CHARACTERIZATION OF GASTRIC MUCOSAL MEMBRANES

X. Immunological Studies of Gastric ($H^+ + K^+$)-ATPase

GAETANO SACCOMANI, HERBERT F. HELANDER, SYLVIA CRAGO, HSUAN H. CHANG, DOUGLAS W. DAILEY, and GEORGE SACHS

From the Laboratory of Membrane Biology and the Department of Microbiology, University of Alabama in Birmingham, Birmingham, Alabama 35294

ABSTRACT

Gastric mucosal homogenates from hog were fractionated by differential and density gradient centrifugation and free-flow electrophoresis. The two major membrane fractions (FI and FII) thus obtained are distinct both enzymically and in terms of transport reactivity. This heterogenicity extends to their antigenic activity. Purified antibodies which were raised against the K^+-ATPase-containing H^+ transport fraction FI were of two types: inhibitory and non-inhibitory. Inhibitory antibodies reduced the K^+-ATPase activity by ~80% and the K^+-*p*-nitrophenylphosphatase activity by ~40% in a concentration-dependent manner, while the small Mg^{++}-dependent component of the enzyme activity was unaffected. Antibodies inhibiting the K^+-ATPase also inhibited H^+ transport. These antibodies did not cross-react with the other major membrane fraction isolated by free-flow electrophoresis, FII, and gave a single band on rocket immunoelectrophoresis. Antibodies against this FII fraction also did not react with the K^+-ATPase and were heterogeneous, giving at least four bands with rocket immunoelectrophoresis and inhibiting both the 5′-nucleotidase and Mg^{++}-ATPase of this fraction. Immunofluorescent staining of tissue sections showed that the FI was derived from the parietal cell of gastric tissue and was localized to the supranuclear area of the cell. Staining of isolated rat gastric cell suspensions by FI antibodies confirmed the selectivity of the antibody and showed a polar, plasma membrane localization. FII antibodies also largely stained the parietal cells in tissue sections. In the 16 hog tissues tested, FI antibodies cross-reacted only with gastric fundus, thyroid and weakly with thymus. Immunoelectronmicroscopy showed that FI antibodies reacted strongly with the secretory membrane at the apical cell surface of the parietal cells and at the secretory canaliculi, weakly with the apical surface of the zymogen cell, and not with the basal-lateral surface of the cells. Thus, the proton-translocating ATPase is localized in the parietal cells and in the region postulated to be the site of acid secretion.

KEY WORDS gastric mucosa · parietal cell · gastric membranes · K^+-ATPase · acid secretion · immunofluorescence · immunoelectronmicroscopy

Although mammalian gastric mucosa is a single layered epithelium, it is heterocellular and anatomically complicated by the presence of the tubular glands (9, 16). These glands contain scattered

acid-secreting or parietal cells which comprise ~15% of the mucosal volume (7). The parietal cells are structurally polarized and functionally asymmetric. Their cytoplasm contains a large number of tubulovesicles which decrease in number during stimulation of acid secretion and the secretory surface increases in area (7, 8) by formation of microvillus-lined intracellular secretory canaliculi. From this, the parietal cell contains a complex infolded or segregated membrane system which presumably becomes continuous with the apical plasma membrane during acid secretion by formation of the secretory canaliculi which then permeate the entire cytoplasm.

Recently, it has been shown that crude and purified dog (11) or hog (21) gastric membranes are capable of transporting H^+, a process dependent on the hydrolysis of ATP by a K^+-activated ATPase which results in H^+:K^+ countertransport (4, 23). Moreover, free-flow electrophoresis of the gastric membrane fraction enriched in the (H^+ + K^+)-ATPase results in a characteristic separation into three membrane fractions (17). The anodic peak FI (membrane vesicles associated with K^+-ATPase and *p*-nitrophenylphosphatase activity) contains a single 105,000-dalton polypeptide region, whereas the middle peak FII (mostly open membrane forms containing Mg^{++}-ATPase and 5′-nucleotidase) shows several peptides ranging in molecular weight from 115,000 to 20,000 daltons or less. The third peak FIII (a mixture of membrane vesicles and also fragments containing K^+-ATPase and pNPPase activity among others) appears to combine the characteristics of the other two in terms of peptide composition. The ATP-induced H^+ transport is confined to FI (21).

It is important to prove the parietal cell origin of the transport active fraction. It is also of interest to compare cellular and cellular-regional origins of the FI and FII fractions and to determine the organ distribution of the major FI antigen. In this work, we therefore describe a series of immunohistochemical studies on these membrane fractions, using antibodies to these hog gastric membrane fractions.

MATERIALS AND METHODS

Membrane Isolation

Gastric membranes from hog gastric mucosa were prepared as previously described (17). Briefly, mucosal homogenate was centrifuged at 20,000 *g* for 40 min in a Sorvall RC-2 centrifuge (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.), and the resulting supernate was centrifuged at 105,000 *g* for 60 min in a Beckman L-2 ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) to yield the crude microsomal pellet. This pellet was suspended in 0.25 M sucrose-20 mM Tris-Cl (pH 7.4) and distributed by zonal centrifugation using a continuous density gradient made between 7.5% (wt/wt) Ficoll in 0.25 M sucrose and 37% (wt/wt) sucrose. The fraction collected between a density of 1.08 and 1.12 (GII) was diluted with 20 mM Tris-Cl, pH 7.4, and centrifuged at 105,000 *g* for 60 min. The GII pellet was washed twice in 8 mM Tris-8 mM acetic acid containing 0.25 M sucrose, adjusted to pH 7.4 and 2 M NaOH, and subjected to free-flow electrophoresis under the conditions previously reported (17). The resulting fractions were routinely lyophilized and stored at −80°C before use for the antibody study.

Preparation of Antisera to Membrane Protein

Antisera were produced against gastric membrane fractions isolated by free-flow electrophoresis and designated FI, FII, and FIII. Three groups of five New Zealand white rabbits, each weighing ~3 Kg, were obtained from Norwood Farms, Ala., during the 12-mo study period.

Appearing healthy after 2 wk, the rabbits were pre-bled from the central ear vein. The sera were obtained by clotting at 22°C for 1 h and overnight at 4°C, and then centrifuging at 3,000 *g* for 15 min.

2 wk after pre-bleeding, the rabbits were immunized using 1 mg of each protein antigen suspended in 1 ml of physiological saline and mixed with an equal volume of Freund's complete adjuvant. The rabbits received 1.0 ml of the emulsion subcutaneously into 10–15 different sites of the back. Subsequent injections of antigen in Freund's complete adjuvant were administered as described above, biweekly for 8 wk. 5 d after the second injection, the rabbits were bled and the sera were prepared as described above. Thereafter, rabbits were bled weekly, and sera were tested for immunoreactivity towards the respective antigens by double diffusion (15).

Fractionation and Purification of Antisera

Precipitation of γ-globulins from pre-immune sera as well as antisera was carried out by dropwise addition at 4°C of 1 vol of saturated ammonium sulfate solution at pH 7.4 for 1 vol of the serum. The precipitate was stirred for 2 h at 4°C and then collected by centrifugation at 10,000 *g* for 30 min. The precipitate was resuspended in a volume of 20 mM Tris-Cl buffer at pH 7.4 equal to 50% of the original volume of the sera. The precipitation and resolubilization cycle was repeated three times. The final γ-globulin fraction was then dialyzed against 100 vol of 150 mM Tris-Cl buffer, pH 7.4, at 4°C with six changes of the dialysate and a total dialysis period of 72 h. Monitoring of the dialysate by addition of saturated $BaCl_2$ indicated that these conditions were sufficient for the removal of residual ammonium sulfate.

Gamma globulin fractions were additionally subjected to DEAE-cellulose chromatography (5). Results obtained with antibody which had undergone DEAE cellulose purification were indistinguishable from those after ammonium sulfate precipitation alone.

In some experiments, the antibody fractions were further purified by adsorption on the membrane-bound antigens, incu-

bating for 30 min at 37°C and then overnight at 4°C (13). After washing and centrifuging, bound protein was eluted from the membrane by suspending the complex in 10 mM glycine-0.9% NaCl, pH 2.8, and incubating at 22°C for 1 h. After centrifugation, the supernate was dialyzed against 150 mM Tris-Cl, pH 7.4. Aliquots of ~0.5 ml each of pre-immune sera and antibodies were stored at −80°C until use.

Immunochemical Analysis

Double-diffusion gel precipitation was carried out according to the method of Ouchterlony (15) using 1% agarose (Sigma Chemical Co., St. Louis, Mo.) in 0.05 M veronal buffer at pH 8.6. Diffusion was allowed to proceed for 12–24 h in moist atmosphere at 22°C.

Rocket immunoelectrophoresis was carried out according to Laurell (10). Antibodies were mixed with 1% agarose in 0.02 M barbital at pH 8.6 to give a final dilution of 1:175.

A 100 × 100 × 1.5-mm glass plate was overlaid with 17.5 ml of agarose-antibody solution (antibody content 0.22 mg cm^{-2}) and allowed to cool at 22°C. 2.5-mm wells were punched into the gel and 7 μl of antigens in 1% Triton X-100 containing ~5–10 μg of protein were applied. The samples were run at 6 V cm^{-1} for 5 h in a Bio-Rad model 1400 electrophoresis cell (Bio-Rad Laboratories, Richmond, Calif.), using barbital buffer. After electrophoresis, the plate was washed, dried, and stained with 0.25% Coomassie Blue solution in 30% ethanol-7.5% acetic acid.

Chemical Determinations

Protein measurements were performed by the method of Lowry et al. (12). ATPase, 5′-nucleotidase and *p*-nitrophenyl-phosphatase activities were analyzed as previously described (17). The methods of Yoda and Hokin (28) were used to measure the inorganic phosphate released after enzymatic incubation of 37°C, while the method of Torriani (27) was used to measure the *p*-nitrophenol liberated. Substrates used for the biochemical assays were obtained from Sigma. All reagents were the best grade available.

Inhibition of Enzyme Activities by Antisera

To study the effect of the antibodies on the enzyme activities, either fresh or lyophilized, FI, FII, and FIII antigens were usually pre-incubated with their purified antibodies or pre-immune sera as controls. The condition found to be both efficient and effective involved preincubation in the enzyme assay medium before substrate addition, at 22°C for 30 min, using different antigen to antibody or pre-immune sera protein ratios varying from 1:1 to 1:100.

Inhibition by FI Antibody of H^+ Uptake

Experiments were carried out as earlier reported (4). Anti-K^+-ATPase antibody (FI-AB) and pre-immune sera were dialyzed against 150 mM KCl, 5 mM glycl glycine and 2 mM $MgCl_2$, pH 6.1, for 24 h at 4°C with three changes of the dialysate. For the H^+ transport study, FI membrane vesicles were prepared in unbuffered 0.25 M sucrose, following the purification steps indicated under *Membrane Isolation.*

In a typical experiment, 83 μg of FI membrane vesicles (FI antigen) were incubated at 22°C for 30 min with 2.0 mg of FI antibody in a final volume of 0.5 ml. The mixture was then added to 1.5 ml of a medium containing 150 mM KCl, 5 mM glyclglycine buffer, pH 6.1, and 2 mM $MgCl_2$. ATP was then added to give a final concentration of 2×10^{-5} M. Ionophores such as tetrachlorosalicylanilide (TCS) (10^{-6} M) and valinomycin (10^{-6} M) were added in 10 μl methanol. Vesicles alone and vesicles incubated with pre-immune sera were used as experimental controls.

The measurement of pH change was carried out using a Radiometer pHM 64 pH meter coupled to a Servorecorder with a REA 112 amplifier. The H^+ concentration change was calibrated in each experiment by back titration with 10-μl aliquots of 10^{-3} N HCl.

Cell Separation from Rat Fundic Mucosa

CELL ISOLATION PROCEDURE: Cell isolation and separation was performed following the method of Soumarmon et al. (25) with some modifications. Rats of either sex weighing ~200–250 g were sacrificed by a blow on the head. The stomach was removed, everted, and washed with deionized water. Everted sacs were produced and ligatures were placed at the pylorus, at the antral border, and at the proximal end of the corpus. Pronase (Merck, 70,000 PUK/g) diluted in a solution (A) containing 0.5 mM NaH_2PO_4, 1 mM Na_2HPO_4, 20 mM $NaHCO_3$, 70 mM NaCl, 5 mM KCl, 80 mM glucose, 2 mM EDTA, 2% bovine serum albumin, and 1% antifoam (SE2, from Walker Chemie, Germany), at a final concentration of 1,000 PUK/ml, was injected into the sacs which were incubated in the same medium at 37°C for 30 min. The incubation medium was removed and replaced with a fresh one. Incubation was continued for 30 min at 37°C. The cell suspension was filtered through a nylon stocking and centrifuged at 100 *g* for 10 min. The cell sediment was washed twice in the above medium containing 1 mM $CaCl_2$ and 1.5 mM $MgCl_2$ (solution B) without pronase. After centrifugation, cells were resuspended and kept on ice.

The sacs were placed in Erlenmeyer flasks and incubated at 22°C for 15 min, under gentle stirring. The medium containing cells was processed as above. Fresh solution B was added and incubation was allowed for two additional 30-min periods.

The isolated cells were washed as described above and combined with the first batch, giving the final cell preparation.

ENRICHMENT OF PARIETAL CELLS: Separation of parietal cells from other cell types was achieved by a serial centrifugation technique. All centrifugation steps were carried out at 100 *g* for 45 s. The pooled cell populations were resuspended in 20 ml of solution B and spun down in a glass centrifuge tube (Tube 1). After the first centrifugation, the supernate containing ~50% of all isolated cells was decanted into a centrifuge tube (Tube 2). The cell sediment of tube 1 was resuspended in 20 ml of fresh solution B. Tubes 1 and 2 were centrifuged. The supernate from tube 2 was decanted into a centrifuge tube 3, while the supernate from tube 1 was decanted into tube 2. Tube 1 was filled with fresh solution B. All sediments were resuspended and centrifuged. This procedure was continued until the cells were distributed over seven tubes. The parietal cells were enriched in the seventh tube to ~60%. Parietal cells were identified by the succinic dehydrogenase stain using *p*-nitro tetrazolium blue (3).

Immunohistochemical Studies

TISSUE: Immunofluorescence studies were carried out using the method of Sainte-Marie (22). Tissue blocks of hog mucosa

no more than a few millimeters square were fixed in ice-cold 95% ethanol for 24 h. After dehydration in absolute ethanol, the blocks were cleared in xylene and embedded in paraffin. Rat stomachs were fixed by vascular perfusion with 2% glutaraldehyde in 0.1 M phosphate buffer as previously described (8), and tissue blocks were processed as above. Sections 5–6 μm in thickness were deparaffinized in cooled xylene, hydrated through a series of ethanol-water mixtures, and equilibrated in 50 mM phosphate-buffered saline, pH 7.4, for 30 min. 50–100 μl of antibody (1.5–3.0 mg of protein) was applied to the sections and incubated in moist atmosphere at 22°C for 45 min. After washing in phosphate-buffered saline, they were stained by the "indirect" method (22) with fluorescein isothiocyanate-conjugated goat anti-rabbit γ-globulin, Hyland Lab., Los Angeles, Calif., reconstituted in 1.0 ml of sterile distilled water, using 50–100 μl of a 1:10 diluted solution. After incubation at 22°C for 45 min, sections were washed overnight with phosphate-buffered saline and mounted in buffered elvanol (Monsanto Co., St. Louis, Mo.). Stained sections were examined with a Leitz fluorescence microscope and photographed.

The specificity of the staining was determined by using the preimmune sera as control, following a procedure identical to that used for specific antibody staining.

CELLS: Enriched preparations of rat parietal cells were washed twice in phosphate-buffered saline containing 1% bovine serum albumin and 0.03 Na-azide. 200 μl of cell suspension containing ~1–5 × 10^6 cells were incubated at 22°C for 1 h with 100 μl of antibodies or γ-globulin from non-immunized rabbit. After being spun down at 100 *g* for 5 min, the sediment was washed twice. The pellet was resuspended in 200 μl of phosphate-buffered saline, and 100 μl of 1:10 dilutions of fluorescein conjugated goat anti-rabbit γ-globulin were added. After another 30-min incubation at 22°C, the cells were again washed and the final pellet was dispersed in a few drops of buffer. The suspension was examined with a Leitz fluorescence microscope and photographed.

Electron Microscopy

Cryostat sections were cut from fixed rat stomach at 8–10 μm thickness and attached to egg-albumin-coated glass slides. The sections were exposed to 50 mM Tris-Cl-0.9% NaCl buffer, pH 7.6, for 5 min and then to 3% normal goat serum in 50 mM Tris-Cl-0.9% NaCl buffer, pH 7.6, for 30 min at 22°C. FI antibody diluted to ~3 mg/ml in 1% normal goat serum solution in Tris-saline buffer was applied to the sections and incubated for 48 h at 4°C in a moist atmosphere. Control sections were incubated with rabbit pre-immune serum diluted in the same way. Sections were rinsed three times with Tris-saline buffer and then incubated for 1 h at 22°C with goat anti-rabbit γ-globulin diluted 1:10 with 1% normal goat serum solution in Tris-saline buffer. After rinsing three times with Tris-saline buffer, the sections were exposed for 1 h at 22°C to rabbit peroxidase-antiperoxidase (PAP) (Cappel Lab, Cochranville, Penn.) diluted 1:50 with 1% normal goat serum solution in Tris-saline buffer (26). The sections were washed again three times in Tris-saline buffer and incubated with 0.05% diaminobenzidine and 0.01% H_2O_2 in 100 mM Tris-Cl at pH 7.6 for 8 min at 22°C. After washing in distilled water, the sections were fixed in 5% OsO_4 in distilled water for 30 min at 22°C, rinsed in distilled water, and dehydrated in absolute ethanol. Sections were embedded in Epon-filled gelatin capsules turned upside down and placed on the glass slides.

After removal of the glass in liquid nitrogen, the tissue was sectioned at ~0.12 μm thickness. Without further contrasting, the sections were then studied in a Philips 200 electron microscope at 60 Kv.

RESULTS

Preparation of Gastric Membrane Fraction

Free-flow electrophoresis technique has been successfully used to fractionate in large scale membranes deriving from a crude microsomal preparation obtained by differential and density gradient centrifugation of hog gastric mucosa homogenate. As previously shown (17), three membrane fractions named FI, FII, and FIII are produced, with different enzymatic and compositional characteristics. It should be pointed out that in FI ~90% of the total protein stain is localized at the 105,000-dalton region, whereas FII exhibits a more heterogeneous pattern. FIII appears to be a mixture of FI and FII and will not be described in detail. An adequate separation is therefore obtained between FI and FII to anticipate immunological discrimination.

Preparation and Characterization of Antisera

Antibodies that were obtained from rabbits injected with native membrane preparations (antigens) are referred to as FI-AB, FII-AB, and FIII-AB. The γ-globulin obtained from the antisera was characterized by a variety of immunological techniques. The test antigens employed were native FI, FII, and FIII membrane fractions solubilized in 1% Triton X-100. Fig. 1 shows the immunodiffusion pattern obtained with the antisera to the FI, FII, and FIII antigens. Single precipitin lines fusing at their extremes were formed by FI-AB against FI and FIII (Fig. 1 *a*).

When FII-AB was reacted with FII, two precipitin lines were obtained (Fig. 1 *b*) one of which fused with the precipitin band detected by immunoreaction of FII-AB with FIII. FIII-AB similarly cross-reacted with FI and FIII and gave a faint precipitin band with FII (Fig. 1 *c*).

These results demonstrate that at this stage of purification, although all antisera react with their respective antigens, a reciprocal cross-reaction took place between FI-AB and FIII (or FIII-AB and FI), indicating the presence of a common antigenic protein(s) in the two fractions. Reciprocal cross-reactivity is also observed between FII-AB and FIII (or FIII-AB and FII) while no im-

munoreaction is found between FI-AB and FII or between FII-AB and FI.

When FI-AB was further purified by adsorption to and elution from the enzyme in the native membrane fraction and immunodiffusion was performed against FI, one precipitin band was obtained, while no immunoreactivity was detected against FII and FIII (Fig. 1*d*). An analysis by "rocket immunoelectrophoresis" is shown in Fig. 2*a* for FI-AB and in Fig. 2*b* for FII-AB. It is clear that, while one single precipitin line is formed by reaction of purified FI-AB with its antigen and none were observed against FII and FIII, four "rockets" are produced when FII-AB reacted with FII and two "rockets" are detected when FII-AB reacted with FIII. Thus, FI-AB is monospecific and homogeneous according to both techniques, whereas FII-AB is heterogeneous.

Inhibition of Enzyme Activity by Antisera

Antibody responses of rabbits challenged with FI and FII membrane fractions were assessed by

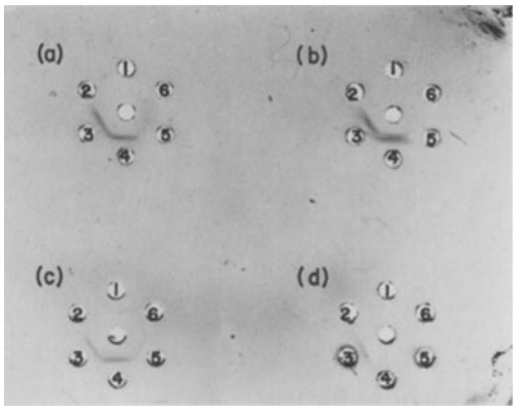


FIGURE 1 Double immunodiffusion in agar gel. (*a*) Central well contained 10 μl of FI-AB_1 (3.0 mg/ml); well 2, control serum; well 3, 10 μl of FI membrane fraction (~1.0 mg/ml); well 4, 10 μl of FIII membrane fraction (~1.3 mg/ml); well 5, 10 μl of FII membrane fraction (~0.9 mg/ml). (*b*) Central well contained 10 μl of FII-AB_1 (2.4 mg/ml); well 1, control serum; well 2, 10 μl of FI; well 3, 10 μl of FIII; well 4, 10 μl of FII. (*c*) Central well contained 10 μl of FIII-AB (1.9 mg/ml); well 2, control serum; well 3, 10 μl of FI; well 4, 10 μl of FIII; well 5, 10 μl of FII. (*d*) Central well contained 10 μl of purified FI-AB_1 (1.5 mg/ml); well 2, control serum; well 3, 10 μl of FI; well 4, 10 μl of FIII; well 5, 10 μl of FII. Immunodiffusion was carried out as described in the text.

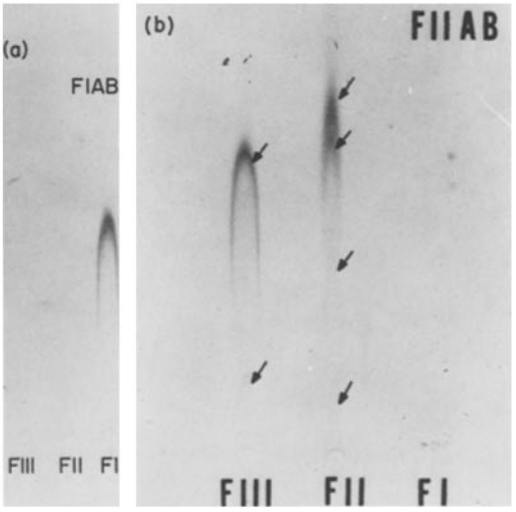


FIGURE 2 "Rocket immunoelectrophoresis" in agar gels containing (*a*) antiserum to FI membrane fraction (FI-AB_1) and (*b*) antiserum to FII membrane fraction (FII-AB_1). FI, FII, and FIII Triton X-100 solubilized membrane fraction (7 μl) at concentrations of 1.0, 0.9, and 1.3 mg/ml respectively were applied in the respective wells. Immunoelectrophoresis was performed as indicated in Materials and Methods. The arrows show the "rockets" produced when FII-AB_1 reacted with FII and FIII fractions.

TABLE I

Effects of Five FI Antibody Preparations on the Enzymatic Activities of FI Gastric Membrane Fraction

	K^+-ATPase		K^+-pNPPase	
Antibody preparation	Antibody* (μg/μg membrane prot.)	% Inhibition (max)	Antibody* (μg/μg membrane prot.)	% Inhibition (max)
FI-AB_1	10	77	6	34
FI-AB_2	10	83	8	38
FI-AB_3	—	0	—	0
FI-AB_4	34	4	—	0
FI-AB_5	—	0	—	0

* Values indicate the concentration of FI-AB_{1-5} at which 50% of maximal inhibition occurred. Experiments were carried out as described in text.

measurement of the ability of γ-globulin fractions of sera from these animals to inhibit the activities of the enzymes characterized in the membrane preparations. Table I summarizes the effect of FI-AB on the K^+-ATPase and K^+-phosphatase activities associated with FI gastric membranes and Table II summarizes the effect of FII-AB on the

Mg^{++}-ATPase and 5′-nucleotidase activities associated with FII gastric membranes.

It can be seen that of the five animals used for immunization with FI two produced antibodies (FI-AB_1 and FI-AB_2) which inhibited the K^+-ATPase and phosphatase activities, while four out of five rabbits immunized with FII produced antibodies (FII-AB_1, FII-AB_2, FII-AB_3, and FII-AB_4) inhibiting Mg^{++}-ATPase and 5′-nucleotidase.

Since, in all instances, immunodiffusion and rocket immunoelectrophoresis show precipitin lines between FI and FI-AB_{1-5}, two types of FI-AB, inhibitory and non-inhibitory, were produced.

TABLE II

Effects of Five FII Antibody Preparations on the Enzymatic Activities of FII Gastric Membrane Fractions

	Mg^{++}-ATPase		5′-Nucleotidase	
Antibody preparation	Antibody* (μg/μg membrane prot.)	% Inhibition (max)	Antibody* (μg/μg membrane prot.)	% Inhibition (max)
FII-AB_1	10	38	12	98
FII-AB_2	15	42	14	92
FII-AB_3	12	38	17	100
FII-AB_4	11	39	12	81
FII-AB_5	—	0	—	0

* Values indicate the concentration of FII-AB_{1-5} at which 50% of maximal inhibition occurred. Experiments were carried out as described in text.

Fig. 3*a* shows K^+-ATPase and K^+-phosphatase activities as a function of increasing FI-AB_1 concentrations. It is evident from the results that the degree of inhibition of enzyme activities was proportional to the concentration of FI-AB_1 with maximal inhibition of ~80% for the K^+-stimulated ATPase and ~35% for the K^+-activated phosphatase. In contrast, the "basal" Mg^{++}-ATPase was not inhibited. Thus, FI-AB_1 is specific for the K^+-dependent enzyme activities.

Fig. 3*b* shows the effect of varying concentrations of FII-AB_1 on the Mg^{++}-ATPase and 5′-nucleotidase activities of FII membrane fractions. It can be seen that the degree of inhibition of the 5′-nucleotidase activity increases up to 100% when FII-AB_1/FII-Ag ratio was 50:1. At the same ratio, the inhibition of the Mg^{++}-ATPase reached ~40% and levelled off at higher antibody/antigen ratios.

Antibody Effect on H^+ Transport in Gastric Vesicles

It has been shown that purified gastric vesicles (FI fraction) are capable of taking up H^+ with the addition of ATP (6).

Fig. 4 shows that compared to the control vesicles, addition of FI-AB_1 or FI-AB_2 produces ~50% inhibition of the H^+ uptake while γ-globulin from non-immunized rabbit was without significant effects. Experiments carried out under the same

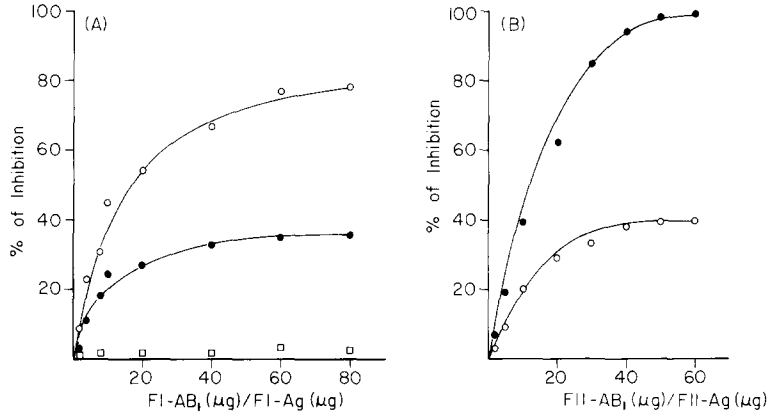


FIGURE 3 Inhibition of the enzymatic activities of FI and FII gastric membrane fractions by varying amounts of their respective antibodies. (A) Inhibition of the "basal" Mg^{++}-ATPase (□——□); K^+-ATPase (○——○) and K^+-pNPPase (●——●) activities of FI membrane fraction by FI-AB_1. (B) Inhibition of the Mg^{++}-ATPase (○——○) and 5′-AMPase (●——●) activities of FII membrane fraction by FII-AB_1. Varying amounts of antibodies were preincubated with the enzymes at 22°C for 30 min in 40 mM Tris-Cl, pH 7.4, before substrate addition. The enzymes were assayed as described in the text.

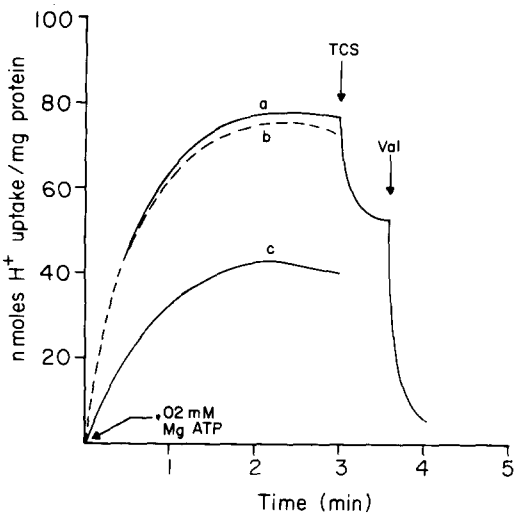


FIGURE 4 Inhibition of the proton transport of the FI gastric membrane vesicles by FI-AB_1. Curve a indicates a control experiment. FI vesicles were added to a solution of 150 mM KCl with addition as in the text, followed by 2×10^{-5} M ATP. At times indicated, tetrachlorosalicylanilide (TCS) and valinomycin (val) were added. Partial dissipation of the H^+ gradient by addition of TCS and complete dissipation of the H^+ gradient by addition of val was achieved. Curve b shows the H^+ uptake by FI vesicles after incubation with normal rabbit serum. Curve c shows the inhibitory effect of FI-AB_1 on the H^+ transport of FI vesicles.

conditions with FII-AB_1, FII-AB_2, FII-AB_3, and FII-AB_4 were negative.

Only the FI-AB_1 or FI-AB_2 which inhibit the K^+-ATPase and phosphatase activities were able to inhibit the H^+ transport by the vesicles. This observation confirms that two types of FI-AB were produced against this class of gastric membranes.

Immunohistochemical Localization of the Gastric Membrane Fractions

Antibodies were used to stain gastric mucosa sections by the indirect immunofluorescence technique. The staining pattern obtained by treatment of the gastric tissue from hog and rat with FI-AB_{1-5} of both types (i.e., inhibiting or not the K^+-ATPase and phosphatase) is illustrated in Fig. 5 *a* and *b*. The fluorescence is confined to the middle third region of the gastric gland, where the majority of the parietal cells is present (3), and can be stated to be localized to the parietal cells on the basis on shape and location of the cells. This strong fluorescence appears to have mainly a supranuclear or apical distribution. Mucosa sections treated with γ-globulin obtained from sera of control rabbits were uniformly negative (Fig. 6).

By electron microscopy the peroxidase staining was strong at the secretory (i.e., the apical and intracellular canalicular) surfaces of the parietal cells (Fig. 7 *a*). A much weaker reaction was seen at the secretory surface of the zymogen cells (Fig. 7 *b*). Lysosomes were regularly stained. In a few instances where the parietal cells had been cut open by the cryostat, a moderate perioxidase stain could be observed at the cut surface. No staining was found in any other cytoplasmic structure. The control sections were not stained at all except for the lysosomes.

The immunofluorescence produced by treatment of gastric tissue sections with FII-AB_{1-5} was weaker but, again, mainly localized in the parietal cells (Fig. 8).

When suspensions of isolated rat gastric cells were treated with purified FI-AB and goat anti-rabbit γ-globulin fluorescence reagent, only the parietal cells show a strong positive staining. On some parietal cells, fluorescence was distributed over the entire surface, but on most of them an asymmetric, polar distribution was found (Fig. 9).

Tissue Specificity

From the data presented it seems that, for FI membrane fraction antibody, we are dealing with a mono-specific antibody which recognized an antigen present in the plasma membrane of the parietal cells from hog and rat gastric mucosa.

The tissue specificity has been determined by the immunodiffusion technique using FI-AB_1, FI-AB_2 against the Triton X-100 solubilized microsomal fractions derived from 16 different hog tissue. Table III shows that, of the tissues surveyed, only three reacted, the gastric mucosa, the thyroid less strongly, and the thymus very weakly. Thus, the FI membrane fraction antigen is tissue selective.

DISCUSSION

The major emphasis in this work is put on the immunological characterization of two enzymatically distinct gastric membrane fractions. On the basis of enzymatic characteristics of the two fractions, FI and FII, only FI contains the gastric ($H^+ + K^+$)-ATPase which is expected to be a parietal cell component. This expectation is based on its absence from the non-acid secreting area of the

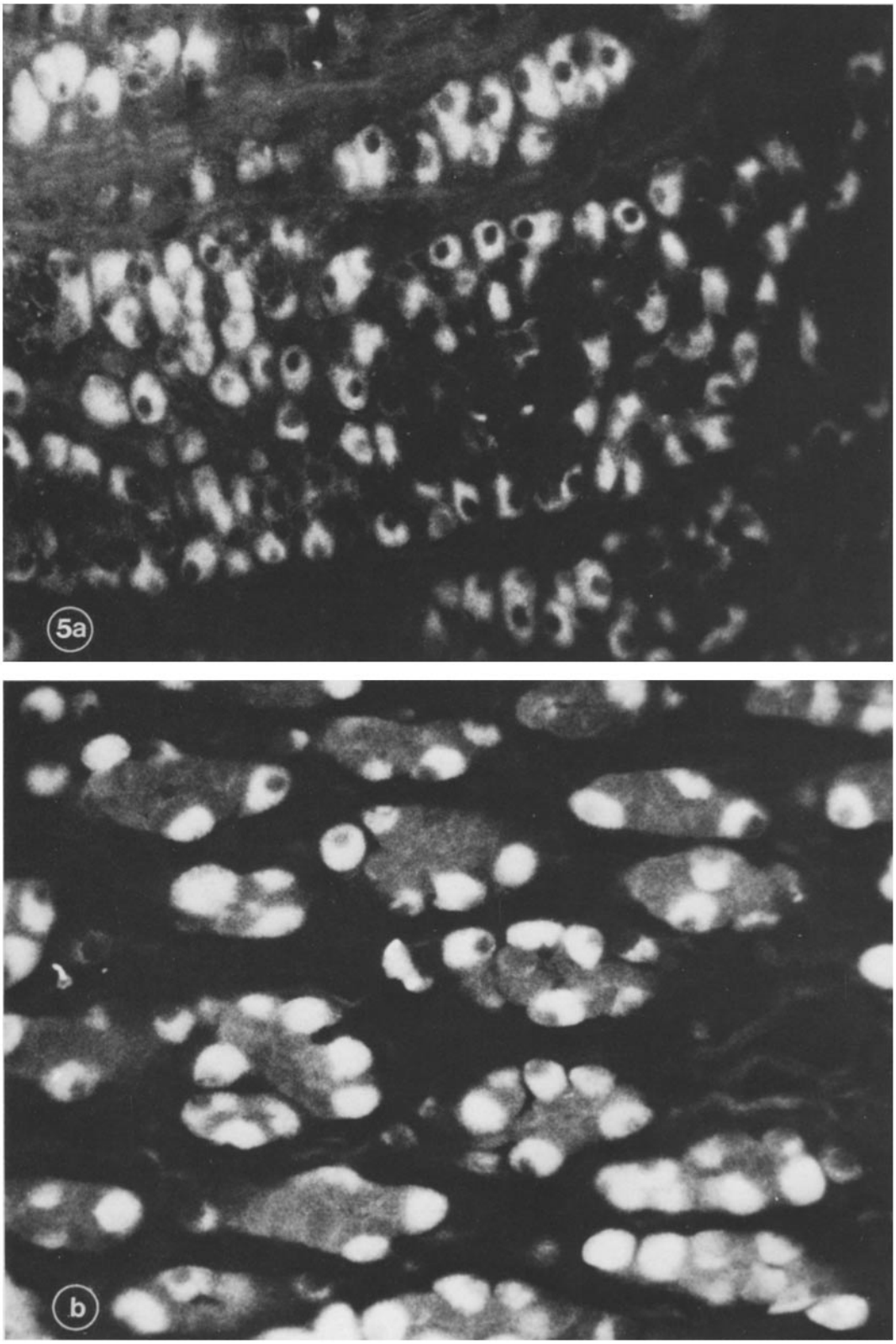


FIGURE 5 Fluorescent antibody staining. (*a*) Hog gastric mucosa stained with FI-AB_1. The majority of the fluorescence is confined to the middle third region of the gastric gland. × 270. (*b*) Survey of the basal portion of rat gastric mucosa after fluorescence staining with FI-AB_1. The position of the stained cells (at the outer rim of the glands and seldom in contact with each other) and their large size indicated that staining was confined to the parietal cells. × 270.

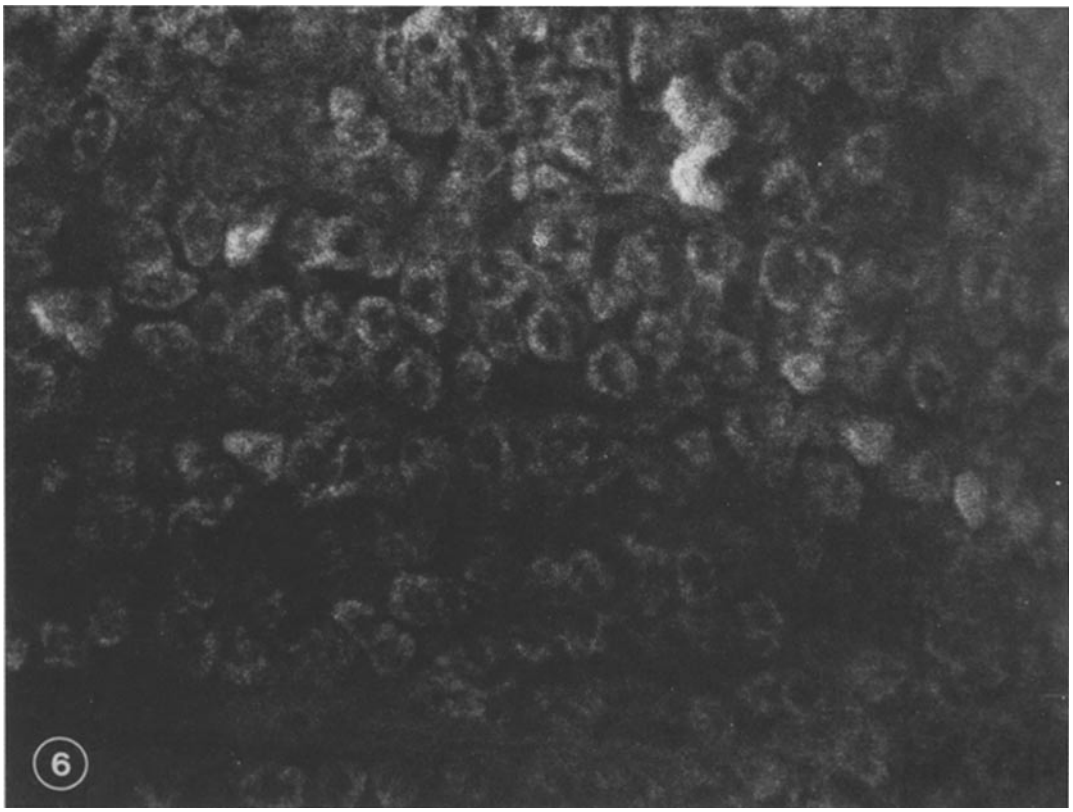
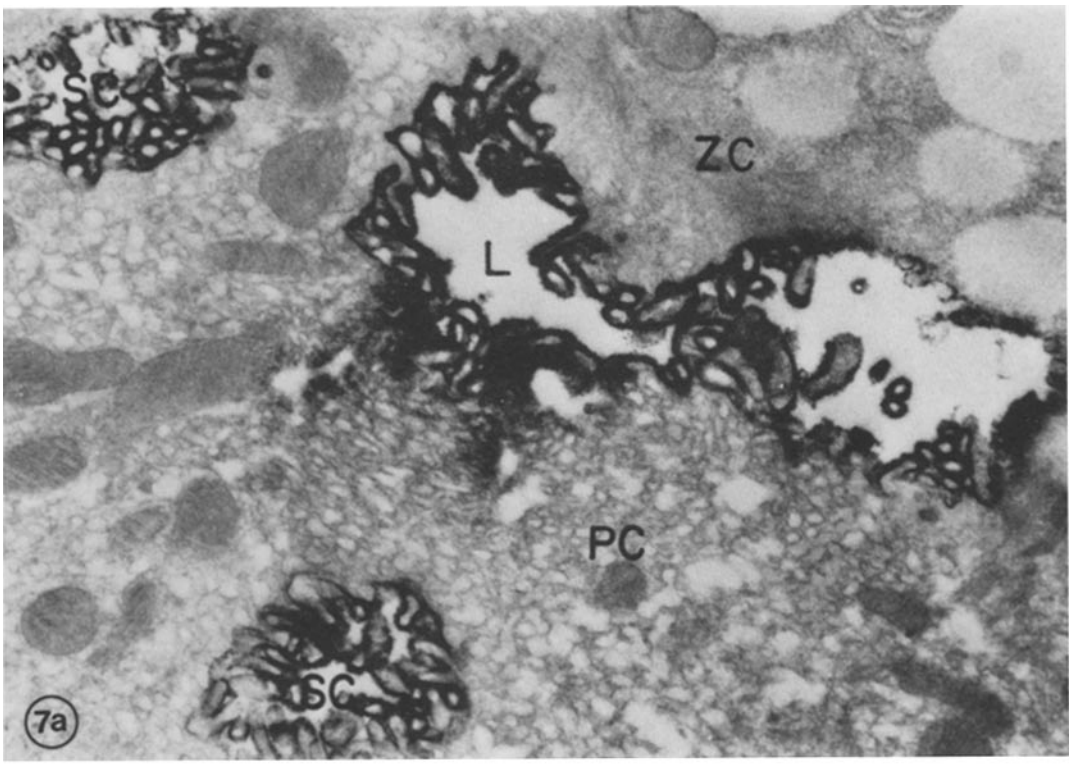


FIGURE 6 Indirect immunofluorescence staining of hog gastric mucosa section treated with normal rabbit γ-globulin as control. × 270.

stomach, the antrum (6), and on its proton transport capability in vesicular form (21, 4).

By precipitation and adsorption techniques, purification of FI antibody to homogeneity on rocket electrophoresis was straightforward. However, from a given group of rabbits, both inhibitory (FI-AB_{1-2}) and non-inhibitory binding (FI-AB_{3-5}) antibodies were produced. The latter type of antibody has been described previously for the (Na^+ + K^+)-ATPase for example (1), as have inhibitory antibodies for this enzyme. Thus, it is not unexpected that more than one class of antibody was produced. Recently, we have shown by tryptic digestion in the absence and presence of protective ligands that the 105,000 M.W. polypeptide on SDS gels probably contains three distinct polypeptides: a catalytic (phosphorylatable and ligand-protectable) subunit; a trypsin-sensitive (ligand-insensitive) subunit, hydrolysis of which reduces enzyme activity; and, finally, a glycoprotein (18). Thus, the antibodies we have generated may be against distinct peptide subunits which comprise the functional ATPase complex. The inhibition of the enzyme activities also discriminates between K^+-ATPase and K^+-pNPPase activities in that inhibition of the former is considerably greater than the latter. We have shown both with phospholipase A_2 treatment (19) and by ligand inhibition of tryptic hydrolysis (18) that the pNPPase and ATPase reaction pathways are not identical. On the other hand, ATP and pNPP are mutually competitive in terms of hydrolysis (unpublished observations). The greater sensitivity of the K^+-ATPase may indicate better accessibility of the ATPase as compared to the pNPPase site in terms of antibody reaction. Similar results have been reported by one group of workers studying antibodies to the (Na^+ + K^+)-ATPase (2). In no instance was significant alteration of Mg^{++}-ATPase activity observed. Similar results have also been described for (Na^+ + K^+)-ATPase antibodies (24). A possible explanation for this finding is that the "basal" Mg^{++}-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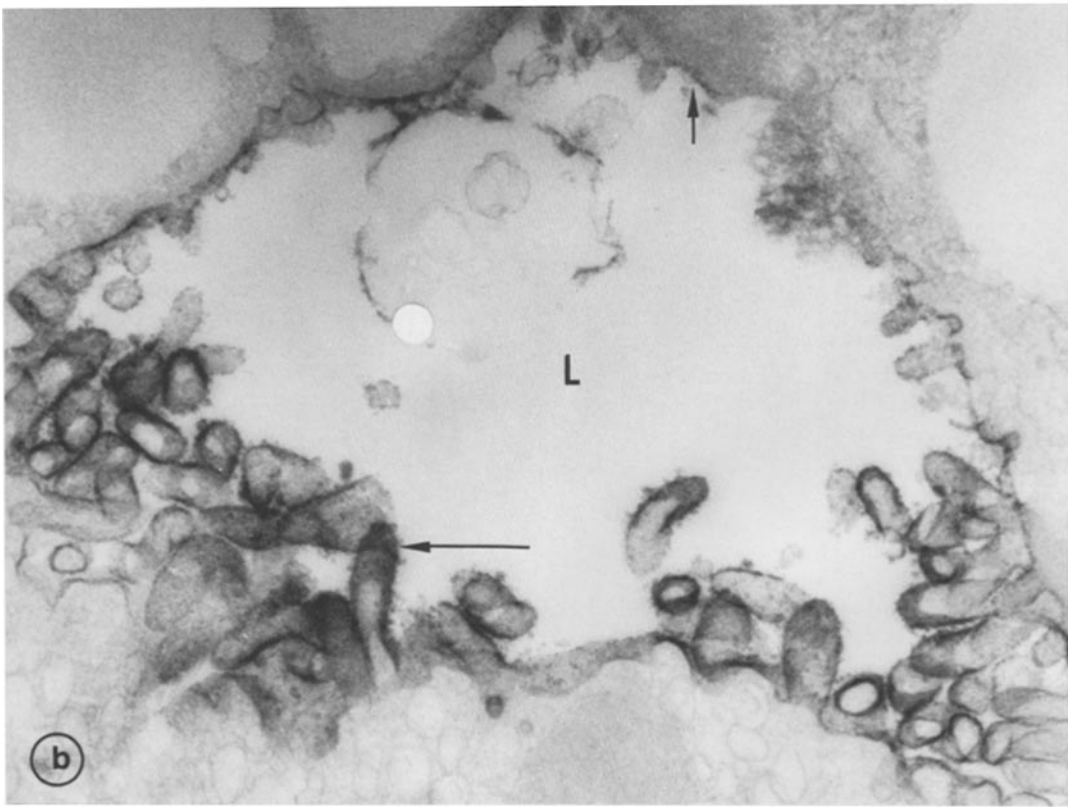
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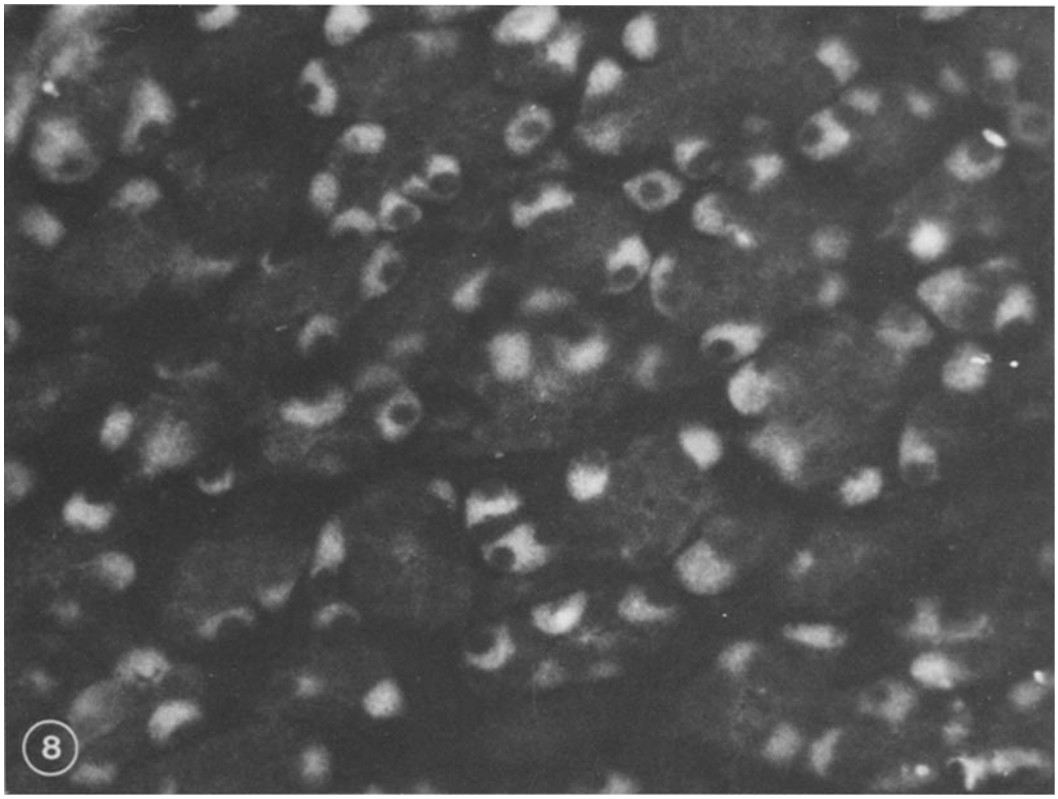

FIGURE 8 Survey of basal portion of rat gastric mucosa after fluorescence staining with FII-AB_1. The staining is much weaker than that obtained using FI-AB_1, but it still appears that only parietal cells stain. × 270.

ATPase present in FI fraction, although related to the $(H^+ + K^+)$-ATPase, is in a different conformation or it uses a different reaction pathway. It is clearly distinct from the Mg^{++}-ATPase present in FII fraction but it may be similar to some Mg^{++}-ATPase found in FIII fractions. Antibodies to this conformation of the $(H^+ + K^+)$-ATPase may exist in the unpurified FI-antibody, accounting for the cross-reactivity with FIII membrane fraction, but it is lost after purification by adsorption to and elution from FI antigen.

The result from immunofluorescence studies of the isolated parietal cells indicate that the FI antibodies are membrane-antibodies selective for the parietal cell. In addition, the histological section studies indicate a localization expected for the intracellular secretory surface of this cell type. Immunoelectron microscopy confirmed the mem-

FIGURE 7 Ultrastructural appearance of FI membrane antigen localization with the PAP procedure. (*a*) Lumen of rat oxyntic gland (*L*) with adjoining parietal cell (*PC*) and zymogen cell (*ZC*). This section was stained with FI-AB_1. The staining reaction is strong along the secretory surface of the parietal cell, both on the apical cell surface (facing the gland lumen) and on the surface which borders the secretory canaliculi (*SC*). A faint staining is also seen on the apical surface of the zymogen cell. There is no antibody reaction in the cytoplasm of either cell. × 15,000. (*b*) This section was treated in the same way as described for *a*. The main reaction is seen on the outer surface of the parietal cell plasma membrane (long arrow) but there is also a weak reaction on the zymogen cell plasma membrane (short arrow). *L*: gland lumen. × 43,000.

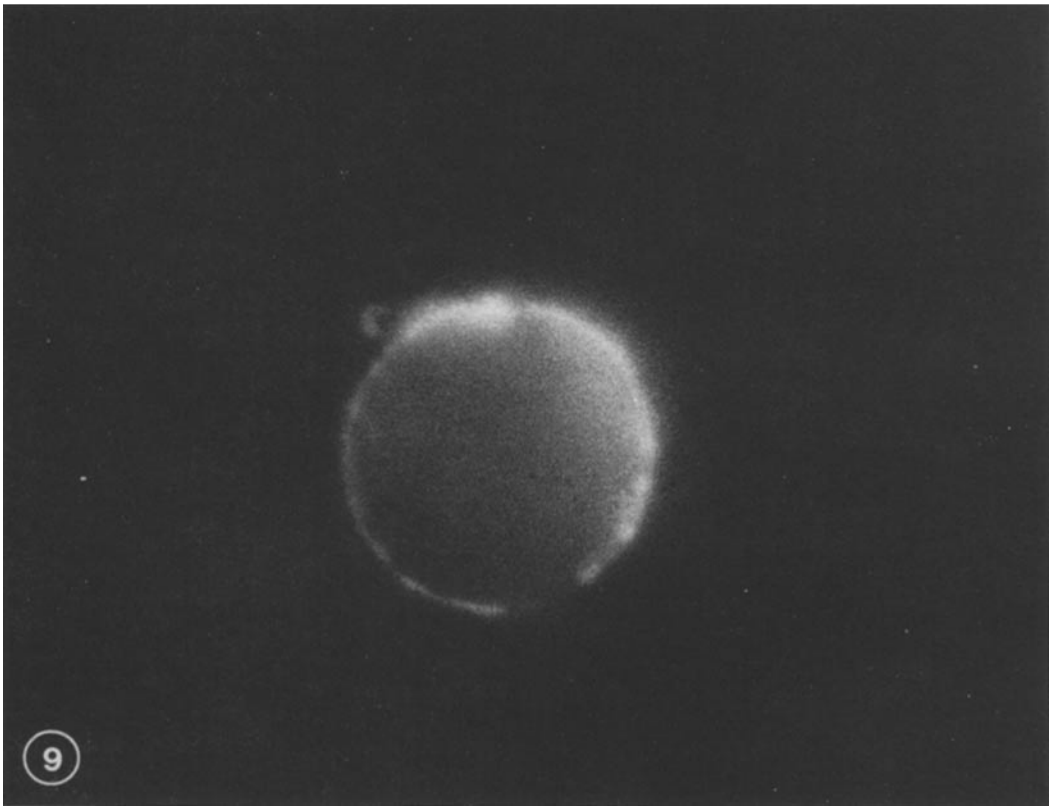

FIGURE 9 Fluorescent antibody staining of isolated rat gastric cells. Parietal cell stained with FI antibody showing a membrane distribution largely localized to one side of the cell. × 775.

brane-selective and cell-selective nature of the FI antibodies. Thus, strong reaction was obtained with the microvilli of the luminal and intracellular canaliculus membrane system of the parietal cell, weak reaction with the apical membrane of the peptic cell, and no reaction with basal-lateral surface. Lack of reaction with the tubulovesicular system may be explained by poor penetration of the antibody into the cytoplasm of the sectioned cell in the thick section and subsequently poor visualization of tubulovesicular staining in the second section used for electron microscopy. This problem of penetration has previously been discussed by Nakane (14).

These studies with hog, rat, and other studies with man (20) show that this class of antibody is species cross-reactive. However, the tissue selectivity is high. The cross-reaction with the thyroid which has both anion and K^+ transport mechanisms may be understandable, but the weak thymus reactivity is more difficult to understand. However, a novel type of gastric secretory inhibitor, a benzimidazole derivative, shows selective toxicity for the thyroid and the thymus (E. Fellenius, and S. E. Sjöstrand, personal communication).

In contrast to the FI antibody, FII antibody had quite different characteristics. Corresponding to the considerable peptide heterogeneity of this fraction, inhomogeneous antibody was obtained. This class of antibodies was quite distinct from the FI antibody. Thus, no cross-reactivity was noted and the antibodies inhibited both the 5′-nucleotidase and the Mg^{++}-ATPase of the FII fraction. This provides conclusive evidence that the Mg^{++}-ATPase in the FII fraction is distinct from the basal Mg^{++}-ATPase of the FI fraction.

The cellular reactivity of the FII-AB was considerably weaker than that of FI-AB. However, again, the parietal cells were the major cell type stained, although also nonparietal cells were observed to stain in hog tissue. This antibody was also species cross-reactive, staining rat parietal

TABLE III

*Immunoreactivity of FI Antibody against Microsomal Fraction Obtained from Different Hog Tissue**

Tissue	Immunoreactivity‡
Fundus	5+
Thyroid	2+
Thymus	1+
Antrum, adrenal medulla, brain, colon	None
Esophagus, heart, kidney, liver	
Lung, pancreas, small intestine	
Spleen, urinary bladder	

* This was established by double immunodiffusion technique in 1%/agarose gels. Microsomal fractions derived from each tissue were solubilized in 1% Triton X-100 at protein concentration of ~1.5 mg/ml. 10 μl of each sample were applied to Ouchterlony plates and reacted with 10 μl of FI-AB_1 or FI-AB_2. For details, see Materials and Methods.

‡ Immunoreactivity was graded from 0 to 5+ based on the intensity of the precipitin lines obtained in double-diffusion experiments.

cells. Thus, although we can conclude that FI-AB, whether inhibitory or not, is parietal cell specific, FII-AB appears rather to be parietal cell selective.

Accordingly, a heterocellular tissue such as gastric mucosa can be manipulated by standard biochemical fractionation techniques to yield selective membrane fractions. One of these fractions transports H^+ and K^+ and is derived from the secretory structure of the parietal cell. This increases confidence in the relevance of this fraction and its constituent ($H^+ + K^+$)-ATPase to the mechanism of H^+ secretion by the stomach. The interesting peptide complexity of this enzyme should be amenable to a more detailed immunological analysis.

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