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CHARACTERIZATION OF GASTRIC MUCOSAL MEMBRANES

IX. FRACTIONATION AND PURIFICATION OF K^+-ATPase-CONTAINING VESICLES BY ZONAL CENTRIFUGATION AND FREE-FLOW ELECTROPHORESIS TECHNIQUE

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Summary

Methods are described for purification of a vesicular membrane fraction of hog gastric mucosa using differential centrifugation, density gradient separation on zonal rotors and free-flow electrophoresis. As a result a fraction is obtained enriched 40-fold in terms of K^+-ATPase and free of any other enzyme marker other than K^+-activated *p*-nitrophenyl phosphatase.

The 5′-nucleotidase and basal Mg^{2+}-ATPase are clearly separated from the latter enzymes.

Osmotic shock, Triton X-100 treatment or K^+ ionophores increased the K^+-ATPase activity in isotonic conditions, but K^+-*p*-nitrophenyl phosphatase is not affected by these treatments, nor is the ATPase activity in the presence of NH_4^+. The results suggest that the electrophoretic fraction contains a major population of tight vesicles, whose permeability to K^+ is rate limiting for the ATPase activity but not for the *p*-nitrophenyl phosphatase activity. It is concluded that K^+ site for the ATPase is internal whereas the K^+ site for the *p*-nitrophenyl phosphatase is external, hence, the K^+ site must be mobile across the membrane.

Introduction

The gastric mucosa is a complex heterocellular epithelium containing as major cell types the surface cell, the mucus neck cell, the parietal cell and the

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Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanasulfonic acid; SDS, sodium dodecyl sulfate.

chief of peptic cell. Current interest centers on the properties of the parietal or acid secreting cell, and fractionation of gastric homogenate has been aimed at producing purified preparations of the parietal cell's apical membrane across which H^+ transport occurs. Plasma membranes of gastric fundic mucosa have been prepared by a variety of techniques in species such as frog [1], necturus [2], dog [3], rabbit [4] and recently, in hog [5]. Perhaps the most purified membrane fractions were obtained using Ficoll sucrose density gradients on zonal rotors, as applied to the dog [3]. Similar techniques applied to the hog produced fractions of equivalent purity, but various manipulations of gradient composition and shape, as well as rate rather than isopycnic centrifugation, did not improve the purity. Recently, free-flow electrophoresis technique has been used to successfully separate the basal and apical membranes of rat kidney tubule cells [6]. In the present work this technique was used to increase the purity of gastric membrane fractions, and optimum electrophoretic separation conditions as well as some morphological and biochemical studies of the isolated fractions are described.

Materials and Methods

Fresh hog stomachs were obtained from the local slaughterhouse. After washing free of contents in tap water, the fundic mucosa surface was flooded with a saturated solution of NaCl, and the surface mucus and most of the superficial cells removed by vigorous wiping with paper towels, leaving mostly parietal and peptic cells. The fundic mucosa was then scraped from the underlying muscular layer and suspended in (approx. 10% tissue, w/w) cold solution of 0.25 M sucrose, 20 mM Tris · HCl buffer at pH 7.4. Homogenization was carried out in a tight-fitting teflon-glass homogenizer (Potter-Elvehjem) with 10 up-down strokes at 2000 rev./min.

Differential centrifugation. The resulting homogenate was centrifuged at 20 000 × *g* for 30 min in a Sorvall RC-2 centrifuge. The pellet was washed once, and the combined supernatants were centrifuged at 78 000 × *g* * for 60 min in a Beckman L-2 ultracentrifuge with a No. 30 rotor to yield the crude microsomal pellet. The remaining material was termed the final supernatant.

Zonal centrifugation. The technique used has been previously described [3]. The 78 000 × *g* pellet was resuspended in 0.25 M sucrose 20 mM Tris · HCl (pH 7.4) and after homogenization was distributed by zonal centrifugation using a continuous density gradient made from 7.5% (w/w) Ficoll 0.25 M sucrose and 37% (w/w) sucrose in 20 mM Tris · HCl at pH 7.4.

Absorbance at 280 nm was used to estimate protein peaks in gradient fractions, while the Lowry method [7] was used in all other instances. Sucrose concentration was measured with an Abbe refractometer. Fractions collected between a density of 1.04 and 1.06 (GI), between 1.06 and 1.08 (GI—GII), between 1.08 and 1.12 (GII), between 1.12 and 1.15 (GII—GIII) and between 1.15 and 1.26 (GIII) were diluted with 20 mM Tris · HCl (pH 7.4) to less than 1.04 density and centrifuged at 78 000 × *g* for 60 min to obtain pellets.

Free-flow electrophoresis. Further fractionation of GI and GII fractions

* *g* value at R_{max} = 105 500.

obtained from zonal separation was accomplished on the FF5 electrophoresis apparatus developed by Hannig [8] (Biomedical Instruments, N.Y.). The electrophoresis buffer (or the separation buffer) was 8 mM Trizma-base, 8 mM acetic acid and 0.25 M sucrose adjusted to pH 7.4 by 2 M NaOH, containing 0.1 mM MgATP. The electrode buffer consisted of 100 mM Trizma-base and 100 mM acetic acid adjusted to pH 7.4 by 2 M NaOH.

Prior to electrophoresis, GI and GII fractions were washed twice in separation buffer and suspended to a final protein concentration of 8 mg/ml. Sample injection rate was 1 ml/h, and 90 fractions were collected at 4°C. The conditions of the runs were as follows: 120 ± 10% V/cm, 147 mA, temperature 7.4 ± 0.2°C and electrophoresis buffer flow 4 ml per fraction per h.

Mobility measurement. The total deflections of individual bands of particles within the separation chamber were recorded photographically. The lateral displacement of particles was made up of one component due to the electrophoretic velocity of the particles and a second component due to the electro-osmotic velocity of the separation buffer. To correct for the electro-osmotic velocity of the separation buffer, a zero mobility marker (separation buffer containing 0.4 M sucrose) was injected into the chamber. The angle of deflection of the bands due to electrophoretic velocity of the particles was measured as the difference between the angles of total deflection of the individual bands and the angle of deflection of the marker. The volume flow rate of the separation buffer was measured directly by collecting of the output of the electrophoretic chamber for measured time intervals. The maximum vertical velocity of the separation buffer was calculated from the volume flow rate and the geometry of the chamber. The separation buffer was assumed to exhibit laminar flow, with a parabolic velocity profile across the narrowest dimension of the chamber.

The mobility of a given band of particles was calculated using the formula:

$$u = \frac{v}{E} (\tan \alpha - \tan \alpha_o)$$

where u = electrophoretic mobility, v = maximum velocity of the buffer solution, E = electric field within the chamber, α = the angle of deflection of the particles bands, and α_o = the angle of deflection of the zero mobility marker.

Electron microscopy. Zonal and free-flow electrophoresis fractions were spun down at 100 000 × g for 1 h. The pellets were fixed with 3% glutaraldehyde for 1 h. After removing the glutaraldehyde by washing with 50 mM Tris · HCl (pH 7.4), the samples were fixed with cold 1% OsO_4 buffered with 50 mM cacodylate (pH 7.4) for 1 h. The pellets were then washed in buffer and dehydrated in increasing concentrations of ethanol. The pellets were placed in propylene oxide for 30 min and then in a mixture of propylene oxide and Epon (1 : 1, v/v) for 12 h. Thereafter the pellets were embedded in pure Epon, polymerized for 40 h at 60°C. Thin sections were studied in a Phillips EM 300 electron microscope.

Enzymatic assays. Substrates used for the biochemical assays were obtained from Sigma Chemical Co. All of the chemicals were reagent grade.

5'-Nucleotidase was measured in a medium containing 3 mM $MgCl_2$, 3 mM AMP and 40 mM HEPES Tris buffer at pH 7.4. ATPase activity was assayed in

a medium containing 2 mM $MgCl_2$, 20 mM KCl or 20 mM $NaHCO_3$, 2 mM ATP (disodium salt), 40 mM Tris · HCl buffer at pH 7.4. Valinomycin was added in 10 µl methanol to give a final concentration of 10^{-5} M, and the effect compared to controls with only 10 µl methanol added. The term "basal ATPase" refers to the activity observed when the only activating ion present was Mg^{2+}. The phosphate released was measured by the method of Yoda and Hokin [9] following incubation at 37°C. K^+-stimulated *p*-nitrophenyl phosphatase was assayed using a medium containing 6 mM $MgCl_2$, 6 mM *p*-nitrophenyl phosphate (ditris salt) and 50 mM Tris/acetate (pH 7.5). The *p*-nitrophenol liberated was measured by the method of Torriani [10]. 5—50 µg of protein was used for the above assays depending on the specific activity of the sample. Other experimental variables are indicated in the figures and tables.

Cytochrome *c* oxidase was measured by the procedure of Cooperstein and Lazarow [11] and succinate dehydrogenase by the method of King [12]. Monoamine oxidase was estimated by a modification of the method of Tabor et al. [13] by following the formation of benzaldehyde spectrophotometrically at 250 nm at 37°C in a medium containing 2.5 mM benzylamine and 50 mM phosphate buffer at pH 7.6, using a Gilford 2400 recording spectrophotometer. RNA was determined according to Munro and Fleck [14] while DNA was assayed by the method of Burton [15].

$^{86}Rb^+$ uptake. For uptake experiments, gastric membrane fractions at a final protein concentration ranging from 0.75 to 2.10 mg/ml were incubated for 72 h at 4°C in a medium containing 40 mM Tris/acetate, 75 mM RbCl with 10 µCi/ml $^{86}Rb^+$, 125 mM sucrose and 2 mM $MgCl_2$ at pH 6.1, the optimum pH for H^+ transport [16]. Separation of the Rb^+-containing vesicles from the incubation medium was achieved by Millipore filtration as previously reported [16]. $^{86}Rb^+$ uptake under these conditions has been demonstrated to reach equilibrium.

Polyacrylamide gel electrophoresis in SDS. Electrophoresis was carried out following the method previously described [3]. Solubilized membranes were fractionated on 6.1 × 80 mm polyacrylamide gels.

A 3.5% stacking gel was prepared in 0.1 M Tris · HCl (pH 6.8). Separation gels were 10% acrylamide prepared in 0.4 M Tris · HCl (pH 8.6). In both cases 2.5% of the acrylamide was *N,N'*-ethylenebisacrylamide. Reservoir buffer was 0.19 M glycine/Tris base at pH 8.6. Gels and reservoir buffer contained 0.1% SDS. Samples applied contained approx. 50 µg of protein. Gels were stained for protein by Coomassie Blue. Molecular weight estimations were made on gels using standard proteins as described earlier [3].

Results

(1) Differential centrifugation. The distribution of the various marker enzymes tested in this work is plotted in Fig. 1 according to the classical method of de Duve [17] and is similar to that found by Forte et al. [5] for his preparation of hog gastric microsomes; it is presented in terms of the 20 000 × *g* (M), 100 000 × *g* (P), and supernatant (S) fractions. In this representation, the specific activity of all the constituents in the homogenate is set at 1; the height of the blocks gives the relative purification achieved over the homoge-

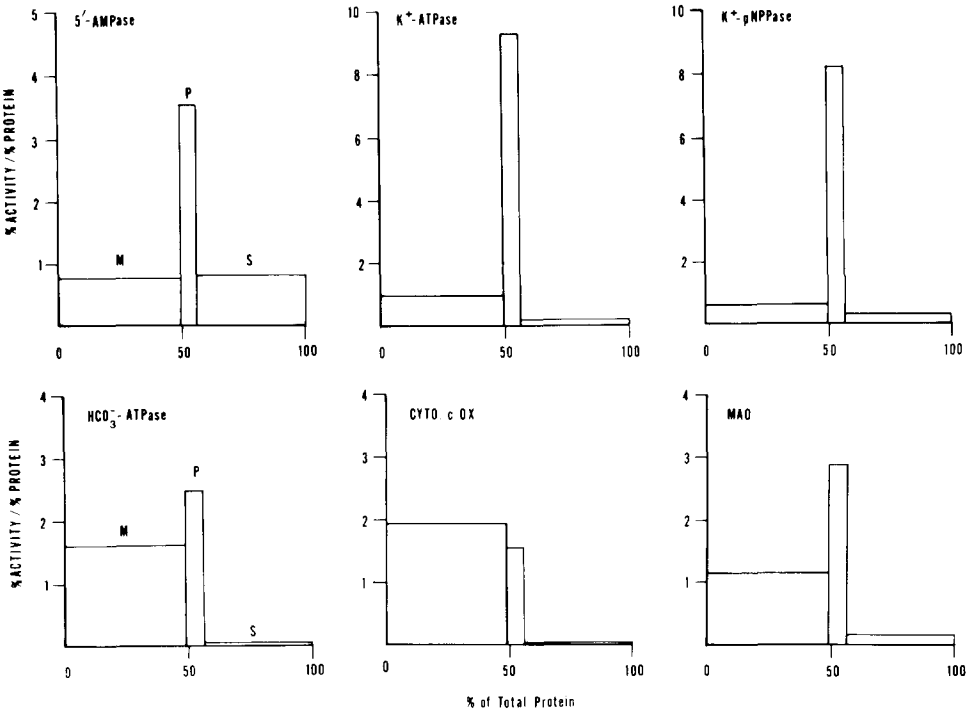

Fig. 1. Differential fractionation of hog gastric mucosa. The subcellular patterns are standardized. ATPase was estimated in presence of 2 mM ATP, 2 mM $MgCl_2$ with 20 mM KCl or $NaHCO_3$, in 40 mM Tris · HCl at pH 7.4. Other assay procedures were as described in Materials and Methods. Fraction M, (20 000 × *g*); fraction P (100 000 × *g*); fraction S (supernatant). pNPPase, *p*-nitrophenyl phosphatase; CYTO c Ox, cytochrome *c* oxidase; MAO, monoamine oxidase.

nate and the surface area of the blocks (relative specific activity × % protein) is the percentage of the constituent recovered in the corresponding fraction.

Two characteristic distributions are obtained at this stage. 5'-Nucleotidase, K^+-ATPase and *p*-nitrophenyl phosphatase are localised in the microsomal fraction, while cytochrome *c* oxidase is associated with the mitochondrial fraction, in good agreement with data reported for gastric mucosa of other species [1,3], including hog [5]. Monoamine oxidase, HCO_3^--stimulated ATPase and Mg^{2+}-ATPase showed a distribution somewhat different from the above markers; although the major portion of these three enzymes were found in the mitochondrial fraction, all showed a relative enrichment in the microsomal pellet. The microsomal fraction accounted for 8.5% of the protein, 31% of the RNA and 0.3% of the DNA of the total homogenate.

(2) Density gradient separation. Separation of the microsomal fraction on density gradients was trimodal, as shown in Fig. 2A, with peaks of protein at densities of 1.05 (GI), 1.11 (GII), and 1.16 (GIII). Material was also detected between the fractions, (GI—GII and GII—GIII).

Mg^{2+}-ATPase and HCO_3^--ATPase showed very similar distributions with maximal enrichment in GIII, but significant activity was detected in GII (28% for HCO_3^--ATPase). The markers for inner mitochondrial membranes, cytochrome *c* oxidase and succinate dehydrogenase, exhibited greater than 90% of their

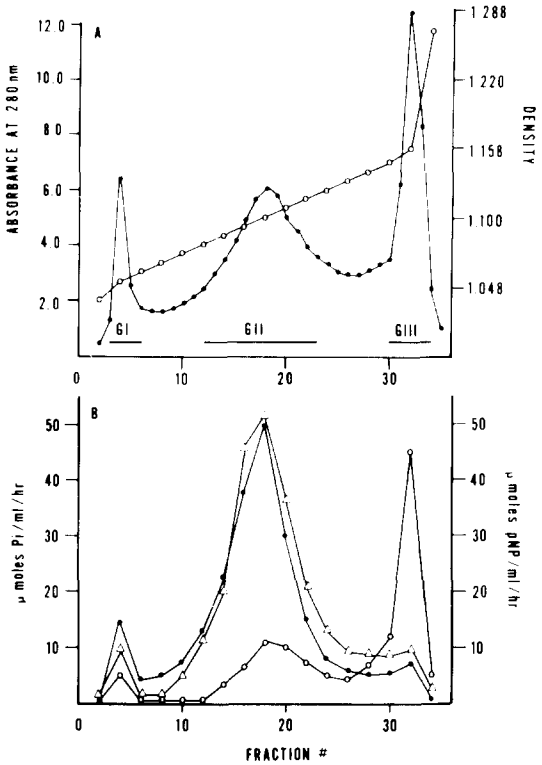

Fig. 2.(A) Fractionation of gastric cell membranes (microsomal pellet) by isopycnic zonal centrifugation on 7.5% Ficoll/0.25 M sucrose and 37% sucrose in 20 mM Tris · HCl at pH 7.4. ●———●, protein distribution; ○———○, sucrose density. (B) Enzymes fractionation by zonal centrifugation. ATPase and *p*-nitrophenyl phosphatase activities are given as μmol of P_i and *p*-nitrophenyl (pNP) released per ml per h. ○———○, Mg^{2+}-ATPase; △———△, K^+-ATPase; ●———●, K^+-*p*-nitrophenyl phosphatase.

activity in GIII, with very little activity in GII and none in GI. Monoamine oxidase showed essentially the same distribution as these mitochondrial markers, in contrast to the Mg^{2+}- or HCO_3^--ATPase. On the other hand, the K^+-*p*-nitrophenyl phosphatase or K^+-ATPase showed a characteristically different distribution; both enzymes exhibited maximal activity and percent distribution in GII, also showing significant activity in GI, but minimal in GIII. 5′-Nucleotidase had a different distribution from the above enzymes, with its activity peak in GI and decreasing thereafter (Figs. 2B and 3). These results are comparable to those reported for rat fundus [18] and other species [1,3].

(3) Free-flow electrophoresis separation. The indication that the GII gastric membrane fraction was enriched almost 19-fold in K^+-ATPase when compared with the starting homogenate, suggested that further purification of the enzyme from others (such as Mg^{2+}-ATPase and 5′-AMPase) could be achieved using the free-flow electrophoresis technique.

In order to preserve the structural and morphological integrity of the membranes, an isotonic isolation and separation medium was used. In the standard moving curtain buffer (8 mM Tris or triethanolamine/acetate at pH 7.4), the GII fraction separated in two poorly resolved major bands. Addition of 0.1 mM

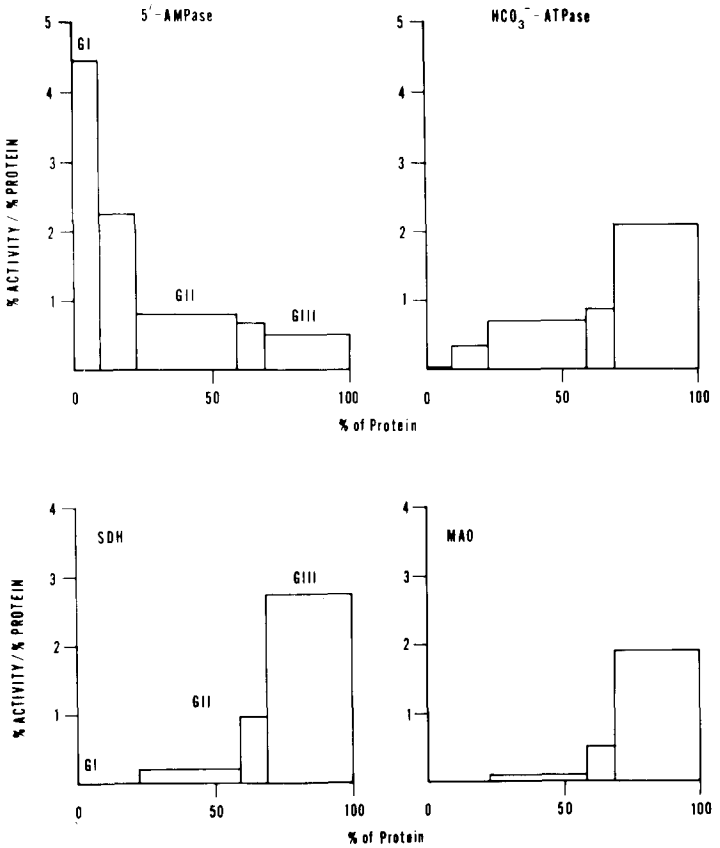

Fig. 3. The distribution of enzymes among the gastric membrane fractions derived from zonal centrifugation. Assay procedures were as described in Materials and Methods. SDH, succinate dehydrogenase; MAO, monoamine oxidase.

MgATP resulted in a much sharper separation into three bands, the first (FI) being displaced towards the anode by ATP, the second (FII) remained stationary. An additional minor peak (FIII) appeared which had the lowest electrophoretic mobility.

Fig. 4 shows a photograph taken during a typical electrophoretic separation of GII gastric membrane preparation. The mobilities of each band were calculated from the angles of deflection, from the observed flow rate of the separation buffer, and from the field strength within the chamber (Table I). The zeta potentials of the three fractions were calculated from the mobility data and buffer characteristic by application of Henry's equation [19]. The method and tabulated data of Loeb and Wiersema [20] were used to determine the surface charge density of FI only since FII and FIII were not closed spheres.

Protein and enzyme profiles of the fractions collected are shown in Fig. 5. K^+-ATPase and *p*-nitrophenyl phosphatase activities are almost entirely associated with FI, although activity is found in FIII, while Mg^{2+}-ATPase and 5'-AMPase are clearly separated from the other enzymes and confined to FII. This purification produced a further 2-fold enrichment in K^+-ATPase with respect to

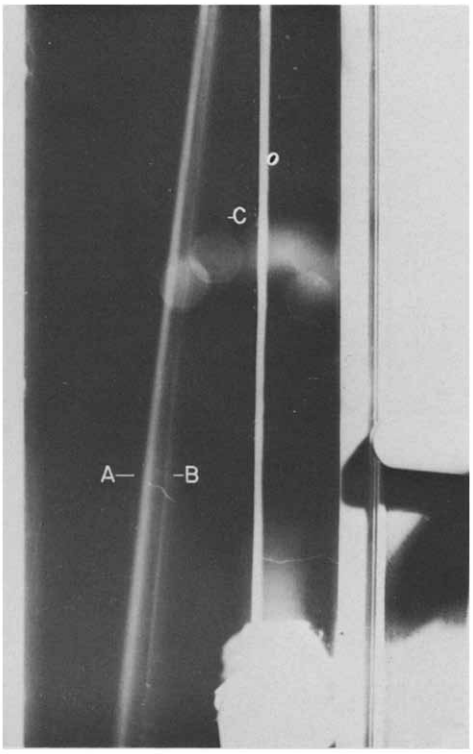

Fig. 4. Bands produced by the electrophoretic fractionation of GII gastric membrane preparation. The bands were photographed through the separating chamber face plate. The line on the right represents the deflection due to the electro-osmotic velocity of the buffer. Separation buffer containing 0.4 M sucrose was injected into the standard separation buffer which contained 0.25 M sucrose. A white thread was superimposed to allow a photographic record. The oblique line (A) is FI band; (B) and (C) indicate FII and FIII bands, respectively.

the original GII zonal fraction (Table II). HCO_3^--stimulated ATPase appeared to be equally distributed in all three protein peaks, the percent of stimulation however, increasing in FI due to the fall in the Mg^{2+}-ATPase level.

TABLE I

ELECTRICAL CHARACTERISTIC OF GASTRIC MEMBRANE FRACTIONS FROM FREE-FLOW ELECTROPHORESIS

Values shown indicate mean of five electrophoretic separation ± S.E.

Fractions	Mobility ($cm^2 \times 10^4/V \cdot s$)	Zeta potential (mV)	Surface charge density ($\mu C/cm^2$)
GII—FI	1.34 ± 0.04	—50.7 ± 1.0	—3.89 ± 0.01
	(1.12 ± 0.02) *	(—42.2 ± 0.5)	(—3.03 ± 0.03)
GII—FII **	1.06 ± 0.02	—40.1 ± 1.7	—
GII—FIII	0.52 ± 0.01	—19.7 ± 0.9	—

* In between parenthesis are represented values where free-flow electrophoresis was carried out in absence of 0.1 mM MgATP into the separation buffer.

** The surface charge density for FII and FIII fractions were not calculated since these fractions appeared to be in non-vesicular form.

TABLE II

PURIFICATION OF GII GASTRIC MEMBRANE FRACTION

Assay conditions as described in Materials and Methods. Activities are expressed in μmol/mg protein per h. (mean of nine fractionations ± S.E.).

Fractions	Mg^{2+}-ATPase	K^+-ATPase *	K^+-*p*-nitrophenyl phosphatase	5'-AMPase
GII (zonal)	19.4 ± 1.8	63.6 ± 2.0	61.2 ± 4.2	2.2 ± 0.3
GII—FI	4.4 ± 1.0	110.7 ± 2.6	68.0 ± 2.5	0.3 ± 0.06
GII-FII	31.0 ± 3.1	20.0 ± 0.7	9.4 ± 1.0	5.4 ± 1.0
GII-FIII	2.8 ± 0.4	50.1 ± 3.2	31.0 ± 2.2	0.1 ± 0.1

* Activity in presence of 20 mM KCl minus the basal rate.

(4) Effect of valinomycin and osmotic shock. Addition of K^+-ionophoretic substances such as valinomycin to the GII membrane fraction produced an approximate 25% increase in the K^+-ATPase activity. Stimulation was much more striking in the FI fraction from electrophoretic separation (about 85%) and it appeared to be entirely associated with this peak. No effect was detected on the K^+-p-nitrophenyl phosphatase activity [28].

In hypotonic conditions (produced by a 10—30-fold dilution of the membrane fractions into a medium lacking sucrose), the K^+-ATPase activity of fraction GII increased by 18% and the valinomycin effect was almost abolished. Osmotically shocked FI showed a 1.7-fold enhancement of K^+-ATPase activity and again, valinomycin treatment did not alter the activity significantly (Table III).

These data suggest that, if a vesicular structure is present in the gastric membranes, the GII vesicle might be more permeable to K^+, and FI vesicles less permeable to the same ion. The K^+ site is therefore on the interior of the vesicle. A final concentration of 0.003% (w/v) Triton X-100 stimulated the K^+-ATPase in GII and FI fractions to the level reached in hypotonic medium and now valinomycin was without effect. Higher concentrations of Triton X-100 inhibited the K^+-ATPase activity. When K^+ was replaced with NH_4^+, since NH_3 appears to be freely permeable across cell membranes [21], in isotonic conditions, an increase in ATPase activity was observed, but valinomycin and osmotic shock were without effect (Table III). Hence the effect of ionophores is indeed due to a limited K^+ penetration of the vesicle. It is important to point out that K^+-*p*-nitrophenyl phosphatase, 5'-AMPase and basal Mg^{2+} activities were not affected by osmotic shock.

(5) Electron microscopy. An electron micrograph of GII membrane fraction is shown in Fig. 6. This fraction consists almost entirely of smooth-surfaced vesicular particles, 0.1—0.2 μm in diameter. Many of the vesicles in this fraction are present in double membrane forms, which often appeared to be two fused membranes. Spherical particles (500 Å), probably mitochondrial fragments, are also seen. Micrographs of thin sections of the FI electrophoretic fraction also exhibited a double membrane structure with occasional granular content. No other cell particles could be detected (Fig. 7). Freeze-fracture confirmed the double membrane structure and also showed the presence of intercalated

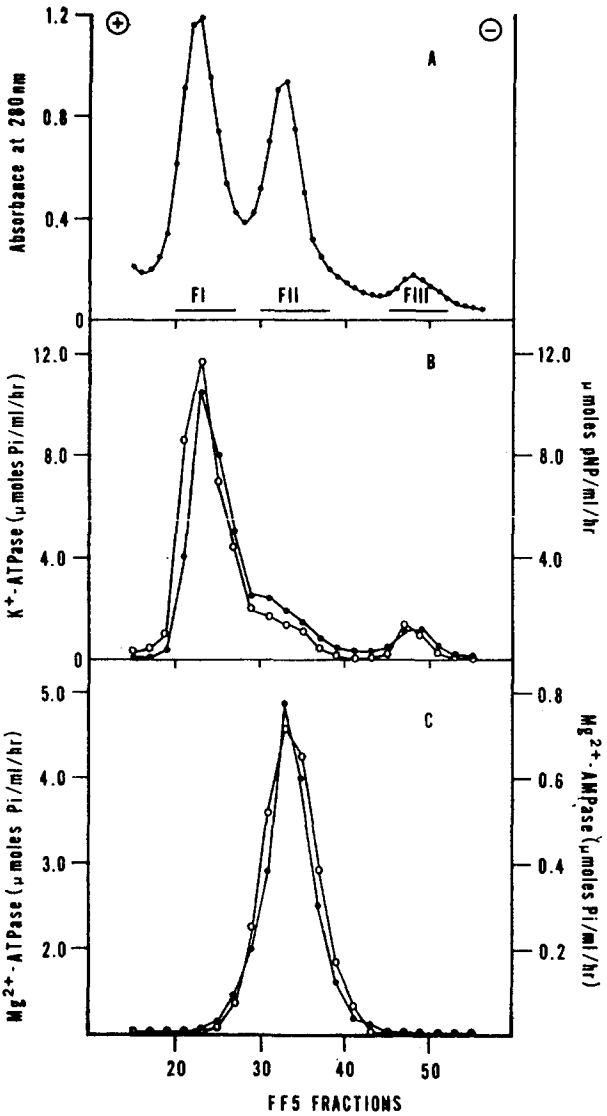

Fig. 5. Separation of GII membrane fraction by free-flow electrophoresis. A, protein distribution in the fractions collected; B, distribution of K^+-ATPase activity (○———○), and K^+-*p*-nitrophenyl phosphatase activity (●———●); C, distribution of Mg^{2+}-ATPase (●———●), and 5′-AMPase (○———○). Separation was carried out using 2 mM Tris/acetate as chamber buffer, containing 0.1 mM Mg^{2+}-ATP at pH 7.4. (See Materials and Methods). pNP, *p*-nitrophenyl

200 Å particles. Fractions FII and FIII showed the presence of both open and closed membrane forms (Fig. 8 and 9) and some contaminating material.

(6) $^{86}Rb^+$ *uptake.* Table IV shows the equilibrium levels of $^{86}Rb^+$ uptake. The total uptake of the original GII fraction was approximately equal to the sum of the relative uptakes (relative uptake = % uptake/% protein) of each free-flow electrophoretic fraction.

FI and FII exhibited $^{86}Rb^+$ uptake, while fraction FIII showed very little in

TABLE III

EFFECT OF ISOTONICITY AND OSMOTIC SHOCK ON THE K^+-ATPase ACTIVITY OF GASTRIC MEMBRANE VESICLES

Activity in presence of the following where appropriate: 2 mM ATP, 2 mM $MgCl_2$, 20 mM KCl, 10^{-5} M valinomycin, 0.003% (w/v), Triton X-100, 0.25 M sucrose in 40 mM Tris · HCl. Activities are expressed in μmol/mg protein per h. (mean of six experiments ± S.E.)

Fractions	Isotonic		Isotonic+Triton X-100	Hypotonic	
	K^+-ATPase	K^+-ATPase + valinomycin	K^+-ATPase	K^+-ATPase	K^+-ATPase + valinomycin
GII (zonal)	52.7 ± 2.2 (66.9 ± 1.5) *	68.3 ± 3.3 (64.4 ± 2.3)	67.4 ± 1.3	63.6 ± 2.0	66.3 ± 1.0
GII—FI	63.7 ± 2.1 (86.4 ± 1.5)	101.5 ± 2.4 (95.6 ± 2.8)	102.2 ± 1.7	110.7 ± 2.6	112.4 ± 1.4
GII—FII	21.2 ± 1.1 (22.7 ± 0.5)	21.4 ± 0.7 (20.4 ± 1.3)	18.6 ± 2.3	20.0 ± 0.7	19.8 ± 2.1
GII—FIII	50.4 ± 3.0 (51.7 ± 1.4)	50.2 ± 2.8 (51.0 ± 2.7)	47.4 ± 3.0	50.1 ± 3.2	51.3 ± 1.7

* Values in between parentheses indicate the ATPase activity when K^+ was replaced with 20 mM, NH_4^+ minus the basal rate.

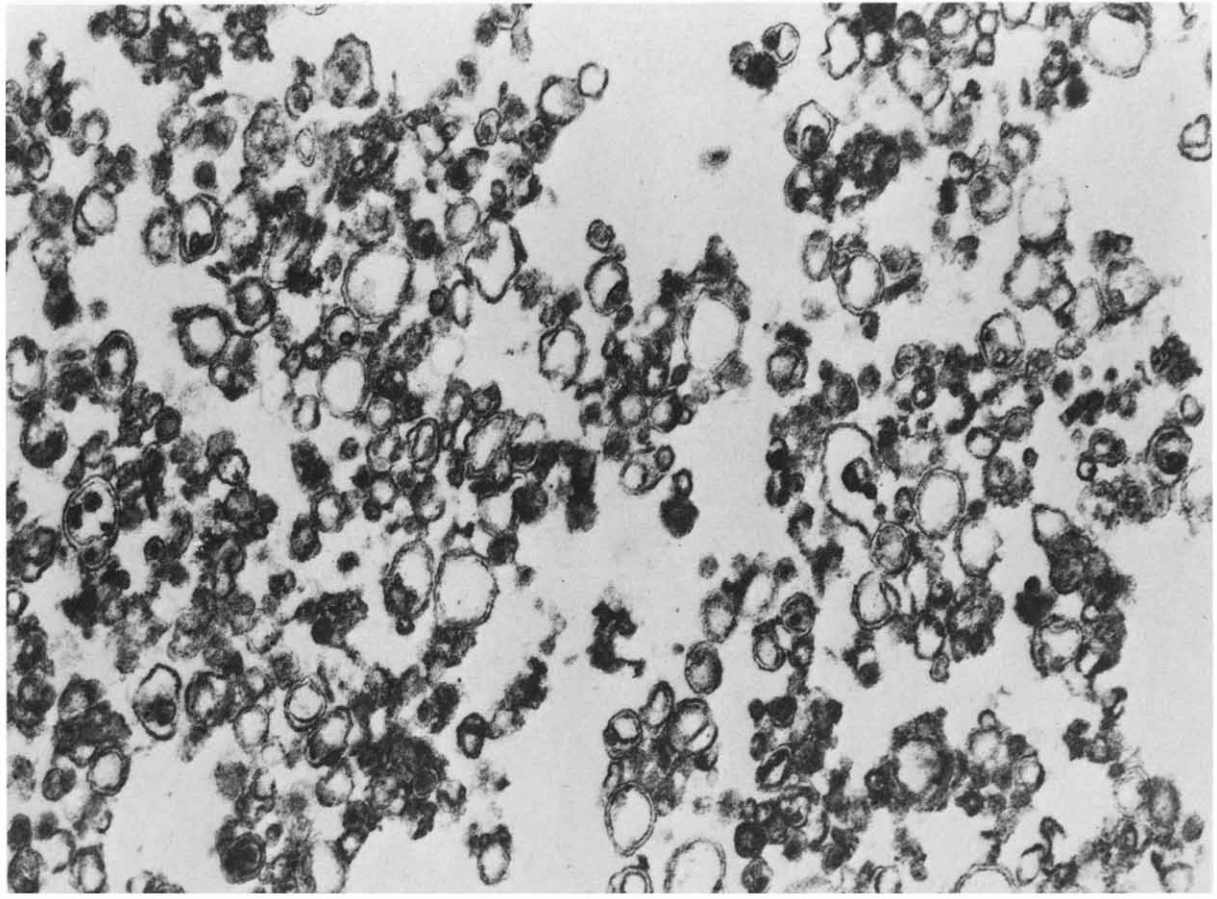

Fig. 6. Electron micrograph of GII membrane fraction. This material consists of smooth-surfaced vesicles with many double membrane forms. In addition particles with morphological appearance as mitochondrial fragments are present (× 48 000).

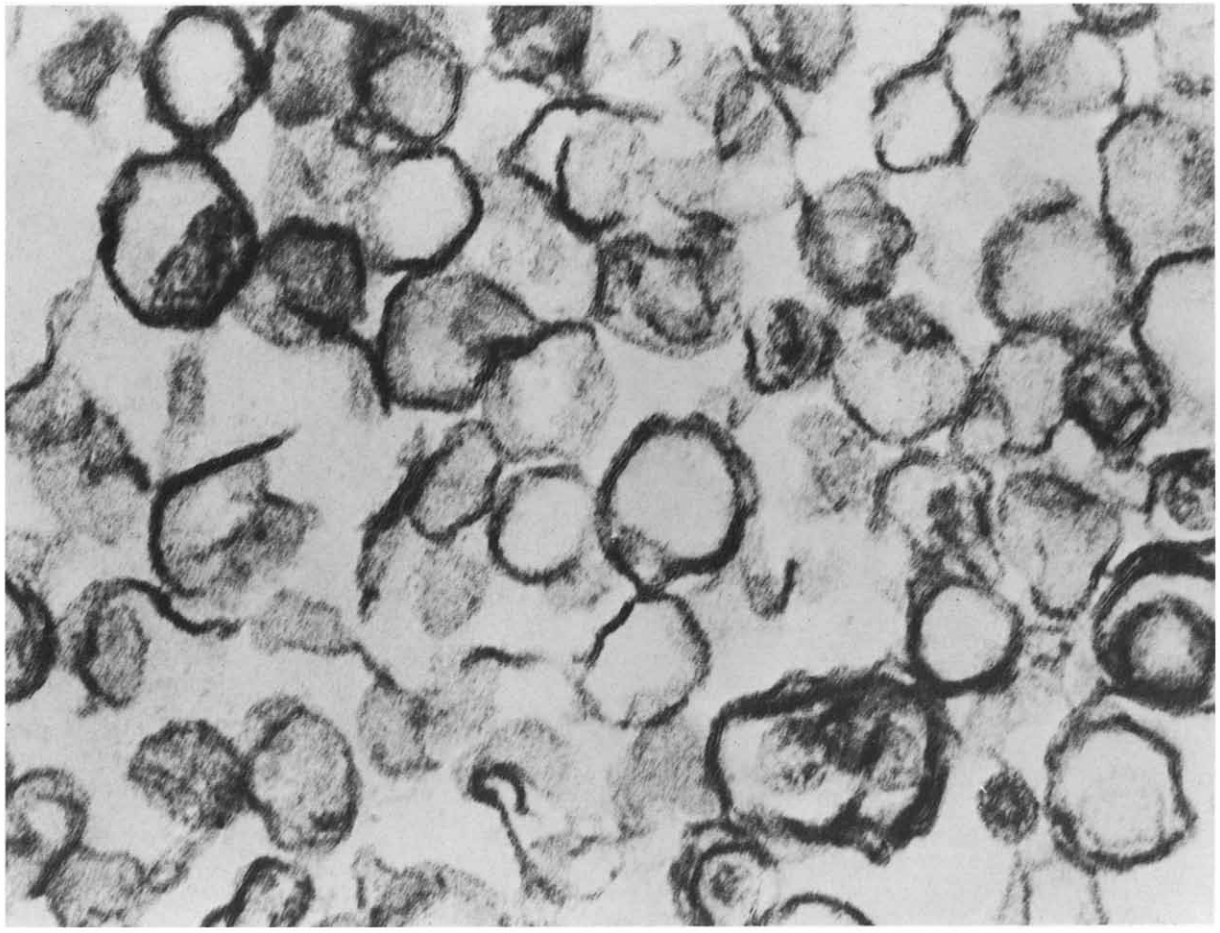

Fig. 7. Electron micrograph of FI electrophoretic fraction. The morphology is similar to that of GII fraction. Contaminations by other cell particles are quite absent (× 96 000).

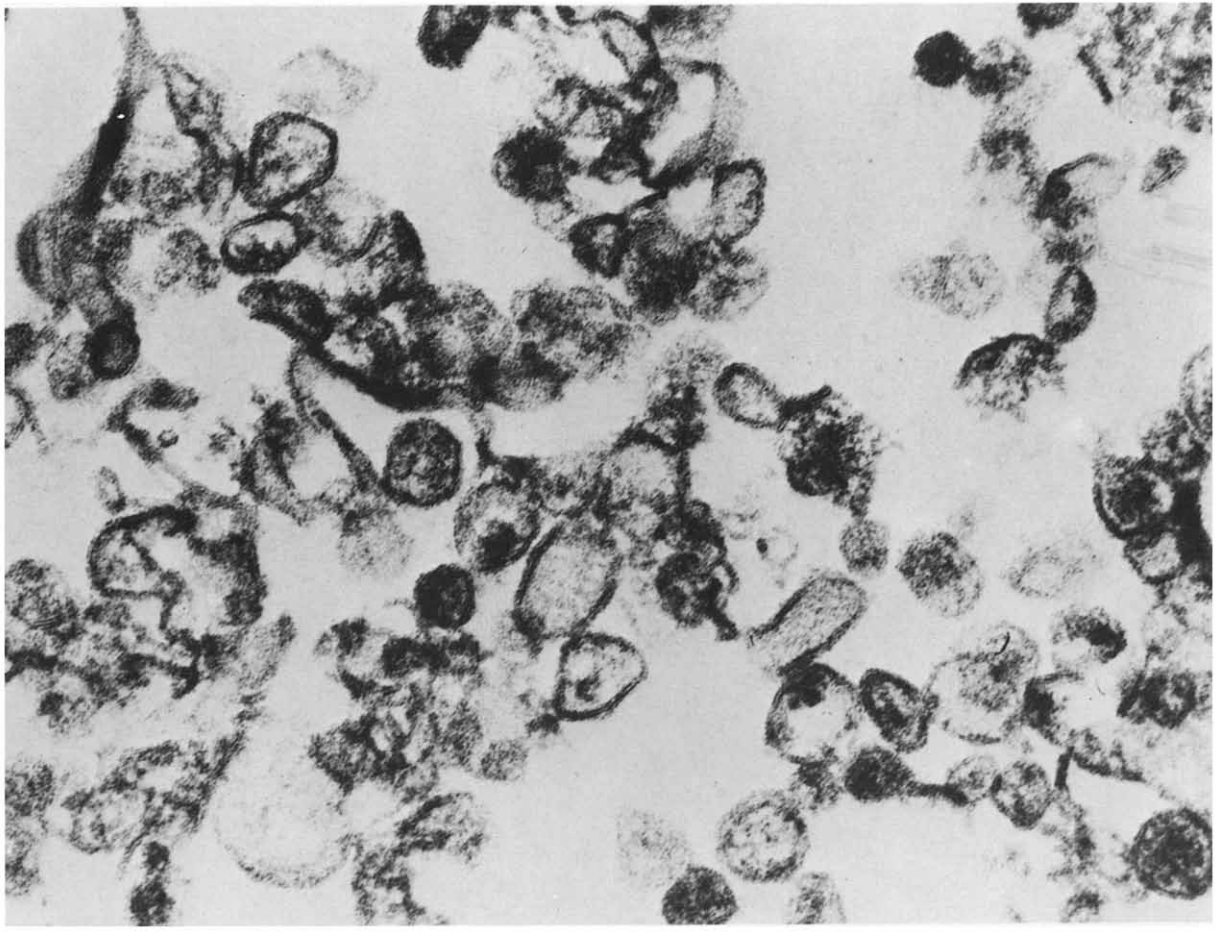

Fig. 8. Electron micrograph of FII electrophoretic fraction. Mostly open membrane forms are present. Note the distinct differences when compared to FI of Fig. 7 (X 96 000).

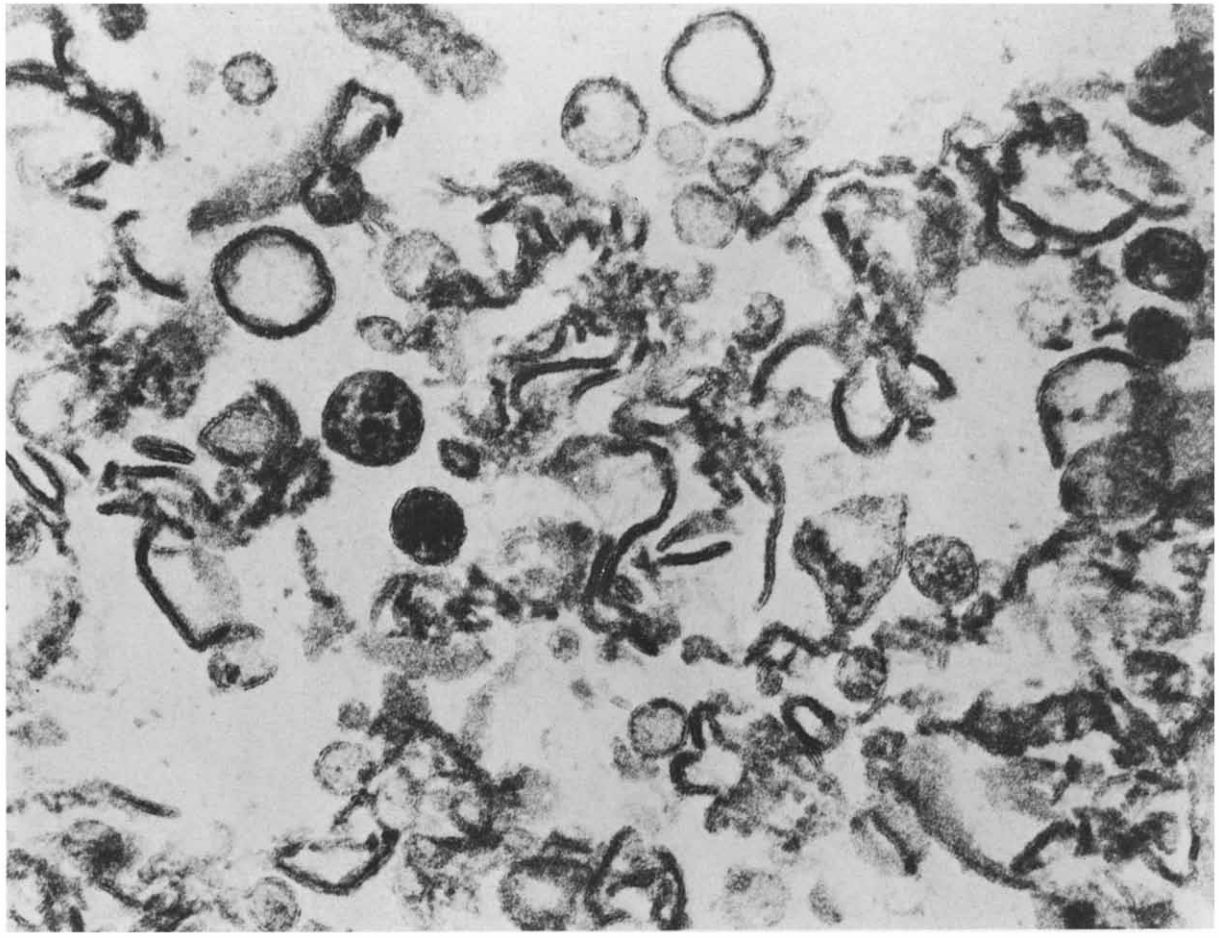

Fig. 9. Electron micrograph of FIII electrophoretic fraction. A mixture of open and closed membranes can be seen (× 16 000).

TABLE IV

$^{86}Rb^{+}$ UPTAKE AND EFFLUX OF GASTRIC MEMBRANE FRACTIONS

Values shown indicate a mean of four determinations.

Fractions	nmol $^{86}Rb^{+}$ uptake/mg protein	nmol $^{86}Rb^{+}$ efflux mg/protein *
GII (zonal)	160	60
GII—FI	235	125
GII—FII	195	8
GII—FIII	75	0

* $^{86}Rb^{+}$ efflux experiments were carried out in the presence of 2 mM ATP, under conditions described in Materials and Methods.

spite of its K^{+}-ATPase activity. For GII and FI fractions, the presence of valinomycin increased the rate, but not the final level, of $^{86}Rb^{+}$ uptake. It appeared that valinomycin enhanced the Rb^{+} permeability of the vesicles [16]. Addition of ATP stimulated H^{+} uptake and Rb^{+} efflux only in the FI fraction [16].

(7) Polyacrylamide gel electrophoresis. In order to compare the peptide composition of the subcellular fractions derived from hog gastric mucosa, the respective fractions were solubilized in SDS-β-mercaptoethanol and subjected

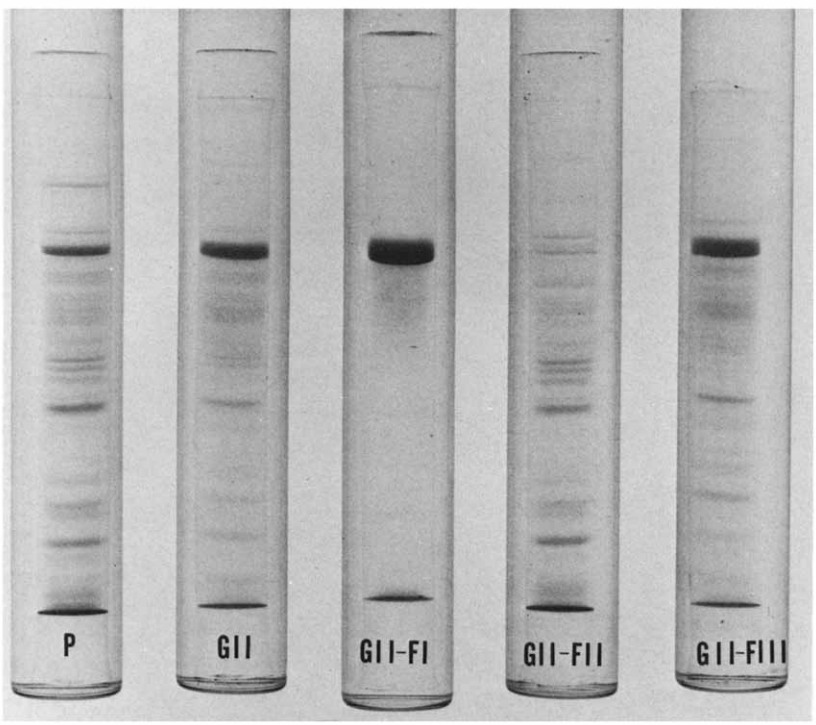

Fig. 10. SDS-polyacrylamide gel electrophoresis patterns of various gastric membrane fractions stained with Coomassie blue. The microsomal fraction P, obtained by differential centrifugation is shown and compared with fractions GII, FI, FII, FIII. Approx. 50 μg of protein were applied to each gel. Molecular weight was estimated by calibration of identical gels with standard protein. Note the increasing purification of the M_r = 100 000 polypeptide region.

to acrylamide gel electrophoresis. Gels stained for protein (Fig. 10) revealed considerable simplification on the polypeptide pattern between the microsomal fraction (P) and the electrophoretic gel purified fraction (FI). It can be seen that, consistent with the enzymatic data presented above, a major polypeptide region of M_r = 100 000 accounts for 23% of the total protein stain in the microsomal fraction and for 75% of the FI fraction. This polypeptide was phosphorylated by [γ-^{32}P]ATP and dephosphorylated in the presence of K^+ [3,5]. On the other hand, FII electrophoretic pattern shows mainly the remaining peptides of the original GII fraction.

Of interest is fraction FIII which shows the presence of a M_r = 100 000 polypeptide region in quite large amounts.

Discussion

Previous studies on purification of gastric microsomal membranes [1,4,5] have used methods similar to those described here, with the exception of zonal isopycnic centrifugation in a Ficoll/sucrose continuous gradient. This technique separates two classes of membranes: GI at density of 1.05 which is relatively enriched in 5′-nucleotidase, and GII at density of 1.11 which is enriched 19-fold in K^+-ATPase and K^+-*p*-nitrophenyl phosphatase with respect to the original homogenate.

The HCO_3^--stimulated ATPase, which has been the subject of much work in the gastric membrane [3,4,18] seemed at first to be located in the denser region of the sucrose gradient where mitochondrial and ribosomal markers were in high activity [5,18]. However, detailed analysis of its distribution indicated that 28% of its activity was still located in GII where only 10% of mitochondrial markers activity was found.

The fractionation procedure described above yielded a satisfactory separation between 5′-AMPase, which is probably associated with other plasma membranes, and K^+-ATPase (or *p*-nitrophenyl phosphate), which is probably associated with apical membrane of the parietal cell surface and the tubulovesicular system [1,5]. Yet it also indicates that densities of the membranes in which these two clases of enzymes are located are perhaps not different enough to allow a complete separation by gradient centrifugation.

In recent investigations [6,23,24] it has been shown that the preparative free-flow electrophoresis technique is capable of effecting separation not possible by other methods. One important conditions for a successful application of this relatively new technique is a difference in the electrical behavior of the material to be purified. In the case of separations of biological membranes, therefore, it is necessary that the membranes have unique electrical surface charges. In most cases, this varying surface charge appears to be related to a functional and morphological difference.

Our initial attempt at the further purification of the K^+-ATPase in GII membrane fraction by free-flow electrophoresis under standard conditions gave indifferent results, probably because of an insufficient difference in charge of the particles involved. However, enzymatic analysis of the fractions collected indicated that some degree of separation had been obtained. It seemed that the inducement of charge perturbation on the system could increase the electro-

phoretic resolution. Since it has been shown that the gastric ATPase can be phosphorylated in absence of cation such as K^+ [22], from the level of phosphorylation (approx. 10^{-9} mol of phosphate/mg protein) and the vesicular volume (2 μl/mg protein) of the membranes, it was possible to calculate a probable increase of 500 negative charges per vesicle. Based on this observation, MgATP was added to the electrophoretic separation medium.

The subsequent change in electrophoretic mobility of the membrane particles, produced by an increase in the negative charge density, resulted in a distinct separation of K^+-ATPase and *p*-nitrophenyl phosphatase from Mg^{2+}-ATPase and 5'-nucleotidase-carrying membrane. However, HCO_3 stimulation of ATPase was present in all the fractions. From the surface charge densities calculated for the FI fraction by the mobility measurements, in presence and absence of MgATP into the separation buffer, it was found that the change in negative charges per vesicle was in good agreement (approx. 600) with the value obtained from phosphorylation experiments.

The morphological appearance of the GII membrane fraction before electrophoretic separation, as seen in the electron microscope was not altered by this technique. The vesicular structure and the double membrane forms observed in GII fraction were also found in FI. On the other hand, the contamination present in GII was clearly separated and primarily confined to FII. Along with the enzymatic data and the electron microscope appearance, the gel pattern of membrane proteins separated by acrylamide electrophoresis tend to confirm the actual fractionation and purification of the gastric K^+-ATPase. The progressive increase in M_r = 100 000 region containing the phosphorylated peptide subunits of the K^+-ATPase and the considerably simplified polypeptide pattern in FI demonstrate the purification produced, when compared to GII and FII fractions.

The free-flow electrophoresis technique appeared also to separate K^+-ATPase present in "tight" vesicles, from K^+-ATPase localised in open membrane fragments. The findings that ionophores such as valinomycin, in isotonic conditions, increase the K^+-ATPase activity of the membrane preparation, suggest that this ionophore enhanced the penetration of K^+ into the interior of the vesicles, eliminating the permeability barrier. This effect appeared significantly larger in fraction FI when compared to GII and it was not observed in fractions FII and FIII.

Based on those data, treatments designed to disrupt membrane vesicles should also be effective. Osmotic shock induced by hypotonic lysis and Triton X-100 treatment [25] were used to alter the tightness of the vesicles. In both cases the K^+-dependent ATPase activity was increased nearly to the valinomycin-expressed rate. It should be noted that increasing the concentration of Triton X-100 increased the ATPase activity up to a maximum and thereafter decreased. These treatments did not affect fractions FII and FIII. $^{86}Rb^+$ uptake studies confirmed the different degree of permeability of the vesicles. The high level of $^{86}Rb^+$ uptake in FI might be explained considering that this fraction could either contain a large percentage of vesicular material per mg of protein, or vesicles less permeable to the ion involved, when compared to the original GII. On the other hand fractions FII and FIII showed less $^{86}Rb^+$ uptake. If the K^+-ATPase was in vesicular form, Rb^+ efflux should occur with ATP addition

[16] as occurs in FI. The lack of efflux in FII and FIII implies either the absence of functional vesicles or vesicles highly permeable to Rb^+.

The results presented so far in this paper indicate that K^+ permeability is a limiting factor in the vesicular gastric ATPase activity. The data obtained by replacing K^+ with NH_4^+ in isotonic conditions supports this point of view. Again, analysis of the kinetics of activation both by K^+ and additional activation by ionophore(s) lead to the same conclusion [22]. The fact that K^+ stimulates both the ATPase and the phosphatase of gastric microsomal membranes raises the question of the relationship between those two enzymes. As for Ca^{2+}-ATPase and ($Na^+ + K^+$)-ATPase [26,27] there is considerable evidence that the phosphatase activity is a property of the ATPase complex. For example, in terms of purification, cation selectivity and action of inhibitors [4,5,16], it would seem that phosphatase and ATPase activity reside on the same enzyme.

The insensitivity to ionophores and to vesicle rupture of the *p*-nitrophenyl phosphatase activity suggests that K^+ penetration is not rate limiting for the activity of this enzyme, in contrast to the ATPase. Rb^+ transport does not occur with *p*-nitrophenyl phosphatase or acetyl phosphate as substrate, hence it is likely that the K^+ site for *p*-nitrophenyl phosphatase activity is readily available to external K^+, whereas the K^+ site for ATPase is internal. Since K^+ is transported by the vesicle and both an internal and external K^+ site are present, the site must be mobile across the membrane.

The double or multilayered structure of many of the membrane forms in GII and FI would suggest that a change in specific enzyme activity might also occur with rupture of the vesicles if the inner vesicle also contained K^+-ATPase. The action of ionophores in achieving maximal activity and the insensitivity of *p*-nitrophenyl phosphatase to loss of vesicle integrity suggests that either ATP or *p*-nitrophenyl phosphate has free access to the inner vesicle, or that this inner vesicle has a different peptide composition from the outer vesicle.

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