Plasma Membrane of the Rat Parotid Gland: Preparation and Partial Characterization of a Fraction Containing the Secretory Surface

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ABSTRACT A plasma membrane fraction from the rat parotid gland has been prepared by a procedure which selectively enriches for large membrane sheets. This fraction appears to have preserved several ultrastructural features of the acinar cell surface observed *in situ*. Regions of membrane resembling the acinar luminal border appear as compartments containing microvillar invaginations, bounded by elements of the junctional complex, and from which basolateral membranes extend beyond the junctional complex either to contact other apical compartments or to terminate as free ends. Several additional morphological features of the apical compartments suggest that they are primarily derived from the surface of acinar cells, rather than from the minority of other salivary gland cell types.

Enzymatic activities characteristically associated with other cellular organelles are found at only low levels in the plasma membrane fraction. The fraction is highly enriched in two enzyme activities—K^+-dependent *p*-nitrophenyl phosphatase (K^+-NPPase, shown to be Na^+/K^+ adenosine triphosphatase; 20-fold) and γ-glutamyl transpeptidase (GGTPase; 26-fold)—both known to mark plasma membranes in other tissues. These activities exhibit different patterns of recovery during fractionation, suggesting their distinct distributions among parotid cellular membranes. Secretion granule membranes also exhibit GGTPase, but no detectable K^+-NPPase. Since Na^+/K^+ adenosine triphosphatase and GGTPase, respectively, mark the basolateral and apical cellular surfaces in other epithelia, we hypothesize that these two enzymes mark distinct domains in the parotid plasmalemma, and that GGTPase, as the putative apical marker, may signify a compositional overlap between the two types of membranes which fuse during exocytosis.

Exocytosis (1) is the process by which products sequestered intracellularly within a membrane-enclosed organelle are released directly into the extracellular space via the fusion of the organelle's limiting membrane with the cell's plasmalemma (2). This process, which has generally been assumed to occur to different degrees in virtually all cell types, has been most actively studied in systems specialized for secretion (e.g., mast cell [3], exocrine pancreas [4], parotid [5], adrenal medulla [6], anterior pituitary [7], intestinal goblet cell [8], and neuromuscular junction [9]). Each of these systems maintains a large and readily identifiable population of storage organelles (secretion granules) which may ultimately discharge their contents when the cells are appropriately stimulated. Studies of this membrane fusion phenomenon have been approximated in vitro using isolated organelles and other simple model systems; however, relatively little is known about the biochemical events and biophysical interactions which operate in vivo to bring about exocytosis in its characteristically highly specific and directed manner.

Our ultimate goal is to probe the mechanism of exocytosis by studying, in vitro, the interactions of the "natural fusion partners" as subcellular fractions. Such an approach would offer the possibility of a detailed and quantitative analysis of those factors which direct and contribute to this complex event. To this intent, we have set out to isolate and characterize the fusion partner membranes involved in exocytosis in the rat parotid.

The rat parotid gland is particularly suited for such studies since: (*a*) an estimated 85–90% of the gland volume consists of acinar (secretory) cells; (*b*) it has already proven amenable to

THE JOURNAL OF CELL BIOLOGY · VOLUME 95 OCTOBER 1982 8-19

subcellular fractionation (10); (*c*) it is particularly rich in secretion granules, one of the fusion partners (11); and (*d*) in contrast to the pancreas (12) and the rabbit parotid (13), potentially harmful lipid and protein hydrolyzing enzymes have not been found to an appreciable extent (11, 14, and unpublished results).

In this report, we will describe (*a*) a simple procedure for the preparation of a rat parotid plasma membrane fraction, the elements of which exhibit contiguous apical and basolateral domains, (*b*) a biochemical survey of this fraction for putative plasma membrane "marker enzymes," (*c*) a comprehensive contamination analysis based on morphologic and enzymatic examination for other cellular organelles and estimation of absorbed secretory protein, (*d*) preparation of a parotid rough microsomal fraction to assess the extent to which it could contribute (as an organelle contaminant) to the marker activities of the plasmalemmal fraction, and (*e*) a limited examination of secretion granules and their membranes for activities enriched in the plasma membrane fraction.

MATERIALS AND METHODS

Definition of Fractionation Media

Medium A: 0.5 mM $MgCl_2$, 1 mM $NaHCO_3$ pH 7.4
Medium B: 0.5 mM $MgCl_2$, 1 mM $NaHCO_3$, 0.7 mM EDTA pH 7.4
Medium C: 0.5 mM $MgCl_2$, 1 mM $NaHCO_3$, 1.7 mM EDTA pH 7.4

Preparation of Parotid Plasma Membranes

The procedure and rationale for preparation of parotid plasma membranes will be presented in the Results section.

Preparation of Parotid Rough Microsomes

A rough microsome fraction was prepared from parotid tissue using the procedure of Adelman et al. (15) with the following modifications: the supernate designated S_4 from the plasma membrane preparation (Results) was used as a starting material. This was spun at 230,000 g_{av}·min in the IEC PR-6000 at 4°C. The resulting pellet was resuspended by 10 strokes in a Dounce homogenizer with loose fitting pestle in ice-cold 1.0 M sucrose in Medium B. The suspension was layered in a discontinuous sandwich gradient with 1.6 M and 2.0 M sucrose layers below, and 0.3 M above; sucrose in each case was dissolved in Medium B. Gradients in Beckman SW27 cellulose nitrate tubes (Beckman Instruments, Inc., Fullerton, CA) were spun at 25,000 rpm for ~18 h. The lowest interface band (1.6–2.0 M sucrose) was collected, diluted with Medium B to 0.37 M sucrose, and pelleted in a Beckman 60Ti rotor at 45,000 rpm for 45 min. The pellets constituting the rough microsomal fraction were resuspended in a small volume (1–2 ml) of supernatant fluid by three strokes in a loose fitting Dounce homogenizer.

Preparation of Parotid Secretion Granules

Secretion granules were prepared by a modification of the procedure worked out using the rabbit parotid gland (14). Purity of this fraction was evaluated by enrichment of α-amylase specific activity as compared to parotid homogenate (this value was 3.8 for greater than five such preparations); and by electron microscopic examination of the fraction which revealed only a small degree of contamination by other organelles (preparation and characterization of parotid secretion granules will be the subject of a subsequent publication).

Chemical Analysis

Protein was determined by a modification of the Lowry method (16) using bovine serum albumin (BSA) as a standard. Phospholipid phosphate was assayed according to Chen et al (17) on chloroform extracts (18) that were washed in 70% perchloric acid (19).

Biochemical Assays

Enzymes (activities are expressed as μmoles product formed/min unless otherwise indicated) were assayed as follows: γ-glutamyl transpeptidase (GGTPase) after Tate and Meister (20); 5′-nucleotidase after Widnell and Unkeless (21); alkaline phosphatase at pH 9.0 after Emmelot and Bos (22) modified by the addition of 1.0 mM ouabain and 1% Triton X-100; alkaline phosphodiesterase I after Touster et al. (23); α-amylase after Bernfeld (24); [^{3}H]UDP-galactosyl transferase by a modification of the assay described by Fleischer (25) including 2 mM ATP and 2mg/ml asialoagalacto-fetuin (26), acid precipitable ^{3}H-label was used as the measure of activity; β-*N*-acetyl glucosaminidase after Findlay et al. (27); monoamine oxidase type A after Castro Costa et al. (28); aminopeptidase after Louvard (29); cytochrome *c* oxidase after Peters et al. (30) using the first order rate constant as a measure of activity (31); NADH-cytochrome *c* reductase after Sottocasa et al. (32) but in all cases including 1.5 μM rotenone plus 0.3 mM sodium cyanide. K^+-dependent *p*-nitrophenyl phosphatase (K^+-NPPase) was assayed by measuring the formation of *p*-nitrophenol (ΔOD_{410}) at 37°C after 30 min. The assay mixture contained 5.0 mM *p*-nitrophenyl phosphate, 0.1 M imidazole pH 7.5, 0.01 M $MgCl_2$, 0.7 mM EGTA, 0.01 M NaCl, and 0.01 M of either KCl or choline chloride.

Preparation of ^{3}H-labeled Secretory Protein

Radioactively labeled biosynthesized secretory proteins were prepared and discharged from rat parotid lobules essentially by the procedure of Castle et al. (14) using ^{3}H-algal protein hydrolysate (ICN, Irvine, CA) at 25 μCi/ml. The radioactivity of the resulting secretory protein mixture used for incubation with plasma membranes was 7.8 μCi/ml. Radioactivity was quantitated by counting sample aliquots dissolved in Liquiscint (National Diagnostics, Somerville, NJ) in a Beckman LS-250 liquid scintillation spectrometer (Beckman Instruments, Inc.).

Processing of Membranes for Microscopy

Plasma membranes and microsomes were routinely fixed in suspension by mixing them with 1 vol of a freshly prepared solution of 2% glutaraldehyde, 2% formaldehyde in 0.2 M Na-cacodylate, pH 7.3. Fixation, postfixation in OsO_4, and subsequent processing for electron microscopic observation were carried out as previously described (14). Micrographs were taken in either a Siemens 101 or a Philipps 301 electron microscope.

Materials

Eagle's minimal essential medium (Earle's salts) was obtained from Gibco (Grand Island Biological Co., Grand Island, NY). EDTA (Aldrich, Milwaukee, WI) was calibrated with a $CaCl_2$ standard solution using a Ca^{2+} electrode. Crystalline BSA was obtained from Armour Pharmaceuticals (Phoenix, AZ); 5′-adenosine monophosphate from Calbiochem (La Jolla, CA); Triton X-100 and [^{3}H]uridine diphosphogalactose from New England Nuclear (Boston, MA). Asialoagalactofetuin was the kind gift of Drs. K. Howell and E. Sztul. [^{3}H]tryptamine was obtained from Amersham (Arlington Heights, IL). Most other stock chemicals were obtained from Sigma Chemical Co. (St. Louis, MO); all were reagent grade. Nitex nylon screen (105 μm^2 mesh) was obtained from Tetko, Inc. (Elmsford, NY).

RESULTS

Isolation of Parotid Plasma Membranes

Plasma membrane fractions prepared from rat parotid tissue have not been described previously; and in the plasmalemmal fractionation schemes developed for the parotid glands of other species (33, 34), the issue of distinct apical and basolateral regions within the plasma membrane has not been addressed. In both rabbit and mouse preparations, the fractions described are heterogenous smooth-surfaced vesicles, and the authors' interpretations have relied heavily on the distribution of putative plasma membrane marker enzyme activities based on the precedent of, and analogy to, rat liver plasma membrane studies. Since exclusive plasma membrane markers in the rat parotid have remained unidentified, success in the development of our isolation procedure was judged by the ability to preserve membranous structures, morphologically recognizable as being plasmalemmal in origin. The protocol (shown schematically in Fig. 1) is fundamentally similar to that developed by Ray (35) for the isolation of the hepatocyte plasmalemma in the form of membrane sheets, but several modifications have been introduced to minimize the difficulty in separating parotid plasma

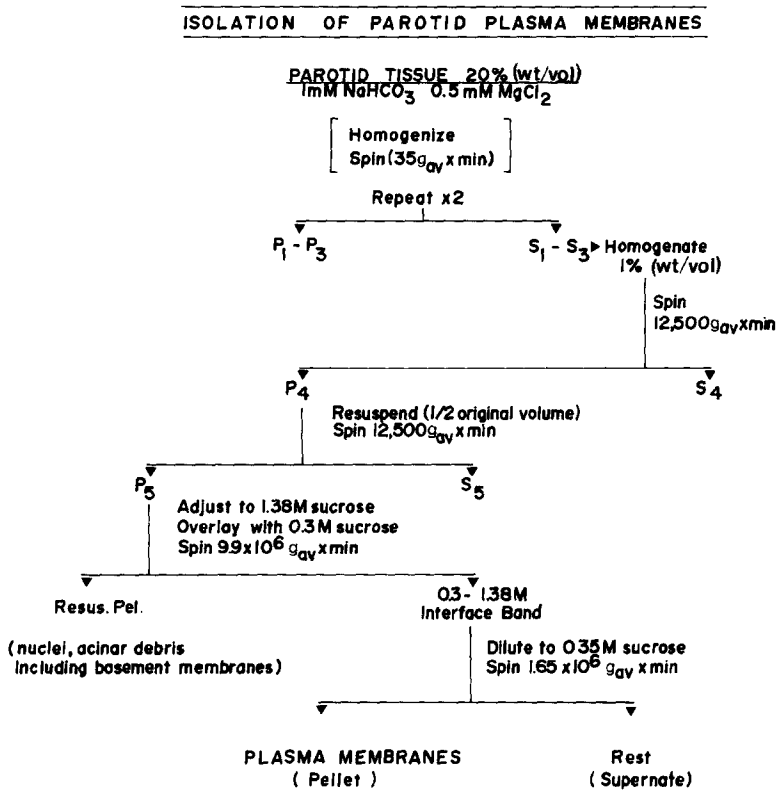


FIGURE 1 Flow sheet for the isolation of the plasmalemmal fraction.

membranes from closely associated connective tissue which heavily invests this epithelium. All steps are done at 4°C.

Male Sprague-Dawley rats (Charles River) weighing 100–125 g were starved overnight and then killed by cardiac incision, under light ether anesthesia. Parotids of 16 rats were excised and immediately immersed into ice-cold oxygenated Eagle's Minimal essential medium (Earle's salts). Associated connective tissue, fat and lymph nodes were dissected away to yield approximately 5.5 g of parotid tissue, which was minced with razor blades and mixed in medium A (0.5 mM $MgCl_2$, 1 mM $NaHCO_3$, pH 7.4) at 20% wt/vol. At this point, the tissue was either (*a*) incubated for 30 min in the presence of 25.5 U of highly purified, protease free collagenase (36, kindly provided by Drs. J. D. Jamieson and M. Sarras) or (*b*) homogenized directly without collagenase. The morphologic appearance, marker enzyme distributions, and extents of contamination are indistinguishable for either procedure. Although data in the tables derive from both procedures, we routinely do not use collagenase so that the possibility of subtle proteolysis may be avoided.

Initial tissue homogenization was achieved in two steps by treatment with a Polytron homogenizer for 15 s at 1,900 rpm followed by 3 up-down strokes with a Brendler teflon pestle homogenizer at 1,300 rpm. The resulting suspension was then spun at 70 *g* for 0.5 min, and the supernatant fluid (S_1) was decanted and saved. The pellet (P_1) contained a large number of unbroken cells in addition to residual connective tissue. To increase the efficiency of homogenization, P_1 was resuspended in the previous volume of Medium A, rehomogenized with the teflon pestle as before, and respun to produce S_2 and P_2. The rehomogenization sequence was repeated once further to yield S_3 and P_3; the latter was discarded.

To completely disrupt the remaining osmotically sensitive intracellular organelles, the supernatant fractions S_1, S_2, and S_3 were pooled, diluted to 1% (wt/vol) in Medium B, mixed, and allowed to stand for several minutes. The large dilution volume and the small amount of net EDTA both serve to reduce organelle aggregation. The suspension was then filtered through four layers of cheesecloth plus one layer of 105 μm^2 mesh Nitex screen, which removes large connective tissue pieces and other debris. The filtrate (termed homogenate) is the basis of assayed enzyme recoveries. Low speed differential centrifugation of the homogenate (825 g_{av} for 15 min in an IEC PR-6000 swinging bucket refrigerated centrifuge) was used to sediment large membrane sheets. The pellet obtained (P_4) consisted largely of nuclei, basement membrane-containing elements, aggregated rough microsomes in addition to plasma membrane sheets. The supernatant fluid (S_4) was saved for assays. P_4 was washed by resuspending with three strokes in a Dounce homogenizer (tight pestle), diluting into one-half the previous volume of Medium B, overlaying the resulting suspension onto 5 ml of 0.3 M sucrose in Medium B (in siliconized 30 ml Corex tubes [Corning Glass, Corning, NY]), and subjecting to recentrifugation under identical conditions (Fig. 1). The 0.3 M sucrose layer was used with the intent of minimizing contamination of the pellet (P_5) with vesiculated membranous elements retained in the supernate (S_5).

To purify the plasma membrane sheets away from residual contaminants, we exploited the anticipated density differences among the structures present in P_5. Preliminary attempts at discontinuous density gradient centrifugation using a range of sucrose concentrations between 1.26 M and 1.58 M, led to the selection of 1.38 M sucrose for the flotation of plasma membranes without concomitant flotation of appreciable nuclear or basement membrane-containing material. The procedure we adopted is as follows: P_5 was resuspended in ~4 ml of Medium B, and then mixed with ice cold 2.2 M sucrose in Medium B to bring the final sucrose concentration to 1.38 M (verified by

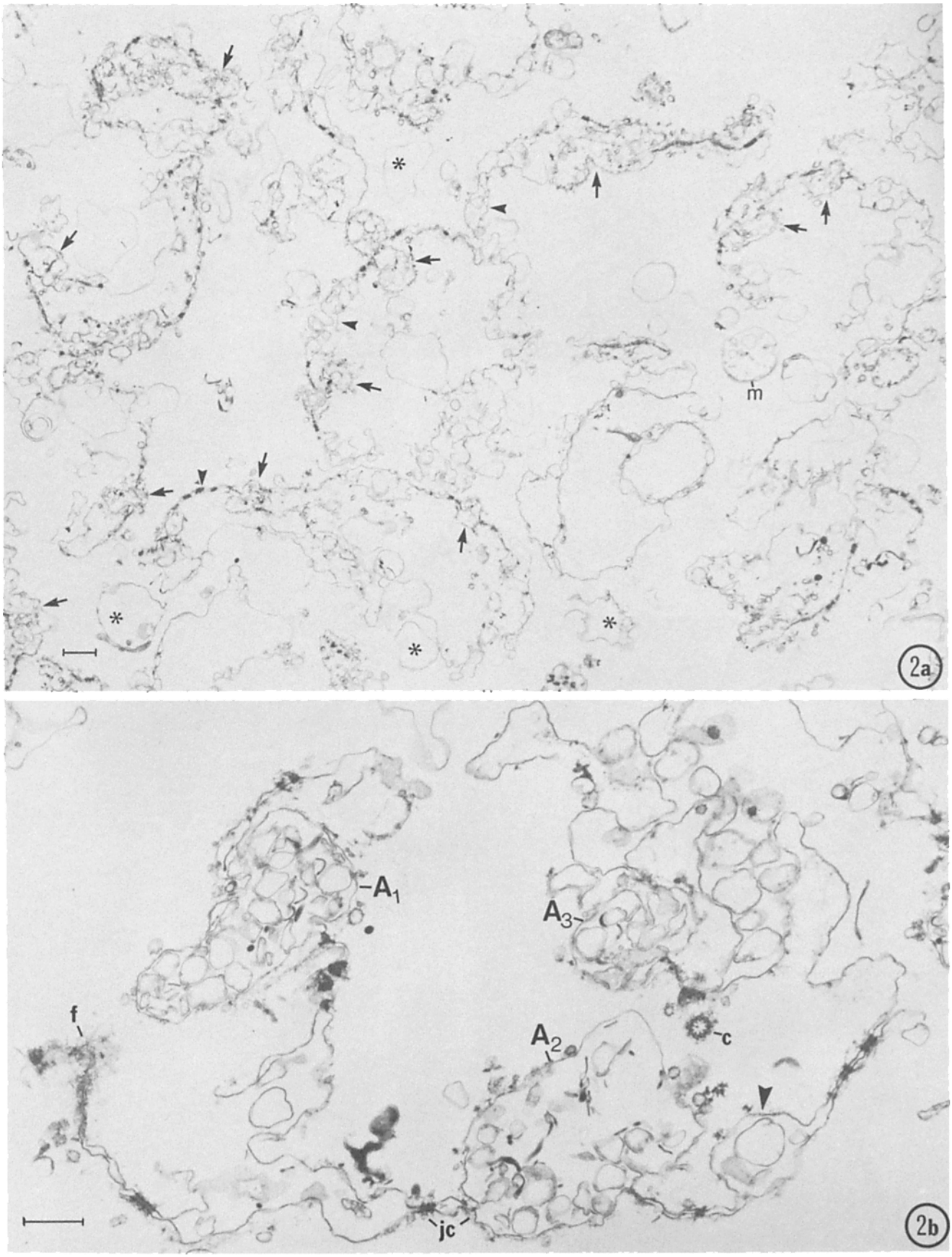
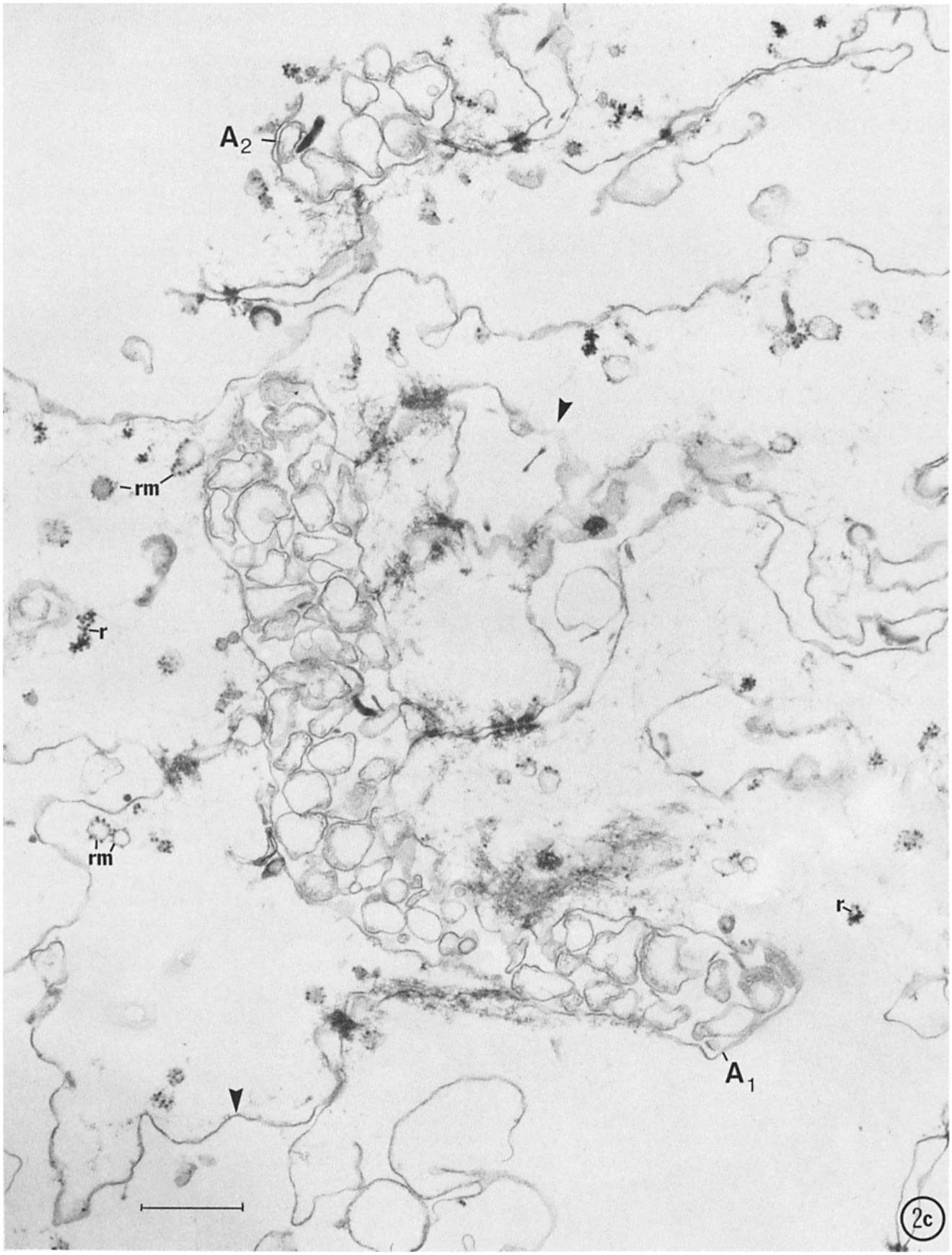


FIGURE 2 Electron micrographs of the parotid plasma membrane fraction. (*a*) At low magnification the fraction is shown to consist predominantly of extended membrane sheets formed by the surfaces of two or more cells that retain an association analogous to that observed *in situ*. Apical regions (arrows) containing remnants of microvilli are linked by intervening stretches of lateral membrane (arrowheads) marked at many points by darkly staining desmosomes. Also present are indistinct membranes of probable plasmalemmal origin (*) frequently in the configuration of large vesicles and showing various degrees of association with the extended sheets. A contaminating damaged mitochondrion (*m*) is seen. Bar, 1 μm. × 6,000. (*b*) At intermediate magnification the membrane of a single cell can be followed continuously (within one sheet) between three different apical regions (A_1–A_3) each of which constitutes a part of the secretory surface adjoined to that of a neighboring cell by elements of the junctional complex (*jc*). Expanded membrane profiles (arrowhead) not bounded by occluding zonules are thought to correspond to lateral



plasmalemmal interdigitations, observed *in situ*. A centriole (*c*) and membrane-associated filaments (*b*) are also evident in this view. Bar, 0.5 μm. × 22,000. (*c*) Elements of the plasma membrane fraction containing apical regions cut longitudinally (A_1) and in cross-section (A_2). Profiles of former microvilli are especially evident in the luminal space, and junctional complexes seen at several points along the boundary of A_1 retain the characteristic organization observed *in situ* with zonulae occludentes apparently intact. The membrane bordering A_2 is not continuous and indicates direct access to the extracellular aspect of the apical domain from the surrounding medium. At many points extended regions of lateral domain (arrowheads) and associated filamentous material may partially restrict access to the cytoplasmic aspect of the apical domain. Rough microsomal (*rm*) and free ribosomal (*r*) contaminants can be seen. Bar, 0.5 μm. × 37,000.

refractive index). Sufficient 1.38 M sucrose in Medium B was added to increase the volume to ~125 ml, and the diluted P_5 was then placed in four nitrocellulose Beckman SW 27 tubes, overlaid (~8 ml each) with 0.3 M sucrose in Medium B, and subjected to centrifugation at 82,500 g_{av} for 2 h. Plasma membranes floated to the interface, while whole and extracted nuclei, basement membranes, microsomes, and some plasma membranes pelleted. The resuspended pellet was saved for assays whereas the "interface band" was collected in a small volume, adjusted to a net EDTA concentration of 1.2 mM, diluted to 0.35 M sucrose by addition of cold Medium C, and given three strokes in a Dounce homogenizer using a tight pestle. The additional EDTA in this medium, as well as further homogenization were used both to reduce membrane aggregation and to liberate nonspecifically attached rough microsomes and free ribosomes from the plasma membrane fragments.

The resulting suspension was then centrifuged at 3.3×10^6 $g_{av} \cdot$min in a Beckman SW41 rotor (Beckman Instruments, Inc.). Plasma membrane pellets were resuspended in a small volume of fresh 0.35 M sucrose in Medium C; the supernatant fluid termed "Rest" was saved for assays. Cytochrome *c* oxidase and NADH cytochrome *c* reductase were always assayed on fresh material upon completion of the preparation; other assay aliquots were frozen at −20°C and assayed without noticeable loss of activity during the following week.

Morphology of the Plasma Membrane Fraction

Fig. 2 *a*, *b*, *c* shows representative electron micrographs of the parotid plasma membrane fraction. Similar to the plasmalemma of the intact secretory cell, distinct regions of apical (luminal) and basolateral membrane are readily seen. The apices frequently appear as compartments which contain microvillar membranes, usually cut in cross section (Fig. 2 *c*). These microvilli are occasionally condensed around a fibrillar core, but more often this core material is extracted during the preparation, giving the membranous projections a more expanded profile. Additional observations of these apices—especially their large numbers of luminal microvilli, their characteristically small diameters when viewed in cross section (approximately the dimensions of a secretion granule), the small number of cells forming their borders, and the considerable number of apical compartments connected into a single membrane sheet (Fig. 2 *a*)—are consistent with an origin of this membrane from the secretory plasmalemma of parotid acinar cells, rather than from duct cell types. The luminal membranes are seen to be separated from the lateral plasmalemmal domain by residual elements of the junctional complex (Fig. 2 *b*). Persisting desmosomal elements render the fraction's lateral membrane recognizable. The most visible contamination of the fraction is by rough microsomes and free ribosomes. However, another major contaminant observed is filamentous material which forms a network around many apices and is often concentrated near desmosomes, where attachments of the filaments are evident (Fig. 2 *c*). A modest degree of plasmalemmal vesiculation can be seen. Surveys for contaminants other than those just mentioned revealed very few mitochondrial, nuclear, lysosomal, or Golgi profiles.

Marker Enzyme Analysis

Each of the fractions generated in the parotid plasma membrane isolation scheme were examined for the presence of enzymatic activities commonly observed in cell surface fractions from other tissues. Quite unexpectedly, several of these putative plasma membrane marker activities were not found to be significantly enriched in the final plasmalemmal fraction (Table I). Most notable among these are alkaline phosphatase and 5′-nucleotidase, whose final recoveries are 0.31 and 0.88%, respectively. While some variability was observed from preparation to preparation in both homogenate activity and plasmalemmal recovery, overall it would appear that these enzymes do not make a major enzymatic contribution to the activity of the acinar cell plasma membrane. Furthermore, the measured alkaline phosphatase in parotid homogenates (0.008 μmol/min per mg protein) is substantially less than that observed from homogenates of other tissues in which extensive plasmalemmal activity has been confirmed cytochemically (rat liver: 0.101 μmoles/min per mg protein [37]). The same is not true, however, for 5′-nucleotidase (present results: 0.031 μmoles/min per mg protein; Rat liver: 0.039 μmoles/min per mg protein [37]). Alkaline phosphodiesterase I, an enzyme which often appears in concert with 5′-nucleotidase (A. L. Hubbard, Johns Hopkins University School of Medicine, personal communication.) also shows a similar distribution in the fractionation scheme reported here, with a low plasmalemmal activity, ≤0.38% of that of the homogenate and enriched only one- to twofold.

Aminopeptidase was tested because this enzyme has been reported to be found in the brush border membranes of kidney and intestine; and has also been histochemically localized to the bile front of the hepatocyte (38, 39). Very little hydrolytic activity toward the leucine-derived substrate was associated with the plasmalemmal fraction (0.27% of homogenate activity), while other peptide substrates have yet to be tried.

The protein yield for the plasmalemmal fraction, as shown in Table II, is unusually low in comparison to that for preparations from other tissues, such as liver, where values of 1–2 mg protein per gram wet tissue weight are characteristically observed (40). We feel this is mainly attributable to the relative paucity of large membrane fragments after vigorous homogenization. However, more gentle approaches, in our hands, failed to disrupt the basement membrane surrounding the acini, hence plasma membrane protein recovery did not improve. Similar findings in fractionating rat parotid have been reported previously (41).

Table II also indicates two enzyme activities which were consistently found to be enriched in this preparation: K^+-dependent *p*-nitrophenyl phosphatase (K^+-NPPase, 20-fold), and γ-glutamyl transpeptidase (GGTPase, 26-fold).

Recovery of Plasmalemmal K^+-NPPase

K^+-dependent NPPase is a conveniently assayed, sodium-independent activity, in most cases attributable to Na^+/K^+ adenosine triphosphatase (42); the assay is especially advantageous when monitoring levels of this enzyme in ouabain-resistant rodent tissues and cell fractions (43). To establish that the rat parotid K^+-NPPase is indeed due to the Na^+/K^+ ATPase, and not merely a K^+-activated phosphatase such as that reported by Forte et al. (44) and others, we have examined a parotid homogenate and its derived plasmalemmal fraction for ATPase and NPPase activities. As shown in Table III, both activities are obtained in the same yield in the plasma membrane fraction. Hydrolysis of either substrate in the absence of K^+ dramatically reduced both activities to background levels (attributable to Mg^{2+} dependent ATPase and phosphatase, respectively). To ascertain further that the plasmalemmal ATP-

TABLE I

Distribution of Putative Plasmalemmal Markers Not Significantly Enriched in the Plasma Membrane Fraction

Sample	Alkaline phosphatase		5′-Nucleotidase		Leucyl aminopeptidase	
	*% Rec**	*RSA§*	*% Rec*	*RSA*	*% Rec*	*RSA*
Homogenate	100 (3.14)	1.0	100 (12.4)	1.0	100 (4.14)	1.0
S_4	76.9	0.80	87.5	0.91	86.1	0.90
P_4	19.5	2.57	10.4	1.36	8.01	1.05
S_5	1.04	0.30	2.70	0.78	4.05	1.16
P_5	15.0	4.34	6.12	1.76	4.78	1.38
Interface band	0.38	0.85	0.88	1.99	0.93	2.10
Resuspended pellet	9.52	3.66	6.99	2.69	4.06	1.56
Plasma membranes	0.31	1.40	0.88	3.82	0.27	1.18
Rest	0.12	0.71	0.11	0.64	0.29	1.74

* Recoveries in each fraction represent the mean value from a minimum of four experiments. Numbers in parentheses represent total homogenate activities (see Materials and Methods).

§ RSA; Specific activity relative to that of homogenate.

TABLE II

Distribution of Protein and Enzyme Activities Associated with Plasma Membranes During Fractionation of Rat Parotid

Sample	Protein		K^+-NPPase		GGTPase	
	*% Rec**	*mg*	*% Rec*	*RSA‡*	*% Rec*	*RSA*
Homogenate	100	398.3	100 (6.52)§	1.0	100 (5.95)	1.0
S_4	95.7	381.3	69.3	0.72	70.9	0.74
P_4	7.60	30.26	23.3	3.07	22.4	2.94
S_5	3.49	13.89	7.85	2.24	8.18	2.36
P_5	3.46	13.78	19.8	5.72	10.8	3.11
Interface band	0.44	1.76	5.15	11.8	8.16	18.5
Resuspended pellet	2.60	10.35	12.4	4.78	4.34	1.67
Plasma membranes	0.23	0.91	4.60	20.1	5.89	25.6
Rest	0.17	0.66	0.34	2.04	0.60	3.66

* Recoveries in each fraction represent the mean value from a minimum of five experiments.

‡ RSA; Specific activity relative to that of homogenate.

§ Numbers in parentheses represent total homogenate activities (see Materials and Methods).

TABLE III

*Comparison of Rat Parotid K^+-dependent NPPase to Na^+-dependent K^+-ATPase **

Sample	NPPase			ATPase		
	μmol/min	Recovery	Ouabain inhibition	μmol/min	Recovery	Ouabain inhibition
		%	%		%	%
Homogenate	6.34	100	—	24.2	100	—
Plasma membrane fraction	0.525	8.28	89.1	1.97	8.14	94.0

* Fractions from a rat parotid plasma membrane preparation were assayed at 37°C in the presence of 50 mM Imidazole HCl pH 7.4, 5 mM $MgCl_2$, and either 3 mM Tris-ATP or 3 mM Tris-NPP. ATPase was measured by a difference value ±90 mM NaCl in the presence of 5 mM KCl; K^+-NPPase was measured by a difference value ±KCl in the absence of NaCl. Ouabain concentration: 2 mM.

ase also requires Na^+ (NPPase does not), we measured ATPase in the presence and absence of this cation, demonstrating a clear sodium dependence. Finally, ouabain inhibited both activities to comparable degrees, yet residual hydrolysis of either substrate was detectable even at a dose of 2 mM, confirming the rat enzyme's known relative insensitivity to ouabain (43).

We have determined that the pH optimum of the rat parotid K^+-NPPase is in the pH range 7.5–8.0, agreeing with that classically determined by Bader and Sen for Na^+/K^+ ATPase (45); this enzyme is also slightly stimulated in the presence of EGTA suggesting that there is no contribution by a Ca^{2+} dependent activity. Approximately 50% of the NPPase activity at pH 7.5 in the parotid homogenate can be attributed to the K^+-dependent enzyme, while the plasma membrane fraction's K^+-dependent activity is ~93% of its total NPPase. This implies that our preparation not only purifies the K^+-stimulated enzyme away from other proteins generally, but away from other NPPases such as nonspecific alkaline phosphatase, which may (see Discussion) be attributable to cell types other than the acinar cell.

Recovery of Plasmalemmal GGTPase

GGTPase levels are determined by monitoring hydrolysis of γ-glutamyl *p*-nitroanilide with transfer of the γ-glutamyl moiety to a peptide acceptor, thus liberating the chromogenic product, *p*-nitroaniline. Using this assay we obtained similar results to those found in other systems (46): the rat parotid enzyme was completely inhibited in the combined presence of 5 mM serine and 10 mM borate; its activity was comparable in both detergent-treated and frozen-thawed samples; and it converted substrate to product at a constant rate for several minutes over a broad range of enzyme concentrations.

Greater than 5% of the homogenate's GGTPase activity could be found in the plasma membrane fraction. Furthermore, the initial distribution of the homogenate's GGTPase and the K^+-NPPase into S_4 and P_4 are strikingly similar. However, the step which results in the greatest plasmalemmal purification, namely the sucrose step-gradient, appears to partially resolve the two plasma membrane activities. Of the K^+-NPPase present in P_5, only 25% floats to the interface band containing the enriched plasmalemmal fraction. In contrast, the GGTPase P_5 partitions such that >70% floats to that interface (while the remainder is found in the resuspended pellet fraction). Consequently, although both of these enzymes appear to mark rat parotid plasma membranes, their distributions somehow differ within the cell and/or the tissue.

Enzyme Activities of Secretion Granule Membranes

Given the specific and exclusive interaction of secretion granule and apical plasmalemma in membrane fusion during exocytosis, we thought it possible that these partner membranes might show compositional similarities. Consequently, we examined preparations of rat parotid secretion granules for three membrane-associated activities found in our plasmalemmal fraction, in the hope of better explaining the distribution of these enzymes within the plasma membrane. Our secretion granule fraction is thought to be representative of the total granule population, since the purified fraction exhibited 22.8% of the tissue amylase activity.

This fraction also displayed 7–8% of the homogenate's GGTPase, ≤0.4% of the homogenate's K^+-NPPase, and 5'-nucleotidase levels that were below detection (essentially 0%). In the two experiments in which the isolated secretion granules were subjected to gentle lysis in a medium containing KCl (47), GGTPase but no K^+-NPPase was found to be associated with the subsequently derived secretion granule membranes.

Contamination of the Plasma Membrane Fraction

Table IV shows the distribution during plasmalemmal purification of the activities of enzymes generally considered to mark organelles other than the plasma membrane. Two of these enzymes, β-*N*-acetyl glucosaminidase and α-amylase, are organelle content proteins and were selected because membrane protein markers have not as yet been described for parotid lysosomal nor granule membranes, respectively. No single enzyme representing organelle contaminants of the plasmalemmal fraction exceeds 0.4% of the homogenate activity.

The secretion granule content marker, α-amylase, is removed >99.99% from isolated plasma membranes. However, as data indicate that amylase is more easily solubilized from membranes than are other parotid granule content proteins (48), we decided to estimate comprehensively the extent of adsorbed secretory proteins by a radioactive mixing experiment. ^3H-labeled rat parotid lobule secretion was prepared as described in Materials and Methods; 8.09×10^6 cpm of this secretory standard was added to 5.55 g rat parotid tissue just before homogenization and routine plasma membrane preparation. Of the 7.8×10^6 cpm measured in the homogenate fraction, only 1.6×10^3 cpm or 0.02% of the label could be found in the final plasmalemmal material. This low degree of contamination by secretory protein is still some 2.5-fold higher than the plasmalemmal α-amylase recovery, suggesting a greater (yet still minor) contamination by other secretory proteins.

For cytochrome *c* oxidase (mitochondrial inner membrane), 0.27% of the homogenate's activity was located in the plasmalemmal fraction. Quantitatively comparable results were obtained for monoamine oxidase (outer mitochondrial membrane); 0.29% of the homogenate activity was found in the plasmalemmal fraction (with slight over-recovery of the homogenate activity found in the summed fractions—109%).

Lysosomal contamination as judged by the yield of β-*N*-acetyl glucosaminidase, was also on the order of 0.3%. Although the recovery of UDP-galactosyl transferase (Golgi) is exceptionally poor in the last step of the isolation, the recovery of thiamine pyrophosphatase, another Golgi marker in this tissue (49) appears to corroborate the ~0.4% level of contamination.

Rough microsomes were monitored by assaying their (rotenone plus cyanide)-insensitive NADH cytochrome *c* reductase activity. Although this enzyme has also been ascribed to mitochondrial outer membranes, Golgi membranes (50, 51), and even plasma membranes of some systems (52, 53), we have taken the 1.76-fold enrichment of this enzyme to indicate primarily a contamination by endoplasmic reticulum. Presumably this enzyme's recovery actually represents the sum of contamination by a variety of organelles. Electron microscopic examination, however (see Fig. 2), confirms rough microsomes

TABLE IV

Marker Enzyme Analysis of Contaminating Organelles in Plasma Membrane Preparation

Sample	Amylase		Cytochrome *c* oxidase*		β-*N*-acetyl-glucosaminidase		UDP-Galactosyl transferase‡		NADH-Cytochrome *c* reductase	
	% Rec§	RSA‖	% Rec	RSA	% Rec	RSA	% Rec	RSA	% Rec	RSA
Homogenate	100 (139,011)¶	1.0	100 (61.5)	1.0	100 (5.26)	1.0	100 (2.64)	1.0	100 (4.45)	1.0
S_4	98.2	1.03	77.1	0.81	93.7	0.98	95.3	1.0	90.4	0.94
P_4	1.1	0.14	15.0	1.97	6.84	0.90	8.0	1.05	9.39	1.24
S_5	0.88	0.25	10.8	3.09	4.02	1.16	4.86	1.39	10.2	2.92
P_5	0.18	0.05	2.65	0.77	1.12	0.32	4.48	1.29	3.18	0.92
Interface band	0.014	0.03	0.54	1.23	0.20	0.44	1.36	3.09	0.89	1.98
Resuspended pellet	0.16	0.06	1.54	0.59	1.31	0.50	2.54	0.98	3.64	1.40
Plasma membranes	0.008	0.04	0.27	1.17	0.29	1.26	0.39	1.70	0.40	1.76
Rest	0.003	0.02	0.04	0.25	0	0	0.05	0.28	0.31	1.90

* Cytochrome oxidase; arbitrary units (see reference 31).
‡ Galactosyl transferase units, nmol/min.
§ Recoveries in each fraction represent the mean value from a minimum of three experiments.
‖ RSA; Specific activity relative to that of homogenate.
¶ Numbers in parentheses represent total homogenate activities (see Materials and Methods).

as the major organelle contaminant of the plasma membrane fraction.

Rough Microsomal GGTPase Contribution to Plasmalemmal Fraction

Because of the preceding contamination analysis, and because cytochemical data obtained from rat pancreas has suggested a partial localization of GGTPase in the rough endoplasmic reticulum (54), we prepared a fraction of highly purified rough microsomes to ascertain how much of the GGTPase activity of our plasmalemmal fraction could be attributed to microsomal contamination. Microsomes were prepared as described in Materials and Methods; Fig. 3 shows typical low and high power electron micrographs of the purified fraction. At all depths from the surface of fixed pellets of microsomes, virtually no organelle contamination of this fraction was visible by electron microscopy. Preparations of microsomes and plasma membranes were assayed side by side for both GGTPase and NADH cytochrome *c* reductase activities. The result of one such representative experiment is presented in Table V. To estimate the maximal contribution of GGTPase associated with rough microsomes to the total activity of the plasmalemmal fraction, we have assumed first, that all of the NADH-cytochrome *c* reductase activity of the plasmalemmal fraction can be ascribed to microsomes, and second, that the microsomal fraction is contaminated to a negligible extent by other organelles. Consequently each unit of microsomal NADH cytochrome *c* reductase present in the plasmalemmal fraction would correspond to a contribution of 0.05 U of microsomal GGTPase within the plasmalemmal fraction's total GGTPase activity. Thus at most, 0.33% of the GGTPase activity of the plasmalemmal fraction can be attributed to microsomal contamination. We think it likely that the majority of the remaining 99.67% of the activity represents bona fide plasmalemmal GGTPase, although some contribution by contaminating organelles other than microsomes cannot be excluded (see Discussion).

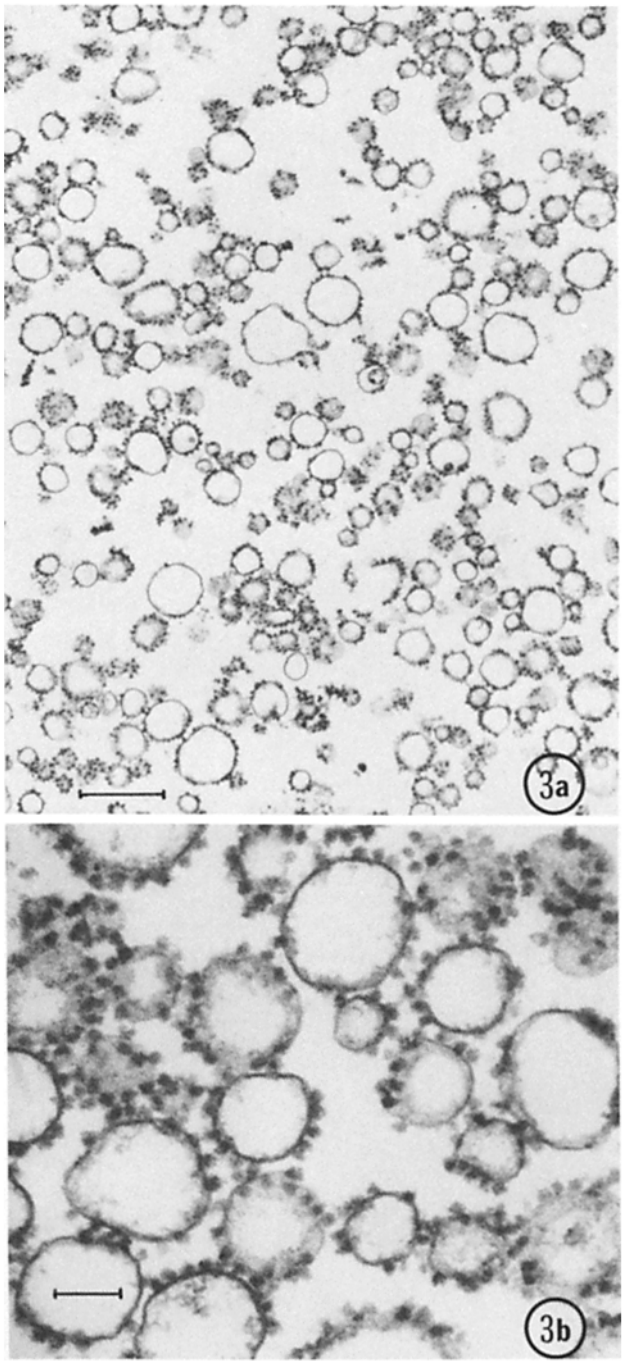


FIGURE 3 The rough microsomal fraction obtained from rat parotid viewed at low (*a*) and high (*b*) magnification. Some degree of aggregation is apparent. The top, middle, and bottom of microsomal pellets have a homogeneous appearance with no visible organelle contamination. (*a*) Bar, 0.5 μm. × 22,000; (*b*) Bar, 0.1 μm. × 85,000.

TABLE V

Comparison of Enzymic Activities in Rough Microsome and Plasma Membrane Fractions

Enzyme activity*	Specific Activity: Plasmalemmal fraction	Specific Activity: Rough Microsome fraction
GGTPase	0.681	0.0052
NADH cytochrome *c* reductase	0.045	0.1056

* Enzyme assays and units of activity are as described in Materials and Methods. Specific activity is expressed as μmoles of product formed/min per μmole lipid phosphate. Data shown is from a single representative experiment.

It is interesting to note that while the specific activity (normalized to lipid phosphorus)[1] of GGTPase is more than two orders of magnitude greater in the plasmalemmal fraction than in the microsomal fraction, the microsomal NADH cytochrome *c* reductase specific activity is only 2- to 3-fold greater than the comparable plasmalemmal specific activity. Although other preparations of the two organelles made side-by-side exhibit somewhat higher ratios of microsomal/plasmalemmal NADH cytochrome c reductase, we feel that this generally low value suggests a reductase activity endogenous to the plasma membrane; also evident in rat liver plasma membrane (52, 53) and Golgi fractions (50, 51).

DISCUSSION

A distinguishing characteristic of epithelial glands is that discharge of exocrine secretory products takes place selectively at the apical cell surface. Electron microscopic studies of isolated parotid plasma membranes show that our fraction includes many apical surfaces adjoined by junctional elements such that

[1] The specific activity measured per unit protein is inappropriate because ribosomal protein is expected to alter the microsomal value selectively. We feel that lipid phosphate is a better estimate of total membrane surface (Table V).

profiles resembling the borders of luminal spaces *in situ* are frequently preserved. Vigorous homogenization and resuspensions during the isolation procedure are required to disrupt connective tissue and reduce membrane aggregation resulting in a large degree of membrane vesiculation seen by electron microscopic observation of fractions S_4 and S_5 (data not shown). Although these disruptive forces reduce the yield of large plasmalemmal fragments, it is our impression that the plasma membrane sheets ultimately obtained by our fractionation scheme tend to be enriched for the secretory surface to a greater degree than for the basolateral plasmalemma. These sheets are likely to be derived from acinar secretory cells (as opposed to other cell types) for the following reasons. First, an estimated 85–90% of the parotid gland volume consists of acinar cells. Consequently, the vast majority of plasmalemmal surface, especially that present in a filtered tissue homogenate, is expected to derive from secretory cells rather than from ducts (since the ducts are larger and more heavily invested with connective tissue, they are expected to be removed to a greater degree by filtration). Second, multiple luminal profiles are often observed in individual sheets of isolated plasma membrane. These profiles are mostly <2 μm in diameter and are largely formed by the apical surfaces of two or three cells. Such an organization most closely resembles the tubular secretory canaliculi constituting the free surface of parotid acinar cells as described for rat and mouse parotid by Parks (55). Therefore, despite the low yield of the isolated fraction, we have assumed as a first approximation that this fraction represents the surface of most, if not all, rat parotid acinar cells.

Biochemical analysis of our fraction suggests that the parotid acinar cell plasma membrane may be enriched with a different enzymic library than are the surfaces of other cells, such as the rat hepatocyte. Consequently, our results contrast with those of workers who have relied on compositional analogies to other tissue types as the basis of interpretation of biochemical data relating to the origins of parotid smooth membrane vesicles (33, 34).

Significantly, histochemical studies of alkaline phosphatase and 5′-nucleotidase in the rabbit parotid (56) have indicated that both of these activities are located primarily in ducts and only secondarily, if at all, on acinar cells. Furthermore, rat parotid alkaline phosphatase has been found to reside exclusively in the capillary endothelium and on the plasma membranes of myoepithelial cells located in the intercalated ducts (57); a more recent cytochemical study demonstrated a similar localization for 5′-nucleotidase (58). These reports appear to be in agreement with the largely negative biochemical findings on these two enzymes presented in Table I.

Our identification of GGTPase in the parotid gland confirms a recent report by Hata (59). This enzyme, first described by Hanes et al. (60) has recently been claimed to possess a variety of catalytic functions involving amino acid, peptide, and glutathione metabolism (61). It has received attention due to the unusually high activities found in kidney from several animal species, e.g. rat, sheep, cow, pig, and man. Further, GGTPase activity has been found in most of the tissues that have been tested; concentrated especially in the mucosal surface membranes of epithelia involved in transport, absorption, and secretion such as the jejunal villi, proximal renal tubules, and epididymis (for a more complete description, see reference 62). Additionally, GGTPase has been shown to be a membrane bound ectoenzyme, not only by histochemistry (54) and immunocytochemistry (63), but also by the demonstration that the enzyme can be released from the brush borders of intestinal and renal cells by treatment with papain (64).

Although no unifying hypothesis concerning this enzyme's physiologic role in such diverse tissues has been confirmed, for those cell types in which structural analogies of the parotid can be drawn, evidence suggests a cytologic localization of GGTPase which is enriched at the luminal surface. The present study extends these findings to show that in the rat parotid gland, this enzyme is also present on secretion granule membranes as well. Our data show also that Na^+/K^+-ATPase (K^+-NPPase), another plasma membrane enzyme, which in other polarized epithelia is found to reside exclusively at the basolateral membrane surface, is absent from these same secretion granule membranes. Furthermore, the plasmalemmal isolation scheme presented in this report is able to distinguish a marked difference in the distributions of the two plasma membrane markers; GGTPase primarily floats to the interface band of the sucrose step gradient, whereas most of the Na^+/K^+-ATPase activity is associated with the pellet. Electron microscope observations of the latter fraction, which reveal fragmented cellular membranes closely apposed to extensive sheets of basal lamina (data not shown), suggest that the resuspended pellet is enriched in basolateral surface.

Taken together, we feel that these data strongly support the hypothesis that the plasmalemmal localization of GGTPase is primarily restricted to the apical domain in unstimulated rat parotid acinar cells, and that Na^+/K^+-ATPase is primarily a basolateral plasma membrane marker. We are now preparing to use immunocytochemical techniques to directly confirm the above hypothesis.

The preliminary biochemical evidence presented in this report points to a compositional overlap between the fusion partner membranes involved in exocytosis in the rat parotid gland. This conclusion contrasts with that of others who have stressed the uniqueness of these two membrane types in other systems (nerve terminal [65]; adrenal medulla, [66]; sea urchin egg [67]). Several points are pertinent in evaluating this difference. First, high levels of organelle cross-contamination in the isolated fractions may bias the interpretation of data. This appears unlikely for our plasmalemmmal preparation since the hyposmotic homogenization procedure is expected to lead to the removal of the contents and membrane fragments of lysed secretion granules into the supernatant fractions S_4 and S_5. Second, secretion granule membrane fractions have long been noted for their contamination by residual secretory proteins (48, 68). Consequently, enzymatic activities, antigens, or polypeptides viewed as SDS gel bands that are associated selectively with granule membranes and not with the plasmalemma must be rigorously shown to be bona fide membrane constituents, before being rated as unique. Third, uniqueness and overlap of membrane constituents are not of necessity mutually exclusive, and the extent of each may vary between cell types. Our results support the compositional overlap of GGTPase in parotid secretion granule and plasma membranes; however, the reasonably small amount of activity and large surface area of secretion granule membranes, suggest that the GGTPase specific activity of these membranes is likely to be lower than that of the cell surface. Therefore, the overlap of GGTPase between the parotid fusion partner membranes may be qualitative but not quantitative.

The extent and generality of compositional overlap between the two membranes under investigation may have broad significance with regard to luminal membrane biogenesis and

turnover. GGTPase may prove to be a useful probe in parotid acinar cells for monitoring the movement of intracellular membrane constituents both before and after exocytosis.

Contamination of the plasmalemmal fraction by other organelles only minimally alters the strength of conclusions which may be drawn from the data presented. Clearly, rough microsomal elements do not contribute very much GGTPase to either the plasma membrane fraction or to the cell itself. However, as the recovery of UDP-galactosyl transferase in the final step of the plasmalemmal isolation is incomplete, and in the absence of a purified parotid Golgi fraction, we cannot rule out a small but possibly significant Golgi contribution to the GGTPase activity of parotid acinar cells and of our plasma membrane fraction.

Finally, it is apparent that these plasma membranes have maintained many of their metabolic capabilities, as judged by the excellent stepwise recoveries of their diverse enzymatic functions throughout the isolation procedure. We have not however, analyzed either the extent of lipolysis or the lipid composition of the plasma membrane; it will be interesting to consider such issues in future studies.

We are grateful to Dr. George E. Palade for valuable discussion and continuous encouragement throughout the course of this research. We wish to thank Hans Stukenbrok for his assistance in preparation of samples for electron microscopy, and we also thank Pam Ossorio and Cindy Davis, respectively for preparation of photographs and typing the manuscript.

This research was supported by research grants GM26524 and AM29868 from the National Institutes of Health. Peter Arvan is supported by GM07205 and these studies constitute work in progress toward fulfillment of the requirements for the Ph.D. degree.

Preliminary reports on these findings were presented at the twenty-first annual meeting of the American Society for Cell Biology (69).

Received for publication 7 April 1981, and in revised form 21 June 1982.

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