J. Membrane Biol. 59, 135–142 (1981)

The Journal of
Membrane Biology

An Enriched Preparation of Basal-Lateral Plasma Membranes from Gastric Glandular Cells

David J. Culp and John G. Forte*
Department of Physiology-Anatomy, University of California, Berkeley, California 94720

Summary. A procedure is described for the preparation of a membrane fraction enriched in basal-lateral plasma membranes from gastric mucosa. Gastric glands isolated from rabbit were employed as starting material, greatly reducing contamination from non-glandular cell types. The distribution of cellular components during the fractionation procedure was monitored with specific marker enzymes. $(Na^+ + K^+)$-ATPase, ouabain-sensitive K^+-stimulated *p*-nitrophenyl-phosphatase and histamine-stimulated adenylate cyclase were used as markers for basal-lateral membranes. These three markers were similarly distributed during both differential and equilibrium density gradient centrifugation. The enriched membrane fraction contained more than 30% of the total initial activities of the three basal-lateral membrane markers which were purified better than 11-fold with respect to protein. $(Na^+ + K^+)$-ATPase activity was resolved from the activities of acid phosphatase, pepsin, Mg^{2+}-ATPase, cytochrome *c* oxidase, NADPH-cytochrome *c* reductase, glucose-6-phosphatase, $(K^+ + H^+)$-ATPase, DNA and RNA.

Electrophysiological and ionic flux studies have been widely used to investigate the transporting properties of *in vitro* gastric mucosa. Consequently, various ionic transporting mechanisms have been proposed such as $Cl^- - Cl^-$ exchange diffusion, $Cl^- - HCO_3^-$ exchange, active Cl^- transport as well as active Na^+ and H^+ transport [e.g., 13, 16, 36]. Such studies have necessarily treated the mucosa as a uniform sheet, failing to sufficiently localize the transport with respect to cell type or plasma membrane orientation.

Similarly, direct pharmacological investigations of gastric secretory control mechanisms have usually been limited to the study of membrane-bound hormone receptor sites from the total homogenate or crude low-speed pellets of whole mucosal scrapings [10, 11, 41, 48]. The results of such studies are overshadowed by possible interference from intracellular constituents.

An alternative approach to the investigation of membrane-bound hormone receptor sites and ion transporting mechanisms in the gastric epithelium is to study a well-defined population of plasma membranes from specific cell types. Ultimately, this approach may lead to the identification of such mechanisms and their characterization at the molecular level. Indeed, this approach has led to the characterization of a gastric K^+-stimulated ATPase, an enzyme specifically associated with the apical and tubulovesicular membranes of gastric parietal cells [6, 17], which may be the mechanism of active H^+ transport [24, 37].

However, little attention has been given to basal-lateral plasma membranes of the gastric epithelium. Therefore, the present study was designed to prepare a membrane fraction enriched in basal-lateral plasma membranes from gastric glandular cells. As starting material we used isolated gastric glands from the rabbit. They represent an easily obtainable supply of functionally intact gastric cells free of contamination from both surface epithelial and connective tissue cell types [3].

The fractionation of basal-lateral membranes as well as other subcellular components during differential and density gradient centrifugation was determined by the distribution of specific marker enzymes. The fractionation of gastric glands resulted in a membrane fraction that yielded more than 30% of the total initial activities of the three basal-lateral membrane markers enriched better than 11-fold with respect to protein.

* *To whom reprint requests are to be addressed.*

0022-2631/81/0059-0135 $01.60

Materials and Methods

Isolation of Glands

Male albino rabbits (New Zealand White) weighing 4–6 lb were used. Glands were isolated according to the procedure of Berglindh and Öbrink [3] with one minor exception. We found that the minced mucosa needed to be incubated in collagenase solution for only 30–45 min instead of 90 min to separate a large number of gastric glands. Prior to homogenization the isolated glands were collected by centrifugation into a preweighed 15-ml conical test tube. The supernatant was aspirated away and the pellet weighed. A preparation of glands from a single rabbit stomach yielded 2–3 g wet weight corresponding to a dry weight of 150 to 250 mg.

Gland Morphology and Viability

The gross morphology of the isolated gastric glands was studied using phase-contrast microscopy. Nitrotetrazolium dye-staining of the glands was by a modification of the procedure described by Nachlas et al. [34]. An aliquot of glands suspended in respiratory medium was diluted with an equal volume of 0.5 mg/ml nitrotetrazolium blue stain in 50 mM sodium phosphate plus 50 mM sodium succinate, pH 7.6, and incubated under 100% O_2 for 100 min at 37 °C before examination. The glands were tested for viability using the erythrosin dye exclusion techniques described by Berglindh and Öbrink [3]. Oxygen consumption was measured with a Clark oxygen electrode.

Gland Fractionation

A diagram summarizing the standardized gland fractionation procedure is shown in Fig. 1. All solutions and containers were kept at 0–4 °C and pH 8.0. Isolation buffer (I.B.) consisted of 250 mM sucrose and 5 mM tris (hydroxymethyl)-aminomethane (Tris)-HCl. Density gradient buffer (D.G.B.) was made up of 250 mM sucrose, 5 mM Tris-HCl and 5 mM Na_2EDTA. Pre-weighed, pelleted gastric glands (1.5 g wet weight) were resuspended with 5 mM Tris-HCl to a volume of 40 ml in a Dounce homogenizer and immediately triturated by 15 strokes with the tight-fitting pestle. The homogenate was diluted with an equal volume of 500 mM sucrose containing 5 mM Tris-HCl and centrifuged at 2,500 rpm for 8 min in a Beckman JA-20 rotor. The supernatant (S_1) was collected with a Pasteur pipette and stored on ice. The pellet (P_1) was resuspended

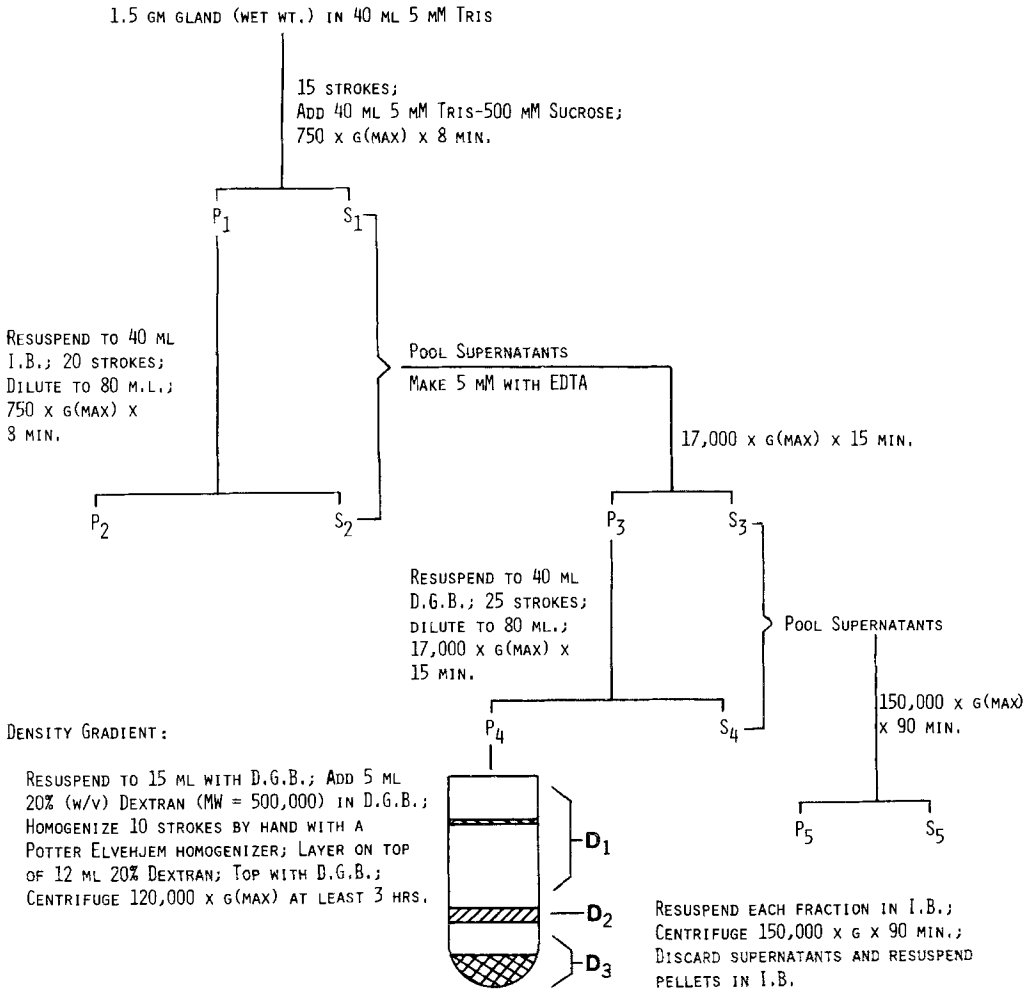

Fig. 1. Procedure for the preparation of enriched basal-lateral membrane fraction. All solutions were 0–4 °C. The isolation buffer (I.B.) contained (in mM): sucrose, 250; and pH 8.0 Tris-HCl, 5. The density gradient buffer (D.G.B.) contained (in mM): sucrose, 250; NaEDTA, 5; pH 8.0 Tris-HCl, 5. Unless indicated all homogenizations are with a Dounce homogenizer with a tight-fitting pestle

to 40 ml with I.B. and rehomogenized with 20 strokes in the Dounce homogenizer. The suspension was diluted with 40 ml of I.B. and centrifuged again as described above. The pellet (P_2) was collected and resuspended in I.B. The supernatant (S_2) was pooled with the S_1 supernatant and brought to 5 mM EDTA. The pooled supernatants were then centrifuged in the JA-20 rotor at 12,000 rpm for 15 min. The supernatant (S_3) was carefully decanted and kept on ice. The collected pellets were resuspended in 40 ml D.G.B. and rehomogenized with 25 strokes. The suspension was immediately mixed with an equal volume of D.G.B. and centrifuged as just described. The supernatant (S_4) was pooled with S_3 and centrifuged for 90 min at 35,000 rpm using a Beckman Type-35 rotor yielding a pellet, P_5, and a supernatant, S_5. The P_4 pellet was prepared for density gradient centrifugation by resuspending to 15 ml with D.G.B. and mixing with 5 ml of 20% (wt/vol) dextran (mol wt 500,000) made up in D.G.B., then homogenizing with 10 strokes by hand in a 20-ml glass-Teflon Potter Elvehjem homogenizer. This suspension was carefully layered on top of a cushion of 12 ml 20% dextran-D.G.B., topped with D.G.B. and centrifuged in a Beckman SW-27 rotor at 27,000 rpm for at least 3 hr, usually overnight. The fractions at the 0–5% and 5–20% dextran interfaces, D_1 and D_2 respectively, and the pellet, D_3, were collected and resuspended in I.B. using a 12-ml syringe with a flat-tipped, 15 mm long, 16 gauge needle. All three fractions, D_1, D_2 and D_3, were diluted to roughly 60 ml with I.B. and centrifuged at 35,000 rpm for 90 min in the Type 35 rotor. The supernatant was discarded and the pellet resuspended in I.B. All fractions were stored at −20 °C.

Analytical Methods

$(Na^+ + K^+)$-ATPase. $(Na^+ + K^+)$-ATPase was measured in 1 ml of medium containing 5–30 μg protein, 50 mM Tris (pH 7.4), 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 3 mM Na_2–ATP, 0.56 μM DCCD (Dicyclohexylcarbodiimide), and either with or without 1 mM ouabain (strophanthin-G). Prior to assay, fractions were sonicated for 1 min using a Biosonik III sonicator (Bronwill Scientific) set at intensity #35, with the thin probe. The reaction was started by the addition of ATP and proceeded for 10 min at 37 °C before termination with 1 ml of ice-cold 14% trichloroacetic acid. Inorganic phosphate was determined by the method of Sanui [39]. All samples were run in duplicate. The difference in activity between the samples with and without ouabain was taken as a measure of $(Na^+ + K^+)$-ATPase. DCCD was added to the medium to inhibit a large percentage of background Mg^{2+}-ATPase activity due to mitochondrial ATPase. More protein could then be added per assay, increasing the ratio of ouabain-inhibited ATPase activity to total ATPase activity, especially in the crude low-speed fractions. This assay procedure was tested with a purified fraction of canine renal $(Na^+ + K^+)$-ATPase obtained from Sigma Chemical Co. Our assay procedure resulted in 102% of the ATPase activity specified by Sigma and 98% of this activity was inhibited by 1 mM ouabain. DCCD at 0.56 μM had no effect on activity.

$(K^+ + H^+)$-ATPase. $(K^+ + H^+)$-ATPase was distinguished from $(Na^+ + K^+)$-ATPase by its insensitivity to ouabain and Na^+ ions [14, 17]. The reaction medium was the same as for $(Na^+ + K^+)$-ATPase except 100 mM NaCl was exluded from the medium and 1 mM ouabain was added to all samples. $(K^+ + H^+)$-ATPase activity was taken as the difference in activity between samples with and without 10 mM KCl. DCCD at 0.56 μM had no effect on the activity of $(K^+ + H^+)$-ATPase purified from pig fundic mucosa according to the method of Forte et al. [15].

Mg^{2+}-ATPase. Mg^{2+}-ATPase was measured under the same conditions as $(Na^+ + K^+)$-ATPase except 100 mM NaCl, 10 mM KCl and DCCD were excluded from the medium. Ouabain (1 mM) was added to all samples.

Ouabain-Sensitive K^+-Stimulated p-Nitrophenylphosphatase. Ouabain-sensitive K^+-stimulated *p*-nitrophenylphosphatase (ouabain-sensitive K^+-*p*NPPase) was assayed in the medium defined by Nagai et al. [35] with and without 1 mM ouabain.

Histamine-Stimulated Adenylate Cyclase. Histamine-stimulated adenylate cyclase was assayed according to the procedure described by Salomon et al. [38] with minor modifications. 10–50 μg protein were incubated in 100 μl of medium, pH 7.5, containing 25 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM 1-methyl-3-isobutylxanthine, 100 U/ml creatine phosphokinase (Sigma), 20 mM creatine phosphate (Sigma) and 1 mM [α^{32}P]-ATP (40–50 cpm/pmole, New England Nuclear). Samples were incubated for 10 min at 30 °C. Stimulation by fluoride was determined in samples with 10 mM NaF added. Maximal stimulation by histamine was at 1 mM with a dose response similar to that reported by Dozois et al. [11]. Stimulation by histamine was therefore determined in samples with 1 mM histamine added. The reaction was stopped by the addition of 100 μl of stopping solution (2% sodium dodecyl sulfate, 40 mM ATP, 1.4 mM cAMP, pH 7.5). After vortexing, 850 μl (20,000 cpm) of ^{3}H-cAMP (New England Nuclear) was added and the mixture decanted into a column of 1 ml (50% wt/vol) Dowex 50W-X4, 200–400 mesh (Biorad). The eluate from this and a subsequent addition of 3 ml H_2O were discarded. The Dowex column was then placed over a column of 0.6 g neutral alumina AG7, 100–200 mesh (Biorad) and 3 ml of H_2O added to the Dowex column. The eluate was discarded, the Dowex column removed and 4 ml of 0.1 M imidazole-HCl, pH 7.5, added to the alumina column. The eluate was collected in scintillation vials with 12 ml Aquasol (New England Nuclear) for counting. ^{32}P blanks were less than 0.0003% of the total added ^{32}P and recovery of ^{3}H was better than 70%. Blanks consisted of samples with stopping solution added prior to enzyme.

Cytochrome c Oxidase. Cytochrome *c* oxidase was measured by the spectrophotometric method described by Smith [42] with the modification that 0.1% Triton X-100 was added to medium to measure latent enzyme activity.

Nucleic Acids. RNA was determined by the procedure suggested by Munro and Fleck [32]. DNA was extracted from protein in the acidified alkaline digest from the RNA isolation procedure as suggested by Davidson [9]. The acidified alkaline digest pellet was resuspended in 1 ml of 0.5 N perchloric acid and incubated at 80 °C for 2 min, then centrifuged while hot and the supernatant withdrawn by a pasteur pipette and diluted to 5 ml with water. The optical density was read at 265 nm. Purified DNA from calf thymus (Sigma) was used to prepare a standard curve.

Other Markers. Acid phosphatase was measured by the procedure described by Mircheff and Wright [28]. Pepsin activity was assayed by the method of Anson and Mirsky [1]. Glucose-6-phosphatase was measured according to the method of Lauter et al. [23]. NADPH-cytochrome *c* reductase was assayed by the procedure of Sottacosa et al. [45] and protein was measured according to the method of Bradford [5].

Results

Isolated Gastric Glands

Our isolated gastric gland preparations were morphologically and functionally similar to those described

by Berglindh and Öbrink [3]. Under the phase-contrast microscope the majority of glands were 0.4 to 0.7 mm in length with parietal cells accounting for a large proportion of the total cell volume as determined after staining with nitrotetrazolium blue. Except for one or two cells per gland, the glands excluded the dye erythrosin and increased oxygen consumption in response to dibutyryl-cAMP [2].

Rationale of Homogenization Procedure

Since the yield of glands from a single rabbit stomach was limited (100–150 mg protein) we had to apply a homogenization technique that was harsh enough to disrupt a large percentage of glandular cells while being mild enough to prevent pertubation of intracellular components. After much experimentation with various homogenization media and cell-disrupting devices we adopted the procedure described in Materials and Methods. This procedure resulted in approximately 95% cell disruption with the release of numerous intact nuclei and large membranous sheets, presumably basal-lateral membranes. We reasoned that the release of basal-lateral membranes in large sheets would aid in their separation during subsequent differential centrifugation from endoplasmic reticulum, tubulovesicles and apical-plasma membranes which traditionally fragment into small vesicles during homogenization [15, 46]. We used an alkaline pH (8.0) and a low ratio of tissue (wet weight) to medium volume to help prevent further breakdown of the membranous sheets and to minimize the extent of organellar damage [12]. Furthermore, the homogenate was immediately brought to isotonicity with sucrose to help prevent further perturbation of released cytoplasmic components during subsequent manipulations. The addition of 5 mM EDTA to the low speed supernatant (S_1+S_2) greatly reduced the aggregation of particulate matter with the large membrane fragments and facilitated the dispersal of the intracellular membrane markers, $(K^+ + H^+)$-ATPase and glucose-6-phosphatase, from the P_4 pellet to fraction P_5.

Differential Centrifugation

Table 1 lists the distribution of enzyme markers after our standard differential centrifugation procedure. Fraction P_4 contained 60% of the total initial activities of both $(Na^+ + K^+)$-ATPase and ouabain-sensitive K^+-pNPPase as well as nearly 78% of the histamine-stimulated adenylate cyclase. These markers for basal-lateral membranes were separated from 80% or more of the total initial activities of $(K^+ + H^+)$-ATPase, acid phosphatase, glucose-6-phosphatase and pepsin. Less than 10% of the total RNA and 14% of the DNA distributed into fraction P_4.

Both mitochondrial markers (Mg^{2+})-ATPase and cytochrome *c* oxidase were distributed in similar patterns; nearly 85% of the cytochrome *c* oxidase and over 70% of the Mg^{2+}-ATPase were associated with

Table 1. Distribution of markers after differential centrifugation[a]

n	Marker	P_2	P_4	P_5	S_5	Initial recovered specific activity
(11)	Protein	12.7±0.4	24.2±0.8	15.5±0.6	47.7±1.0	
(9)	$(Na^+ + K^+)$-ATPase	16.4±2.0	59.5±3.2	24.3±2.7	n.d.	1.33 ± 0.10
(2)	Ouabain-sensitive K^+-*p*NPPase	14.4±1.9	60.6±2.8	25.1±0.9	n.d.	0.43 ± 0.09
(2)	Histamine-stimulated adenylate cyclase	11.3±0.3	77.6±4.3	11.2±4.6	n.d.	13.50 ± 1.37
(9)	$(K^+ + H^+)$-ATPase	8.7±1.2	20.2±3.4	71.1±3.8	n.d.	4.94 ± 0.60
(5)	Mg^{2+}-ATPase	23.1±2.7	71.3±3.0	5.6±1.0	n.d.	56.8 ± 19.5
(5)	Cytochrome *c* oxidase	15.0±3.4	81.5±3.4	3.5±1.0	n.d.	2.64 ± 0.51
(1)	Acid phosphatase	5.6	7.8	13.2	73.4	1.79
(2)	Pepsin	3.3±1.1	2.8±0.2	1.6±0.3	92.5±0.6	5.79 ± 1.21
(2)	Glucose-6-phosphatase	6.0±1.5	18.7±1.3	43.9±1.2	31.5±1.1	0.105± 0.017
(3)	NADPH-cytochrome *c* reductase	10.6±1.9	53.8±6.1	35.7±3.2	n.d.	0.100± 0.012
(1)	RNA	12.6	9.8	16.7	60.9	35
(1)	DNA	44.3	13.6	42.1	n.d.	184

[a] Values presented are the average of the percentage of the total activity for the indicated marker. Because the homogenate was extremely difficult to sample, the total activity was taken as the sum of the activities of all four fractions. Therefore the initial recovered specific activity is the result of dividing the total activity by the total mg protein. Numbers in parentheses indicate the number of preparations (*n*) in which the enzyme activity was measured. For cases where $n>3$, values are the mean±SEM; when $n=2$ values are the mean ±1/2 range. Samples lacking detectable activity are indicated by the abbreviation n.d. Histamine-stimulated adenylate cyclase activity is in pmol·mg protein^{-1}·min^{-1}; DNA and RNA are in units of μg·mg protein^{-1}; pepsin is in mega units·mg protein^{-1}; cytochrome *c* oxidase is in units of K(min^{-1})·mg protein^{-1}; NADPH-cytochrome *c* reductase units are ΔOD_{550}·mg protein^{-1}·min^{-1}. All other enzyme activites are expressed as μmol·mg protein^{-1}·hr^{-1}.

fraction P_4 while less than 6% of either marker distributed into fraction P_5. The cofractionation of $(Na^+ + K^+)$-ATPase activity with mitochondrial markers during differential centrifugation is not unique to the gastric glands and has been reported for the scraped hog fundic mucosa [15] and the rat intestinal epithelium [28]. The large percentage (54%) of NADPH-cytochrome *c* reductase in P_4 is most likely a result of the dual role of this enzyme as a marker for both endoplasmic reticulum and outer mitochondrial membranes [8, 22].

Density Gradient Centrifugation

Various density gradient centrifugation media such as sucrose, Percoll, Ficoll and dextran were investigated for the separation of basal-lateral membranes from contaminating cellular components in fraction P_4. Dextran gave the best resolution of $(Na^+ + K^+)$-ATPase activity from other subcellular markers. We experimented with various stepped gradients rather than continuous gradients because the dextran solutions were extremely viscous and therefore difficult for our gradient maker to handle.

Centrifugation of fraction P_4 on a discontinuous dextran gradient resulted in three distinct fractions labeled D_1, D_2 and D_3. Fraction D_1 at the 0–5% dextran interface consisted of a very thin band of diffuse white material. Equilibrated at the 5–20% dextran interface was a dense band of tinted white, membranous material which we labeled D_2. Fraction D_3 consisted of the large, thick, brown pellet.

The distribution of the total initial activities of all markers and their purification with respect to protein after density gradient centrifugation is presented in Table 2. Fraction D_2 yielded 30% or more of the total initial activities for each of the three basal-lateral membrane markers. The yield of markers for other subcellular components in fraction D_2 ranged from 0.4% for pepsin to 9.3% for $(K^+ + H^+)$-ATPase. Furthermore, only 2.8% of the total initial protein distributed to fraction D_2 resulting in comparatively large purification factors with respect to protein for each of the three basal-lateral membrane markers (11–13). The purification factors for all other markers were less than 3.3.

The three basal-lateral membrane markers in fraction D_2 were largely separated from most of the major contaminants present in fraction P_4. From 62% to 69% of the total recovered activities of the basal-lateral membrane markers from the dextran gradient were collected in fraction D_2. On the other hand only 3% and 7% of the recovered mitochondrial markers, cytochrome *c* oxidase and Mg^{2+}-ATPase, respectively, were distributed in D_2. More than 89% of the recovered mitochondrial markers sedimented in the pellet D_3. Similarly, only 12% of the recovered NADPH-cytochrome *c* reductase activity was present in fraction D_2 while 86% was recovered in D_3.

The effectiveness of the density gradient centrifugation procedure can be further judged by the recovery of various enzymatic and chemical markers within the gradient bands. As shown in Table 2 the recovery of marker activities from fraction P_4 was usually better than 70% except for RNA, pepsin and histamine-

Table 2. Distribution of markers after density gradient centrifugation[a]

n	Marker	D_1	D_2	D_3	% Recovery from P_4	Purification factor
(9)	Protein	0.6±0.1	2.8±0.2	16.5± 1.1	81.2± 0.9	1.0
(6)	$(Na^+ + K^+)$-ATPase	2.6±0.6	31.2±2.6	15.5± 6.0	81.2± 4.5	11.1
(2)	Ouabain-sensitive K^+-*p*NPPase	2.0±0.8	29.9±9.6	15.9± 2.0	78.2±14.2	10.7
(2)	Histamine-stimulated adenylate cyclase	0.8±0.4	36.2±4.9	15.7± 4.4	52.7± 0.6	12.9
(4)	$(K^+ + H^+)$-ATPase	1.5±0.4	9.3±2.9	7.3± 3.5	74.1± 4.0	3.3
(2)	Mg^{2+}-ATPase	1.6±1.0	3.5±1.7	43.4± 1.2	67.5± 3.5	1.2
(5)	Cytochrome *c* oxidase	n.d.	2.7±0.4	76.8±12.7	96.0±11.8	1.0
(1)	Acid phosphatase	0.6	2.4	3.9	89.5	0.9
(1)	Pepsin	0.2	0.40	0.8	50.9	0.1
(2)	Glucose-6-phosphatase	1.6±0.5	4.4±1.8	10.4± 1.3	83.4± 9.8	1.6
(3)	NADPH-cytochrome *c* reductase	0.9±0.4	5.4±1.0	38.0± 1.4	82.3± 3.6	1.9
(1)	RNA	0.5	1.5	3.0	50.6	0.5
(1)	DNA	n.d.	1.6	7.8	69.1	0.6

[a] Values are the average of the percentage of the total activity for the indicated marker. Numbers in parentheses indicate the number of preparations (*n*) in which the enzyme activity was measured. For cases where $n>3$, values are the mean ± SEM; when $n=2$ values are the mean ± 1/2 range. Purification factors are calculated by dividing 2.8% yield of protein in D_2 into the % yield of each marker in D_2. Samples lacking detectable activity are indicated by the abbreviation n.d.

Table 3. Secretagogue control of $(Na^+ + K^+)$-ATPase[a]

	Histamine 10^{-7} M	cAMP 10^{-5} M	Acetylcholine 10^{-4} M
Reported data [29–31]	31 ± 6 (6)	51 ± 13 (11)	200
Fraction D_2	98 ± 2 (8)	96 ± 3 (8)	94 ± 3 (8)

[a] Values presented are the averages of the percent of control activity ± SEM. Numbers in parentheses are the number of experiments in which the enzyme activities were measured.

stimulated adenylate cyclase. A large proportion of both RNA and pepsin activity may have been lost to the supernatant during subsequent washing of each fraction after density gradient centrifugation. This is suggested by the large distribution of these two markers into fraction S_5 during differential centrifugation. This observation may be accounted for by the great proportion of RNA in soluble form in the cytoplasm of chief cells [19] and by the disruption of fragile zymogen granules during the isolation procedure. On the other hand, histamine-stimulated adenylate cyclase activity rapidly decreased with time, losing nearly 80% of its activity in only 48 hr in liquid nitrogen. Therefore, the extra manipulation of the samples plus the time involved in density grandient centrifugation may have contributed to the decreased yield of histamine-stimulated adenylate cyclase activity.

Secretagogue Control of $(Na^+ + K^+)$-ATPase Activity

It has been reported that gastric $(Na^+ + K^+)$-ATPase activity from a crude cell-free fraction is altered by various gastric secretagogues [29–31]. We tested the effects of histamine, cAMP and acetylcholine on the $(Na^+ + K^+)$-ATPase activity in fraction D_2 (Table 3). There were no significant effects on $(Na^+ + K^+)$-ATPase activity by either of the three secretagogues.

Discussion

We have developed a procedure for the isolation of a membrane fraction from gastric glandular cells enriched in basal-lateral membranes as defined by multiple enzymatic markers. The enzymatic markers used to follow the fractionation and purification of basal-lateral membranes were $(Na^+ + K^+)$-ATPase, ouabain-sensitive K^+-pNPPase and histamine-stimulated adenylate cyclase.

Criteria for Basal-Lateral Membrane Markers

There is substantial evidence to justify the use of $(Na^+ + K^+)$-ATPase as a marker for basal-lateral membranes. First, by analogy to the small intestine, a related but more highly characterized epithelium in which $(Na^+ + K^+)$-ATPase is restricted to the basal-lateral membrane [25, 28, 47]. Furthermore, much electrophysiological evidence from *in vitro* mammalian gastric mucosa has led to the postulation of a Na^+ pump at the basal-lateral membrane responsible for active Na^+ reabsorption and the maintenance of an intracellular ionic milieu necessary for Na^+-dependent Cl^- secretion [16, 26].

As an additional indicator of the Na^+-pump we monitored the fractionation of ouabain-sensitive K^+-stimulated pNPPase. This enzyme is closely associated with plasma membranes, inhibited by ouabain [35] and participates in the second step of the $(Na^+ + K^+)$-ATPase reaction sequence [18]. Unlike the K^+-dependent phosphatase activity associated with the gastric $(K^+ + H^+)$-ATPase which is insensitive to ouabain [14, 17], the ouabain-sensitive K^+-stimulated pNPPase was used as a marker for basal-lateral membranes, acting as an additional indicator of $(Na^+ + K^+)$-ATPase activity.

Adenylate cyclase has been widely used as an enzyme marker for plasma membranes [33, 44, 49, 50]. Furthermore, one would assume, according to the "second messenger" hypothesis for cAMP, that hormone-sensitive receptor sites for stimulating adenylate cyclase would be present on the basal-lateral plasma membrane of effector cells. Correspondingly, in the gastric mucosa histamine, a potent secretagogue of acid secretion, has been shown to be mediated by cAMP [7, 21, 43].

Purity of Basal-Lateral Membranes

All three enzymatic markers for the basal-lateral membrane tended to distribute in a similar pattern during both differential and density gradient centrifugation resulting in similar purification factors with respect to protein. The co-purification of these three markers suggests a common localization of these enzymes lending support to our assumptions of their basal-lateral membrane origin. The co-purification of these markers has been reported for other epithelia such as the small intestine [33, 49] and thyroid [50].

A purification factor of 11 for $(Na^+ + K^+)$-ATPase with respect to protein is a respectable number when compared to reported purification factors for other tissues. Mircheff et al. [27] lists the purification factors of $(Na^+ + K^+)$-ATPase for a

number of different procedures reported in the literature for the isolation of basal-lateral membranes of the small intestine; numbers range from 5 to 20. Similarly, Solyom and Trams [44] lists $(Na^+ + K^+)$-ATPase purification factors for a variety of tissue plasma membranes; for example, purified liver plasma membranes have purification factors ranging from 9 to 27, brain (synaptic membrane) 2.6 to 15, olfactory epithelium 46, and thyroid 10 to 80.

In relation to $(Na^+ + K^+)$-ATPase the basal-lateral membranes of fraction D_2 were essentially free of contamination from RNA, DNA, acid phosphatase and pepsin; $(Na^+ + K^+)$-ATPase was purified by factors of 20, 19, 13 and 78 to these markers, respectively. Mitochondrial contamination was very low as the membranes were purified ninefold with respect to Mg^{2+}-ATPase and 11-fold with respect to cytochrome *c* oxidase. Purification factors of 7 and 6 for glucose-6-phosphatase and NADPH-cytochrome *c* reductase, respectively, represent only a slight contamination by membranes of the endoplasmic reticulum.

The apical and tubulovesicular membranes of the parietal cells represent the largest contaminant of fraction D_2. Quantitative morphometric analysis on the ultrastructure of dog parietal cells have shown that the combined area of the apical and tubulovesicular membranes is about 7 times greater than the basal-lateral membrane area [20]. Thus the apical and tubulovesicular membranes represent an extremely large pool of potential contaminating membranes. Nonetheless, differential centrifugation was able to resolve nearly 60% of the $(Na^+ + K^+)$-ATPase activity from almost 80% of the $(K^+ + H^+)$-ATPase. But when applied to a density gradient of dextran, both $(K^+ + H^+)$-ATPase and $(Na^+ + K^+)$-ATPase membranes from fraction P_4 are heterogeneous and share some common equilibrium densities. This may be explained by the fact that the tubulovesicles are plasma membrane-like in lipid composition. Sen and Ray [40] have reported that the tubulovesicular membranes have a cholesterol to phospholipid molar ratio of greater than 1, similar to that of plasma membranes. Furthermore, Black et al. [4] have inferred from freeze-fracture studies that the tubulovesicles fuse with and become an integral part of the apical surface. Therefore, the tubulovesicles may actually represent a reserve pool of apical plasma membranes which are called into action during the initiation of acid secretion. Nevertheless, the basal-lateral membranes of fraction D_2 are purified more than threefold with respect to $(K^+ + H^+)$-ATPase.

Fraction D_2 represents a preparation of glandular cell membranes well-enriched in basal-lateral membranes yielding more than 30% of the total initial $(Na^+ + K^+)$-ATPase purified more than 11-fold with respect to protein. This preparation is free of significant contamination from major intracellular organelles, although there is a slight contamination from tubulovesicular membranes. Future investigations will be concerned with the role of the basal-lateral membrane in the hormonal control of gastric secretion as well as the identification and characterization of inherent ion transporting mechanisms.

References

1. Anson, M.L., Mirsky, A.E. 1932. The estimation of pepsin with hemoglobin. *J. Gen. Physiol.* **16**:59
2. Berglindh, T., Helander, H.F., Öbrink, K.J. 1976. Effects of secretagogues on oxygen consumption, aminopyrine accumulation and morphology in isolated gastric glands. *Acta Physiol. Scand.* **97**:401
3. Berglindh, T., Öbrink, K.J. 1976. A method for preparing isolated glands from rabbit gastric mucosa. *Acta Physiol. Scand.* **96**:150
4. Black, J.A., Forte, T.M., Forte, J.G. 1980. Structure of oxyntic cell membranes during conditions of rest and secretion of HCl as revealed by freeze-fracture. *Anat. Rec.* **196**:163
5. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248
6. Chang, H., Saccomani, G., Rabon, E., Schackmann, R., Sachs, G. 1977. Proton transport by gastric membrane vesicles. *Biochim. Biophys. Acta* **464**:313
7. Chew, C.S., Hersey, S.J., Sachs, G., Berglindh, T. 1980. Histamine responsiveness of isolated gastric glands. *Am. J. Physiol.* **238**:G312
8. Comte, J., Gautheron, D.C. 1978. The markers of pig heart mitochondrial sub-fractions: I. The dual location of NADPH-cytochrome *c* reductase in outer membrane and microsomes. *Biochimie* **60**:1289
9. Davidson, J.N. 1957. Some critical comments in the chemical estimation of nucleic acids in tissues. *Exp. Cell Res. Suppl.* **4**:260
10. Dousa, T.P., Code, C.F. 1974. Effect of histamine and its methyl derivatives on cyclic AMP metabolism in gastric mucosa and its blockage by an H_2 receptor antagonist. *J. Clin. Invest.* **53**:334
11. Dozois, R.R., Wollin, A., Rettmann, R.D., Dousa, T.P. 1977. Effect of histamine on canine gastric mucosal adenylate cyclase. *Am. J. Physiol.* **232**:E35
12. Evans, H.W. 1978. Preparation and characterization of mammalian plasma membranes. *In:* Laboratory Techniques in Biochemistry and Molecular Biology. T.S. Work and E. Work, editors. p. 11. North-Holland Publishing Company, New York
13. Forte, J.G. 1969. Three components of Cl^- flux across isolated bullfrog gastric mucosa. *Am. J. Physiol.* **216**:167
14. Forte, J.G., Forte, G.J., Saltman, P. 1967. K^+-stimulated phosphatase of microsomes from gastric mucosa. *J. Cell Physiol.* **69**:293
15. Forte, J.G., Ganser, A., Beesley, R., Forte, T.M. 1975. Unique enzymes of purified microsomes from pig fundic mucosa. *Gastroenterology* **69**:175
16. Forte, J.G., Machen, T.E., Öbrink, K.J. 1980. Mechanisms of gastric H^+ and Cl^- transport. *Annu. Rev. Physiol.* **42**:111
17. Ganser, A.L., Forte, J.G. 1973. K^+-stimulated ATPase in purified microsomes of bullfrog oxyntic cells. *Biochim. Biophys. Acta* **307**:169

18. Glynn, I.M., Karlish, S.J.D. 1975. The sodium pump. *Annu. Rev. Physiol.* **37**:13
19. Helander, H.F. 1964. Ultrastructure of gastric fundus glands of refed mice. *J. Ultrastruct. Res.* **10**:160
20. Helander, H.F., Hirschowitz, B.I. 1972. Quantitative ultrastructural studies on gastric parietal cells. *Gastroenterology* **63**:951
21. Katsumata, Y., Yagi, K. 1976. Distribution of adenyl cyclase sensitive to histamine in rabbit gastric mucosa. *Biochem. Pharmacol.* **25**:603
22. Kulkoski, J.A., Weber, J.L., Ghazarian, J.G. 1979. NADPH-cytochrome *c* reductase in outer membrane of kidney mitochondria. *Arch. Biochem. Biophys.* **192**:539
23. Lauter, C.J., Solyom, A., Trams, E.G. 1972. Comparative studies on enzyme markers of liver plasma membranes. *Biochim. Biophys. Acta* **266**:511
24. Lee, H.C., Breitbart, H., Berman, M., Forte, J.G. 1979. Potassium-stimulated ATPase activity and hydrogen transport in gastric microsomal vesicles. *Biochim. Biophys. Acta* **553**:107
25. Lewis, B.A., Elkin, A., Michell, R.H., Coleman, R. 1975. Basolateral plasma membranes of intestinal epithelial cells: Identification by lactoperoxidase-catalyzed iodination and isolation after density perturbation with digitonin. *Biochem. J.* **152**:71
26. Machen, T.E., Silen, W., Forte, J.G. 1978. Na^+ transport by mammalian stomach. *Am. J. Physiol.* **234**:E228
27. Mircheff, A.K., Sachs, G., Hanna, S.D., Labiner, C.S., Rabon, E., Douglas, A.P., Walling, M.W., Wright, E.M. 1979. Highly purified basal lateral plasma membranes from rat duodenum. Physical criteria for purity. *J. Membrane Biol.* **50**:343
28. Mircheff, A.K., Wright, E.M. 1976. Analytical isolation of plasma membranes of intestinal epithelial cells: Identification of Na, K-ATPase rich membranes and the distribution of enzyme activities. *J. Membrane Biol.* **28**:309
29. Mózsik, G. 1970. Direct inhibitory effect of adenosine monophosphates on $Na^+ - K^+$-dependent ATPase prepared from human gastric mucosa. *Eur. J. Pharmacol.* **9**:207
30. Mózsik, G., Kutas, J., Nagy, L., Tárnok, F., Vizi, F. 1978. Interrelationships between the cholinergic influences, gastric mucosa $Na^+ - K^+$-dependent ATPase, ATP, ADP, ions of gastric juice and basal secretion in patients. *Acta Physiol. Scand. Special Suppl.* p. 199
31. Mózsik, G., Nagy, L., Tárnok, F., Jávor, T., Kutas, J. 1974. Effects of histamine on transport ($Na^+ - K^+$-dependent) ATPase system prepared from human gastric mucosa. *Pharmacology* **12**:193
32. Munro, H.N., Fleck, A. 1966. The determination of nucleic acids. *In:* Methods of Biochemical Analysis. David Glick, editor. Vol. 14, p. 113. John Wiley and Sons, Inc., New York
33. Murer, H., Amman, E., Biber, J., Hopfer, U. 1976. The surface membrane of the small intestinal epithelial cell. I. Localization of adenyl cyclase. *Biochim. Biophys. Acta* **433**:509
34. Nachlas, M.M., Tsou, K.C., Souza, E. de, Cheng, C.S., Seligman, A.M. 1957. Cytochemical demonstration of succinic dehydrogenase by the use of a new *p*-nitrophenyl substituted ditetrazole. *J. Histochem. Cytochem.* **5**:420
35. Nagai, K., Izumi, F., Yoshida, H. 1966. Studies on potassium dependent phosphatase; its distribution and properties. *J. Biochem. (Tokyo)* **59**:295
36. Rehm, W.S., Sanders, S.S. 1975. Implication of the neutral carrier $Cl^- - HCO_3^-$ exchange mechanism in gastric mucosa. *Ann. N. Y. Acad. Sci.* **264**:442
37. Sachs, G., Chang, H.H., Rabon, E., Schackmann, R., Lewin, M., Saccomani, G. 1976. A nonelectrogenic H^+ pump in plasma membranes of hog stomach. *J. Biol. Chem.* **251**:7690
38. Salomon, Y., Londos, C., Rodbell, M. 1974. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541
39. Sanui, H. 1974. Measurement of inorganic orthophosphate in biological materials: Extraction properties of butyl acetate. *Anal. Biochem.* **60**:489
40. Sen, P.C., Ray, T.K. 1979. Characterization of gastric mucosal membranes: Lipid composition of purified microsomes from pig, rabbit, and frog. *Arch. Biochem. Biophys.* **198**:548
41. Simon, B., Kather, H. 1979. Prostaglandin-sensitive adenylate cyclase in human gastric mucosa. *Pharmacology* **19**:96
42. Smith, L. 1955. Spectrophotometric assay of cytochrome *c* oxidase. *In:* Methods of Biochemical Analysis. David Glick, editor. Vol. 2, p. 427. John Wiley and Sons, Inc., New York
43. Soll, A.H., Wollin, A. 1979. Histamine and cyclic AMP in isolated canine parietal cells. *Am. J. Physiol.* **237**:E444
44. Solyom, A., Trams, E.G. 1972. Enzyme markers in characterization of isolated plasma membranes. *Enzyme* **13**:329
45. Sottocasa, G.L., Kuylenstuerna, B., Ernster, L., Bergstrand, A. 1967. An electron transport system in mitochondrial outer membrane. *J. Cell Biol.* **32**:415
46. Steck, T.L. 1972. Membrane isolation. *In:* Membrane Molecular Biology. C.F. Fox and A.D. Keith, editors. p. 76. Sinauer Associates, Stamford, Connecticut
47. Stirling, C.E. 1972. Radioautographic localization of sodium pump sites in rabbit intestine. *J. Cell Biol.* **53**:704
48. Sung, C.P., Jenkins, B.C., Racey Burns, L., Hackney, V., Spenney, J.G., Sachs, G., Wiebelhaus, V.D. 1973. Adenyl and guanyl cyclase in rabbit gastric mucosa. *Am. J. Physiol.* **225**:1359
49. Walling, M.W., Mircheff, A.K., Van Os, C.H., Wright, E.M. 1978. Subcellular distribution of nucleotide cyclases in rat intestinal epithelium. *Am. J. Physiol.* **235**:E539
50. Wolff, J., Jones, A.B. 1971. The purification of bovine thyroid plasma membranes and the properties of membrane-bound adenyl cyclase. *J. Biol. Chem.* **246**:3939

Received 5 September 1980