator independent) of the ($H^+$,$K^+$)-ATPase might differ primarily by the state of $E_2\sim P$ forms. Our observation (Bandopadhyay et al., 1987) that the activator enhances the sensitivity of the ($H^+$,$K^+$)-ATPase to $K^+$ would be consistent with such an idea since the $K^+$-dependent $E\sim P$ breakdown would be the rate-limiting step for the increased enzyme turnover. It may be noted, in this connection, that the activator does not alter the $E\sim P$ level of either subfraction but considerably enhances the turnover of the enzyme (unpublished observation), presumably by stimulating both the formation and breakdown of $E\sim P$. The precise mechanism of vanadate inhibition of the two forms, however, remains to be elucidated. It may be noted that the $Mg^{2+}$-ATPase activity of the purified turtle bladder membrane vesicles, capable of generating a pH gradient and implicated to be involved in urinary acidification, has recently been resolved into two different types based on their differential sensitivity toward vanadate and *N*-ethylmaleimide (Youmans & Brodsky, 1987).

Additional evidence for the presence of two forms of ($H^+$,$K^+$)-ATPase is based on their responses to micromolar $Ca^{2+}$. Thus, the activator-stimulated ($H^+$,$K^+$)-ATPase is totally obliterated by 4 $\mu$M $Ca^{2+}$ while the activator-independent form shows no such sign of inhibition by $Ca^{2+}$. The gastric microsomal ($H^+$,$K^+$)-ATPase system has previously been demonstrated to be critically dependent on both the phospholipid microenvironment and association with the endogenous activator protein (Sen & Ray, 1980; Nandi et al., 1983c) for optimal activity and function. The microsomal phosphatidylcholine molecules having a relative abundance of saturated fatty acids and poor in unsaturated fatty acids were specifically implicated to be acting as the boundary lipids for optimal performance (Nandi et al., 1983c). Hence, the two forms of the ($H^+$,$K^+$)-ATPase, namely, the activator-dependent and -independent forms, are likely to occur primarily due to the differential makeup of the boundary lipids of the membrane-associated ($H^+$,$K^+$)-ATPase complex.

It is noteworthy in this connection that even though the two monovalent cation transporting ATPase systems such as the gastric ($H^+$,$K^+$)-ATPase and ($Na^+$,$K^+$)-ATPase share nu-

merous commonalities (Ray & Nandi, 1985, 1986; Nandi et al., 1988; Das et al., 1987; Ray & Chakrabarti, 1988) the two forms of the former enzyme reported here are characteristically different than those of the $\alpha$ and $\alpha(+)$ forms reported for the latter (Sweadner, 1979). Thus, contrary to differential inactivation of the ($Na^+$,$K^+$)-ATPase forms by ouabain, *N*-ethylmaleimide (Sweadner, 1979), and pyrithiamine (Matsuda et al., 1984), the ($H^+$,$K^+$)-ATPase forms are differentiated on the basis of their ability to be regulated by the endogenous activator.

*Calcium Ion as a Possible Physiological Switch.* The importance of $Ca^{2+}$ as a second messenger in gastric acid secretion is now increasingly appreciated (Chew & Brown, 1986; Negulescu & Machen, 1988). Recent elegant studies by Negulescu and Machen (1988) demonstrated transient mobilization of $Ca^{2+}$ from similar but unidentified intracellular stores by both carbachol and histamine, the latter effect appearing to be mediated at least in part by cAMP.

The observed dramatic effects of physiological concentrations of $Ca^{2+}$ on the ($H^+$,$K^+$)-ATPase pump will be consistent with the role of $Ca^{2+}$ as an intracellular switch in gastric $H^+$ transport (Banerjee et al., 1987). The fact that the apical plasmalemmal ($H^+$,$K^+$)-ATPase is solely dependent on the endogenous activator for activity would make this a more plausible and fascinating one. Thus, one could visualize an on–off mechanism based on the concentration of $Ca^{2+}$ at or very near the cytosolic microdomain of the apical plasmalemmal activator-dependent ($H^+$,$K^+$)-ATPase pump, $Ca^{2+}$ up to 1 $\mu$M facilitating the onset and beyond 1 $\mu$M the cessation of gastric $H^+$ transport. Such an idea will be totally consistent with the proposed (Bandopadhyay et al., 1987) role of the endogenous activator and $Ca^{2+}$ acting as members of a cytosolic signal-transducing cascade system for the secretagogue-stimulated gastric $H^+$ transport.

*Flow of Membranes during Secretagogue Stimulation.* Our data demonstrate that flow and fusion of the intracellular tubulovesicles to the apical plasmalemma occur during increased secretory activity of the cells. Since both the phospholipid and protein profiles of the apical and tubulovesicular membranes are so radically different, it is unlikely that a direct transfer of the tubulovesicles onto the apical plasmalemmal site will occur. On the other hand, in view of the presence of both the activator-insensitive and -sensitive forms in the tubulovesicles and the exclusive presence of the latter in the apical plasmalemma, it is highly likely that some modification of the tubulovesicles could occur prior to their incorporation into the apical plasmalemma. The modification could occur either through the addition of new phospholipids or through exchange of existing phospholipids or both. Thus, one could visualize such conversion of the tubulovesicle occurring through a series of localized modifications or differentiation during transition of the tubulovesicles prior to their incorporation into the secretory plasmalemma. It is likely that some endomembranes of specialized functions may be involved in such a differentiation process. For instance, the participation of lipid exchange mechanisms associated with such endomembrane elements could largely account for the observed differences in the components of fatty acids and bases between the apical and the tubulovesicular membranes. Besides the endomembranes, some cytosolic phospholipid exchange proteins (Helmkamp et al., 1974; Bloj & Zilversmit, 1977; Negli et al., 1986; Nichols, 1988) may also be involved in a rapid exchange during the process of membrane modification. Various intracellular lipid exchange mechanisms are, thus, likely to play critical roles during the flow–differentiation–fusion events following secretagogue stimulation of the parietal cells. For the fusion to occur in a site-directed organized manner, some kind of coated vesicle may also be involved. It is noteworthy that small coated vesicles containing a single lipoprotein particle are reported (Kartenbeck et al., 1977; Ehrenreich et al., 1973) to be present in appropriate configurations at the periphery of certain cells, suggesting fusion with the plasmalemma. A systematic search for similar kinds of coated vesicles, as well as the endomembranes mentioned earlier, should be made in the parietal cells.

Besides the necessary changes in phospholipid composition, insertion of various characteristic intrinsic proteins such as $K^+$ and/or $Cl^-$ transporter(s) and intrinsic factor transporter must also occur in order to constitute the functional apical plasmalemma. We need to know the mechanisms of insertions of the functional proteins. Also, whether such insertion of different functional proteins occurs prior to or following fusion of the tubulovesicles into the apical plasmalemma remains to be found out.

## Conclusion

The present study unveils the unique characteristics of the secretory plasmalemma and intracellular tubulovesicles of the acid-secreting cells from pig and rabbit fundus. In the latter species, a histamine-induced mobilization of the tubulovesicular membranes and a consequent increase in the population of secretory plasmalemma were also demonstrated to occur. The present approach, thus, opens up the possibility of future studies on membrane migration in other sytems (Stanton et al., 1981; Karnieli et al., 1981; Lewis & deMoura, 1982; Madson & Tisches, 1983; Gluck et al., 1982; Wade, 1986; Youmans & Brodsky, 1987) where an analogous membrane trafficking has been suggested to occur during secretory events.

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**Registry No.** ATPase, 9000-83-3; vitamin $B_{12}$, 68-19-9; histamine, 51-45-6.

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