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Chemiosmotic Properties of Isolated Secretory Granules from Parotid Glands of Normal and Isoproterenol Treated Rats

Peter Raymond Arvan
Yale University.

CHEMIOSMOTIC PROPERTIES OF ISOLATED SECRETORY GRANULES FROM PAROTID GLANDS OF NORMAL AND ISOPROTERENOL TREATED RATS

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PETER ARVAN

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ABSTRACT

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Secretory granule fractions were prepared isoosmotically from the parotid glands of normal rats and from rats that had received repeated injections of isoproterenol. These granules, purified extensively, are stable after isolation, making it possible to study biophysical parameters which may relate to their role in intracellular transport and secretion.

Normal parotid granules have an internal pH of ~6.8 and a low ionic permeability with respect to internal buffering capacity. By contrast, granules from the treated rats have an internal pH ranging from ~7.7 in (0.3 M) sucrose media to ~7.1 in (100 mM) KCl media; suggesting a large ionic permeability with respect to buffering capacity. These intragranular pH values are the highest reported for any secretory vesicle type. The major content protein within control granules is α-amylase ($pI \leq 7$), while the major species in granules from the treated rats are the proline-rich proteins ($pI > 10$). Intragranular pH values probably differ because of these compositional differences.

Normal parotid granules have a low H^+ permeability and cannot utilize ATP either to acidify their interior or to create an inside-positive membrane potential. ATP-stimulated parotid granule lysis (cited by others as evidence implicating a granule H^+-ATPase) is mimicked entirely by non-hydrolyzable ATP analogs, and thus most likely does not depend on ATP hydrolysis.

Parotid granules isolated from isoproterenol-treated rats, however, exhibit a measurable ATP-dependent acidification which is abolished by proton ionophores. The granules are also able to utilize ATP in creating a more inside-positive membrane potential. These effects depend on ATP hydrolysis.

The underlying mechanism which permits the expression of electrogenic H^+-translocating ATPase activity in these granules (but fails to do so in the case of normal parotid granules) has not yet been found. An hypothesis is presented from which a strategy in approaching this question may be planned.

Dedication

To Dave Castle, my friend and adviser

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ABBREVIATIONS

$[K^+]_{in}$	= intragranular potassium
pH_{in}	= intragranular pH
$[K^+]_{out}$	= potassium concentration of the medium
pH_{out}	= external medium pH
ΔpH	= $pH_{out} - pH_{in}$
$\Delta\mu_{H^+}$	= transmembrane electrochemical potential for H^+
$\Delta\pm$	= transmembrane electrical potential
MES	= 2-(N-morpholino) ethanesulfonic acid
PIPES	= piperazine-N, N'-bis (2-ethanesulfonic acid)
HEPES	= N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid
MOPS	= morpholinopropanesulfonic acid
TPP^+	= tetraphenylphosphonium cation
Nbd-Cl	= 7-chloro-4-nitrobenz-2-oxa-1,3 diazole
AMP-PNP	= Mg^{++}-β -imidoadenosine-5'-triphosphate
CCCP (FCCP)	= carbonyl cyanide p-tri chloro (fluoro) methoxy phenyl hydrazone
EGTA	= [Ethylene-bis (oxyethylene-nitrile)] tetracetic acid
GAG's	= glycosaminoglycans
PRP's	= proline-rich proteins

INTRODUCTION

GENERAL REMARKS

The continued attempts of biologists to redefine complex and dynamic cell functions in terms of ordered sequences of distinct stages, so far represent our best efforts in understanding cellular machinery and its mechanisms of action. The process of cellular protein secretion is comprised of a sequence of stages within membrane-bounded compartments in which exportable macromolecules are initially sequestered, chemically modified, and concentrated. The termination of this sequence is exocytosis, the fusion of vesicular secretory organelles with the plasma membrane, resulting in expulsion of intravesicular content into the extracellular space (1). This is neither the sole intracellular fusion event, nor is it used to achieve exclusively one task (e.g., communication, remodeling of the environment, excretion, etc.). Rather, all cells have adapted it to suit their own purposes (2).

The investigations to be described in this dissertation stem from an underlying interest in the exocytotic mechanism. One advantage in this pursuit is the availability of cell types for study which have become so specialized in their secretory capacity, that they retain a population of intracellular secretory vesicles (or granules) as a storage compartment. Since the cell surface membrane (which in polarized cells may be subspecialized) and secretory vesicle membranes are fusion partners in exocytosis, such cells offer two important opportunities: 1) to observe <u>in situ</u> each of the fusion partners before as well as during the exocytotic event (3,4,150), and 2) to obtain and study the fusion partners as isolated fractions. I have begun to explore this latter possibility by investigating the character of plasma membranes (see Appendix) and secretory granules (to be discussed further) isolated from

the rat parotid gland. A long term objective of my studies is to obtain a detailed understanding of these fusion partners as isolated fractions, and eventually to create an assay in vitro for the physiologically relevant membrane fusion between them.

The very existence of some cells which possess a defined intracellular pool of secretory vesicles (such as the parotid gland) raises the issue that constraints may be placed on these cells, such that they are not free to release the contents of more than a few of their vesicles under resting conditions. This is in contrast to other cell types [e.g. plasma cells (171), fibroblasts (172), and less differentiated cultured cells] where secretory discharge is rapid and continuous. Despite this difference, I am assuming as a working hypothesis (for the present) that the discharge mechanism is fundamentally the same in cells of all kinds.

Following their formation at the level of the Golgi complex, the secretory vesicles can be regarded as one of the discrete stages in cellular protein secretion. However, even within this stage, secretory vesicles may possess two distinct dynamic functions. First is their ability to concentrate, package and in some instances process compartmentalized proteins. Second is their ability to accumulate, store and in some instances synthesize small molecules; this may be distinguished from the first group of functions in that it involves translocation across membranes distinct from those of the endoplasmic reticulum. Different types of secretory vesicles appear to emphasize to different degrees either (or both) of these dynamic functions.

The following selected review of morphological and biochemical information pertaining to secretory vesicles is organized in relation to

the two granule functions just described. It is intended to provide a basis for considering possible ways in which these functions might operate according to common mechanisms.

CONCENTRATION, PACKAGING, AND PROCESSING OF COMPARTMENTALIZED PROTEINS

Morphologic studies testify to the diversity of secretory vesicles in different cell types. These structures exist in a considerable range of sizes, from ~0.09 μm diameter [for synaptic vesicles of the Torpedo electric organ, (5)] to ~1.30 μm [for rabbit parotid secretion granules, (6)] and larger. Such size differences may be physiologically meaningful. With some exceptions [that may in part relate to granule maturation, (7)] secretory vesicles are spherical structures. As the radius of a sphere is increased, the volume increases in greater proportion than does the surface area. Thus, large granules provide for the release of large quanta of granule content, such as is required for salivation or secretion into the intestinal lumen, while release of small vesicles may be more useful in situations where great extracellular sensitivity to the secretion may exist (e.g., in the synapse).

The electron microscopic appearance of vesicle (granule) contents is influenced by a number of factors. Differences in chemical composition lead to variations in structural preservation by fixatives (152), retention of heavy metal stains (112), and the extent of formation (153) of macromolecular complexes [even paracrystalline arrays (10)]. Further, in some cases condensed content appears to be shrunken away from the surrounding membrane (9); however, it remains unclear to what extent this appearance reflects the exposure of granules to hyperosmotic

aldehyde-containing fixatives followed by processing solutions of lower osmolality.

Regardless of these uncertainties, the differences in electron opacity between granule types (and within the same granule type at different stages of maturation) give the impression that the extent of packaging within the storage compartment may vary (even during the life of a granule). Such an impression is further fostered by two related observations: 1) secretory granules have a higher buoyant density and protein:lipid ratio than do other organelles in the secretory process, and 2) pulse-chase/autoradiography studies in specialized secretory tissues indicate the highest concentration of autoradiographic grains over the granules. Thus it seems reasonable to consider granule formation as a packaging process that entails the gradual concentration of the compartmentalized secretory products.

Biochemical examinations of isolated secretory vesicles carried out over the past thirty years have improved our understanding of the makeup of these organelles. One of the earliest of such studies (11) concluded that since zymogen granules disappear from pancreatic exocrine tissue upon secretion, and since granules are never found in pancreatic juice, that enzymes held in the form of insoluble complexes within zymogen granules must dissolve prior to, during, or after their release into the pancreatic ducts. Since that time the concept of granule packaging has been amply rephrased, but with only minor gains in understanding of how this is accomplished. Several theories have been proposed. According to Hokin, pancreatic zymogens would be coupled to "matrix protein" in a pH dependent fashion, so that when granule content is exposed to the more alkaline pancreatic juice, the insoluble complexes would dissolve (11).

Indeed, insoluble complexes have also been reported to exist in mast cell granules (12) and in pituitary prolactin-containing granules (13), where demembranated granules (treated with detergent) still retain the sedimentation and electron dense staining characteristics of untreated granules.

Further biochemical studies of prolactin-containing granules (14) revealed the presence of glycosaminoglycans (GAG's, some of which are sulfated), which could play a role in granule packaging. Most of the sulfated GAG's, (which are approximately 50-fold less by weight than prolactin) are composed of heparan sulfate and chondroitin sulfate. Their participation in core complexation is suspected since growth hormone-containing granules [which appear to lack these same sulfated GAG's, (15)] do not sediment as demembranated cores in experiments similar to those described above. Sulfated GAG's have been localized in the zymogen granules of the guinea pig pancreas (16) [also found in pancreatic secretion (17)], and in a wide variety of other granule types (for a review of this literature, see ref. 19). Further, a pancreatic fraction rich in sulfated GAG's has been used to precipitate chymotrypsinogen A in vitro (18); it was concluded that this interaction is ionic in nature, possibly like that found in zymogen granules. The basis for such interactions can be rationalized from the results of Tartakoff et. al., which showed that pancreatic zymogen granule content proteins possess an average pI of ~9.2, whereas the major sulfated GAG in these granules was found to have a pI of ~3.4 (17). Thus at low ionic strength, charge interactions are to be expected between the two. However, the isoelectric points of secretory GAG's of other tissues are not yet established, and information about the charge of secretory vesicle content proteins is sparse.

In contrast to the preceding example, adrenal chromaffin granule content proteins have predominantly low isoelectric points (20), largely due to the acidic glycoproteins chromogranin A and dopamine β-hydroxylase (21). In addition these granules are known to contain sulfated GAG's (22); which raises the question whether such GAG's function in the same way in all secretory granules. Several lines of reasoning seem to suggest that the role of GAG's in packaging may be small in many granule types. First, the levels of these molecules vary widely, being quite low in the insoluble complex of prolactin-containing granules discussed above [It should be noted, however, that some proteoglycans (e.g. those of the extracellular matrix) are known to occupy very large hydrodynamic volumes relative to their molecular weights (23), and should similar properties apply to intragranular GAG's, the high mass ratios of protein:GAG in some granules might not be inconsistent with a role for the latter in granule packaging]. The second point is that while GAG's present in most secretory vesicles so far have not been identified with physiologically significant functions after release, the discharge of heparin from mast cells argues that GAG's in some granules must be considered primarily as secretory products destined to function externally. The third point is that previously mentioned; that content proteins would have to be overwhelmingly cationic in all granule types for precipitation with acidic GAG's to be a universal mechanism of storage.

Finally, it is probably too simplistic to view packaging as merely electrostatic immobilization, since recent evidence suggests that the contents of chromaffin granules (reviewed in ref. 24) behave more as a non-ideal solution. This is especially remarkable for this particular granule type, which is known to possess (in addition to proteins) low

molecular weight constituents at a combined concentration greater than 0.7 M (25), yet has a measured internal osmotic pressure (in isolated granules) of ~320 mOsm (26). Thus, ionic interactions may reduce the osmolality of granule contents, but the resultant granule core may not be in a solid (i.e. precipitated) form. The existence of such interactions would be expected to depend on the composition of the granule contents.

Specific Content Proteins in the Secretory Vesicle Compartment

The composition of granule content protein is unique to its cell type. Only a small fraction of these proteins observed by polyacrylamide gel electrophoresis have identified functions, and gel patterns of content proteins from different secretory vesicles bear little resemblance to each other. Of those content proteins which do have known functions, most are enzymes designed to act on substrates which they will encounter only after their discharge from the cell.

In contrast, it appears that some enzymes involved in the processing of exportable products along the secretory pathway can and do express their activities while within the storage organelles. This area remains poorly understood, since it has been difficult so far to distinguish activities in the condensing vacuoles and secretory granules from those in the Golgi stacks. Thus in specialized secretory cells, while enzymatic functions such as terminal glycosylation and sulfation are still thought to be "true Golgi" activities, further processing of proteins in the storage organelles may occur. Such processing activities include 1) a thiol-sensitive protease which appears to cleave just to the C-terminal side of two successive basic amino acid residues, 2) a carboxypeptidase B-like activity, 3) α-amidation of glycine-extended

peptides, and 4) acetylation of N-terminal amino acids. Specific examples of these activities will now be discussed briefly.

The proteolytic processing of proinsulin to insulin was initially believed to be a Golgi function (28) based on kinetic arguments; but it has been more recently established by cell fractionation studies that the majority of the converting activity is found in the insulin granules proper (29). Despite the objection that such converting activity in isolated granule fractions represents contamination by lysosomal cathepsin B; such arguments have recently been refuted by the demonstration that inhibitors of the two enzymes differ significantly (30). Fletcher et. al. (30) have further fractionated their isolated granules (by freeze-thaw lysis) into soluble and particulate subfractions. Although much of the convertase is found in the soluble supernate (in a higher specific activity), these workers believe that a smaller fraction is membrane-associated. Since it has been shown (31) that lysed granules may in some instances require vigorous treatments (not tried on the insulin-containing granules) to eliminate latency and adsorption of content proteins to membranes, further experimentation is required to define the actual fraction of the convertase that is truly bound to membranes.

Similar activities have been recently reported to be involved in the intragranular proteolytic processing of proglucagon (30), proopiomelanocortin (32), and proneurophysins (36). The characteristics of these peptidases appear so far identical to the proinsulin convertase. The possible identity of these proteases is further underscored by the elegant transfection experiments of Moore et. al. (33), in which cultured cells that normally store ACTH (a proteolytic product of pro-

opiomelanocortin) are genetically manipulated to express the proinsulin gene. These cells are able to process the proinsulin intracellularly to generate a secretable protein that appears to have the properties of mature insulin.

Since this protease activity is intragranular, yet does not catalyze the final modification in the production of mature insulin, it may be inferred that another (carboxypeptidase B-like) activity is likely to be active within storage granules as well. Such an activity has been observed in fractions of lysed insulin granules (34), adrenal chromaffin granules (35), and may be present in granules of the anterior pituitary (154). This activity has been distinguished from a similar lysosomal activity by the selective stimulation of the former by $CoCl_2$ (155). Unfortunately, these activities are at low enough levels that secreted forms of the enzymes have not yet been detected.

Still other enzymes which modify secretory proteins inside granules have been detected, such as α-amidase (37,38), as well as the N-terminal acetylation enzymes (156). All of these acitivities are of great importance in the production of active ACTH, α-MSH, and enkephalins from proopiomelanocortin. The extent to which these activities are involved in the processing of "unrelated" content proteins in other granules is not yet known. Obviously, this is an area of continued interest, as perhaps other Golgi-associated activities, such as those involved in carbohydrate processing may be found outside the Golgi stacks [It seems unlikely that the well established digestive glycosidases (e.g. α-amylase) could play a physiologic role in intragranular processing, since their high copy numbers in the granules and their substrate specificities are far more consistent with their actions as secretory species].

Finally, active enzymes may exist within the granule core which are not related to macromolecular processing. The best studied example of this is dopamine β-hydroxylase (39). However, the extent to which this protein is truly a content enzyme has been controversial; it is clearly a major component of the chromaffin granule membrane (21). Whether other intragranular activities of this class exist is not known.

ACCUMULATION AND STORAGE OF IONS AND SMALL MOLECULES

While the transmembrane movement of macromolecules is thought to be completed in the endoplasmic reticulum, transmembrane movement of small molecules occurs in (at least some) secretory vesicles. Several examples will be discussed.

The passive permeabilities of granules to cations have not been extensively studied except in isolated chromaffin granules and their membranes, where such permeabilities tend to be low (44,50). Indirect measurements in other granule types (e.g. by granule lysis) seem to indicate this same tendency. These low permeabilities have been invoked to explain the low K^+ content of some granules (48-50) despite a cytosolic level which is above 100 mM.

Perhaps the most thoroughly studied intragranular cation is Ca^{++}. Rat parotid secretory granules, the subject of the present study, are known to possess a significant amount of Ca^{++}, measured at similar levels in both granule lysates and discharged secretion (40). The level of 60 nmol Ca^{++} per mg protein can be translated into an approximate value for the total intragranular Ca^{++} concentration of >10 mM (41). Although intragranular Ca^{++} was originally postulated to participate in the maintenance of intragranular storage complexes (40), more recent experiments showing that the calcium ionophore A23187 can elicit the

removal of >90% of prelabeled (24 h) intragranular Ca^{++} without affecting granule integrity (42) apparently contradicts this view. It should be pointed out however that A23187 is known to catalyze only electroneutral exchange of Ca^{++} either for other divalent ions or for two monovalent ions [especially H^+, (43)]. Thus, the observed stability could conceivably reflect ion exchange in the postulated storage complex.

High levels of Ca^{++} are also found in adrenal chromaffin granules, estimated to be between 20 - 30 mM, or 60% of that found in whole tissue (44). Roughly comparable concentrations have been observed in isolated platelet α-granules and dense-granules (49,46) and isolated zymogen granules of the guinea pig pancreas (47); an even higher concentration was reported for isolated granules from a rat insulinoma (48). Of course, intragranular free Ca^{++} is much lower than the total granule Ca^{++} (50), but is probably still very much higher than that of the cytoplasm (49). The observations that these organelles do not appear to lose their calcium upon isolation, and that Ca^{++} is not tightly bound to granule content (42,49), seem to support the idea that granule membranes have a Ca^{++} permeability which is quite low [Such impermeability has been demonstrated clearly for the chromaffin granules (50)]. If such a low Ca^{++} permeability were a general property of secretory vesicles, then these vesicles would have to accumulate internal Ca^{++} via a primary active transport process, or alternatively, regulate Ca^{++} permeability so that influx might occur (perhaps coupled to other ion gradients).

One possible Ca^{++} uptake mechanism in chromaffin granule ghosts has been described (58) involving Ca^{++}/Ca^{++} and Ca^{++}/Na^+ exchange. This mechanism was found to be energy independent, thus it is difficult to

imagine how chromaffin granules could use this mechanism to achieve their high level of internal Ca^{++}, unless there existed a high in:out Na^{+} gradient to favor Ca^{++} accumulation (and/or major internal Ca^{++} binding). An inward-directed "sodium pumping ATPase" activity could produce such a gradient, but no such activity has been located in secretory granules, even when it has been specifically examined (122). Alternatively, Michaelson et. al. have reported ATP-stimulated uptake of Ca^{++} into cholinergic synaptic vesicles from Torpedo (59). It appears that this Ca^{++} uptake mechanism is similar to that produced by the Ca^{++}-ATPase of sarcoplasmic reticulum, since Ca^{++} transport was inhibited by mersalyl (Ca^{++} pump inhibitor) yet only slightly affected by valinomycin or FCCP (at doses of 50 μm). Further, even these effects of the ionophores on Ca^{++} transport may be complicated by the influence of shifting ion gradients on $\Delta\Psi$ or ΔpH. Nonetheless, as in the case of the secretory granules of the rat parotid, virtually all the $^{45}Ca^{++}$ accumulated by the cholinergic synaptic vesicles is releasable by addition of A23187; suggesting that most of the Ca^{++} accumulation is maintained by transmembrane transport rather than by tight binding to the vesicle interior.

Although the role of Ca^{++} in the secretory vesicle interior is not well established, there is precedent for the function of cations in intragranular packaging. Specifically, a useful model for metal ion complexes in the granule interior is that of zinc found in pancreatic β-cell granules. This ion is at high levels in the insulin-containing granules, and is known to form hexameric complexes with purified insulin (51). Further, a correlation between the pH sensitivity of these complexes to solubilization and the pH sensitivity of β-granules to lysis

has been reported (52). The generality of such complexes in secretory vesicles continues to be an open question.

In contrast to the few studies of granule cations, a large number of studies concerning granule permeabilities to anions have been reported. These permeabilities may be substantial, depending on the anion in question. In chromaffin granules and their membranes, anionic permeabilities increase in the order fluoride = acetate < chloride < iodide < thiocyanate (60), with thiocyanate permeability being 50 - 100 times greater than that of chloride (44). Some variations in the relative permeabilites have been observed, probably depending upon the conditions employed (61), but similar results have been obtained in other isolated granule types, such as the pancreatic zymogen granules (62). However, the significance of anion-selective permeability in isolated granules has not been established. Although an hypothesis involving anion influx in exocytosis has been advanced (63), more recent studies indicate that permeant anions are not necessary for exocytotic discharge to occur from stimulated (permeabilized) adrenal chromaffin cells (64).

Several secretory vesicle types possess high levels of ATP, including chromaffin granules [125 mM, (54)] and synaptic vesicles isolated from Torpedo electric organ [which contain comparable concentrations (151,53)]. Although synaptic vesicles of the central nervous system may store ATP as a bona fide neurotransmitter (55); in non-synaptic storage vesicles the ATP is thought to be an effective packaging agent (46,56), interacting with amines such as serotonin and norepinephrine as well as calcium. It may be that such packaging effects are limited primarily to those granules which accumulate large quantities of biogenic amines, since levels of ATP in granules which

specialize in macromolecular discharge [such as insulin-containing granules, (48)] are much lower than in chromaffin vesicles.

Relatively little is known about the means of accumulation of intragranular ATP. Winkler and colleagues initially reported seven years ago the existence of an ATP uptake system in partly purified adrenal chromaffin granules. These studies are based on uptake of either [^{3}H]ATP or [^{32}P]ATP into gradient fractions which contain the catecholamine peak (80). This uptake was inhibited by the addition of either 0.2 mM atractyloside (which is known to inhibit the mitochondrial ATP/ADP exchanger), 1 mM N-ethylmaleimide, or 250 μM CCCP (an extremely high dose). From these and other studies (81), these workers concluded that ATP uptake into chromaffin granules is coupled to an electrical potential across granule membranes. However, serious objections concerning the purity of the fractions studied and the reproducability of the results have not been answered to date. In contrast, Torpedo synaptic vesicles have been shown to contain a membrane protein which can be photoaffinity labeled (157) with azido-ATP (which also inhibits ATP accumulation) and which binds (158) [^{3}H]atractyloside. The mechanism of this vesicle transporter has not yet been elucidated.

Catecholamine Uptake as a Model for Small Molecule Accumulation

By far the best characterized small molecule uptake system is that for biogenic amines. Early studies of the uptake of epinephrine, norepinephrine, and dopamine into isolated chromaffin granules were interpreted to reflect accumulation within granules as a result of intragranular acidity, as in the case of methylamine (65). However, the ability of reserpine to selectively block uptake of biogenic amines (and not methylamine), and the inability of ammonium ions to alter the

transmembrane distribution of catecholamines [yet ammonium addition drastically reduces concentration of intragranular methylamine, (66)], suggests that both uptake and storage of catecholamines proceed through a mechanism distinct from that of methylamine.

Other studies showed clearly that agents which do not alter intragranular acidity but which do influence granule transmembrane potential, have marked effects on catecholamine accumulation (67). Since the transmembrane potential is influenced by the transmembrane pH gradient (68), biogenic amine uptake is likely to reflect both parameters. Yet reserpine has been shown to affect neither of these (67). The results are most consistent with the idea that unlike methylamine, which may permeate membranes directly through the lipid bilayer, catecholamines permeate granule membranes through a specific transporter, which itself is influenced by transmembrane potential and pH gradients.

Indeed, catecholamine uptake has been shown to be mediated by a specific carrier which exhibits stereospecificity, saturation kinetics, and inhibition by reserpine (69). It seems likely that this carrier is a bona fide membrane protein since the reserpine-sensitive transport activity has been solubilized from chromaffin granule membranes with detergent, and reconstituted into liposomes (71). Although the detailed mechanism of action of this carrier has not been elucidated, results presented by Johnson et. al. (70) are consistent with a model of translocation in which the amine with no net charge is accumulated in exchange for the efflux of a single proton; or one in which a protonated amine is exchanged for two internal protons. Both models predict that amine uptake will be coupled to transmembrane ΔpH and $\Delta\Psi$.

Scarpa and colleagues have also shown that addition of ATP to chromaffin granules results in inward proton translocation (to be discussed further in the following section), which enhances their ability to do chemiosmotic work such as catecholamine accumulation (45). The presence of substantial amounts of thiocyanate (permeant) ion fails to influence ΔpH while $\Delta\Psi$ (inside positive) is collapsed. By contrast, ammonia entry into the granules reduces ΔpH but increases $\Delta\Psi$. These observations indicate that the result of H^+ pumping can be manifested either as a ΔpH or a $\Delta\Psi$, depending on the experimental conditions used. Other experiments have shown that proton ionophores block not only the ATP-dependent generation of an inside positive $\Delta\Psi$ (67), and increases in ΔpH (95), but also the uptake of catecholamines as well (45). Quantitative studies of such uptake into "ghosts" of chromaffin granules have established firmly that the amine accumulation is driven by a chemiosmotic force equivalent to $\Delta\Psi - 2\Delta pH$. Therefore, catecholamine accumulation is critically dependent on energy coupling in the membrane and is markedly enhanced by the addition of ATP.

Since chromaffin secretory vesicles are known to possess $\sim$0.5 M catecholamine (54), and cytosolic levels have been estimated at $\sim$20 μM (24), a catecholamine concentration ratio of $\sim$25,000:1 (in:out) must exist in the cell. The magnitude of this accumulation has been cited as proof for intragranular storage complexes (96). However, it is evident that a ΔpH of 1.7 units [the internal pH of granules *in situ* is 5.3, (93)] and a steady state $\Delta\Psi$ of $\sim$60 mV [approximately this value is obtained *in vitro*, (45)] would result in a net driving force for amine accumulation of $60 - 2(-60)(1.7) = 264$ mV (at 25° C); the catecholamine concentration ratio (in:out) resulting from this potential energy is in fact 25,119.

Similar chemiosmotic forces drive uptake of serotonin in isolated platelet granules (72) [and membrane vesicles derived therefrom (73)] and in synaptic vesicles (74,85). Thus, such a transporter may be a general property of granules which store large quantities of biogenic amines. As a result of this facilitated accumulation mechanism, uptake of biogenic amines should be favored in secretory organelles over other organelles which possess an acidic interior.

That ATP hydrolysis is a major energy source for this carrier-mediated, FCCP-sensitive amine uptake is demonstrated by the experiments of Aberer et. al. (81) in which norepinephrine uptake into chromaffin granules was substantially inhibited by the presence of β,γ-imidoadenosine triphosphate (AMP-PNP). This ATP analog cannot be used as a substrate for the granule H^+-ATPase (97), and thus it serves as a very useful tool to distinguish energy transduction (ion conduction obligatorily coupled to ATP hydrolysis) from other ATP dependent effects.

Non-amine transport systems in secretory vesicle membranes are less well documented, and studies are mostly restricted to isolated chromaffin granules and their membranes. One such example is the electron transport system mediated by cytochrome b-561 (75), also known as chromomembrin B (76). Evidence has been presented that this transmembrane protein is a bona fide granule constituent and is the second most abundant protein in the chromaffin vesicle membrane (77). Njus and colleagues have recently demonstrated that this cytochrome is involved in transmembrane electron transfer reactions, using ascorbate as a redox partner (75). A similar protein to chromomembrin B has been found in

isolated vesicles from sympathetic nerves in the rat vas deferens (78) and in platelet dense granules (79).

A POSSIBLE RELATIONSHIP BETWEEN MACROMOLECULAR PACKAGING AND SMALL MOLECULE ACCUMULATION: OSMOREGULATION AND THE GRANULE H^+-ATPase

The limited number of known functions of secretory vesicle membranes may indicate that these vesicles are simpler than many other organelles [perhaps reflected by their low protein:lipid ratio; e.g., see ref. 82]. Indeed, the overriding functions of these organelles are two: storage and secretion. While specific macromolecules of the granule membrane have been postulated to confer secretory capability (83), proteins of the granule membrane have not yet been proposed to influence storage/packaging, and this mechanism remains a mystery. Nevertheless, granule membranes or their immediate precursors are good candidates to be involved in the concentrating process thought to occur in condensing vacuoles (2).

Energy Could be Involved in Granule Maturation

Jamieson and Palade (8) reported that pancreatic condensing vacuole "conversion" to mature zymogen granules (measured in granule fractions) was inhibited ~14% when cellular ATP levels were reduced to ~5% of that of controls. A far more substantial inhibition (~43%) was seen by quantitative autoradiography of pancreatic slices *in situ*. Even this level of inhibition is less than the "lock-gate" effects seen in ATP-dependent intracellular transport from endoplasmic reticulum to Golgi complex (2). However, in view of limitations in the quantitation process noted by these workers (8), we cannot be certain to what extent the condensing vacuole conversion process is independent of cellular energy, or whether it merely requires energy at lower threshold levels or in other forms.

We do know that in many tissues, roughly spherical condensing vacuoles are larger in diameter than their mature granule counterparts. Therefore, simple geometric consideration require that the surface area of maturing condensing vacuoles must be reduced to a considerable degree, and their volume must be reduced to an even larger degree. The surface area reduction has long been postulated to occur by removal of membrane in the form of coated vesicles (84), however, the small radius of these vesicles implies that they cannot play a significant role in volume reduction. It is generally assumed that this latter process must be achieved by efflux of water from the newly-formed granules.

Indeed, water extrusion has been postulated to occur (in an energy-independent manner) as a result of reduced osmotic activity following the aggregation of granule proteins into an insoluble matrix (2). However, some simple calculations indicate that such protein aggregation could account for only a small part of the water extrusion process. Measurements of exchangeable internal water space of secretory vesicles (at approximately isotonic conditions) vary from ~2 µl H_2O/mg protein [cholinergic synaptic vesicles, (85)] to ~4 µl/mg protein [neurohypophyseal granules, (86)] and ~4.5 µl/mg protein [chromaffin granules, (24)]. Pollard et. al. reports that water inside chromaffin granules that exchanges with ^3H_2O [4.35 µl/mg protein, (87)] is much less than the total intragranular water space of ~12 µl/mg protein (87); thus, the water space measurements cited may be underestimates. Using 5 µl/mg protein as a reasonable value and 25 kilodaltons as an estimate of the average protein molecular mass, then the average protein concentration within the hypothetical secretory vesicle is 4×10^{-8} mol/5×10^{-6} liter = 8 mM, providing all protein molecules are free in solution. Should these molecules form an insoluble aggregate, their combined

concentration should decline only by this amount. If the internal water volume were higher than 5 µl/mg protein [as claimed by Pollard et. al., (87)] or the average protein molecular mass were higher than 25 kD, then the combined intragranular protein concentration would be less than 8 mM. If we assume the other extreme for internal water volume (i.e., 1 µl/ mg protein), then this concentration would still be only ~40 mM, and should contribute only ~40 mOsm assuming ideal behavior. In the event of non-ideal behavior, Njus et al (24) report that the osmotic contribution of (chromaffin) granule core constituents is not higher, but in fact either equal to or lower than that predicted in ideal solution.

In the rat incisor ameloblast, Leblond and co-workers have claimed that condensing vacuole surface area is reduced by ~ one third in the process of granule formation (84); this corresponds to a volume reduction of ~50%. It does not seem possible that a decrease in osmotic activity of 8 - 40 mOsm could result in a halving of the condensing vacuole volume. Such a large volume (water) reduction requires a commensurate decrease in the number of osmotically active particles -- more likely to be small molecules and/or ions.

Mechanisms Controlling Transmembrane Water Flow

Studies over the past 30 years have revealed that organelles are able to use the concentration gradients of selected molecules across their limiting membranes to regulate water flow via osmotic gradients. A different concentration of the same molecule across the membrane results in a chemical gradient of stored energy. In the case of ions, different concentrations across the membrane produce both a chemical gradient and an electrical gradient. Several examples of passive processes (and concomitant water movements) utilizing these gradients will be

considered first. Each case is based on studies of secretory vesicles *in vitro*, and for this reason, only water influx (rather than efflux) will be discussed. The (chemiosmotic) conditions prevailing *in vivo* are likely to be quite different, and will be considered (as active processes) in the subsection following this one.

The first example is the observed lysis of secretory granules. Such an effect has been frequently noted in isolated granule preparations and suggests a larger number of osmotically active particles inside than out. This behavior, however, can be explained as a consequence of the membrane (Donnan) potential created by multiply-charged, non-permeant intragranular molecules (such as proteins), and its influence on the passive distributions of permeant charged molecules across the membrane. The result is an increased internal concentration of ions (cations plus anions) over that of the surrounding medium. Several deductions can be made by invoking Donnan theory to explain granule lysis (89). First, the vesicles need not have so many osmotically active impermeant molecules to induce lysis; they need have only sufficient internal fixed charges to induce ionic redistribution and development of hydrostatic pressure which can rupture the membrane. Second, this increased hydrostatic pressure can be mediated only by the presence of permeant ions; a similar incubation of vesicles with impermeant ions or in non-ionic media would not result in potential-driven transmembrane water movement (89). This latter deduction agrees well with the observation that isolated secretory granules are far more labile in salt-containing media than in sucrose media (90), even when the granules are prepared in isoosmotic solutions [obviously the osmotic stress is even greater if

granules are transferred from media of higher to lower osmolality (26), and less when the opposite transfer is effected (91)].

A second example is based on the use of ionophores to cause passive ion flux across vesicle membranes (and thus to drive water flow). Since influx of free ions will increase the osmolality of the vesicle interior, ionophore induced vesicle swelling and/or lysis is a good indicator of the magnitude and direction of ionic gradients. Thus, when isolated chromaffin granules are incubated in media containing the potassium salts of permeant anions; the addition of valinomycin greatly accelerates the rate of granule lysis (60). This suggests that the intragranular K^+ concentration is lower than that of the medium and that valinomycin enhances the inward flow of K^+ [in the presence of permeant anions, this results in an increased intravesicular water space, (44)].

Similar intravesicular volume expansion of chromaffin granules has been achieved using nigericin in a K^+-containing medium (50). Initially this result may appear surprising since nigericin can only exchange monovalent cations across the membrane, and thus is not expected to lead to increased osmotic activity in the granule interior. However, the explanation lies in the fact that nigericin is catalyzing the exchange of external K^+ for internal H^+. Since the internal buffering capacity of the granules is extremely high (66), most of the H^+ exiting the granules derive from a large reservoir of bound protons. By contrast there is no evidence of intragranular K^+ binding; thus the exchange of bound ions for free ions leads to increased internal osmotic activity and water influx. Support for this interpretation is found in the results of Phillips (44), who showed that the osmotic lysis of chromaffin granules elicited by K^+ and nigericin was independent of the anion used, whereas

valinomycin-stimulated lysis was strictly dependent on the presence of a permeant anion.

Comparable results in the swelling of chromaffin vesicles have been observed using monensin in a sodium containing medium, (92). These examples then, show that (passive) movements of ions down their concentration gradients are one means of converting chemical potential energy into a (pressure x volume) form of work on the granules, by H_2O flux.

The H^+-Translocating ATPase as an Osmoregulatory Enzyme

In each secretory vesicle type in which it has been looked for to date, an inward-directed H^+-translocating ATPase has been found. It has been recently claimed that both low intragranular pH and a membrane-bound electrogenic H^+ translocating ATPase are features common to secretory vesicles (93). The acidic interior is likely to be created both by passive proton influx [as a result of an internal-negative Donnan potential] and active influx [in which the ATP-utilizing proton pump drives electrochemical H^+ accumulation], (94). Evidence for both mechanisms has been obtained in isolated chromaffin granules, [depending upon the absence or presence of added ATP] (68). The H^+ pump attains an even greater importance in (chromaffin) secretory vesicles, since passive permeabilities to other cations are low (44,50).

A central advance in conceptualizing the mechanism of H^+-ATPases was the realization that the action of these enzymes is limited only by the total electochemical potential for H^+, i.e., the protonmotive force (88). Further, as pointed out repeatedly (45,98), the presence or absence of permeant counter-ions is the major factor in determining whether the H^+-ATPase will generate principally the chemical (ΔpH), or

the electrical ($\Delta\Psi$) component of this force. Casey et. al. have shown that the ATP-induced generation of a ΔpH by addition of permeant anions obliges water flow into the vesicle interior (99). Such a mechanism is completely analogous to the osmotic lysis experiments using KCl and valinomycin discussed previously (60), except that in the present case, an active rather than passive mechanism drives water flow (via an osmotic gradient) into the granules. Both influx mechanisms have been validated by extensive investigation.

In contrast, when permeant anions are absent, secretory vesicles may utilize the H^+-ATPase to generate large internally-positive membrane potentials. This circumstance is likely to be most representative of that inside the cell, since recent data indicate that chromaffin granules *in situ* (unlike those observed *in vitro*) are very osmotically insensitive despite their continual exposure to cytoplasmic levels of ATP and chloride (159). The "permeant anions absent" situation can be maintained only by removal of the appropriate anions or by regulating anionic permeability of the granule membrane.

The internal-positive potentials generated by the H^+-ATPase also have osmotic consequences. As noted in the discussion of the osmotic lysis experiments using nigericin and monensin, transmembrane movements of unbound cations will oblige water movements. If unbound cations are present in the granule interior, an inside-positive potential will cause those which may permeate the membrane to exit. Thus, an inward-directed H^+ pump in combination with a high internal buffer capacity could catalyze the reverse of the nigericin effect described above: inward proton movement (and binding) in exchange for unbound cations (e.g., K^+). Such an effect could reduce the internal osmotic activity and result in dehydration of the intravesicular space.

In theory then, the same H^+ pump could drive intragranular osmotic increase (with permeant anions) or decrease (without permeant anions). Such an hypothesis concerning osmotic regulation by the electrogenic H^+-ATPase is especially attractive since 1) it proposes alternate uses of the pump without increasing the complexity of the existing system, 2) it has precedent in the known regulation of cytosolic water by cell-surface ion pumps (170), 3) it provides a possible explanation for the presence of such a pump in granules which are not involved in small molecule accumulation, and 4) deductions may be drawn from it which are testable: First, it predicts that unbound cations will be found only at low levels inside secretory granules. Second, in operating to reduce internal water, H^+-ATPase activity would be useful only in the early stages of maturation of granules that function primarily in the storage of macromolecules --- whereas after extrusion of unbound cations, depletion of excess internal water, and possible complexation of the core; further H^+ pump activity would become unnecessary (providing passive permeabilities to ions remain low). In those mature vesicles which maintain high ionic permeabilities, and in those involved in small molecule (e.g. catecholamine) accumulation, sustained H^+ pump activity would be necessary. This hypothesis provides a special rationale for examining systems other than amine-secreting cells for H^+ pumps associated with secretory vesicles of both mature and immature varieties.

One additional point (previously implied) should be expressly clarified. A major distinction must by drawn between the presence of an acidic granule interior and the presence of H^+ pump activity. Maintenance of internal acidity (measured as ΔpH) in the isolated chromaffin granules has been repeatedly shown to be independent of ATP,

and even independent of the presence of proton ionophores (99,45,68). Such acidity is maintained largely by the inside-negative Donnan potential and high internal buffering capacity. This is why the isolated granules can accumulate catecholamines or Ca^{++} (with A23187) even in the absence of added ATP, and as such represents passive (facilitated) chemiosmotic work. Since many storage granules possess high protein concentrations, buffering capacity is likely to be generally high in these organelles. This further implies that the measureable internal pH may be primarily a function of the major buffering species contained within the isolated granules -- in chromaffin granules, the acid-base behavior of these species alone (ATP and chromogranin A) very accurately predicts the pH of the granule interior (24). As a result, ΔpH in chromaffin granules is unaffected by changes in $\Delta\Psi$ (45), while $\Delta\Psi$ is closely coupled to ΔpH (68). Obviously, the internal pH of secretory granules may still be influenced by the presence of permeant ions, both in isolation and in the cell. However, the internal pH of chromaffin granules within the cell has been estimated at values quite close to those for isolated granules (93). Thus, with a large internal buffering capacity, the effect of proton pumping and/or permeable ions on the intragranular pH may be one of only small magnitude.

Other Hypotheses Concerning Intragranular Acidity and H^+-ATPase

The earliest observations of the low internal pH in adrenal chromaffin granules led to several hypotheses as to its significance. In 1976, Johnson and Scarpa suggested that the ΔpH was related to catecholamine uptake (65). Later, with the discovery that insulin-containing granules and neurohypophyseal granules also possess a low pH_{in}, the "purpose" of this internal acidity was suggested to provide an

appropriate environment for proteolytic converting enzymes (with acid pH optima) to function (30). Pollard and coworkers alternatively proposed that exocytosis could be explained by ATPase-driven H^+/anion cotransport into the granules at their time of meeting the plasma membrane (91). Recently, a variation on this notion was put forward, suggesting H^+/cation countertransport as the osmotic driving force for exocytosis (100). Similarly, others claim that acidity is essential for granules to maintain internal storage complexes in an osmotically quiescent state (101).

Finally, Moore et. al. have suggested most recently that sorting of ACTH precursors into storage granules may require a low intravesicular pH (115). They showed that chloroquine treatment (which raises the internal pH in acidic compartments) of a cultured pituitary cell line results in the rapid release of ACTH precursors which apparently have not been packaged into secretory granules. This effect appears to represent an enhancement of a pre-existing non-granule secretory pathway in these cells.

Of these hypotheses, only the pH dependence of catecholamine uptake into the storage vesicles of amine-secreting cells has gained strong experimental support. Although they are not mutually exclusive, the generality and significance of other theories remain to be proved.

THE PRESENT EXPERIMENTAL SYSTEM: PAROTID SECRETORY GRANULES

Rat parotid secretory granules are perhaps less-well characterized than either the existing neuroendocrine (chromaffin) or exocrine (pancreatic zymogen) granule models. However, features which favor their study include a large size, high buoyant density (favoring purification) and low levels of proteolytic or lipolytic enzyme activities (82).

Further, these granules are abundant in the cell and undergo massive exocytosis upon stimulation with β-adrenergic agonists (102). As a result of such pharmacologic stimulation, cells can be synchronized in their restoration of intracellular granules in the secretory cycle (103).

The granule contents are largely proteinaceous, although only five enzyme activities have been characterized therein. They are α-amylase, ribonuclease, deoxyribonuclease (27) as well as peroxidase (104) and "non-specific" esterase (105) activities. In addition, the granules contain several proline-rich proteins which have no known enzymatic functions (106); other polypeptides are identified on SDS-polyacrylamide gels (82).

The rat parotid gland is known to increase in size by hypertrophy and hyperplasia when the animals receive repeated daily doses of isoproterenol (107). Under these conditions secretory granules also undergo changes of their size (increases, 108), electron opacity (decreases, 108), and content composition (109). Especially affected are α-amylase, which declines (per unit gland weight) 3 to 4 fold (109), and the proline-rich proteins, which increase about 10 - 20 fold (110). Observations of parotid secretory granules from these chronically-stimulated rats (which will be considered further in chapter 3) serve as an excellent example of the cellular control of gene expression (111), intragranular content packaging (112), and membrane biogenesis (113).

In the following chapters I intend to show that the rat parotid secretory granules provide an especially favorable system, not only for investigating the mechanism of exocytosis, but also for exploring the mechanism of granule maturation which may be common to all secretory vesicle types.

CHAPTER 1

OSMOTIC PROPERTIES AND INTERNAL pH OF ISOLATED RAT PAROTID SECRETORY GRANULES

Introduction to Chapter 1

Secretory granules are highly specialized organelles whose functions include the accumulation and storage of contents within a limiting membrane while inside the cell, and ultimately, the controlled discharge of these contents into the extracellular environment via exocytosis. Although considerable effort has been invested into elucidating the biochemical mechanisms of these processes, they are still poorly understood. Recently, however, several independent laboratories have established that many secretory granule types possess an internal pH well below that found in the cell sol. Intragranular acidity serves as a driving force for accumulation of biogenic amines (66, 114), and has been suggested to play a role in intragranular content processing (30) and storage (115), as well as exocytotic discharge (91). A wide variety of secretory granule types including those of adrenal medullary chromaffin cells [~5.5 (65)], platelets [~5.7 (116)], mast cells [~6.0 (117)], insulin-containing pancreatic islet cells [~5.9 (118)], and dense granules of the anterior- [~6.0 (119)] and posterior- [~5.8 (120)] pituitary maintain a low pH_{in} at physiologic external pH's. Many granules are also thought to exhibit common selective membrane permeabilities to specific anions such as Cl^- (44, 60, 62). These similarities have led some to believe that all types of secretory granules are acidic. If true, such generality might imply common biochemical mechanisms in the functioning of secretory granules.

The system chosen for the present study is the rat parotid gland, in which acinar cells rapidly and quantitatively secrete the contents of their granules in response to β-adrenergic stimulation (103). The parotid secretory granules, which occupy ~31% of the acinar cell volume (113), are large (~1 μm diameter), and dense, thus facilitating their isolation as a subcellular fraction by standard techniques (121). Since the ultimate goal of our laboratory is to study membrane interactions between secretory granules and plasma membranes (122) in vitro, it was of interest to learn whether the previously mentioned granule properties are also found in the rat parotid, since such properties possibly could influence the granule-plasmalemmal interaction. To this intent, I have examined the influence of medium osmolality on isolated parotid granules, their susceptibilty to lysis, their internal buffer capacity, and their internal pH. The data reveal that despite certain similarities shared by parotid and other granule types with respect to the former three properties; the parotid intragranular pH is considerably higher (~6.8). These investigations also serve as the basis for examining the effects of Mg-ATP on isolated parotid granules, which is the subject of the following chapter. Taken together, these studies lend support to the concept that neither intragranular acidity nor granule transmembrane proton pumping are important in exocytosis, and further suggest that greater differences between granule types exist than has previously been suspected.

Part of this work has been presented in abstract form (123).

Methods

Isolation of Parotid Secretory Granules - Secretory granules were purified by a modification of the differential centrifugation procedure

of Schramm and Danon (124) as follows: Parotids of 150g male Sprague-Dawley rats (starved overnight) were homogenized at 10% (w/v) in filtered 285 mM sucrose which was saturated with argon (to minimize lipid peroxidation). Homogenization was achieved in two steps, with a Tekmar tissumizer at 1900 rpm followed by 3 up-down strokes in a Brendler teflon pestle homogenizer at 1300 rpm. The sedimented pellet from a brief (35 g-min) centrifugation was rehomogenized as above, combined with the initial supernate and filtered through 1.0 mm^2 nylon mesh. This homogenate was then centrifuged at 9,400 g-min to sediment nuclei; the supernate was recentrifuged at 19,000 g-min to yield a crude granule pellet. This pellet was resuspended in an identical volume to that of the homogenate, centrifuged first at 7,500 g-min, and the supernatant fluid recentrifuged at 19,000 g-min to yield a pellet which when resuspended represented the final parotid granule fraction.

Electron Microscopy of the Secretory Granule Fraction - Granules were fixed in aldehydes, post-fixed in OsO_4, dehydrated in ethanol and embedded in Epon as previously described (121).

Biochemical Assays - α-amylase; cytochrome c oxidase; NADH-cytochrome c reductase; γ-glutamyl transpeptidase; UDP-galactosyl transferase; and β-N-acetyl glucosaminidase were all assayed as described previously (122). Protein was determined with fluorescamine as described by Udenfriend et al (125) using bovine serum albumin as a standard.

Internal Aqueous Volume and Internal pH of Isolated Parotid Granules - These determinations were performed according to Rottenberg (126) using $[^3H]_2O$/$[^{14}C]$sucrose and $[^3H]_2O$/$[^{14}C]$methylamine or sodium $[^3H]$acetate/ $[^{14}C]$sucrose in parallel granule suspensions. Final concentration and radioactivity of each probe was as follows: $[^{14}C]$sucrose - 7.4 μM, 2.5 μCi/ml; $[^{14}C]$methylamine - 100 μM, 3.0 μCi/ml; $[^3H]$acetate -

Table I

Recoveries and Relative Specific Activities of Marker Enzymes in Parotid Secretory Granule Isolation

	Protein	α-Amylase	Cytochrome c-oxidase
Homogenate activity	248.5 (mg)	150,260.	46.6
Relative specific activity in granule fraction	6.58 mg	4.08	0.53
% recovered in granule fraction	2.65 (5)	10.8 (4)	1.41 (4)

Results shown are extracted from the balance sheets of 5 separate fractionation experiments. Cytochrome c oxidase units are proportional to the first order rate constant as described by Max et al. (177); UDP galactosyl transferase units are pmoles/min; all other enzyme activites are expressed in μmoles/min. Relative specific activites refer to specific activites in the granule fraction with respect to the homogenate. Data shown are mean values; values in parentheses are the number of determinations.

Table 1 (Continuation)

NADH cytochrome c-reductase	γ-glutamyl transpeptidase	UDP-Galactosyl transferase	β-N-acetyl glucosaminidase
6.02	2.09	667	3.62
0.21	1.39	0.13	0.64
0.56 (3)	3.68 (3)	0.34 (2)	1.69 (3)

1.4 μM, 2.7 μCi/ml; [^{3}H]water - 2.0 μCi/ml. Incubations were terminated by centrifugation for 2 min at 11,500 xg in a Beckman microfuge. Samples of each supernatant solution were taken for scintillation counting, external pH measurement, and protein or α-amylase determination. Pellets were solubilized either in 1% sodium dodecyl sulfate or in 0.2% Triton X-100; samples were removed for scintillation counting and either protein or α-amylase measurement. Scintillation counting of double-labeled samples and subsequent calculations are described elsewhere (126). Osmotic pressures of various test media were either calculated[1] or measured using a Wescor 4100A vapor pressure osmometer. The instrument was always pre-calibrated with standards of known osmolality.

Measurement of Extent of Lysis of Isolated Secretory Granules - Granule lysis was determined by the fraction of total granule content marker not sedimented in a 2 min centrifugation at $\geq$ 10,000xg in a Beckman microfuge. Choice of content marker was either α-amylase and/or total protein. In comparative experiments, results using either marker were similar. Valinomycin (Sigma Co.) when used, was added immediately after granule addition to the pre-mixed media. Valinomycin was dissolved in ethanol, whose final concentration did not exceed 0.5% in any incubation.

Light Microscopy with Acridine Orange - Cells and/or secretory granules were incubated for 10-30 min at 25°C in well-buffered media containing acridine orange at either 1.5 μM (for cells) or 50 μM (for granules), in a manner similar to that described by (127). A drop of suspension was examined on albumin-coated slides with uncoated cover slips, using a Zeiss photomicroscope outfitted with epifluorescence illumination (exciter filter, BG12; barrier filter 53).

Materials - Materials are identical to those listed in Chapter 2.

Figure 1. Low-Power Electron Micrograph of the Parotid Secretory Granule Fraction.

A) The upper portion of the pellet (top) reveals expanded membrane profiles in part deriving from the nuclear envelope (arrow) as identified by the presence of pore complexes. Occasional mitochondria and plasmalemmal profiles can also be seen. (x 12,000)

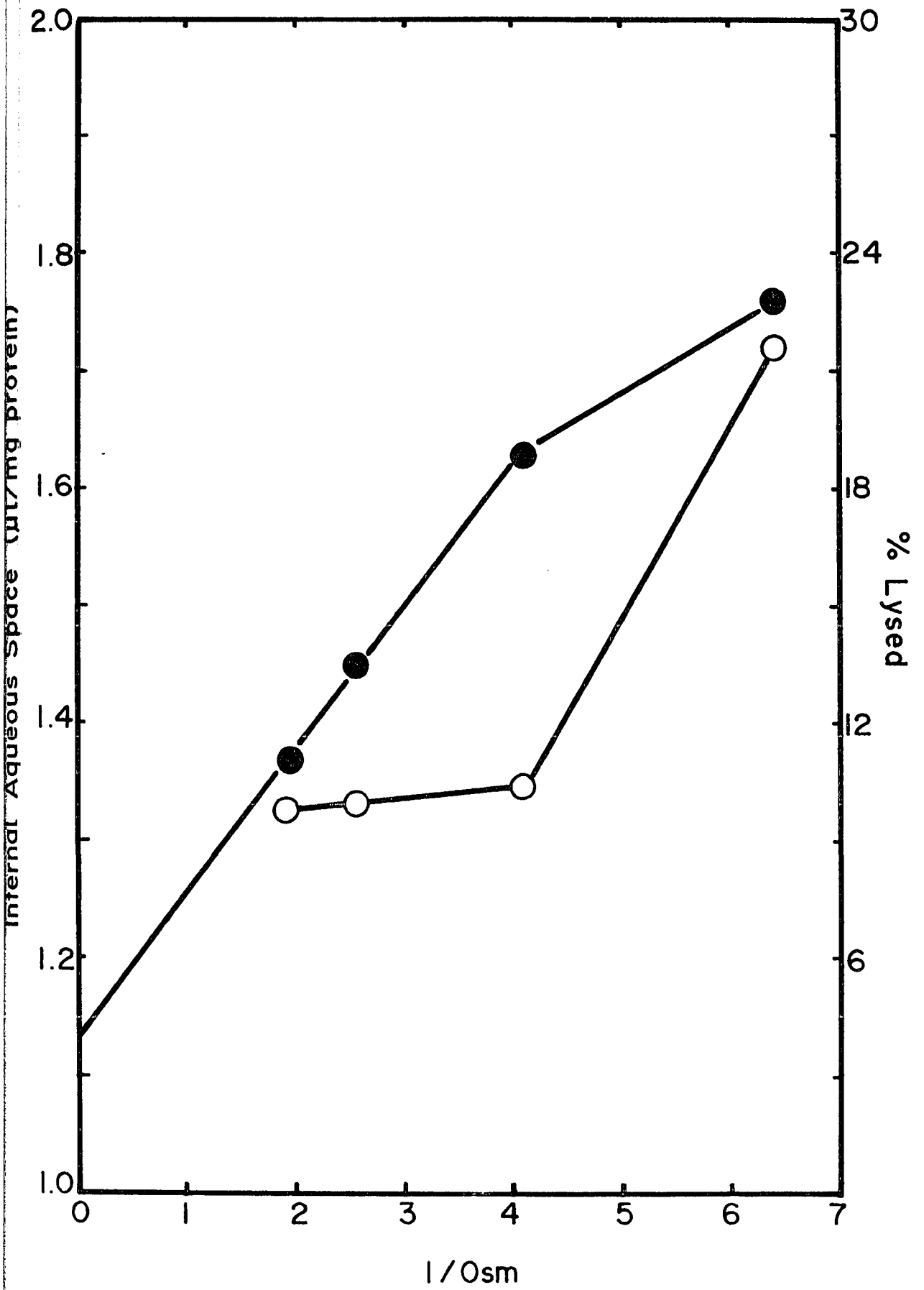
B) The lower portion of the pellet (bottom) is primarily secretory granules, sectioned through different planes. A few rough microsomes (arrowhead) and unidentified small vesicles may be seen. (x 12,000).

Results

Isoosmotic Purification of Parotid Secretory Granules - Transfer of parotid granules from a hyperosmotic isolation medium into isoosmotic test media results in marked granule lysis (42). Similar manipulation has been shown to create irreversible structural changes in isolated adrenal chromaffin granules (26). Despite the recent success of our laboratory in isolating a very highly enriched fraction of rat parotid secretory granules by sucrose gradient centrifugation (82), the above concerns preclude such an isolation procedure for physiologic studies. Consequently, it was decided instead to modify the differential centrifugation technique of Schramm and Danon (124), in order to maintain isoosmolality throughout the granule isolation. While this procedure results in a somewhat less purified secretory granule fraction than reported in (82), the fraction is still predominantly granules obtained in favorable yield and with improved stability over that of other procedures (128, 129). Such a procedure also has the advantage of yielding the final granule pellet in only 90 min of fractionation time (with all steps carried out at 4° C), thus minimizing possible enzymic inactivation. A biochemical analysis of the parotid secretory granule fraction is shown in Table I. The data therein reveal a relative depurification in the granule fraction with respect to the homogenate of mitochondrial (cytochrome c oxidase), endoplasmic reticulum (NADH-cytochrome c reductase), Golgi (UDP-galactosyl transferase), and lysosomal (β-N-acetyl glucosaminidase) marker enzymes. The γ-glutamyl transpeptidase activity in the granule fraction probably reflects both a limited contamination of the granule fraction by plasmalemmal elements as well as activity contributed by the granules themselves, since this marker is

Figure 2. Osmometer Behavior of Isolated Rat Parotid Granules.

Parotid granules (125 μg protein) were incubated for 45 min at 25°C in 100 μl of 50 mM MES-NaOH buffered sucrose, pH 6.2. [^{14}C]sucrose-excluding (internal) aqueous space was measured as described in Methods. Medium osmolality was varied by changing the external concentration of sucrose; osmolalities plotted were calculated according to footnote 1. Granule lysis was determined as described in Methods using total protein as a granule content marker. Closed circles - internal aqueous space; open circles - fraction of granules lysed. Each point is the mean of duplicate determinations from two experiments.

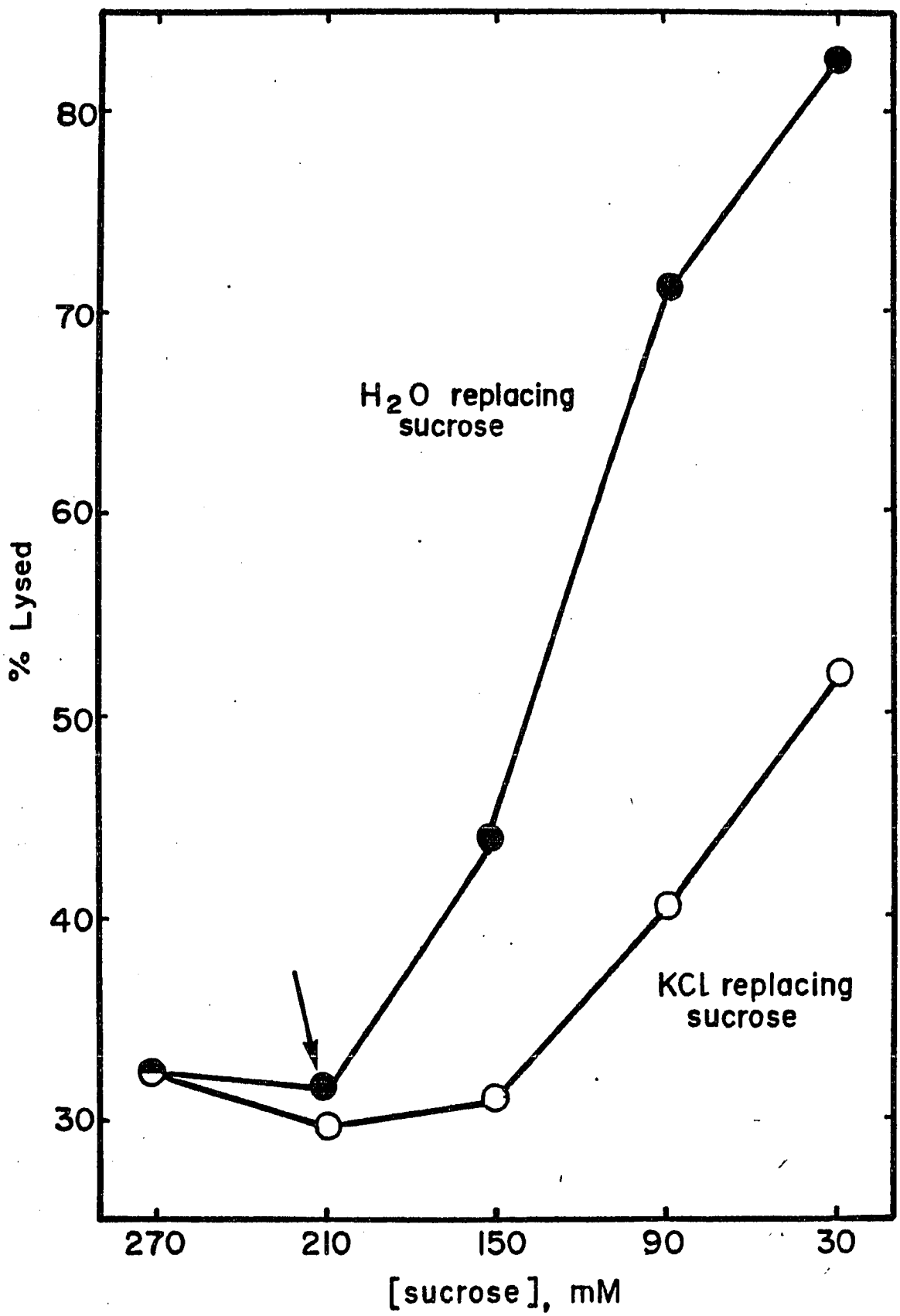


shared by both granule membranes and luminal plasma membranes (122). α-Amylase, taken as the secretory granule-specific marker, is recovered in ~11% yield and purified ~4-fold (Table I). To establish the reliability of these assays, all intermediate fractions generated in the granule isolation procedure were tested; total recoveries for each activity ranged from 92-107% of the values found in the homogenate.

Fig. 1 is a low power electron micrograph of a fixed pellet from the granule fraction. Two representative views are shown; one at the top of the pellet and a second nearer to the bottom. The upper part of the pellet reveals the presence of nuclear membranes not detected biochemically, which appear to be the only significant structures of larger size than secretory granules. Since in most cases these large membranes display discontinuities and thus do not have the appearance of sealed vesicles, they are not likely to possess a [^{14}C]sucrose excluding volume. Other biochemically-detectable contaminants are observed at low levels. Unlike the contaminating organelles, the isolated secretory granules appear to be morphologically unchanged in comparison to their appearance in situ, having a continuous surrounding membrane and well-preserved internal contents.

Osmotic Properties of Isolated Parotid Granules - Osmotic properties of parotid granules were investigated in order to establish the ability of these vesicles to behave as osmometers and to assess granule stability under conditions of hypoosmotic stress. The results of the Van't Hoff plot (Fig. 2) indicate that the intragranular water space decreases in response to increasing osmolality (decreasing 1/Osm) of the suspending medium. At osmolalities >245 mOsm (1/Osm <4), these decreases in the intragranular aqueous space are linear in the Van't Hoff plot, but do not extrapolate to zero volume at infinite sucrose

Figure 3. Effect of Hypoosmotic Stress and Salt on the Stability of Parotid Granules Isolated in a Non-ionic Medium. Secretory granules (350 units amylase activity) were incubated for 30 min at 37°C in 200 µl of 20 mM sodium PIPES buffer, pH 6.8 with decreasing concentrations of sucrose as shown. Sucrose was replaced with water (closed circles) or with increasing volumes of 150 mM KCl so that medium osmolality remained constant (open circles) at ∿310 mOsm. The arrow indicates a medium osmolality of 250 mOsm (H_2O replacement). Granule lysis was measured as described in Methods, using α-amylase as granule content marker. Each point is the mean of triplicate determinations in a single experiment; standard deviation was <3.1 for all values shown.



concentration. Thus, similar to other types of isolated granules (86), they do not behave as "perfect osmometers". In addition, when the osmolality is reduced below ~245 mOsm, the intragranular water space does not change linearly and there is a concomitant increase in granule lysis. Even these conditions however, fail to disrupt the majority of parotid granules (Fig. 2).

To further examine granule osmotic lysis, the experiment shown in Fig. 3 was performed. The lysis of granules incubated in a buffered sucrose (310 mOsm) solution was compared to that of granules suspended in solutions in which sucrose was replaced either by water or by an isoosmotic KCl solution. Even before sucrose replacement, a small fraction of granules was lysed (this basal lysis, which varies in extent between experiments, is not fully understood). As sucrose is replaced with water, the hypoosmotic stress induces progressive granule lysis which ultimately exceeds 95% below 50 mOsm (124). Replacement of sucrose by equivalent isoosmotic increments of KCl (Fig. 3) also results in a progressive granule lysis (of a lesser magnitude). This latter effect was prominent only at concentrations of KCl > 60 mM. Similar effects have been previously observed in parotid and other granule types (82,62,130) and have been postulated to be caused by a significant granule membrane permeability to chloride and other ions with resultant osmotic lysis (62).

To investigate whether the effects of various anions on parotid granule stability were similar to those reported for other granule types, I designed experiments patterned after those of Dolais-Kitabgi and Perlman (60) in which anionic permeabilities of the chromaffin granule membrane were compared indirectly by measuring granule lysis stimulated by potassium salts of various anions in the presence of

Table II

Effect of Anions on Lysis

of Rat Parotid Secretory Granules

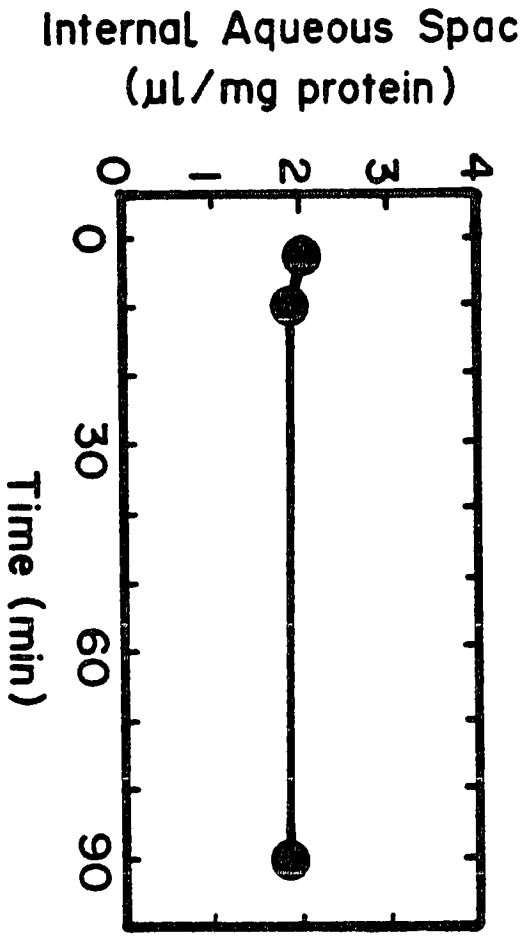
	% Lysed
Sulfate	11.0 ± 0.6
Phosphate	15.4 ± 2.5
Gluconate	16.3 ± 1.0
Isethionate	19.0 ± 2.0
Acetate	22.0 ± 0.2
Chloride	23.5 ± 2.0
Thiocyanate	50.0 ± 0.1

Parotid granules (350 units amylase activity) were incubated in 25 mM PIPES-NaOH, pH 6.8 at 37°C for 30 min in 200 µl of media containing the K^+ salts of the listed anions, all at concentrations of 150 mM plus 10 µM valinomycin. Several experiments using KAc and KCl under these conditions showed that the omission of valinomycin resulted in about 7 to 10% less granule lysis than was observed upon valinomycin addition. Granule lysis was measured as described in Methods, using α-amylase as the granule content marker. Data shown are the mean values ± standard deviation from triplicate determinations.

valinomycin. This K^+ ionophore is included in order to insure that the counter-ion (anion) permeability is rate limiting. The results of a representative experiment are shown in Table II. Several points deserve mention. First, anions promote granule lysis in the order: sulfate < phosphate $\leq$ gluconate $\leq$ isethionate $\leq$ acetate $\leq$ chloride << thiocyanate. Other anions tested in granule lysis assays include glutamate (comparable to gluconate), and other halides: $Cl^- \leq F^- < Br^-$ (all less than SCN^-). Second, the order of anions is comparable (although not identical) to that reported for other granule types (60, 62). However, parotid granules appear to be more stable than, for example, isolated bovine chromaffin granules in which catecholamine release (used as an index of lysis) is ~50% after only 5 min at 30°C in a 150 mM KCl-containing medium (60), as compared to the 23.5% amylase release under the present conditions (Table II). Such differences could reflect: a generally reduced anion permeability in the parotid granules (catecholamine may permeate directly across the granule membrane); differences in the choice of lysis markers; or other factors related to the relative stabilities of the packaged contents. Finally, the order of anions which promote granule lysis was essentially unchanged by the omission of valinomycin (although the extent of lysis was decreased). In more than 50 individual measurements using 8 of the above anions (without valinomycin), Na^+ and K^+ supported parotid granule lysis equally well; while in the presence of valinomycin, granule lysis was always higher in K^+ salts[2] (data not shown).

Internal pH and Buffering Capacity of Isolated Rat Parotid Secretory Granules - To obtain a quantitative determination of parotid intragranular pH, the method of Rottenberg (126) was employed, which is based on the distribution of tracer quantities of radioactive weak acids

Figure 4. Measured Intragranular Water Space as a Function of Time. Secretory granules were incubated in a medium of ~280 mOsm under conditions described in Figure 2. [^{14}C]sucrose was added to all samples at zero time. Each point is the mean of triplicate determinations in a single experiment.



and bases that are assumed to permeate across the membranes of vesicular structures only in their neutral forms, and which requires a reliable estimation of intragranular water space. To confirm that the volume of granule pellets which exclude [^{14}C]sucrose is indeed a reliable estimate, I measured the internal aqueous space over a 1.5 h period while in the continuous presence of [^{14}C]sucrose. In a buffered 280 milliosmolal medium, measured intragranular volume changes only ~10% over this time period (Fig. 4). At higher osmolalities, the change in measured internal volumes over the same time period was even less than that found at 280 mOsm (not shown). These small but consistent decreases in intragranular water space may reflect a low degree of permeability of the granules to [^{14}C]sucrose, however we cannot rule out that subtle intragranular volume changes are indeed occurring. In order to minimize the effect that these apparent volume changes would have on intragranular pH determinations, internal volume measurements were always made under conditions of time and medium composition identical to that in which internal pH was tested, rather than to rely on averaged volume values. In spite of this precaution, I obtained a range of intragranular pH values with a mean value of ~6.8 (Table IIIA) using the equilibrium distribution of [^{14}C]methylamine (which accumulates in granules suspended in alkaline media). Similar values of internal pH were found (Table IIIB) using independent measurements with [^{3}H]acetate, another probe which accumulates instead within granules incubated in acidic media. Intragranular pH values simultaneously measured using [^{14}C]methylamine and [^{3}H]acetate were nearly identical (provided $pH_{out} \cong pH_{in}$). This close agreement renders significant binding of either probe unlikely. To my knowledge, this nearly neutral pH value is the highest thus far reported for any secretory granule type.

Table III

Internal pH of Isolated Parotid Granules as Measured by the Distribution of Weak Acids and Bases

	pH_{out}	Added Anions	pH_{int}
A	6.70	--	6.45
	7.10	--	6.67
	7.20	--	6.71
	7.00	Cl^-	6.88
	7.40	--	6.95
	7.70	--	6.97
	7.00	SO_4^{2-}	7.01
B	6.40	--	6.74

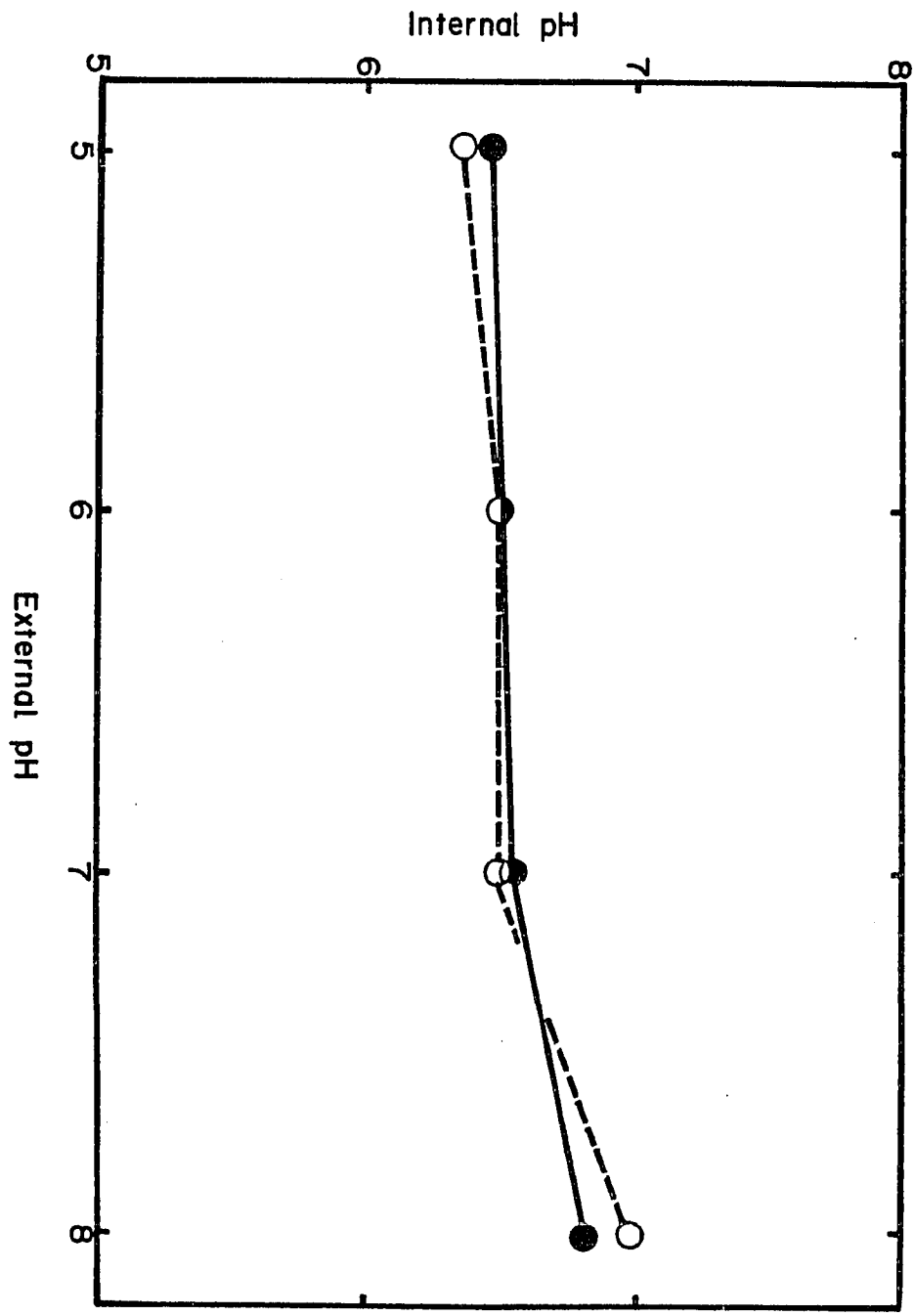
A) Parotid granules were incubated from 30 - 60 min at 25°C in 50 mM MOPS or PIPES buffered sucrose in parallel [^{14}C]sucrose and [^{14}C]methylamine containing samples. Incubations also included [^{3}H]water as described in Experimental Procedures. K_2SO_4, when added, was 50 mM; KCl was 75 mM. The range of values shown derive from 4 different granule preparations; all values are the mean of duplicate samples.

B) Granules were incubated as in (A), except in 50 mM MES buffered sucrose containing [^{3}H]acetate instead of [^{14}C]methylamine.

While preliminary experiments using both probes suggested that neither time of incubation nor choice of (sulfonic acid) buffer influenced the intragranular pH measurement (not shown), it appeared that two factors did have a small effect on these determinations. First, the addition of anions from the series listed in Table II tended to slightly decrease intragranular pH in the same order as they supported granule lysis[3]; and second, the internal pH tended to shift slightly toward the medium pH with time. To systematically analyze this latter observation, I examined the internal pH of parotid secretory granules over a 1000-fold range of external H^+ concentrations (Fig. 5). The results indicate that the measured pH_{in} drifts toward pH_{out}, suggesting some permeability of the granules to protons (Fig. 5). However the addition of proton ionophores to granules incubated under similar conditions had minimal effect on the measured ΔpH (see next chapter). Thus, it appears that buffering by granule contents and/or limited ionic permeability are primarily responsible for the rather fixed intragranular pH seen in Fig. 5.

To assess the intragranular buffering capacity, isolated parotid granules were challenged with an alkaline load (66, 119), and the subsequent rise in their internal pH was determined by the [^{14}C]methylamine and [^{3}H]acetate distribution techniques. The data (Fig. 6) indicate an increasing intragranular pH with increasing concentrations of external amines; changing the base (from ammonia to methylamine) or the external pH (from 7.65 to 8.05) results in little change in the shape of the curve. By calculating the concentration of amines which actually become protonated in the intragranular space, a base-titration is obtained from which the parotid granule buffer capacity may be estimated. These results suggest that an 85-140 mM change in intragranular H^+ is needed

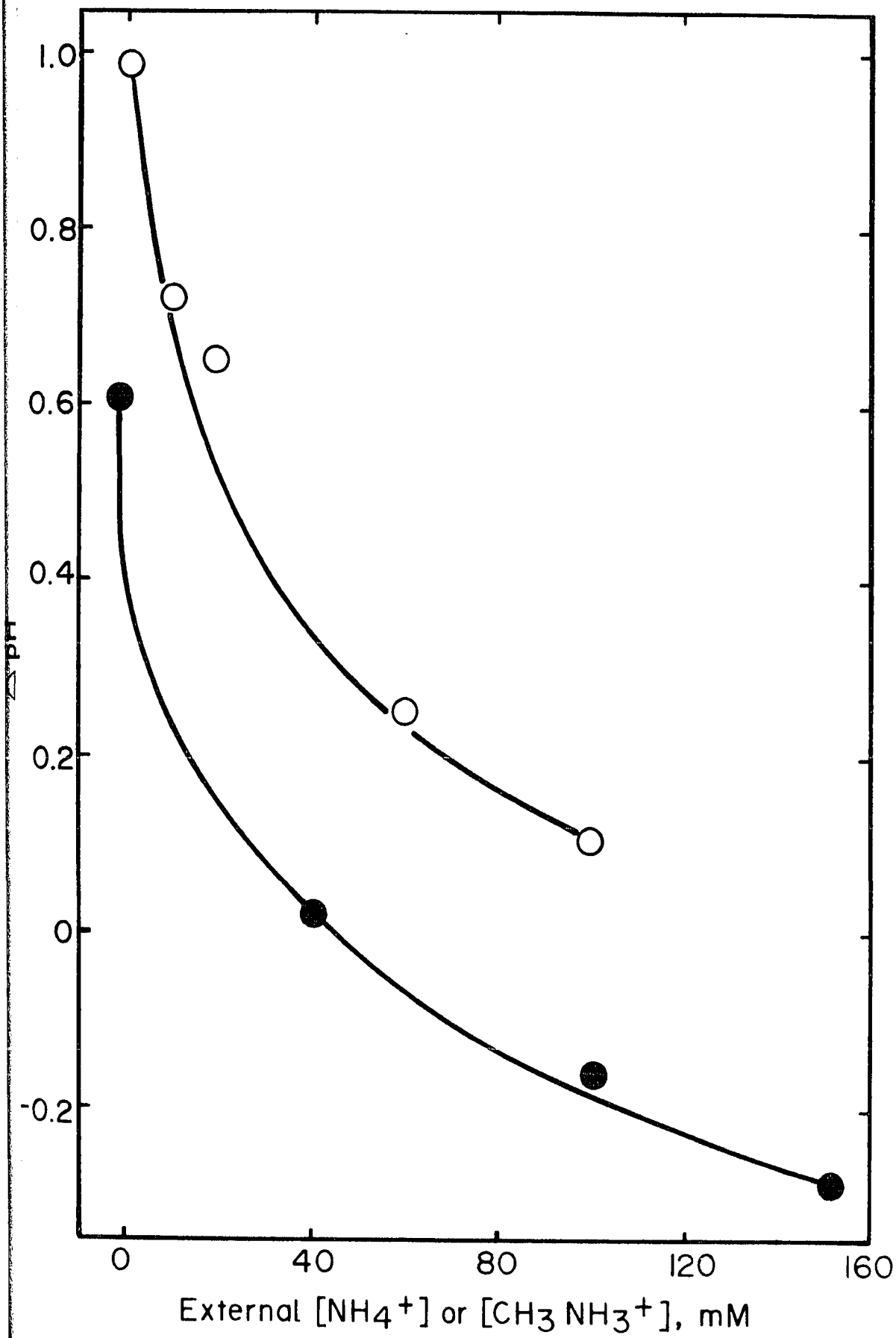
Figure 5. Internal pH of Isolated Rat Parotid Secretory Granules as a Function of Medium pH. Granules were incubated in either 50 mM MES-NaOH (pH's 5.0, 6.0) - or 50 mM HEPES-NaOH (pH's 7.0, 8.0) - buffered sucrose at 25°C. Intragranular pH was measured as described in Methods using the distribution of either [^{3}H]acetate or [^{14}C]methylamine as a probe of ΔpH. Closed circles - 10 min determination; open circles - 60 min determination. Each point is the mean of duplicate determinations in a single experiment.



to change the parotid intragranular pH 1.0 unit (from the data using ammonium and methylammonium, respectively). This high degree of buffering is quite similar to that reported for bovine adrenal chromaffin granules [92 mM H^+ bound/pH unit and ~70-210 mM H^+ bound/pH unit (131,66)]. Unlike the chromaffin granules however, parotid granules are not known to contain high concentrations of ATP (a principal buffer in the chromaffin granule core); hence, I am presuming that buffering in parotid granules derives primarily from their proteinaceous contents. Results comparable to the above studies were obtained by ordinary pH titrations of parotid granule contents after lysis of granule pellets in distilled water (not shown). The initial pH of parotid granules lysed in this manner ranged from 6.95-6.75, depending on the extent of aqueous dilution of the lysed granules (from 10-50 fold larger than the intragranular volume). Further dilution resulted in pH values which progressively approached that of distilled water (pH = 5.7). Thus the pH of the parotid granule contents are similar in both packaged (intact) or soluble (lysed) form.

The results in Fig. 6 also disagree with the assertion by Pollard et. al. that the alkalinization of the granule interior by ammonia continues until $\Delta pH = 0$ (132). Since the [^{3}H]acetate distribution technique is far more sensitive than is the ^{31}P nuclear magnetic resonance method (131, 132) to intragranular alkalinization in the range of pH_{in} >6.5, these data clearly show that ammonia in sufficient concentration can elevate the intragranular pH to values higher than the pH of the medium. This result supports the premise as stated by several investigators, that amine accumulation ceases not when $\Delta pH = 0$, but when the neutral form of the amine achieves equilibrium across the membrane (66,93,95). Obviously, another limit to the alkalinization may occur if

Figure 6. Dose-dependent Effects of Ammonium and Methylammonium on the Granule Transmembrane ΔpH. Results shown represent 2 separate experiments using different preparations of parotid granules for the different treatments. Closed circles - $(NH_4)_2SO_4$ for 30 min at 25°C. The medium was buffered with 50 mM MOPS, pH's 7.67, 7.65, 7.64 and 7.63, respectively. Open circles - $(CH_3NH_3)_2SO_4$ for 10 min at 37°C. The medium was buffered at pH 8.05 which remained constant at all concentrations of methylammonium. $\Delta pH = pH_{out} - pH_{in}$, with pH_{out} measured by electrode and pH_{in} determined as described in Methods using the distribution of [^{14}C]methylamine or [^{3}H]acetate.



the pH of the intravesicular space approaches the pK_a of the amine itself. Methylammonium, which has a pK_a more. Protein was determined with fluorescamine as described by Udenfriend et al (125) using bovine serum albumin as a standard. to levels higher than that of the medium (data not shown). These increasing concentrations of external amines were noted incidentally to increase progressively the extent of parotid granule lysis.

Visual Estimation of Intragranular pH In Situ vs. In Vitro - Since the nearly neutral pH of isolated parotid secretory granules with that of other granule types which share several physical properties with parotid granules, it was a matter of concern whether parotid granule isolation had caused a change in their internal pH from that found inside the cell. To test this possibility, I exploited both the known ability of the fluorescent acridine dyes to distribute as weak bases in a manner similar to that of methylamine, and the large size of parotid granules, to visualize their appearance under the fluorescence microscope. These dyes have been used to estimate the internal pH of mast cell and insulin-containing pancreatic granules (117, 127). I chose acridine orange, whose fluorescence at low concentrations appears green; but becomes orange due to its metachromatic behavior at higher concentrations (133). The staining of the acidic granules of the rat anterior pituitary and rat peritoneal mast cells was compared (Table IVA) to that of the nearly neutral granules of rat parotid acinar cells in situ (since the former are also of large size and can be resolved by light microscopy). Thus, while mast cell granules and pituitary dense granules in situ appeared to be acidic as judged by their ability to concentrate the acridine dye and produce an orange fluorescence, parotid granules in situ remained green, consistent with their nearly neutral pH discussed

Table IV

Fluorescent Appearance of Secretory Granules

Treated with Acridine Orange

	pH_{ext}	Pituitary	Parotid	Mast cell
A	cytosolic	orange	green	orange
B	7.85	--	orange	--
	6.80	--	yellow-green	--
	6.30	--	green	--

A) Mechanically-dissociated rat pituitary and parotid cells were examined with acridine orange in oxygenated HEPES or bicarbonate buffered minimal essential medium, pH 7.4, as described in Methods. Mast cells were similarly incubated in a phosphate-buffered (pH 7.0) Tyrode's solution containing 2 mg/ml glucose and 1 mg/ml gelatin.

B) Isolated rat parotid granules were examined with acridine orange in sucrose media buffered at varying pH's with Na phosphate, as described in Methods.

previously. To substantiate that the orange appearance of the former granule types results from internal acidity and not from binding of acridine orange to the granule contents, it was observed that agents known to raise intragranular pH within cells [e.g. $(NH_4)_2SO_4$, (93)] changed the fluorescent staining of the acidic granules from orange to green. Finally, I examined isolated parotid granules treated with acridine orange at different medium pH's, to establish conditions necessary (pH_{out} >7.85) to achieve a sufficient internal concentration of the dye to produce an orange granule staining (Table IVB). Despite the relative insensitivity of this technique with respect to the previous measurements using radioactive pH probes, these studies enable one to establish a limit to the in situ granule ΔpH. If this ΔpH were in the range of 1 pH unit, the granules would display an orange fluorescence, as do lysosomes in the same cells. Thus the internal pH of parotid granules inside the cell and that examined after granule isolation is roughly the same.

Discussion of Chapter 1

Parotid secretory granules, owing to their large size and density, are especially well suited to rapid purification from other cell components by differential centrifugation in isoosmotic sucrose. Employing such a procedure results in the isolation of a granule fraction of reasonable purity (Table I, Fig. 1), which is osmotically responsive (Figs. 2,3), exhibits a substantial internal buffer capacity (Fig. 6), and apparently has selective permeability to anions in an order comparable to that of other granule types (Table II). However, the nearly neutral intragranular pH of parotid granules (Table III, Fig. 5) contrasts distinctly with that of other more acidic granule types

(65,91,116-120). Although the parotid granules appear to be an exception in this respect, it seems very likely that the measured intragranular pH is a valid measurement, since this value is corroborated by both the distributions of weak acids and weak bases. Further, such a value appears to be comparable both *in vitro* and in the cell, since *in situ* studies with the weak base acridine orange indicate that the parotid intragranular pH is similar to that of the cytoplasm.

In the following chapter, it is demonstrated that unlike adrenal chromaffin granules, isolated rat parotid granules do not show evidence of significant H^+ translocating ATPase activity. The difference in internal pH values between the two granule types could reflect this activity difference. However, both granule types are able to maintain a stable internal pH *in vitro* because they possess low membrane ionic permeabilities, and high intragranular buffering capacities which are presumably due (at least in part) to the proteins contained within the granules.

Summary of Chapter 1

Secretory granules of the rat parotid have been purified rapidly by differential centrifugation in isoosmotic sucrose. Despite low-level contamination by other organelles, this fraction is predominantly secretory granules, based on biochemical and morphological characterization. Measurements of intragranular water space show that isolated granules swell in hypoosmotic media, and begin to release α-amylase and other content proteins below osmolalities of ∿250 mOsm. Exposure of granules to selected anions also causes lysis, increasing in the order sulfate < phosphate ≤ gluconate ≤ isethionate ≤ acetate ≤ chloride << thiocyanate. The internal pH of isolated parotid granules is ∿6.8 as measured by the distributions of either [^{3}H]acetate or [^{14}C]methylamine. Varying the external medium pH from 5.0 to 8.0 resulted in little change in the measured internal pH. Parotid granules have a substantial internal buffering capacity: a decrease of 85 - 140 mM intragranular H^{+} is needed to raise the internal pH by 1.0 unit. Finally, results obtained using acridine orange staining suggests that the secretory granule internal pH *in situ* is similar to that measured in isolated granules.

CHAPTER 2

ISOLATED PAROTID SECRETORY GRANULES DO NOT CONTAIN SIGNIFICANT H^+-TRANSLOCATING ATPase ACTIVITY

Introduction to Chapter 2

Investigators in a number of laboratories have recently obtained evidence that the membranes of several types of secretory granules contain an inward-directed H^+-translocating ATPase [e.g., chromaffin granules (45,67,68,95,132,135-137), platelet dense granules (72,73, 119), neurohypophyseal granules (120,134)]. Its activity results in the generation of an ATP-dependent transmembrane electrochemical H^+ gradient, consisting of an electrical potential ($\Delta\Psi$, interior positive) and a pH difference (ΔpH, interior acidic). Further, in chromaffin granules, this proton pump provides the driving force for biogenic amine uptake (45,67) as well as for osmotic lysis of granules (99), the latter under conditions approaching those in which granule acidification occurs (95).

In the preceding chapter reporting biophysical properties of rat parotid secretory granules, it was shown that in contrast to several acidic granule types, isolated rat parotid granules have an internal pH near neutrality. This pH value ($\sim$6.8) is maintained by a low ionic permeability of the granule membrane and by a high intragranular buffering capacity. Previously, it has been suggested (128) based on the observation of an apparent ATP-stimulated granule lysis (128,139,140), that parotid secretory granules possess an H^+ pump analogous to that studied in adrenal chromaffin granules. This proposal seemed at variance with a neutral intragranular pH.

In an attempt to test for the existence of an ATP-driven proton translocase in parotid granules, I examined four of its possible manifestations: a) ATP-induced changes in the internal pH of isolated parotid secretory granules; b) ATP-generated shifts of the granule transmembrane potential; c) ATP hydrolyzing properties of parotid granules in comparison to those of bovine chromaffin granule membranes and parotid mitochondria; and d) ATP-dependent lysis of granule suspensions [a reexamination of earlier reports (128,139,140)]. No evidence was found by these methods to support the existence of significant electrogenic or electroneutral H^+-translocating ATPase activity in parotid secretory granules. Although it was possible to reproduce a limited lysis of granule suspensions by addition of ATP (140), this effect is apparently independent of ATP hydrolysis and consequently does not support the notion that an active proton pump is present. Some of this work has been presented in abstract form (123).

Methods

Isolation of Rat Parotid Secretory Granules - Granule fractions were prepared essentially as described in the preceding chapter. Rat parotid post-microsomal supernate was made by filtration of the post-granule supernate through a 1.2 μm^2 Millipore filter, followed by repeated 200,000 g-min centrifugations of the filtrate until no further visible sediment was obtained.

Internal pH of Isolated Parotid Granules - Intragranular pH was measured as described previously (126); the calculated [^{14}C]methylamine concentration ratios were based upon internal aqueous space measurements made simultaneously and under identical experimental conditions to the pH measurements [radioactive probe concentrations were identical to

those listed in Chapter 1]. The aqueous volume determinations were always normalized to pellet protein values (i.e., µl/mg protein) assayed by the method of Udenfriend et. al. (125). External medium pH was measured with a Radiometer electrode and pH meter immediately after the termination of granule incubations by centrifugation. The (centrifugation plus subsequent processing) time required to complete granule incubations was approximately five minutes; this delay is not included in the listed times for Tables or Figures.

Electrical Potential Measurements Across the Membranes of Isolated Secretory Granules - Three methods were used to estimate granule transmembrane potentials. The primary technique was based on the equilibrium distribution of [^{86}Rb]Cl in the presence of 10 µM valinomycin (126). Rubidium was used in tracer concentrations, at a final specific radioactivity of 0.5 µCi/ ml. A second technique utilized the equilibrium distribution of tracer quantities of [^{3}H]tetraphenylphosphonium bromide (TPP^+). In both cases the preparation of samples and subsequent incubations were analogous to those used for internal pH measurements. The concentration ratios (intragranular/medium) for each radiolabeled potential probe were based upon internal volume measurements done in parallel (as described in previous chapter) however, the time required to achieve equilibrium with [^{3}H]TPP^+ was far longer than with [$^{86}Rb^+$]/valinomycin. Conversion of the (in:out) ratios of radiolabeled pH and potential probes into an electrical potential value was done by applying the Nernst equation; $\Delta\Psi = -60 \log [^{86}Rb^+_{in}/^{86}Rb^+_{out}]$ (at 25°C). In the third method potential changes were monitored by fluorescence using the dicarbocyanine dye diS-C_3(5), in a manner similar to that used for red blood cells [e.g. see (141)]. For quantitative measurements, the $^{86}Rb^+$/valinomycin technique was exclusively used (with the latter two

procedures reserved only to provide confirmatory evidence) because both TPP^+ and diS-C_3(5) exhibit high levels of binding to granule membranes and/or contents. Ionophores when used, were added in absolute ethanol, whose level never exceeded 0.5% in any incubation.

Assessment of ATP Hydrolyzing Activity - Whole granules and samples containing granule membranes were pre-incubated for 10 min at 37° with 105 mM KCl, 5.56 mM NaCl, 3.33 mM $MgSO_4$, 2.78 μM FCCP and 5.56 mM PIPES buffer, pH 6.8. After this time, buffered Na_2ATP containing tracer quantities of either [α-^{32}P]ATP or [γ-^{32}P]ATP were added; final concentration of Mg-ATP = 3 mM, KCl = 95 mM, NaCl = 5 mM, FCCP = 2.5 μM and PIPES buffer = 25 mM, pH 6.8. Final volume of incubation was 200 μl. Efrapeptin (final concentration, 1 μM), Nbd-Cl (final concentration, 20 μM) and sodium vanadate (final concentration, 50 μM) were included in pre-incubations when used. Sodium vanadate was briefly boiled immediately prior to use (to favor conversion of meta to ortho-vanadate forms). Reactions were stopped either by collecting membranes by centrifugation or by acid precipitation with (~11%) TCA. A 5 μl aliquot of the soluble fraction from each incubation was spotted on polyethyleneimine-cellulose coated thin-layer plates and ATP, ADP, AMP, and P_i were resolved by one-dimensional chromatography (142) in 1M ammonium formate:1N HCl (68:32 v/v). Spots were located under short-wave UV light (for non-radioactive standards and samples) or by autoradiography (of plates containing radioactive spots). In the latter case, the spots were excised from the plate and counted in a Beckman liquid scintillation counter in the presence of Liquiscint (National Diagnostics). The fraction of total ATP hydrolyzed was assumed to equal the fraction of total counts recovered in either [α-^{32}P]ADP or

[γ-^{32}P]P_i, depending on the original substrate. Background hydrolysis of ATP was calculated by examining the fraction of [^{32}P]ATP hydrolyzed in control incubations without membranes. Values obtained with either tracer were quite similar (differences between the two methods were always <2.0%).

Extent of Lysis of Isolated Parotid Secretory Granules - The release of α-amylase (143), a granule content marker, into the soluble supernate was used as an index of granule lysis as described in the preceding chapter.

Materials

[^{3}H]TPP^+ was a gift from Dr. R. Kaback. Parotid mitochondria isolated according to (82), were the kind gift of Dr. R. Cameron. Bovine chromaffin granule membranes were prepared according to (136); efrapeptin was donated by Dr. R.L. Hamill, Lilly Research Laboratories; Nbd-Cl, FCCP, ATP, β, -imidoadenosine-5'-triphosphate (AMP-PNP), valinomycin, and other stock chemicals were from Sigma Company. [^{3}H]acetate, [^{14}C]methylamine and [^{3}H]water were from New England Nuclear; [^{14}C]sucrose was from ICN; [^{86}Rb]Cl was from Amersham.

Results

Determination of Anionic Permeabilities of Isolated Rat Parotid Secretory Granules - The two most straightforward parameters that have been exploited in order to identify inward-directed electrogenic H^+-ATPases of certain granule types are the acidification of the granule interior and the shift of granule transmembrane potentials to more inside-positive values in the presence of ATP. The detection of acidification is favored by the presence of permeant anions to reduce the buildup of internal-positive potentials that limit further net proton

influx. By contrast, the observation of ATP-generated transmembrane potential changes relies on minimizing the presence of permeant anions (95). Thus, in order to properly design experiments measuring ΔpH or $\Delta\Psi$, some advance knowledge of relative anionic permeabilities of the parotid granule membrane is essential. Although indirect evidence (based on the ability of the K^+ salts of various anions to support granule lysis in the presence of valinomycin) suggested that the order of anionic permeabilities in parotid granules was roughly comparable to that of chromaffin granules (previous chapter) a method was sought which could more directly compare the respective anionic diffusion-potentials.

For this purpose, the equilibrium distribution of $^{86}Rb^+$ was examined. This cation, free to cross membranes treated with valinomycin, will tend to accumulate in the intragranular space if a sufficient number of anions diffuse inwardly to cause a shift in the granule transmembrane potential to more inside-negative values. The technique is advantageous because the $^{86}Rb^+$ probe achieves rapid equilibrium [within seconds, according to (126)]. Its major disadvantage is the ability of valinomycin to simultaneously disturb the transmembrane K^+ gradient. To minimize this latter effect, preliminary studies were undertaken to determine the concentration of external K^+ that would create no apparent shift in the granule transmembrane potential; at 1.1 mM K_2SO_4 (2.2 mM K^+), the $^{86}Rb^+$ (in:out) ratio was found to be approximately 1.0. This K^+ concentration, which is in good agreement with the parotid intragranular K^+ level as estimated by atomic absorption spectroscopy, was also found to be close to the K^+ null point [at which no fluorescence change is detected (141) upon valinomycin addition] using the dicarbocyanine dye, diS-C_3(5). Thus, care was taken

Table I

Effect of Anions on Parotid Granule Transmembrane Potential

Anion	Relative $\Delta\Psi$
Sulfate	0
Isethionate	-10.0
Acetate	-10.3
Chloride	-23.5
Thiocyanate	-51.1

The equilibrium distribution of $^{86}Rb^+$ in the presence of 10 μM valinomycin was used as a measure of the ability of each of the anions listed to generate inside-negative diffusion potentials. Granules were incubated for 10 min at 25°C in the presence of 0.5 mM K_2SO_4 and 50 mM anions added as their sodium salts. Incubations also contained 50 mM MOPS buffer, pH 7.2 and 131 mM sucrose [except for the sulfate-containing sample which included only 81 mM sucrose (to maintain equal osmolality in all samples)]. The assignment of 0 mV to the relative $\Delta\Psi$ of the sulfate-containing sample was arbitrary. Values were calculated from the mean of duplicate determinations of (in:out) $^{86}Rb^+$ concentration ratios as described in Methods; differences between all duplicate samples were <8%. The $^{86}Rb^+$ concentration ratio (in:out) in sulfate medium was 0.45.

to maintain the external K^+ concentration between 0.5-4 mM in subsequent experiments.

To examine anionic permeabilities, the Na^+ salts of various anions (at 50 mM) were added in the presence of 0.5 mM K_2SO_4 to the granule suspending medium. As shown in Table I, the relative decrease in granule transmembrane potential proceeds in the order sulfate < isethionate $\leq$ acetate < chloride << thiocyanate. Based on these data and on the increasing lytic effects observed for this same sequence of anions on isolated rat parotid granules (previous chapter), the parotid granule membranes can be taken to be "freely permeable" to thiocyanate, of "limited permeability" to chloride and virtually "impermeable" to sulfate.

Effect of ATP on Intragranular pH - Initial experiments employing ATP concentrations between 1.0 - 10 mM failed to provide any evidence for internal acidification of parotid granules in either gluconate or chloride media. Indeed early experiments using the [^{14}C]methylamine distribution technique showed small ($\sim$0.1 pH unit) increases in apparent intragranular pH after incubation with ATP. However, these early experiments required further consideration of a number of key points. First, ATP hydrolysis, even in the absence of proton pumping, will tend to change the pH of the external medium. To counteract this, I a) increased the buffering of the medium to 50 mM using sulfonic acid buffers whose pKa's were quite close to the pH used in the particular experiment; b) measured ΔpH by the distribution of [^{3}H]acetate, using conditions similar to that employed by Thayer and Hinkle (144) such that little or no change in the medium pH ($\sim$6.15) would occur; and c) carefully measured the medium pH after the granule incubation, and used this pH_{out} to calculate the intragranular pH value. These corrections

Table II

ΔpH Across Membranes of Isolated Parotid Granules:

Effect of Anions, ATP

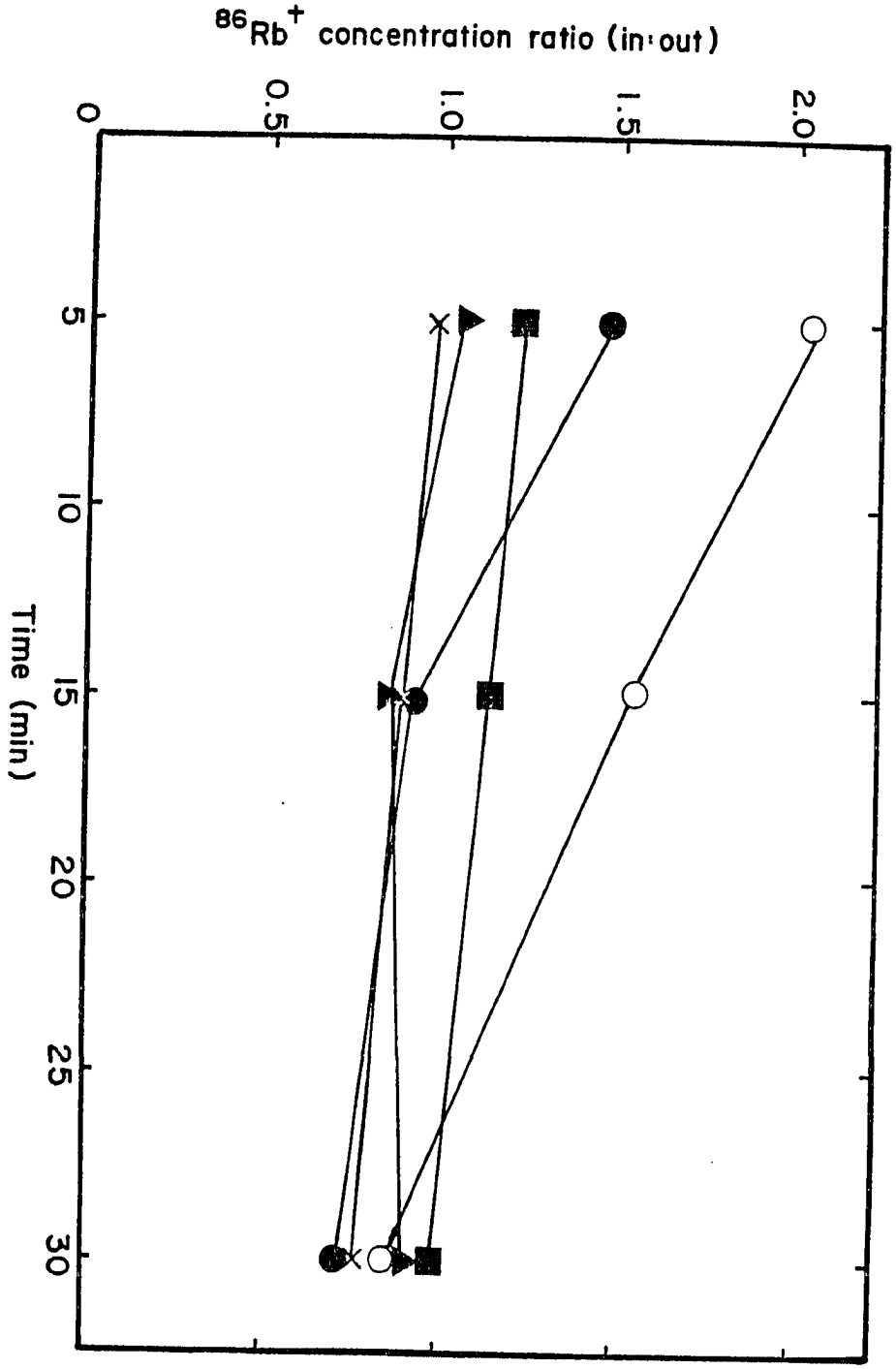
		10 min measurement		30 min measurement	
Anion	ATP	$pH_{out} - pH_{in}$ =	ΔpH	$pH_{out} - pH_{in}$ =	ΔpH
SO_4^{2-}	-	6.96 - 6.86 =	0.10	6.94 - 6.81 =	0.07
SO_4^{2-}	+	6.90 - 6.84 =	0.06	6.86 - 6.81 =	0.05
Cl^-	-	6.97 - 6.87 =	0.10	6.94 - 6.73 =	0.21
Cl^-	+	6.88 - 6.75 =	0.13	6.86 - 6.73 =	0.13
SCN^-	-	6.97 - 6.77 =	0.20	6.95 - 6.69 =	0.26
SCN^-	+	6.90 - 6.72 =	0.18	6.87 - 6.63 =	0.24

Aliquots of secretory granules were incubated for the indicated times at 25°C either in medium containing 10 mM $MgSO_4$ and 10 mM Na_2ATP, or 16.7 mM Li_2SO_4 (to match the osmolality of the ATP-containing medium). The incubations also included 50 mM PIPES buffer (at the medium pH's listed), 295 mM sucrose and either 30 mM K_2SO_4 or 45 mM KCl or KSCN. Parallel experiments under these conditions (using thin layer analysis of the nucleotides, as described in Methods) revealed that < 50% of the added ATP was hydrolyzed over the 30 min time course. Vapor pressure measurements of these media confirmed an osmolality of ~550 mOsm. Values were calculated from the mean of duplicate determinations of (in:out) [^{14}C]methylamine ratios as described in Methods. Unlike the results shown, chromaffin granules incubated under conditions similar to those described above (addition of ATP in a hyperosmotic, chloride-containing medium) have been found to acidify their interior approximately 0.3 pH units (95).

resulted in the elimination of any apparent alkalinization of the intragranular space (with ATP), but still failed to show acidification.

The second point relates to uncertainty about the time needed for maximum acidification to occur. While Casey et. al. (95) found that the chromaffin granule interior was maximally acidified at times $\geq$20 min, we could find no change in intragranular pH induced by ATP at any times between 5 - 60 minutes, despite that thin-layer analyses of adenine nucleotides after such incubations revealed that > one-half of the added ATP remained unhydrolyzed over this time period. The final point alludes to the possibility that we could be using anions which are either insufficiently permeant to allow development of ΔpH, or so permeant that coupled H^+-anion influx would lead to osmotic granule lysis (95). Since parotid granule intactness was always $\geq$90%, absence of detectable acidification due to granule lysis seems unlikely in our experiments. However, to evaluate this last point comprehensively, possible acidification was examined in hyperosmotic media using anions -- SO_4^{2-}, Cl^-, SCN^- -- that span the permeability spectrum. The results (Table II) for granules incubated 10 and 30 min in the presence and absence of 10 mM ATP using the [^{14}C]methylamine distribution technique indicate at most a minor acidification in one sample containing ATP. Granule intactness in all samples was >90% (except the 30 min sample containing ATP and KSCN, which was 89.2% intact). The lower intragranular pH values in some ATP containing samples seem to be related primarily to decreases in medium pH. However, since it was not possible to maintain perfectly the same pHout in the presence and absence of ATP, ΔpH values are not strictly comparable. Nonetheless, a substantial intragranular acidification was not observed under any of the conditions tested.

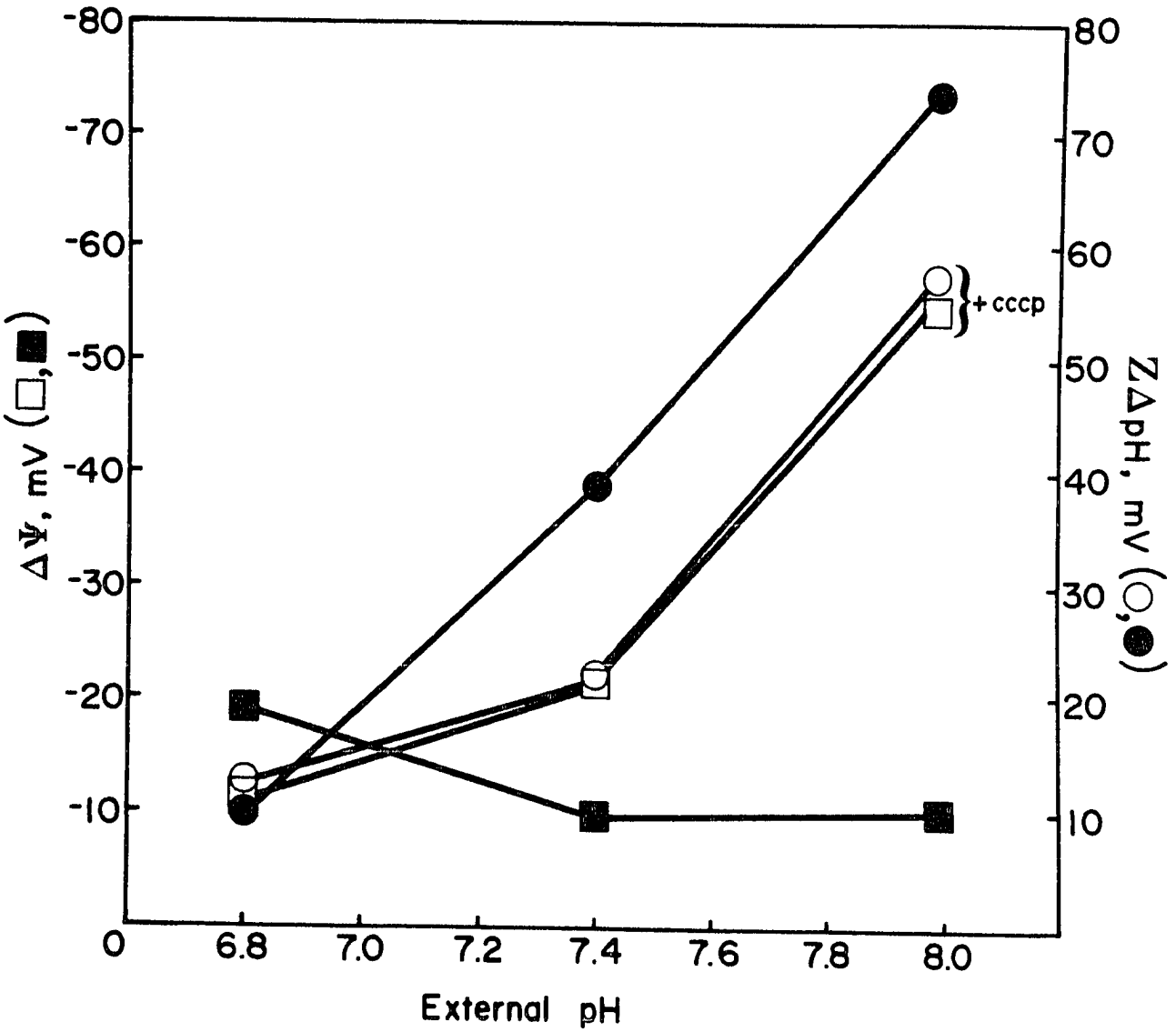
Figure 1. Time Course of $^{86}Rb^+$ Accumulation into Isolated Parotid Secretory Granules. Granules were incubated with $^{86}Rb^+$ and valinomycin as described in Methods at 25°C for the times indicated in media containing ~190 mM sucrose, 0.5 mM K_2SO_4, and 50 mM MOPS plus the following additions: (○), 8.33 mM Li_2SO_4, final pH = 6.79; (●), 5.0 mM $MgSO_4$ and 5.0 mM Na_2ATP, final pH = 6.85; (■), 5.0 mM $MgSO_4$, 5.0 mM Na_2ATP and 10 μM CCCP, final pH = 6.84; (▲), 5.0 mM Na_2 ATP and 3.33 mM Li_2SO_4, final pH = 6.88; (x), 5.0 mM $MgSO_4$ and 5.0 mM AMP-PNP, final pH = 6.83. Each point is the mean of duplicate determinations in a single representative experiment.



Effect of ATP on the Electrical Potential Across the Membranes of Isolated Secretory Granules - Being unable to detect substantial acidification with ATP, I attempted to examine effects of ATP on the granules' transmembrane potential, since the influx of fewer protons is needed to increase $\Delta\Psi$ (interior positive) than is necessary to acidify the well-buffered intragranular space. The distribution of $^{86}Rb^+$ in the presence of valinomycin was utilized for dectecting changes in $\Delta\Psi$. Rubidium partitions almost exclusively into the aqueous phase and in the presence of 20 mM external K^+, the $^{86}Rb^+$ concentration ratio obtained (<0.2 in:out) suggested that its binding to granules was insignificant.

Preliminary experiments examining the time course of $^{86}Rb^+$ distribution in the presence and absence of ATP indicated minor differences in potential ($\sim$10 mV) seen only within the first 30 minutes. To ascertain whether these small initial effects were due to proton translocation dependent on ATP hydrolysis, the experiment shown in Fig. 1 was performed. Granules were incubated in one of five test conditions containing low levels of impermeant anions. The effects of ATP were compared to those of either buffer alone, AMP-PNP (an ATP analog which is not hydrolyzed by parotid granules), and ATP added in the absence of Mg^{2+}. Additionally, one set of granules received both ATP and CCCP, an ionophore expected to dissipate the H^+ electrochemical gradient. The experiment was performed at pH 6.8, both because this value is in the pH range used previously in studies which suggested the presence of a parotid granule ATPase (128), and because it is sufficiently close to the parotid granule internal pH so as to minimize the effects of CCCP addition on transmembrane potential. The data indicate a gradual decline from initial values of the intragranular/medium $^{86}Rb^+$ concentration ratios under all conditions. The cause of this decline, although

Figure 2. Generation of $\Delta\Psi$ by Imposition of ΔpH. Granules were incubated for 10 min at 25°C in a medium containing 10 μM valinomycin, 0.5 mM K_2SO_4, 190 mM sucrose and 50 mM MOPS buffer at the pH's listed. Sets of 6 samples were divided into three pairs for internal aqueous space ([^{3}H]water; [^{14}C]sucrose), ΔpH ([^{3}H]water; [^{14}C]methylamine), and $\Delta\Psi$ ([^{3}H]water; $^{86}Rb^+$) measurements. Values for $Z \cdot \Delta pH$ and $\Delta\Psi$ were calculated from the Nernst equation as described in Methods, where $Z = 2.3RT/F$. Differences between duplicate values in all cases were < 12.5%; the mean values are shown. (●,■), control; (○,□), plus 10 μM CCCP.



unknown, is consistent with a slow redistribution of other ions following granule addition to the reaction mixture.

It is quite clear that at any given time, only small differences in the $^{86}Rb^+$ distributions are observed among all of the test conditions. The transmembrane potential of granules incubated in ATP containing media is only slightly influenced by the presence of CCCP (squares, Fig. 1). In addition, the calculated $\Delta\Psi$ from the measurements made at 30 min in the presence of the non-hydrolyzable ATP analog (crosses) differs by only 2 mV from that caused by ATP itself, suggesting that the observed (in:out) $^{86}Rb^+$ ratios do not depend on ATP hydrolysis. By 60 min, no difference in $\Delta\Psi$ was found between the ATP treated granules and those containing buffer alone (not shown). Since proton pumping would be expected to generate inside-positive potentials within minutes (45,135,137,145), and since such potentials should dramatically reduce the intragranular concentations of permeant cations (68), these data signify an inability of parotid granules to transduce the energy of ATP hydrolysis into an electrical potential via proton translocation.

Proton Permeability of the Membranes of Isolated Parotid Secretory Granules - One possible explanation for the inability to detect ATP-driven proton translocation is that the H^+ conductance in the membrane of isolated parotid granules is high, and thereby dissipates the electrochemical H^+ gradient. To assess H^+ conductance, various transmembrane proton gradients were experimentally created by altering medium pH, and both the $^{86}Rb^+$ and [^{14}C]methylamine concentration ratios were examined at equilibrium in the presence and absence of CCCP. The ionophore is expected to increase granule membrane H^+ permeability, and in these samples, the overall transmembrane potential should be dominated by the H^+ equilibrium potential. Consequently the total

electrochemical gradient for H^+ should = 0, since ΔpH should be roughly equal and opposite to $\Delta\Psi$ (68,45).

The results shown in Fig 2 demonstrate that, in the absence of CCCP, the granule membrane H^+ permeability is low, and that CCCP addition brings $\Delta\Psi$ into equilibrium with ΔpH. As the external pH is varied over the range from 6.8 to 8, the ΔpH increases both in the presence and in the absence of CCCP, although the change is slightly more pronounced without the ionophore (open vs. closed circles, Fig. 2). In the presence of CCCP, $\Delta\Psi$ (measured by $^{86}Rb^+$ distribution) closely follows $Z\Delta pH$ (where $Z = 2.3RT/F$) as expected if the granule membrane acts as an H^+ electrode (similar results are obtained without valinomycin, using the probe $[^3H]TPP^+$ to measure electrical potential, as described below). The observed behavior also indicates, as predicted by earlier measurements, that the amount of K^+ present in the granule is small with respect to the intragranular buffering capacity. In the absence of CCCP, however, an increase in $Z\Delta pH$ from 10 to 73 mV (acid inside) failed to increase the magnitude of $\Delta\Psi$, which actually decreased from -19 to -10 mV. In this case (Fig. 2), $\Delta\Psi$ is probably controlled by the K^+ equilibrium potential (since all samples are incubated in the presence of valinomycin). These data clearly indicate that isolated parotid granules have an H^+ conductance which is far lower than in CCCP-treated granules.

To be certain that the presence of valinomycin did not adversely affect measurement of H^+ conductance, similar measurements were made using the lipophilic cation $[^3H]TPP^+$ (see Methods). The protocol for measuring $\Delta\Psi$ in relation to ΔpH (using $[^3H]TPP^+$ and $[^{14}C]$methylamine) differed as follows: a) valinomycin was not included in granule samples; b) granules were incubated for 60 min to permit the $[^3H]TPP^+$

Table III

Inhibitor Sensitivity of ATPase(s) from Chromaffin Granule Membranes and Parotid Secretory Granule Fraction

Source of ATPase(s)	Vanadate plus Efrapeptin	Nbd-Cl	Released P_i (nmoles/min)	% of total
	-	-	3.17	100
Chromaffin	+	-	2.38	75
Granule Membrane Fraction	-	+	0.608	19
	+	+	0.403	13
	-	-	1.80	100
Parotid	+	-	0.522	29
Granule Fraction	-	+	1.85	102
	+	+	0.520	29

Both fractions were incubated with 3 mM Mg-ATP and 2.5 μM FCCP as described in Methods. Protein of the parotid fraction was higher than that of the chromaffin fraction, but amounts of each fraction assayed were chosen on the basis of activity rather than protein. Values shown are the mean of duplicate determinations, differences between duplicate measurements were all <2.0%.

concentration ratio to achieve a stable value; and c) background binding of the [^{3}H]TPP$^+$ probe had to be subtracted. However, the results obtained by this method were the same as that shown in Figure 2 except that no decline in [^{3}H]TPP$^+$ concentration ratios with increasing ΔpH was observed. These experiments suggest that it is very unlikely that the inability to detect changes in the H^+ electrochemical gradient reflects a high intrinsic H^+ conductance of parotid granule membranes.

Preliminary Characterization of ATPase Activity in the Rat Parotid Granule Fraction - While the absence of significant proton pumping in isolated parotid granules suggests that they lack the H^+-ATPase, the possibility existed that this ATPase was present, but was not coupled to H^+ pumping. Consequently, ATP hydrolysis by the parotid granule fraction was tested to see if it showed similar characteristics to that of the ATPases of either chromaffin granule membranes or parotid mitochondria. To this intent, the inhibitory abilities of three molecules were exploited: a) sodium vanadate, known to inhibit many types of ATPases which proceed through the formation of a phosphoenzyme intermediate (146), but having no effect on either chromaffin granule or mitochondrial membrane ATPases (147); b) efrapeptin, an inhibitor of the mitochondrial ATPase (138), but ineffective on known granule ATPases (147,148); and c) Nbd-Cl, known to inhibit mitochondrial ATPase at high doses (149), but recently shown to cause extensive inhibition of chromaffin and platelet granule ATPases in the 1-20 μM range.[4] ATP hydrolysis was assayed in the presence of 2.5 μM FCCP to minimize possible inhibitory effects of ATP-generated H^+ gradients on further ATPase activity.

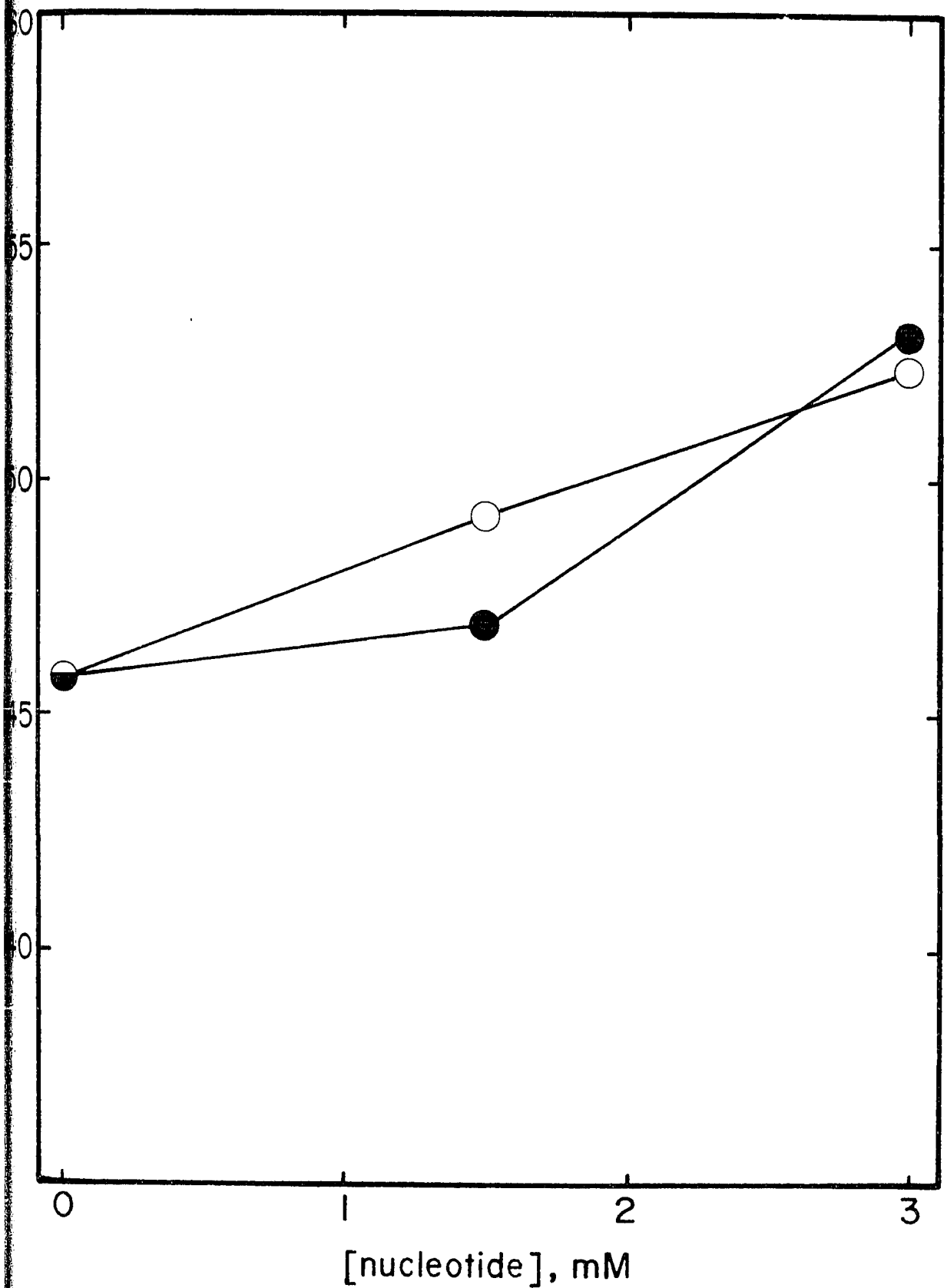
Initial studies of the actions of the three inhibitors on parotid mitochondria established that while vanadate had no demonstrable effect,

efrapeptin inhibited ~90% and Nbd-Cl (20 μM) ~2.0% of the ATPase activity. The parotid granule fraction, however, expressed an ATPase activity which was 50-75% inhibited by the combined presence of vanadate and efrapeptin (with the individual vanadate and efrapeptin sensitive proportions approximately equal but variable between preparations), while Nbd-Cl had no effect. To directly compare the ATPase of the parotid secretory granule fraction to that of bovine chromaffin granule membranes purified according to (136), I performed the experiment shown in Table III. The results reveal a striking difference in the inhibitor susceptibility of the ATPase(s) between the two fractions; namely, while ATP hydrolysis by chromaffin membranes is reduced > 80% by Nbd-Cl, the parotid granule fraction has no ATPase component which is sensitive to inhibition by this compound. However, both fractions have a component which is susceptible to the efrapeptin-vanadate combination, and both possess a residual ATPase activity after the combined addition of all three inhibitors. The significance of these latter activities has not yet been determined.

To rule out that rat parotid cells might contain an inhibitor (either soluble or granule membrane-bound) of H^+-ATPases of the chromaffin type (which could prevent ATPase expression in previous assays), I examined the ATPase activity of mixtures of either the parotid granule fraction or parotid post-microsomal supernate with chromaffin granule membranes (to see if the combined activities were equal to or less than the sum of those of the individual fractions). No inhibition was detectable by either parotid fraction (not shown).

Extent of Lysis of Isolated Parotid Secretory Granules in ATP-Containing Media - The preceding data which fail to provide evidence for

Figure 3. Effect of ATP and AMP-PNP on Parotid Granules Lysis. Granules were incubated at 37°C for 10 min in a medium containing Tris-HCl, pH 7.4 and either ATP (open circles) or AMP-PNP (closed circles) at the concentrations shown plus 3 mM $MgCl_2$, 100 mM KCl and approximately 30 mM sucrose (from the granule suspension). A small amount of Li_2SO_4 was added as needed to maintain all samples at identical osmolalities. Granules lysis was measured as described in Methods. Each point represents the mean of triplicate determinations in a single experiment.



a parotid granule proton pump contrast with results obtained for other granule types and also appear at variance with reports of ATP-induced lysis of rat parotid granules [(128,139,140), recently postulated to be the chemiosmotic result of a granule membrane proton pump (128)]. Indeed our own studies showed only a 1-2% increase in the lysis of parotid granules with ATP (up to 5 mM) above that observed for control samples (no ATP) in chloride-containing, phosphate-buffered media of 300-350 mOsm (10-30 min, 37°C).

To resolve these apparent discrepancies, I carefully reviewed the original data of Ishida and colleagues (139,140) and discovered three significant points: a) their granule test media had an osmolality (~265 mOsm) approaching that which may cause granule lysis due solely to hypoosmotic stress (see previous chapter). Since this media also contained 100 mM KCl [which further serves to destabilize isolated parotid granules, (chapter 1)], it is not surprising that even the control granules were only ~50% intact (139). b) The actual fraction of the granule population which responded (by lysis) to ATP was only 6-16% in different experiments (139). c) The effect of ATP was abolished by either the pre-treatment or simultaneous treatment of parotid granules with EGTA (1 mM). Indeed, these authors concluded that such lytic effects of ATP on parotid granules were dependent on trace concentrations of Ca^{2+} (139,140). Such a calcium requirement has not been noted in the ATP-induced osmotic lysis of chromaffin granules mediated by an inward-directed proton pump (99). From these three points it was suspected that ATP-induced parotid granule lysis might be based on a mechanism different from the osmotic lysis of chromaffin granules observed by others (99,60).

To directly test this possibility, I repeated the parotid granule lysis studies employing an incubation medium identical to that used by Ishida et. al. (140), but simultaneously examined granule samples in which the non-hydrolyzable ATP analog, AMP-PNP, was included instead of ATP (Fig. 3). The 7% increase in granule lysis in the presence of 3 mM ATP (Fig. 3) is in reasonable agreement with that found by Ishida et. al. (140). However, this modest effect is entirely mimicked by AMP-PNP. One-dimensional thin-layer separations of the adenine nucleotides confirmed that after granule incubations, all of the analog-containing nucleotide co-migrated with an authentic AMP-PNP standard (not shown). Thus, the effect of AMP-PNP could not be due to its metabolism. Conversely, the effect of ATP was not likely to be limited by its metabolism, since 50-75% of the original ATP remained unhydrolyzed after the granule incubations. Finally, the effect of EGTA was examined; our data confirms that of Ishida et al (140) namely, that the presence of 1 mM EGTA inhibited the effects of both ATP and the non-hydrolyzable analog on parotid granule lysis (not shown).

Discussion of Chapter 2

Considerable effort is being made in several independent laboratories to delineate the physiologic role of granule membrane ATPases in the cell types where they have been found. Through intensive investigation of adrenal medullary chromaffin granules both in vitro and in situ, several excellent studies have revealed that these granules possess an electrogenic, inward-directed H^+ translocating ATPase which is primarly responsible for the generation of internal positive membrane potentials (68); the ability to further acidify the granule interior (95); the ability to increase intragranular osmolality and hence cause

osmotic lysis (99); and perhaps most importantly, the ability to accumulate biogenic amines (45). In addition, several hypotheses have been put forward which cite the properties of intragranular acidity and/or granule membrane H^+-ATPases as factors common to secretory granules of all cell types (93), in the thought that such factors may share a general role in processes related to content storage and discharge (91,100,101,115). However, since the granules from different cell types show major differences in their respective contents, it is virtually certain that a subset of granule properties will vary from one type to another.

The major function of parotid acinar cells is to discharge an exocrine secretion rich in high molecular weight macromolecules, rather than a neuroendocrine secretory function like that observed in adrenal chromaffin cells. The results of the present analyses for the presence of a chomaffin-like proton pump associated with isolated granules from the rat parotid are uniformly negative: a) despite the absence of permeant anions, the parotid secretory granules do not show an ability to use ATP to shift their transmembrane potential to more inside-positive values (Fig. 1). Such an effect cannot be explained by a granule membrane proton leak for two reasons. First, the H^+ permeability of parotid granules appears to be quite limited (Fig. 2); and second, even granules with a measurable H^+ permeability are able to generate an internal-positive potential in the presence of ATP, if they possess an electrogenic H^+-translocase (68). b) Despite the presence of permeant anions, the parotid granules are unable to acidify their interior (Table II). c) The characteristics of the ATP hydrolysis by the parotid granule fraction differ drastically from those of purified

adrenal chromaffin granule membranes, most obvious with respect to their lack of sensitivity to the inhibitor Nbd-Cl (Table III). The relatively large amount of efrapeptin/vanadate-sensitive ATPase in the fraction may well be attributable to organelles which contaminate the granules [especially mitochondria, plasma membranes and nuclear membranes, (see chapter 1)]. d) The granule lysis data which have been cited to support such an ATPase are likely to represent a different, Ca^{2+}-sensitive effect which does not require ATP hydrolysis (Fig. 3 and refs. 139,140). In contrast to studies of isolated chromaffin granules (99), studies of α-amylase release in comparison to optical density changes, as indices of lysis of granules in suspension, fail to corroborate the accuracy of the latter method [as used by (128)] for parotid granules (not shown).

The failure to find a proton pump in these granules is apparently not due to the manner of their purification, since similar isolation procedures do not inactivate the H^+-ATPase of adrenal chromaffin granules (87); nor does it appear to be due to any active ATPase inhibitor either in parotid granule or cytosolic fractions. However, the absence of proton translocase activity in these mature granules isolated from unstimulated parotid glands, does not rule out the possibility that such an activity in a subpopulation of granules or in granules at different stages of maturation might exist. Indeed, experiments to be discussed in the following chapter have demonstrated ATP-dependent acidification of granules prepared from parotid glands of rats which have been chronically stimulated by repeated intraperitoneal injections of isoproterenol; the possible significance of these findings will be discussed.

Regardless of these considerations, the present studies suggest that both intragranular pH and granule membrane H^+-translocases are

properties which may vary between the secretory granules of different cells. In addition, these results tend to support the conclusions of Holz et. al. (93), that electrochemical H^+ gradients across the secretory granule membrane are not immediately involved in exocytosis.

Summary of Chapter 2

Isolated rat parotid secretory granules have been examined for the possible presence of an ATP-driven H^+ translocase activity using several approaches. First, the transmembrane pH difference measured by the equilibrium distribution of either [^{14}C]methylamine or [^{3}H]acetate was not substantially affected by ATP in the presence of membrane-permeating anions. Second, despite a low intrinsic H^+ permeability of parotid granule membranes, no significant changes in transmembrane potential could be detected. Third, ATP-induced lysis of parotid granules was minor (~7% of total), and independent of ATP hydrolysis. Finally, ATP hydrolase activity of the parotid granule fraction was not susceptible to inhibition by 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (Nbd-Cl) at a concentration which decreased the measured ATPase of purified chromaffin granule membranes by more than 80%. It is concluded that there is no evidence to support the existence of significant electrogenic or electroneutral H^+-ATPase activity in isolated parotid granules despite a previous report suggesting that such an activity elicited granule lysis *in vitro*.

CHAPTER 3

ISOLATED SECRETORY GRANULES FROM PAROTID GLANDS OF CHRONICALLY-STIMULATED RATS POSSESS AN ALKALINE INTERNAL pH AND AN ELECTROGENIC H^+-ATPase ACTIVITY

Introduction to Chapter 3

Results in previous chapters showed that secretory granules isolated from the parotid glands of normal rats differ markedly in two respects from granules isolated from other cell types. First, the parotid granules possess a pH_{in} ($\sim$6.8) higher than any other secretory granule reported thus far; and second, they fail to exhibit significant H^+-translocating ATPase activity. A potential limitation of the experiments conducted (detection of ΔpH and $\Delta\Psi$ in the isolated granules) was the use of a granule fraction constituting only $\sim$11% of the total tissue amylase activity and thus possibly inadequately representing the entire granule population of acinar cells. A point of special concern is whether the fraction studied could have been enriched selectively in granules of a particular age (older as opposed to younger) along the continuum of formation originating at the Golgi complex.

A model has been outlined (in the main Introduction of this dissertation) which hypothesizes that a secretory vesicle not primarily involved in small molecule (e.g., catecholamine) accumulation might possess an electrogenic H^+ pump in its early stages of maturation, in order to create an inside-positive membrane potential which would drive unbound cations (and water) out of the vesicle interior. After energy-dependent dehydration is completed, the remaining granule could modify its membrane by 1) maintaining or establishing very low passive ionic permeabilities, and 2) removing or inactivating its H^+-ATPase.

This hypothesis is consistent with the observed absence of significant H^+-ATPase activity in the isolated parotid granules just described, but it predicts that early forms of the same granules (which can be considered the functional equivalent of condensing vacuoles) will express proton pump activity. However, the number of early granule forms in the resting parotid cell is unknown, and so far no convincing means of separating subpopulations of different aged secretory granules has been described. Consequently, it is not yet possible to test directly this hypothesis in the parotid (or other) system.

In the absence of a direct test, two indirect approaches have been undertaken. First, studies of secretory vesicles isolated from parotid glands of normal rats which are restoring their granule population following complete degranulation induced by isoproterenol (102) have been initiated. Second, a detailed examination of granules purified from rat parotid following chronic β-adrenergic stimulation has been conducted. In the latter case (the results of which are reported in this chapter), the isoproterenol treatment used causes extensive hypertrophy and hyperplasia of the parotid gland (107), an exceptional increase in the volume of the secretory granule compartment of acinar cells [~13.5 fold (113)], and the appearance of granules that bear a striking morphological resemblance (large size and low electron opacity) to condensing vacuoles (112). These characteristics coupled with the rapid secretory turnover as a consequence of the pharmacologic stimulation suggested that granules of such tissue might approximate immature forms. Therefore, investigation of possible H^+-ATPase activity using an isolated fraction of these granules was considered appropriate.

In this report I will describe a) a procedure for the rapid isolation of these unusual granules (in isoosmotic media), b) a partial morphologic and enzymologic characterization of the granule fraction, to assess its purity and representative nature, c) a preliminary description of the secretory granule content species and amino acid composition, d) the internal volume and pH of these granules, and e) the finding of an electrogenic H^+-translocating ATPase activity in the isolated granule fraction.

These observations could point to a link in the functioning of the membranes of secretory granules which are involved in small molecule accumulation and those involved in macromolecular packaging.

Methods

Chronic β-Adrenergic Stimulation of Rats - Intraperitoneal injections of d,l-isoproterenol [30mg/ kg/ day, in 0.15 M NaCl (0.3 ml)] were administered to rats for 3 to 10 successive days. Routinely, rats injected for 10 days [which achieves near-maximal gland enlargement, (160)] were used for intragranular pH measurements and studies of H^+-ATPase; injections were usually given between 10 a.m. - 2 p.m.. After the last injection, animals were starved 16 - 22 h before sacrifice. The effects of prolonged starvation on parotid granules of normal rats (161) were not observed in these stimulated rats.

Biochemical Assays - α-amylase; cytochrome c oxidase; γ-glutamyl transpeptidase; β-N-acetyl glucosaminidase and UDP-galactosyl transferase were assayed as previously described (122). Protein was assayed with fluorescamine as described by Udenfriend et. al. (125) using bovine serum albumin as standard.

Amino Acid Analysis - Parotid granule content proteins (described in the text) and 10 nmol of norleucine (used as an internal standard) were subjected to acid hydrolysis in 6 N HCl for 20 h. The amino acid content of these samples were then quantitated using a Durrum D-500 amino acid analyzer.

Microscopy - Parotid tissue from normal and chronically-stimulated rats was fixed by immersion in aldehydes as described (121). Isolated parotid granule fractions were fixed in suspension in aldehydes (82); tissue and granule fractions were then post-fixed in OsO_4, dehydrated in ethanol and embedded in Epon (121). Light micrographs were taken of methylene-blue stained thick section from Epon blocks, using a Zeiss photomicroscope and electron micrographs of thin sections stained with uranyl acetate and lead citrate were taken on a Philips 300 electron microscope.

SDS-Polyacryamide Gel Electrophoresis - Reduced and alkylated polypeptides were resolved by electrophoresis on gradients of 8 - 16% polyacrylamide with a 4% polyacrylamide spacer gel, using the Laemmli discontinuous system (173). After electrophoresis, gels were processed directly for silver staining (174), and photographs were taken immediately thereafter.

Internal Aqueous Volume and Internal pH of Isolated Granules - These determinations were performed in a manner identical to those for isolated parotid granules from normal rats (chapter 1), except that [^{14}C]sucrose (marker of the excluded H_2O volume of granule pellets) was added (in a volume of 2 µl) just prior to the termination of incubations by centrifugation, rather than at the beginning of the incubation. This modification limits the length of exposure of granules to [^{14}C]sucrose, thus reducing problems of permeation into the granule interior. Both

[^{14}C]methylamine and [^{3}H]acetate were used as probes of ΔpH (126). The equilibrium distributions of these probes were calculated from internal aqueous space values measured in parallel with the ΔpH determination. Concentrations of radioactive probes used were the same as described previously (chapter 1).

Secretory Granule Transmembrane Potentials - Measurements of electrical potential were made using the equilibrium distribution of $^{86}Rb^{+}$ in the presence of 10 μM valinomycin (as described in chapter 2). ΔΨ was calculated using the Nernst equation. All incubations were carried out at 25° C.

Materials - Materials are the same as those listed in chapter 2.

Results

Isolation of Parotid Secretory Granules from Chronically Stimulated Rats - Although the interest in the enlarged parotid secretory granules from chronically-stimulated rats has been longstanding (162), these granules have not been prepared previously as an isolated fraction. One study indicated no success in obtaining a fraction with sedimentation characteristics similar to those from control parotid glands (163).

In an attempt to increase granule yield without compromising granule purity, the Percoll-density gradient centrifugation procedure developed for the isoosmotic isolation of chromaffin granules (164) was modified for the parotid gland. Percoll contributes less than 12 mOsm to the osmolality of the present isolation medium. All steps are carried out at 4° C.

Male Sprague-Dawley rats (150 - 225 g) were killed by cardiac incision (under ether anesthesia). The enlarged parotid glands of two or thee rats were excised, cleaned of connective tissue, minced thoroughly

with razor blades, and gently homogenized (with four strokes in a Brendler teflon pestle homogenizer at 1,300 rpm) in ~33 ml (~15% w/v) of ice cold sucrose (0.3 M), MOPS (2 mM, pH 6.9), and $MgCl_2$ (0.2 mM). The low ionic strength of the fractionation medium is thought to minimize organelle aggregation. The resulting suspension was then spun at 300 x g for 2.5 min; unbroken cells, nuclei, and some secretory granules sediment under these conditions. The supernatant fluid was saved; the pellet was rehomogenized in another ~33 ml of fresh medium and respun as before. This supernate was pooled with the first, adjusted (with a 200 mM EDTA stock solution) to a net EDTA concentration (EDTA minus Mg^{++}) of 1.0 mM, filtered through one layer of nylon screen (20 μm^2 mesh), and homogenized with three strokes in a Dounce homogenizer (tight pestle) with the intent of minimizing any possible organelle aggregation. This homogenate (nuclei removed) forms the basis for assayed enzyme recoveries.

The homogenate (~66 ml) was mixed with two volumes of buffered-Percoll medium: 0.3 M sucrose, 86% (v/v) Percoll, 1 mM EDTA, and 2 mM MOPS, pH 6.9. This mixture was loaded into 8 polycarbonate centrifuge tubes and spun in a Beckman 60Ti rotor at 15,000 rpm for 30 min (with brake on). Granules were banded in the lower (denser) quarter of the self-formed gradients; most other organelles were near the top. Granule bands were pooled, mixed, reloaded into two polycarbonate centrifuge tubes, and spun again in the 60Ti rotor at 25,000 rpm for 30 min (brake on). A small amount of contaminating material, mostly mitochondria, could be floated away from the granules during this second centrifugation. The granule bands were again collected, and the remaining gradient fluid was pooled and saved for assays. The granule-rich band was then diluted at least 5-fold with a solution containing

Figure 1: Light Micrographs of (methylene-blue stained) Rat Parotid Tissue and Secretory Granule Fractions. Fractions were prepared as described in Methods and in chapter 1. Magnification in all cases is x 970. a) Normal rat parotid tissue. Secretory granules occupy ~31% of acinar cell volume (113). b) Secretory granule fraction from normal parotid gland; section near the bottom of the pellet. c) Parotid tissue from a rat injected intraperitoneally with isoproterenol x 10 d. Note the enlargement of the acinar cells, nuclei, and secretory granules, which now occupy > half the cell volume (113). d) Parotid secretory granule fraction from chronically-stimulated rats; a section near the top of the pellet. The entire thickness of the granule pellets has a homogeneous appearance. Note the size difference between these structures and those shown in b).

0.3 M sucrose, 1 mM EDTA, 2% polyethyleneglycol, and 2 mM MOPS, pH 6.9; which serves to reduce the buoyant density of the medium and favors disaggregation of Percoll from the granule surfaces. Granules were then pelleted by centrifugation at ~2,300 x g for 15 - 30 min. Granule pellets were white, similar in appearance to those from control animals (chapter 1).

Purity and Recovery of Enlarged Secretory Granules - Fig. 1 is a group of light micrographs of parotid tissue from chronically-stimulated and normal rats, and granule fractions isolated therefrom (using the current isolation procedure and that described in chapter 1). Differences in size and staining characteristics are apparent between the granules both *in situ* and *in vitro*. No structures other than granules can be seen to occupy a significant volume fraction in the pellet of granules from stimulated rats. Quantitation of the contaminant volume fraction (at the electron microscope level) of these pellets is in progress (however, this value is expected to be <5% of total volume of structures present). Evidently, the morpholgic appearance of these granules in isolation is quite similar to that seen *in situ*.

An analysis of marker enzymes is shown in Table I. Even though levels of α-amylase per granule decline in the stimulated parotid glands, the recovery of this marker (still presumed to be exclusively in secretory granules) is >20% of that of the homogenate. Other known secretory enzymes (e.g. DNAase, RNAase) also decline in the stimulated glands (107); however, they were not assayed in the present fraction. The 3.5-fold increase in relative specific activity of α-amylase is quite high, in view of the observation that secretory granules of the chronically-stimulated parotid occupy more than half the acinar cell volume (113). Thus the fraction appears to be very pure; and since less

Table I

Distribution of Protein and Enzyme Activities in Preparation of Parotid Secretory Granules from Chronically-Stimulated Rats

		Protein	Amylase	γ-glutamyl transpeptidase
Homogenate	Recovery	100	100	100
	R.S.A.	1.0	1.0	1.0
Remaining Gradient	Recovery	88.4%	56.3%	80.9%
	R.S.A.	--	0.637	0.916
Granule Band	Recovery	13.1%	42.8%	22.4%
	R.S.A.	--	3.25	1.70
Final Supernate	Recovery	7.20%	22.8%	13.9%
	R.S.A.	--	3.17	1.93
Granule Pellet	Recovery	5.83%	20.4%	14.0%
	R.S.A.	--	3.50	2.40
Total Recovery		101%	99.5%	109%

Results shown are from four preparations of granules (each value is the mean of at least three determinations). Total recovery represents the summed fraction activities ÷ homogenate activity. Homogenate activities were: Amylase 49,300 μmol/min; γ-glutamyl transpeptidase, 20.8 μmol/min; β-N-acetyl glucosaminidase, 4.35 μmol/min; cytochrome c oxidase, 110 units [proportional to the first order rate constant as described in (177)]; UDP-galactosyl transferase, 2.1 nmol/min. Relative specific activites (R.S.A.) refer to specific activites in the granule fraction with respect to the homogenate.

Table I (Continued)

β-N-acetyl glucosaminidase	cytochrome c oxidase	UDP-galactosyl transferase
100	100	100
1.0	1.0	1.0
90.6%	98.4%	108%
1.03	1.11	1.23
6.42%	1.69%	2.08%
0.488	0.128	0.158
5.08%	1.28%	1.93%
0.705	0.179	0.268
1.32%	0.611%	0.577%
0.226	0.105	0.099
97.0%	100%	110%

than 50% of the homogenate amylase can be recovered in the gradient interface [an indication that a significant fraction of amylase has been lost due to mechanical rupture of granules, (124)] the recovery of the remaining intact granules after homogenization may approach 50%. The incomplete sedimentation of amylase in the final centrifugation step (Table I) is unexplained; it may reflect heterogeneity in granule size, partial granule lysis, or unusual sedimentation properties [noted previously (163)].

γ-glutamyl transpeptidase, a common marker of granule and plasma membranes (122), also exhibits an unusual distribution in this preparation. Of the activity present in the density gradient, >20% is associated with the granule band. This level is unlikely to reflect plasmalemmal contamination because 1) the medium density in this portion of the gradient is expected to limit greatly the penetration by plasmalemmal elements, and 2) electron microscopic observation fails to give evidence of significant plasmalemmal contamination. In the final differential centrifugation, the γ-glutamyl transpeptidase of the granule interface partitions between the supernate and pellet in proportions equal to those of α-amylase. Thus, these data are consistent with the notion that the granule compartment in the chronically-stimulated rat parotid gland possesses γ-glutamyl transpeptidase activity (most likely to a higher degree than in normal parotid tissue), but not to the exclusion of other (probably plasmalemmal) organelles.

The measurements of β-N acetyl glucosaminidase, cytochrome c oxidase, and UDP-galactosyl transferase suggest that the specific activities of these lysosomal, mitochondrial, and Golgi markers are 4.5-fold, 9.5-fold, and 10-fold lower than those of the homogenate, respectively. No attempt was made to analyze recovery of elements of the

Table II

Absolute and Relative Amino Acid Composition of the Contents of Parotid Secretory Granules from Normal and Chronically-Stimulated Rats

	Normal		Isoproterenol (10 d)	
	Absolute (n moles)	% amino acid	Absolute (n moles)	% amino acid
ASX	26.0	11.7	30.6	5.6
THR[a]	8.6	3.9	4.0	0.7
SER[b]	19.1	8.6	18.4	3.4
GLX	29.4	13.2	137.3	25.1
PRO	36.1	16.2	156.7	28.7
GLY	34.5	15.5	115.6	21.2
ALA	12.1	5.4	8.7	4.5
VAL	9.8	4.4	4.5	0.8
MET	2.2	1.0	0.5	0.1
ILE	4.9	2.2	2.9	0.5
LEU	16.4	7.4	10.6	2.0
TYR	5.0	2.2	2.3	0.4
PHE	7.8	3.5	6.7	1.2
HIS	4.4	2.0	8.6	1.6
LYS	7.3	3.3	14.8	2.7
ARG	9.0	4.1	24.1	4.4
Total[c]	222.8	100%	546.4	100%

Samples equivalent to 25 µg BSA by fluorescamine assay were hydrolyzed for 20 h in 6 N HCl with 10 nmoles norleucine as an internal standard; they were then analyzed for amino acid content. [a,b] Analyses were corrected for the destruction of threonine and serine (5% and 10%, respectively). [c] Tryptophan and cysteine are not detected by this procedure, but each is nearly absent from proline-rich proteins of the rat parotid gland (166).

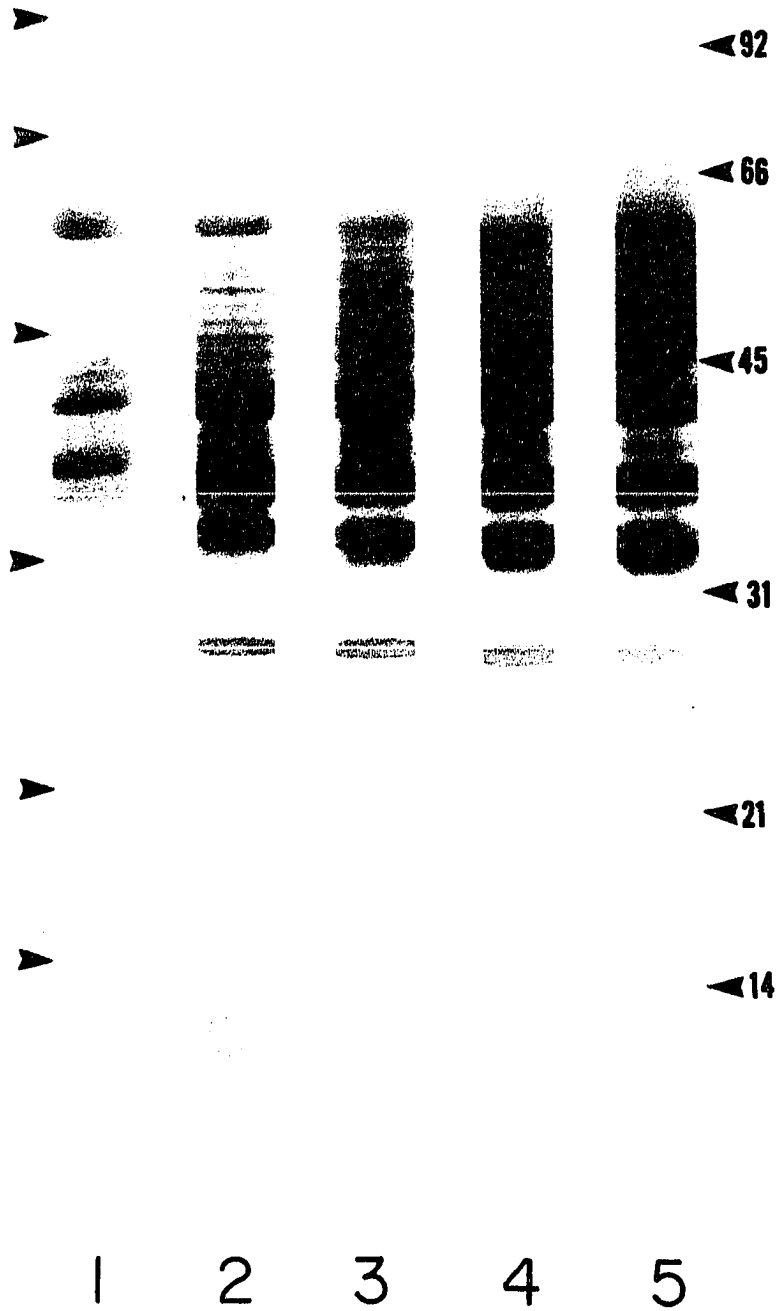
endoplasmic reticulum (observed to be present in very small amounts by electron microscopy).

Effect of Sustained Isoproterenol Treatment on the Granule Content Proteins of the Rat Parotid - Several groups have reported that chronic isoproterenol treatment results in a marked change in the contents of rat parotid secretion, as judged by the polypeptides in parotid saliva (108,163,165). More specifically, Carlson and colleagues have shown that soluble proteins (from a freeze-thawed homogenate) of the chronically-stimulated parotid gland consist largely of proline-rich proteins [PRP's, (166)]. These proteins can be considered (110) in two groups: an acidic protein (which is glycosylated and phosphorylated, pI = 4.8), and a family of (> 6) basic PRP's (largely non-glycosylated, pI's >10). The basic species greatly predominate, making up more than 50% of the total glandular proteins in the chronically-stimulated parotid; their levels progressively increase on each day of treatment with isoproterenol [from days 0 - 10, (167)].

Since the PRP's are found in cannulated parotid secretion from the chronically-stimulated rats (166), their intracellular localization is expected to be within the secretory granules. To examine this possibility, isolated rat parotid granules were subjected to three successive lysis treatments: freeze-thaw, hypoosmotic shock (by aqueous dilution), and brief sonication in a bath-type sonicator. The lysed granules were spun for 172,500 g x min (tiny pellets were obtained); aliquots of the supernatant fluid were taken for protein assay and amino acid analysis.

Table II shows the absolute and relative amino acid content of the lysed parotid granule fractions from control and 10 d injected rats. In each case the analyses were conducted on amounts equivalent to 25 μg of

Figure 2: Effect of Successive Isoproterenol Injections on Protein Composition of Parotid Secretory Granules. Rats received 0 (lane 1), 3 (lane 2), 6 (lane 3), 8 (lane 4) or 10 (lane 5) daily injections of isoproterenol. Secretory granules were isolated in each case according to the procedure outlined in the text. Approximately 25 µg granule protein (determined as described in the text) was loaded in each lane. Electrophoresis was performed as described in Methods. Arrows represent locations of molecular weight standards, in kilodaltons.



a BSA standard by fluorescamine assay (a rough calculation assigning a molecular weight of 110 g per mole amino acid indicates that the combined protein weight from control granules is ~24 μg). Remarkably, the sample from the treated rats had an amino acid content nearly 2.5-fold greater than that of the control. Increases in only three of the amino acids (GLX, PRO, GLY) accounted for >95% of this discrepancy.

Since most of the granule content protein from the treated rats consists of the basic PRP's (pI > 10), it is useful to note that while the fraction of total amino acids represented by the basic amino acids (LYS plus ARG) is not increased, the percentage of GLU + GLN nearly doubles (Table II). Since it has been shown that a very high fraction of the GLX content of the PRP's is in fact glutamine (110), the alkaline nature of these proteins may be due in large part to their decreased fraction of glutamic carboxyl groups. Consistent with this observation is the large quantity of ammonia released during acid hydrolysis of the "10 d content" proteins (data not shown); ammonia is known to be liberated from glutamine during this process.

It should be pointed out that the above analysis utilized H_2O solubility as a selection criterion for the granule content proteins. Consequently, secretory proteins adsorbed to membranes may have sedimented. Indeed, Castle and Palade have shown that some parotid content proteins (particularly PRP's) have a strong tendency to adsorb to granule membranes, and require high-salt washes for complete removal (31). Therefore, the data shown in Table II may tend to underestimate slightly the PRP contribution to the granule contents.

To visualize directly the granule polypeptides on SDS polyacrylamide gels, granules from control rats, and from those injected with isoproterenol for 3, 6, 8, and 10 days, were lysed either as just

described, or were solubilized directly in SDS. Both lysates and SDS-solubilized granules were assayed for protein content using fluorescamine and the protein values were individually corrected based on amino acid analysis of each lysate sample. For electrophoresis, 25 μg of "corrected granule protein" was loaded per lane of the polyacrylamide gel which resulted in the silver-stained electrophoretogram shown in Fig 2. The difference (error) in quantity of proteins in each lane is estimated at no more than 12%.

The polypeptide pattern for the control granules (Fig. 2) is quite similar to that observed for cannulated parotid secretion from normal rats (82). However, with increasing days of isoproterenol treatment, the granule proteins are observed to change markedly, with the α-amylase band decreasing, and many other bands (including a major band of similar mobility to α-amylase) increasing. Many of the bands are clearly the same PRP's which have been identified on gels of extracts of chronically-stimulated parotid tissue (167). However, a large number of other molecules (especially a series of bands with seeming periodicity in the region of 40 - 50 kilodalton apparent molecular weight) are also seen. The nature of these bands are presently unknown, although they are not identified in the [^{3}H]proline labeling experiments of Muenzer el. al. (166).

Internal Aqueous Volume and pH of Isolated Granules from Chronically-Stimulated Parotid Glands - A dozen preparations of isolated parotid granules from isoproterenol (10 d) treated rats were incubated for 30 min at 25° C (in various media of 300 - 350 mOsm) in the presence of [^{3}H]water to determine their internal aqueous space. Fourteen independent measurements (each based on duplicate samples) were made.

Table III

Internal pH of Parotid Granules from Rats Chronically-Stimulated with Isoproterenol

	[^{14}C]methylamine measurement	[^{3}H]acetate measurement	$^{86}Rb^{+}$/valinomycin + CCCP measurement
pH_{out}	7.17	7.13	7.10
pH_{in}	7.72	7.70	7.74

Granules were incubated for 30 min at 25°C in 0.3 M sucrose plus 50 mM MOPS at the pH's indicated. Probe concentrations were calculated from internal aqueous space values measured in parallel; values are the mean of duplicate samples. Valinomycin, CCCP were 10 μM.

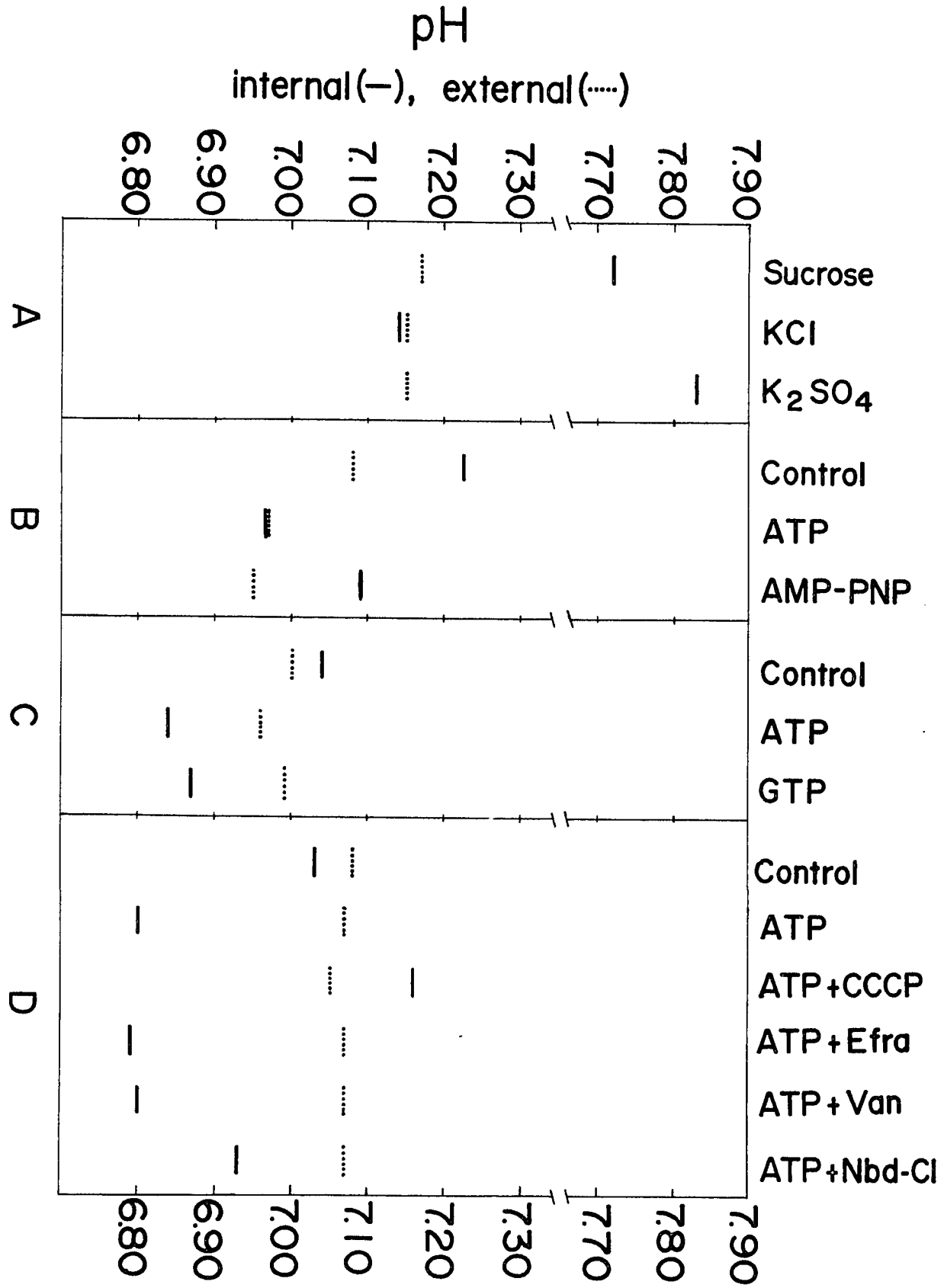
Combining data from measurements made in high ionic strength (usually containing 100 mM KCl) and in sucrose media, gave a mean internal space of ~3.6 µl/ mg granule protein; however individual values obtained over the range (3.1 µl/ mg to 4.2 µl/ mg) seemed to depend on the specific conditions employed. Incubation in KCl media generally led to a higher internal volume measurement, and was also noted to result in a somewhat higher extent of granule lysis (data not shown).

Initial intragranular pH measurements were made in 50 mM MOPS-buffered sucrose media; Table III shows values of pH_{in} determined by three independent measurements for granules isolated from rats injected for 10 d with isoproterenol. Two of the measurements, based respectively, on the equilibrium distribution of a weak base ([^{14}C]methylamine) and a weak acid ([^{3}H]acetate) gave a very similar value (pHin = 7.7).

The third measure of intragranular pH, is based of the combined use of the proton ionophore CCCP and $^{86}Rb^{+}$ in the presence of valinomycin. Under these conditions the H^{+} equilibrium potential will be the dominate the membrane potential (providing that the influence of valinomycin on other cation movements is small). Since the ΔpH of parotid granules treated with 10 µM CCCP is equal (in magnitude) and opposite (in direction) to $\Delta\Psi$ (chapter 2), the $^{86}Rb^{+}$ equilibrium distribution can be "translated" into a value for ΔpH. Using this approach the calculated internal pH is quite comparable to the other two measurements (Table III).

Reducing the ionic strength of the medium still further (by decreasing the buffer concentration from 50 mM to 5.0 mM) had a negligible effect on the measured intragranular pH of 7.7. In light of these data, the statement that normal parotid granules (pH_{in} = 6.8)

Figure 3: External and Internal pH's of Parotid Granules from Chronically-Stimulated Rats. Isolated granules from four different preparations were incubated either 20 min (c,d) or 30 min (a,b) at 25 °C with either [^{14}C]methylamine (a,c,d) or [^{3}H]acetate (b) as probes of ΔpH. All values represent the mean of duplicate determinations. Incubation media (~350 mOsm) contained the following: a) 50 mM MOPS-NaOH, and either sucrose alone, or replaced isoosmotically (in part) with 75 mM KCl or 50 mM K_2SO_4. b) 5.0 mM MOPS-NaOH, sucrose replaced isoosmotically by 25 mM KCl and 45 mM K_2SO_4; Mg-ATP and Mg-AMP-PNP, when used, were 10 mM. c) 50 mM MOPS-NaOH, sucrose replaced isoosmotically by 100 mM KCl; Mg-ATP and Mg-GTP, when used, were 10 mM. d) 50 mM MOPS-NaOH, sucrose replaced isoosmotically with 100 mM KCl; Mg-ATP, when used, was 10 mM; CCCP was 10 μM. ATPase inhibitors were preincubated with granules for 10 min at 25 °C. Final concentrations of inhibitors were: efrapeptin, 1 μM; sodium vanadate, 50 μM, Nbd-Cl, 20 μM. External pH - dotted lines; Internal pH - solid lines.



contain an internal pH higher than any other type of granule had to be revised. However, further evaluation was required to test, first, whether these measured values were affected by the absence of added salts, and second, whether these unusual granules have an internal buffer capacity comparable to that estimated for granules isolated from normal rat parotid glands (chapter 1).

To address these issues, measurements of intragranular pH were repeated in three different media. The results shown in Fig. 3a indicate that the ionic composition of the medium has a large effect on the measured internal pH. Specifically, incubation of the granules from treated rats in a potassium chloride medium results in a sharp decline in the pHin. In contrast, a similar incubation in a medium containing potassium sulfate results in an increase in intragranular pH (above the level seen in buffered sucrose, Fig. 3a). The data suggest the following important and related properties of these unusual granules. First, in contrast to normal parotid granules (chapter 1), the extent of ion permeation in relation to internal buffering capacity is quite large. Consequently, under conditions where the inward diffusion of anions is likely to exceed that of cations (e.g., Cl^- entry > K^+ entry) pH_{in} decreases; but where cation entry is expected to predominate [e.g., K^+ entry > (SO_4^{2-} x 2) entry] pH_{in} increases. Thus, my results imply that these ions permeate the granules at different rates [in the order Cl^- > K^+ > (SO_4^{2-} x 2)] and that H^+ may also permeate the granule membrane.

The observation that incubation of the isolated granules in a choline chloride medium (choline is thought to permeate membranes only very slowly) results in a measured pH_{in} < 6.8 (data not shown) provides additional support for this view. In this case the pH_{in} more closely resembles that measured in granules from normal rats and raises the

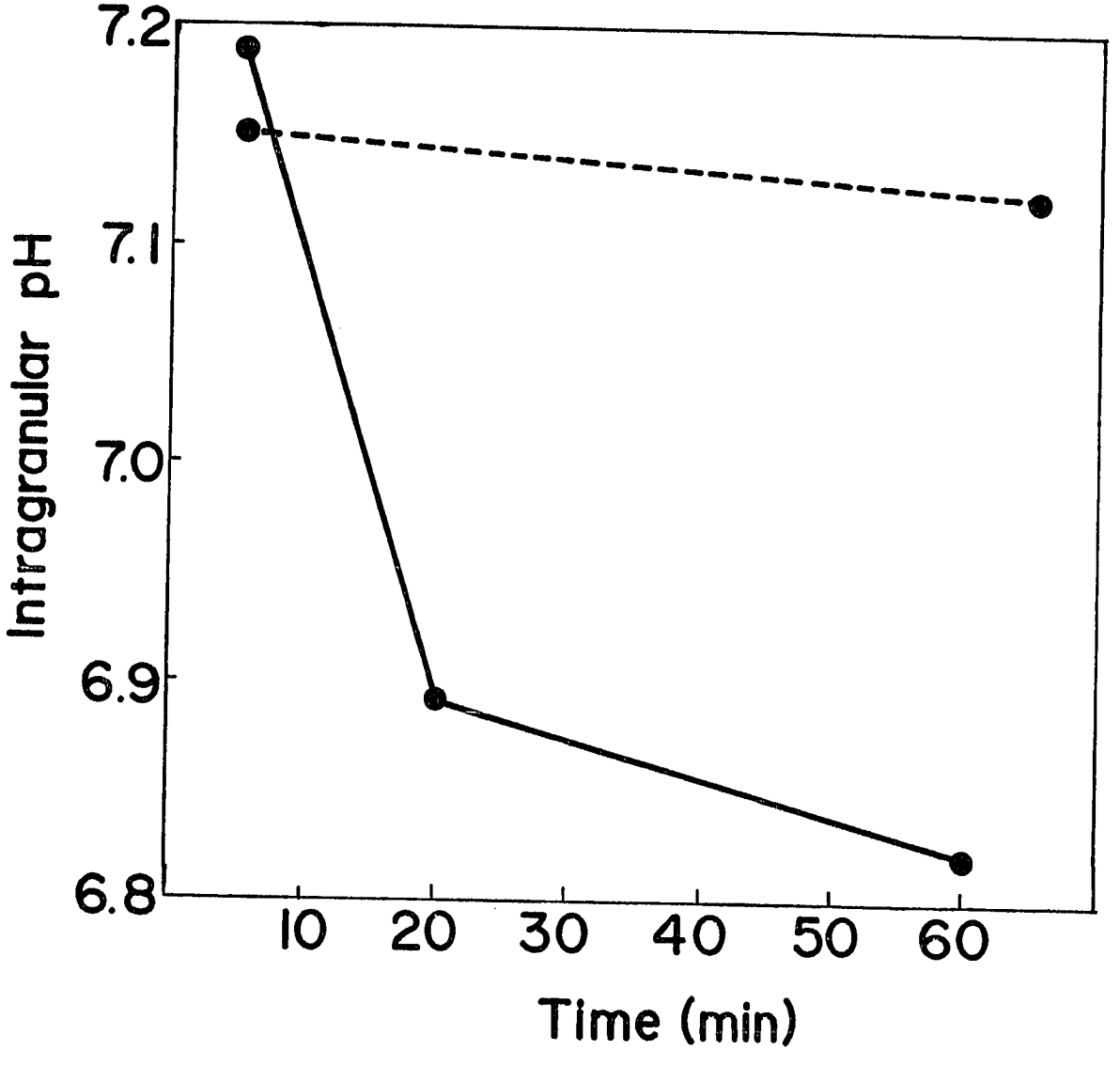
possibility that granules from treated rats may not, in all cases, maintain a higher internal pH.

Since in situ staining of normal parotid tissue using acridine orange indicated that normal parotid granules have an internal pH similar to that of the cytosol and similar to that measured in isolated parotid granules (pH_{in} = 6.8), I tried similar experiments with this dye using chronically-stimulated parotid tissue and granules isolated therefrom. Unfortunately, in both cases, the granules failed to take up enough dye to obtain satisfactory staining. While this observation is consistent with an elevated intragranular pH both in situ and in vitro, differences in acridine orange binding might easily account for the different appearances of granules from normal and treated rat parotid glands.

Effect of ATP on Intragranular pH - Isolated parotid granules from the treated rats were tested for possible ATP-driven H^+ translocation into the granule interior. In each of 10 granule preparations, different incubations with ATP in doses > 1mM resulted in a measurable acidification of the intragranular space (generally an ATP concentration of 3 mM or 10 mM was employed).

Experiments on three preparations of granules are shown in Fig. 3(b - d), where both medium pH and intragranular pH are recorded. The pH of the external medium tended to be more constant in 50 mM MOPS buffer (Fig. 3c and 3d) than in 5.0 mM MOPS (Fig. 3b). In each case ATP addition caused an increase in the transmembrane pH difference relative to that of control samples. Some variability was observed in the magnitude of the acidification (calculated by subtracting the ΔpH of the control sample from the ΔpH in the ATP-containing sample) with a range

Figure 4: Time Course of ATP-Dependent Acidification of Parotid Granules from Chronically-Stimulated Rats. Isolated secretory granules were incbated in a medium (~350 mOsm) containing sucrose isoosmotically replaced by 100 mM KCl, and 50 mM MOPS-NaOH, pH = 7.10. External medium changed no more than 0.03 pH units over an hour incubation. ΔpH was determined in each case by the distribution of [^{14}C]methylamine. Mg-ATP, when added, was 10 mM. Broken line - control; solid line - plus ATP.

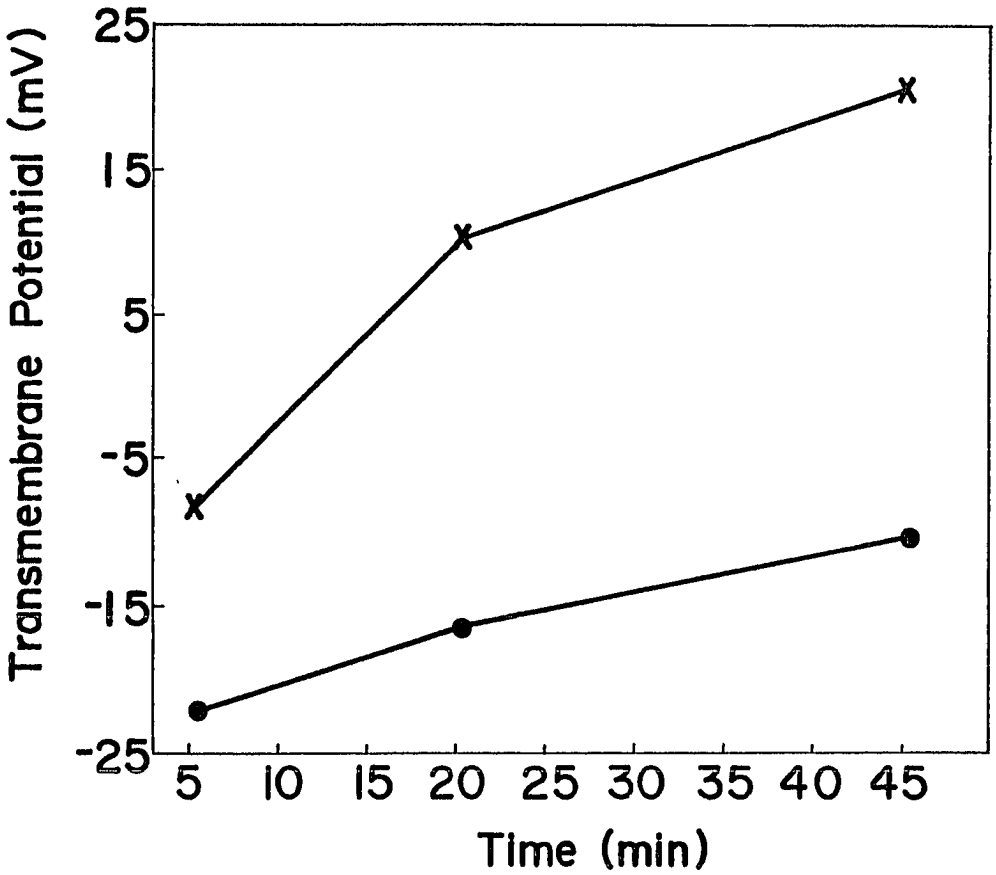


from 0.1 to > 0.4 pH units, depending on the conditions used. Typically, an ATP-dependent acidification of ~0.2 pH units was observed. Of particular significance is the observation that addition of AMP-PNP (the non-hydrolyzable ATP analog) did not result in a significant increase in ΔpH (Fig. 3b); this appears to exclude possible effects of ATP other than those which depend on ATP hydrolysis (such as possible changes in the passive permeabilities to ions) in producing the intragranular acidification. Although a pH_{in} lower than that of the control granules is seen upon addition of AMP-PNP in Fig. 3B, it parallels decrease in medium pH in this poorly-buffered medium, and is felt to be coincidental since this decrease was not observed in media of higher buffering capacity. In contrast, the effect of GTP addition was found to largely substitute for that of ATP (Fig 3c).

Fig. 4 shows the time dependence of the ATP-induced acidification. Most of the effect is observed within 20 min, while only a small decline in intragranular pH was seen in control granules over a 65 min period. The pH_{in} seen at 2 h after ATP addition was no lower than that observed at 1 h. Prolonged (> 1 h) incubation in KCl-containing media, however, was noted to result in extensive granule lysis.

The effects of various ATPase inhibitors and uncouplers were examined. Efrapeptin, at a dose which inhibits > 90% of parotid mitochondrial ATPase (chapter 2) failed to influence the ATP-dependent acidification of these granules. Sodium vanadate, a broad spectrum inhibitor of ATPases [which are phosphorylated in their catalytic cycle, (146)] was also without effect. In contrast, the addition of CCCP, the proton ionophore, completely abolished the intragranular acidification seen in the presence of ATP. Indeed, ionophore addition resulted in a granule pH elevated above control values (Fig. 3d); this effect rules

Figure 5: Effect of ATP on the Transmembrane Potential of Isolated Parotid Granules from Chronically-Stimulated Rats. Isolated secretory granules were incubated for the times indicated (at 25 °C) in a medium containing 0.3 M sucrose, and 5 mM MOPS-NaOH, pH 7.27. The external medium changed no more than 0.05 pH units over the time course of the incubation. $\Delta\Psi$ was determined in each case by the distribution of $^{86}Rb^{+}$ in the presence of 10 μM valinomycin. Mg-ATP and Mg-AMP-PNP were each used at 3 mM final concentration. Closed circles - plus AMP-PNP; crosses - plus ATP.



out that the observed acidification is due to passive H^+ movements. A partial inhibition of ATP-dependent acidification was obtained by the addition of Nbd-Cl. The magnitude of this inhibition in repeat experiments was extremely variable, ranging from none (in one experiment) to complete (in one experiment). The cause of this variability is not known.

Measurement of Transmembrane Potential - In experiments conducted in preparation for studying the ionic requirements of this ATP-dependent acidification, I found unexpectedly that a complete replacement of salts by a sucrose medium failed to have any inhibitory effect on the magnitude of the ATP-stimulated ΔpH. This result was obtained regardless whether [^{14}C]methylamine or [^{3}H]acetate was used as a probe of ΔpH (data not shown). Since unimpaired acidification in a non-ionic medium could be indicative of an electroneutral proton pump, it was necessary to establish directly whether or not the H^+-translocating ATPase was electrogenic. Therefore, I examined the possible development of a granule transmembrane potential with ATP, using the established $^{86}Rb^+$/valinomycin technique (chapter 2).

An important limitation is inherent in such a measurement. Namely, omission of permeant anions is required in order to create an ATP-generated $\Delta\Psi$ instead of ΔpH (chapter 2). Under these conditions, the intragranular pH is approximately 7.7 (when $pH_{out} \cong 7.15$, Table III). Since H^+ may permeate the granule membrane (Fig. 3a), the alkaline interior could result in inward-directed proton diffusion (down its concentration gradient) which is likely to generate an inside-positive membrane potential (the same direction as that which would be seen with H^+ pumping).

In spite of this limitation, a demonstrable change in $\Delta\Psi$ (becoming more positive inside) was seen in ATP-containing samples versus granules incubated with AMP-PNP (Fig. 5). The magnitude of this potential is ~30 mV at 20 - 45 min, which is less than that seen for chromaffin granules [~70 mV at 40 min, (45)], but much greater than that seen in normal parotid granules [~2 mV at 30 min, chapter 2]. A gradual drift (Fig. 5) in both samples to more inside-positive values was observed, consistent with proton diffusion into the granule interior[5]. These data indicate that an inside-positive potential (to the extent of 30 mV) is caused by ATP addition to granules; the effect is apparently dependent on ATP hydrolysis.

Discussion of Chapter 3

Chronic β-adrenergic stimulation of the rat parotid gland results in structural and compositional changes in the secretory granules which can be analyzed in greater detail using an isolated granule fraction. The fraction obtained is composed almost totally of granules (Fig. 1), with very low levels of contamination by other organelles (detected biochemically). Further it contains a significant fraction of the tissue amylase at a high relative specific activity.

The protein composition of these organelles is not adequately reflected by protein assays (165) because of the unusual nature of the granule content species (163). In fact, the present work clearly shows that the fluorescamine assay (125) systematically under-detects the protein in the 10 d injected rat parotid granules (when compared to controls) by a factor approaching 2.5-fold (Table II), based on the assay of total protein in comparison with parallel samples in which

amino acid contents of these granules were quantitated. Most of the under-detected proteins are especially rich in glutamine, proline and glycine (Table II). Indeed, such proteins (PRP's) have been isolated and characterized from chronically-stimulated rat parotid tissue (110,166), where they represent ~50% of the total glandular protein.

Additionally, an increase in other secretory granule material is seen in silver-stained gels of parotid granules from the treated rats. In particular, a series of bands reminiscent of the "sulfated staircase" of proteoglycans described by Moore et. al. (168) is progressively enhanced by repeated isoproterenol injections (Fig. 2). It is tempting to speculate that these bands represent sulfated GAG's which are required for effective packaging of the basic PRP's. Studies will be conducted in the future to evaluate this suggestion.

The internal pH of granules from chronically-stimulated rats was found to be ~7.7 when measured in a sucrose-containing medium (Table III). The direct implication of this finding is twofold: 1) an acidic interior is not required of all granules (further underscoring the results obtained with normal parotid granules), and 2) these granules from chronically-stimulated rats possess net fixed-positive internal charges, consistent with the observed enrichment of basic proteins inside the granules. Such a finding strongly supports the notion that intragranular pH is influenced to large degree by content molecules, just as is seen in the chromaffin granules (24).

However, unlike chromaffin or normal parotid granules, these unusual granules have a larger ionic permeability with respect to internal buffering capacity (Fig 3a). As a consequence of this property and the inability to stain with acridine orange, it is not possible to state with certainty what pH prevails inside the granules *in vivo*. However, in

a medium containing approximately the intracellular levels (175,176) of K^+ (115 mM) and Cl^- (25mM), isolated granules had an internal pH of >7.2 (Fig. 3b).

In addition, the parotid granules studied in this report were noticeably influenced by the presence of ATP. Their abilities to generate a more inside-positive membrane potential (Fig. 5) and to acidify their internal space by mechanisms which depend upon ATP hydrolysis (Fig. 3b) suggest that the effects of ATP are manifested through the action of an electrogenic H^+-translocating ATPase. The complete abolition of acidification with the proton ionophore CCCP is consistent with this view (Fig. 3d). Additional studies have revealed an ATP hydrolase activity associated with the granule fraction, which unlike normal parotid granules (chapter 2), is inhibited by 20 μM Nbd-Cl (which acts as a partial inhibitor of acidification, Fig. 3d). In recent experiments this activity has been purified further by the preparation of a granule membrane subfraction (data not shown). Additional studies will be needed to prove that the inhibition of both ATP hydrolase activity and proton pump activity is mediated through the same membrane protein(s).

The H^+-ATPase activity described herein is unlikely to be due to organelle contamination of the granule fraction for three reasons: 1) The volume fraction of contaminating organelles is expected to be lower than 5% of the volume of all structures present in the fraction. An acidification of 0.2 pH units, if ascribed to the contaminants, would represent a selective acidification in these structures of > 4 pH units. 2) Efrapeptin and sodium vanadate do not affect ATP-stimulated acidification as would be expected if this ATPase were related either to the

mitochondrial F_1-ATPase (138) or to an enzyme (e.g., Ca^{++}-ATPase, Na^+/K^+-ATPase or H^+/K^+-ATPase) which forms a phosphorylated intermediate during its catalytic cycle (146). 3) The purity of this granule fraction with respect to contamination by parotid lysosomes (based on the relative specific activity of β-N-acetyl glucosaminidase) is approximately 3-fold better than that seen in the previously reported preparation of parotid granules from normal rats (chapter 1). However, the latter preparation fails to demonstrate significant H^+-ATPase activity in similarly designed assays (chapter 2).

These results, taken together, suggest that the secretory granules from the treated rats are the source of the H^+-translocating ATPase activity. Such a finding further underscores the apparent discrepancy between the property of intragranular acidity and the presence of proton pump activity. Obviously, the latter activity does not mandate the former property.

Although I have identified and partially characterized an H^+-ATPase activity associated with a population of parotid granules that resembles condensing vacuoles, its relevance to the hypothesis initially presented should be viewed with great caution. First, the similar morphologic appearances of the two organelles may be ascribed to different properties: a) a low concentration of normal secretory protein in condensing vacuoles, and b) the inability of unusual secretory protein to retain stains (110) in the treated rat parotid granules. Second and more importantly, these unusual granules have a measured exchangeable H_2O space which is only ∿ twice that of normal parotid granules [∿3.6 μl/mg protein (this report) vs. ∿1.6 μl/mg protein (chapter 1)] despite a volume increase per granule [based on stereologic measurements, (113)] of ∿6-fold. Hence the <u>fractional</u> water space in the granules from

treated rats is actually lower than that in normal parotid granules. Further, the true fractional H_2O space in the treated rat granules may be lower still, since the protein denominator in the internal volume measurement is an underestimate (Table II). Thus the latter granules should not be viewed as being less well packaged than normal mature granules.

The new appearance of H^+-ATPase activity in the secretory granule compartment after isoproterenol treatment raises important issues about the source of this activity and its regulation. In other experiments (not reported herein), a direct comparison of parotid granules from rats injected 3, 6, and 10 days with isoproterenol showed a progressive increase in their ability to acidify their internal space upon ATP addition. However, in view of the change in intragranular contents during this period (Fig. 2), it remains unclear whether such an increase in acidification results from an increase in proton translocation across the membranes, or a decrease in buffer capacity of the granule interior.

Nevertheless, the present findings seem to signify the introduction (with successive isoproterenol injections) of proton-pump activity into secretory granule membranes (or the disinhibition of latent H^+-ATPase activity). Such an activity could, in normal parotid tissue, be located outside the secretory granule compartment. Since granule membranes are known to be reutilized after secretion, any of the organelles involved in the secretory cycle (especially Golgi, apical plasmalemma, and possible endosomal compartments) could be the source of the H^+-ATPase found in parotid granules from stimulated rats. Recent reports of such H^+ pumps in an isolated Golgi fraction (98), isolated endosomes (169), and in isolated coated vesicles (170) are of particular interest in this

regard. However, given the extensive quantity of new membrane synthesized by stimulated parotid glands (113), the possibility that this new activity reflects membrane biogenesis can not be ignored.

Finally, these results once again reinforce the concept that neither intragranular acidity nor H^+-ATPase activity are likely to be a central factor in exocytosis (93), since the discharge of granules (which lack significant H^+-ATPase activity) from normal parotid glands and of granules (which possess internal fixed-positive charges) from chronically-stimulated glands is observed to be normal in both cases (107).

Summary of Chapter 3

Secretory granules have been isolated from the parotid glands of rats chronically-stimulated with the β-adrenergic agonist, isoproterenol. Using Percoll density-gradients, isoosmolality was maintained throughout the preparation, while purifying extensively the secretory granules (as judged by both morphological and biochemical criteria). These granules are of greater size than the parotid granules isolated from normal rats, and their contents consist largely of a family of unusual cationic proteins that are rich in proline. Unlike the contents of normal parotid granules, SDS-polyacrylamide gels of granules from the stimulated rats reveal significant amounts of these proline-rich proteins, and also indicate the presence of a large number of other bands (which show an apparent periodicity). The measured internal pH of these granules was found to vary considerably depending on the chosen assay medium; in sucrose the granules maintained an internal pH of ~7.7, while in KCl-containing medium their internal pH was measured at ~7.1. This and other information suggests that these granules possess net fixed-positive internal charges, and have a large ionic permeability with respect to intragranular buffering capacity. ATP addition to parotid granules from chronically-stimulated rats resulted in a time-dependent intragranular acidification which was abolished by the addition of proton ionophores. Additionally, an inside-positive transmembrane potential developed after ATP addition which depended upon ATP hydrolysis. These data, coupled with information concerning granule purity and selective inhibitor studies, suggest that the secretory granules isolated from chronically-stimulated rats possess an electrogenic H^+-translocating ATPase activity. The presence of this activity is in striking contrast to that found for isolated granules from the normal

rat parotid (Chapter 2). Such an activity may be an enhancement of underlying membrane processes, such as those involved in granule maturation, membrane recycling, or biogenesis.

CONCLUSION

The investigations described herein have been directed towards gaining an understanding of the organelles which are "fusion partners" in exocytosis. For this purpose, I have examined isolated fractions of organelles from the rat parotid gland -- a polarized epithelium which is specialized for its stimulatable discharge of salivary protein, fluid and electroytes. Although initial studies focussed on the characterization of a fraction which was enriched in the secretory (apical) plasma membrane surface, the main consideration of my dissertation research has been analyzing selected biochemical and biophysical properties of isolated secretory granules.

These latter studies have been guided by the assumption, as a working hypothesis, that the mechanism of exocytosis is similar in all cells. If this hypothesis is correct, then special emphasis must be placed on those properties (or molecules) which are found to be invariant amongst the secretory vesicles. Recently, considerable interest has been aroused by the possiblility that two secretory vesicle properties may be common amongst these organelles, namely, the possession of an acidic interior and an inward-directed proton pump (93). So far, this has been found to be true for the vesicles of embryologically unrelated cell types, including vesicles which either do (116,94) or do not (119,120) play a major role in the accumulation and secretion of biogenic amines. The apparent general distribution of these properties, in combination with recent observations implicating intraorganelle pH in the regulation of specific intracellular events (178,179), has led to the development of a variety of hypotheses concerning the role of acidification in the secretory pathway. These

hypotheses relate to mechanisms of protein sorting, content processing, complexation and storage, and exocytosis; in short, all of the activities characteristically associated with secretory vesicles or granules.

The studies presented herein represent the first detailed biophysical examination of isolated secretory granules from an exocrine cell type. The results obtained were clear-cut: the normal rat parotid granules isolated by differential centrifugation (in isoosmotic sucrose) possess neither an acidic granule interior nor significant H^+-translocating ATPase activity. The intragranular pH value estimated *in situ* (as judged by acridine orange staining) appears to conform closely to the value measured *in vitro*.

From these data, it can be concluded that either the working hypothesis (cited above) is incorrect, or these two biophysical properties (internal aciditiy and H^+-ATPase) are not central to the common mechanism of exocytosis. It is interesting that others have recently reached the latter conclusion, based on the observation of (apparently normal) stimulated exocytotic discharge from adrenal chromaffin cells after reducing (pharmacologically) the large H^+ electrochemical potential which exists across the membranes of the secretory granules (93).

The idea that the H^+-ATPase may be present only in a (large) subset of secretory granule types, suggests that a variable requirement may exist for proton pumping in this compartment. Strong evidence (reviewed in the Introduction of this dissertation) exists to support the action of the H^+ pump in providing a driving force for catecholamine accumulation. However, in granules which do not actively accumulate biogenic amines, this pump is likely to serve a different purpose. If such

pumping is not required for the terminal event in the secretory pathway (exocytosis), then more proximal events must be considered. The recent report of amine accumulation in zymogen granules of the exocrine pancreas *in vivo* (57) is especially interesting in relation to these ideas. This study (57) demonstrates that uptake occurs only in immature granules; although amines were detected in the mature granules, neither amine uptake nor exchange could be observed to occur in these mature forms.

In view of these considerations, I have developed an hypothesis concerning the possible role of H^+-translocating ATPase activity in granule maturation. This model provides for proton pump activity in the immature forms of all secretory vesicles, with its selective removal or inhibition in some of the vesicle types (such as the rat parotid) during and/or after maturation. The initial function of this activity would be to dehydrate the vesicle interior via electrical potential-driven cation efflux; to the extent that H^+ is exchanged for internal cations, some intravesicular acidification may also occur. In some vesicles the presence of H^+-ATPase activity after maturation would provide the energy source for other forms of chemiosmotic work.

The finding (in chapter 3) of an H^+-ATPase in the parotid granules of chronically-stimulated rats neither proves nor disproves the above hypothesis. However, since the methodology employed in the study of the parotid granules in chapter 2 is virtually identical to that used for the treated-rat granules, the latter type serves as a valuable positive control to further confirm the validity and reliability of these commonly-used bioenergetic techniques. These results also underscore the possible significance of the negative findings concerning absence of

intragranular acidity and proton-pump acitivity in the normal parotid granules.

Although the effect of repeated isoproterenol injections is to induce a complete (daily) turnover of secretory proteins and a major amplification of the granule compartment, I have not yet obtained evidence which supports the premise that these unusual granules are analogous to the immature forms of granules from normal parotid acinar cells. Indeed, the fractional water space in these granules (a value which may prove to be a useful parameter in assessing the degree of intragranular packaging) appears to be no higher than that of normal granule controls. Clearly, other approaches will be needed to evaluate the validity of the hypothesis discussed previously.

Two strategies in particular appear especially promising. First, granule restoration after degranulation of normal parotid tissue should be investigated in detail. The chief advantage of pharmacologic agents in inducing massive degranulation may also be a major disadvantage: that of an un-physiologic cellular response to the overload of secretory (granule) membranes in "retrieved compartments", and perhaps in newly-formed granules as well. An attractive alternative to this pharmacologic approach may be the exploitation of the diurnal eating cycle in the rat, leading to naturally amplified restoration of the secretory granule population during non-feeding hours (180). A second complementary approach would be an attempt to subfractionate the parotid (and other exocrine) granules into younger and older groups, to directly examine their differences. In this regard, it may be possible to exploit density differences created by differences in the fractional water space.

Obtaining a detailed understanding of isolated granule properties may not only be helpful in establishing whether granule fractions can be used for *in vitro* studies as an appropriate fusion partner, but also it may yield information concerning other granule functions, such as "sorting", packaging of content, and the maturation process.

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FOOTNOTES

[1] Calculated osmotic pressures are only estimates, based on the assumption that 50 mM buffer contributes ~100 mOsm and that 1 mM sucrose ~1 mOsm. Measured osmolalities are very close to those calculated when [sucrose] < 0.3 M, but fairly small discrepancies are observed for [sucrose] > 0.3 M.

[2] To support the notion that the ion-driven lysis of granules in the presence of valinomycin has an osmotic basis, it is necessary to show that $[K^+]_{out} > [K^+]_{in}$. Based on preliminary studies employing atomic absorption spectroscopy of lysed parotid granules, the intragranular potassium concentration was estimated at ~2.0 mM.

[3] It was noted incidentally that the ΔpH in sucrose-containing media tends to be higher than in salt-containing media. Although this tendency may be attributable to a Donnan effect (30), this possibility has not been systematically evaluated.

[4] Dean, G., Nelson, P.J., and Rudnick, G., manuscript in preparation.

[5] The addition of 10 μM CCCP, which increases H^+ permeability, was also found to result in a more inside positive $\Delta\pm$, as predicted by H^+ diffusion into the alkaline interior. The only way to eliminate such an effect would be to elevate pH_{out} to a value $\geq pH_{in}$. Since it was expected that raising medium pH might result in still higher intragranular pH values, it seemed unreasonable to examine H^+-ATPase activity under such non-physiologic conditions of pH_{out}.

APPENDIX

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

PETER ARVAN and J. DAVID CASTLE
Yale University School of Medicine, Section of Cell Biology, New Haven, Connecticut 06510

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

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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 15 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, 0.0 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. The radioactivity of the resulting secretory protein mixture used for incubations 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 formaldehyde in 0.2 M Na-cacodylate, pH 7.3. Fixation, postfixation and subsequent processing for electron microscopic observation were carried out as previously described (14). Micrographs were taken in either a Siemens or a Philipps 301 electron microscope.

Materials

Eagle's minimal essential medium (Earle's salts) was obtained from Flow (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); adenosine monophosphate from Calbiochem (La Jolla, CA); Triton X-100; [^{3}H]uridine diphosphogalactose from New England Nuclear (Boston, MA). Asialoagalactofetuin was the kind gift of Drs. K. Howell and E. Sztul. [^{3}H]triolein 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 (Elmsford, NY).

RESULTS

Isolation of Parotid Plasma Membranes

Plasma membrane fractions prepared from rat parotid have not been described previously; and in the plasma fractionation schemes developed for the parotid glands of 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 assay interpretations have relied heavily on the distribution of putative plasma membrane marker enzyme activities based on precedent of, and analogy to, rat liver plasma membrane studies. Since exclusive plasma membrane markers in the parotid have remained unidentified, success in the development of our isolation procedure was judged by the ability to prepare membranous structures, morphologically recognizable as plasmalemmal in origin. The protocol (shown schematically in Fig. 1) is fundamentally similar to that developed by Ray (33) 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

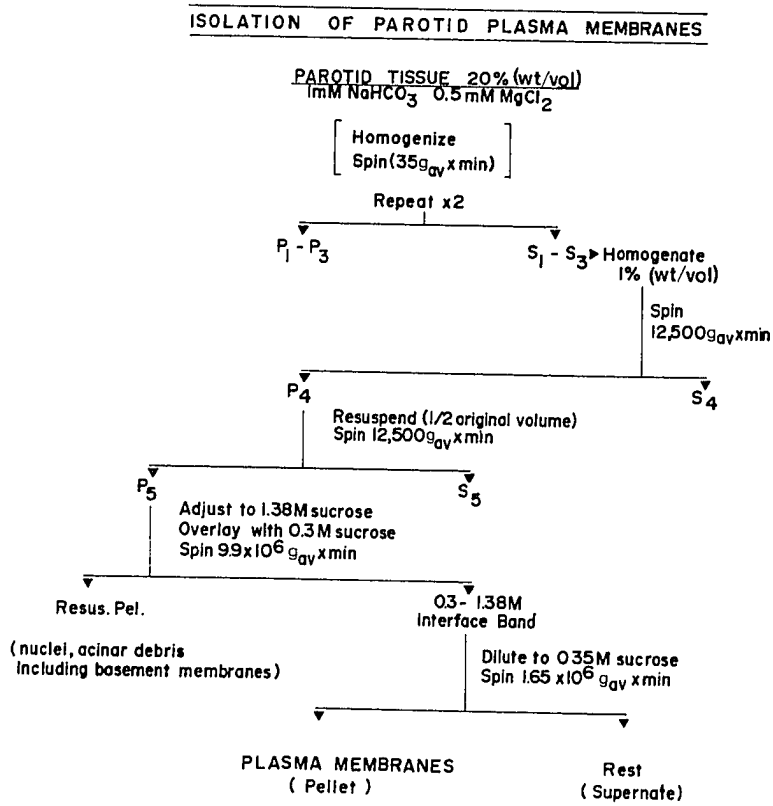


FIGURE 1 Flow sheet for the isolation of the plasmalemmal fraction.

mb anes from closely associated connective tissue which vil invests this epithelium. All steps are done at 4°C.

Ma · Sprague-Dawley rats (Charles River) weighing 100– g were starved overnight and then killed by cardiac inci- n, nder light ether anesthesia. Parotids of 16 rats were ise and immediately immersed into ice-cold oxygenated gle' Minimal essential medium (Earle's salts). Associated ne ive tissue, fat and lymph nodes were dissected away to d : proximately 5.5 g of parotid tissue, which was minced h r :or blades and mixed in medium A (0.5 mM $MgCl_2$, 1 [N :HCO_3, pH 7.4) at 20% wt/vol. At this point, the tissue ei ıer (*a*) incubated for 30 min in the presence of 25.5 U hig ly purified, protease free collagenase (36, kindly pro- d y Drs. J. D. Jamieson and M. Sarras) or (*b*) homoge- d irectly without collagenase. The morphologic appear-e, ı arker enzyme distributions, and extents of contamina- a ar · indistinguishable for either procedure. Although data he ıbles derive from both procedures, we routinely do not co: ıgenase so that the possibility of subtle proteolysis may vo .led.

iti l tissue homogenization was achieved in two steps by tm nt with a Polytron homogenizer for 15 s at 1,900 rpm w d by 3 up-down strokes with a Brendler teflon pestle og nizer at 1,300 rpm. The resulting suspension was then a 70 *g* for 0.5 min, and the supernatant fluid (S_1) was nt d and saved. The pellet (P_1) contained a large number nb oken cells in addition to residual connective tissue. To eas the efficiency of homogenization, P_1 was resuspended e previous volume of Medium A, rehomogenized with the n pestle as before, and respun to produce S_2 and P_2. The mo genization sequence was repeated once further to yield nd P_3; the latter was discarded.

o completely disrupt the remaining osmotically sensitive cellular 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

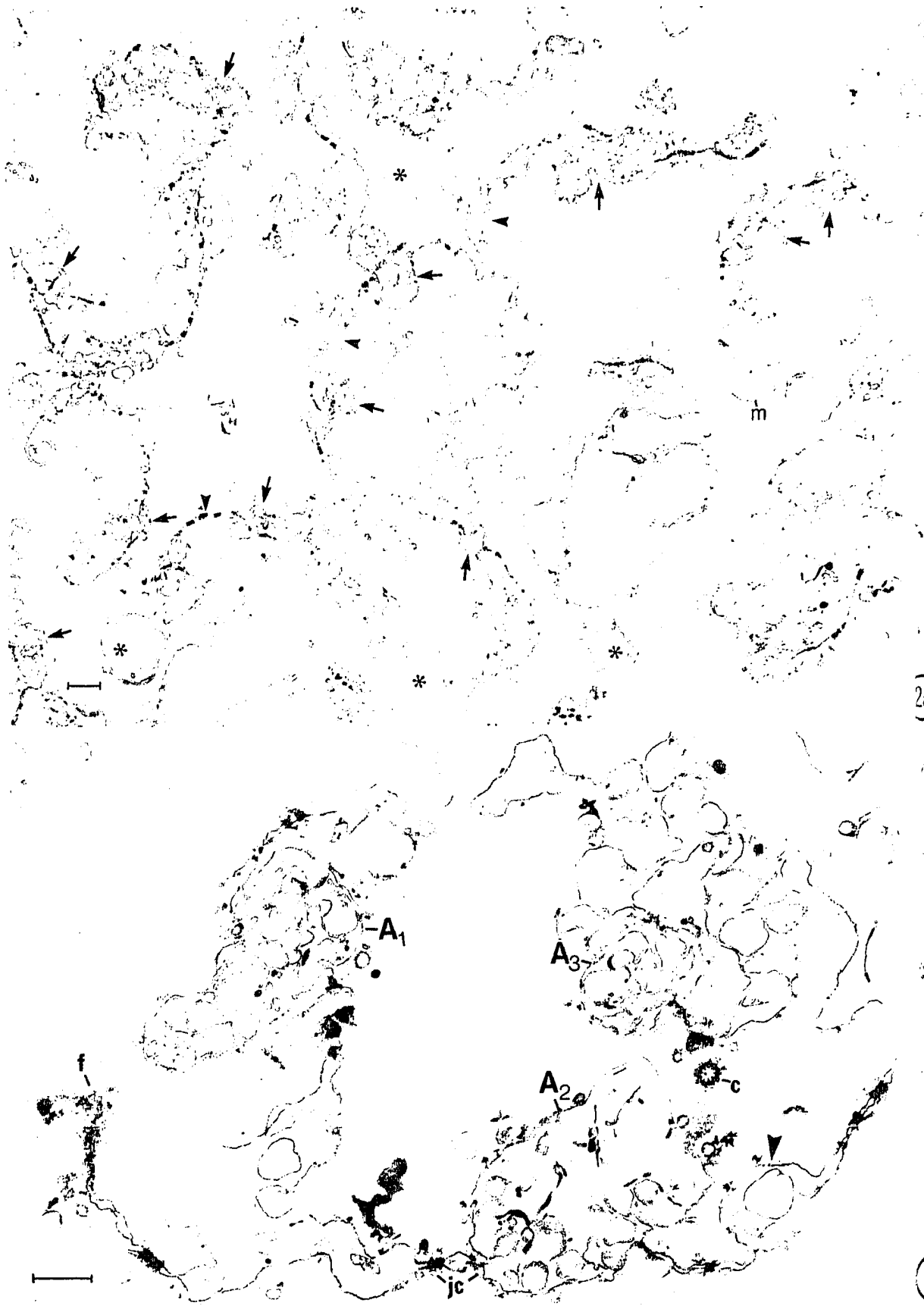
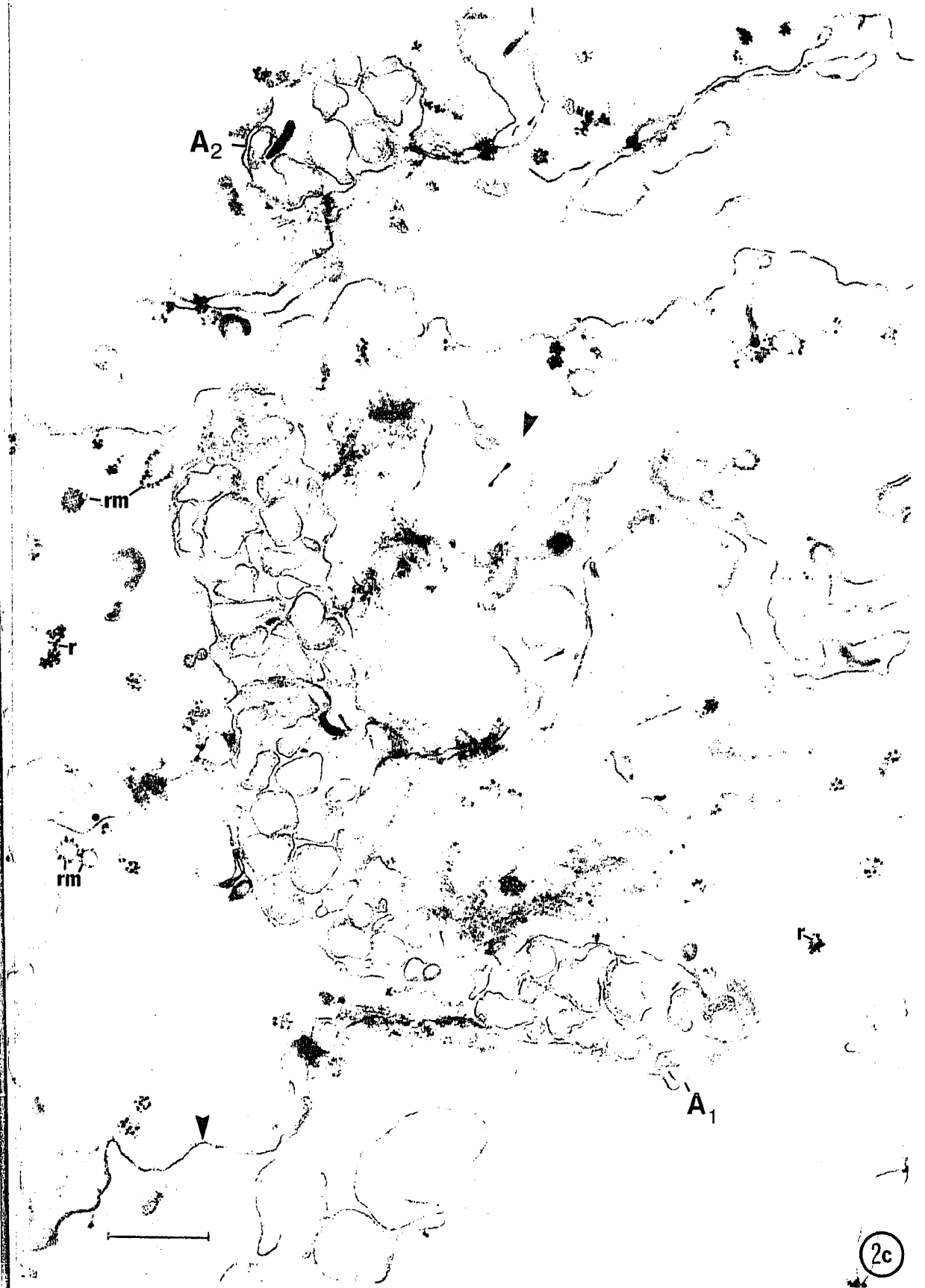


FIGURE 2 Electron micrographs of the parotid plasma membrane fraction. (*a*) At low magnification the fraction is shown to consist predominantly of extended membrane sheets formed by the surfaces of two or more cells that retain an association analogous to that observed *in situ*. Apical regions (arrows) 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 membranous 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 fr tions from other tissues. Quite unexpectedly, several of th putative plasma membrane marker activities were not found be significantly enriched in the final plasmalemmal fract (Table I). Most notable among these are alkaline phosphat and 5′-nucleotidase, whose final recoveries are 0.31 and 0.8 respectively. While some variability was observed from pr aration to preparation in both homogenate activity and pl malemmal recovery, overall it would appear that these enzy do not make a major enzymatic contribution to the acti vity the acinar cell plasma membrane. Furthermore, the m asu alkaline phosphatase in parotid homogenates (0.008 μm l/m per mg protein) is substantially less than that observ l fr homogenates of other tissues in which extensive plasma mi activity has been confirmed cytochemically (rat live 0. μmoles/min per mg protein [37]). The same is not tru ho ever, for 5′-nucleotidase (present results: 0.031 μmoles/ in mg protein; Rat liver: 0.039 μmoles/min per mg prote [3 Alkaline phosphodiesterase I, an enzyme which often pe in concert with 5′-nucleotidase (A. L. Hubbard, Johns H pk University School of Medicine, personal communicati) a shows a similar distribution in the fractionation sch ne ported here, with a low plasmalemmal activity, ≤0.38 of the homogenate and enriched only one- to twofold

Aminopeptidase was tested because this enzyme l be reported to be found in the brush border membranes o idh and intestine; and has also been histochemically loc zed the bile front of the hepatocyte (38, 39). Very little h olv activity toward the leucine-derived substrate was a cia with the plasmalemmal fraction (0.27% of homogena act ity), while other peptide substrates have yet to be trie

The protein yield for the plasmalemmal fraction, a ho in Table II, is unusually low in comparison to that fo re rations from other tissues, such as liver, where valu of mg protein per gram wet tissue weight are characte tica observed (40). We feel this is mainly attributable to th lat paucity of large membrane fragments after vigorous no nization. However, more gentle approaches, in ou an failed to disrupt the basement membrane surroun g acini, hence plasma membrane protein recovery did n prove. Similar findings in fractionating rat parotid h be reported previously (41).

Table II also indicates two enzyme activities wh w consistently found to be enriched in this preparati dependent *p*-nitrophenyl phosphatase (K^+-NPPase, fo and γ-glutamyl transpeptidase (GGTPase, 26-fold).

Recovery of Plasmalemmal K^+-NPPase

K^+-dependent NPPase is a conveniently assayed, diu independent activity, in most cases attributable to adenosine triphosphatase (42); the assay is especially a van geous when monitoring levels of this enzyme in oua in sistant rodent tissues and cell fractions (43). To estab h the rat parotid K^+-NPPase is indeed due to the ATPase, and not merely a K^+-activated phosphatase uch that reported by Forte et al. (44) and others, we have e min a parotid homogenate and its derived plasmalemmal act for ATPase and NPPase activities. As shown in Table I l. b activities are obtained in the same yield in the plasm me brane fraction. Hydrolysis of either substrate in the ab nce K^+ dramatically reduced both activities to backgroun le (attributable to Mg^{2+} dependent ATPase and phosp at respectively). To ascertain further that the plasmalemm AT

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 is so 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 plasmalemmal 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 fo parotid lysosomal nor granule membranes, respectively. N single enzyme representing organelle contaminants of the plas malemmal fraction exceeds 0.4% of the homogenate activity.

The secretion granule content marker, α-amylase, is remove >99.99% from isolated plasma membranes. However, as dat indicate that amylase is more easily solubilized from mem branes than are other parotid granule content proteins (48), w decided to estimate comprehensively the extent of adsorbe secretory proteins by a radioactive mixing experiment. ^{3}H labeled rat parotid lobule secretion was prepared as describe in Materials and Methods; 8.09×10^6 cpm of this secretor standard was added to 5.55 g rat parotid tissue just befor homogenization and routine plasma membrane prepar tion Of the 7.8×10^6 cpm measured in the homogenate fra tion only 1.6×10^3 cpm or 0.02% of the label could be found the final plasmalemmal material. This low degree of contamin tion by secretory protein is still some 2.5-fold higher tha the plasmalemmal α-amylase recovery, suggesting a greate (ye still minor) contamination by other secretory proteins.

For cytochrome *c* oxidase (mitochondrial inner memb nel 0.27% of the homogenate's activity was located in the pl ma lemmal fraction. Quantitatively comparable results we ob tained for monoamine oxidase (outer mitochondrial em brane); 0.29% of the homogenate activity was found he plasmalemmal fraction (with slight over-recovery of t ho mogenate activity found in the summed fractions—109

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

Rough microsomes were monitored by assaying their none plus cyanide)-insensitive NADH cytochrome *c* red activity. Although this enzyme has also been ascribed tochondrial outer membranes, Golgi membranes (50, 51 even plasma membranes of some systems (52, 53), we taken the 1.76-fold enrichment of this enzyme to in primarily a contamination by endoplasmic reticulum. Pr ably this enzyme's recovery actually represents the s contamination by a variety of organelles. Electron micro examination, however (see Fig. 2), confirms rough micro

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

* 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).

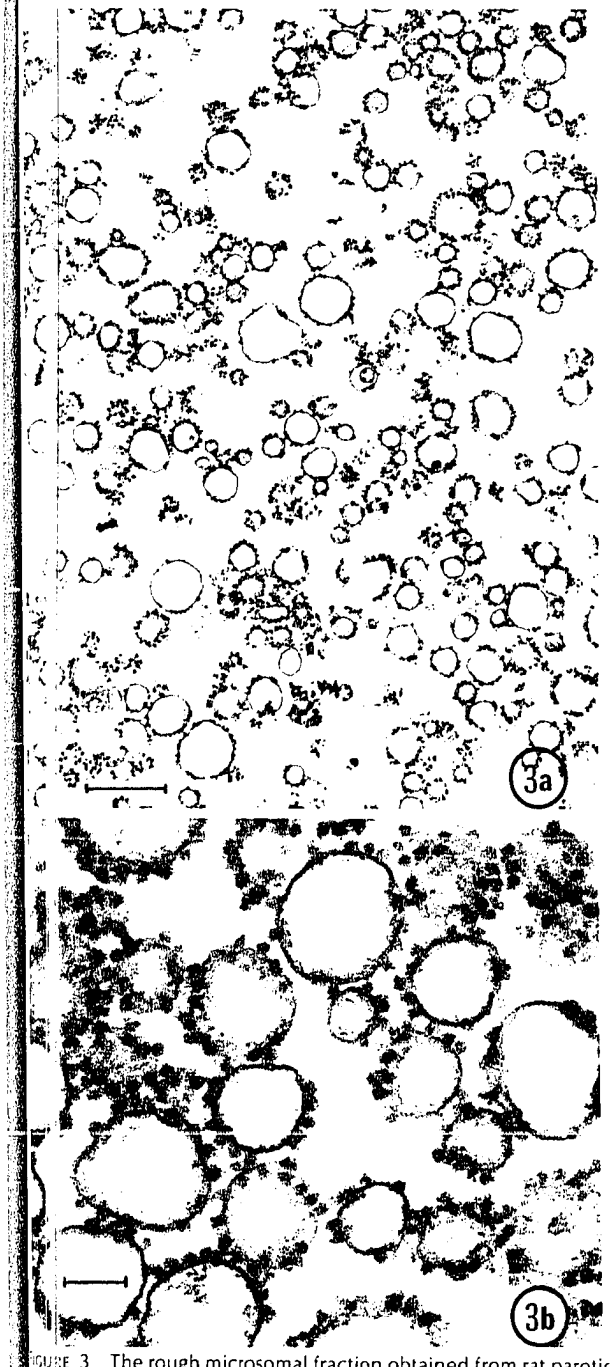


FIGURE 3 The rough microsomal fraction obtained from rat parotid viewed at low (*a*) and high (*b*) magnification. Some degree of aggregation is apparent. The top, middle, and bottom of microsomal pellets have a homogeneous appearance with no visible organelle contamination. (*a*) Bar, 0.5 μm. × 22,000; (*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 intestina and renal cells by treatment with papain (64).

Although no unifying hypothesis concerning this enzyme physiologic role in such diverse tissues has been confirmed, io those cell types in which structural analogies of the parotid ca be drawn, evidence suggests a cytologic localization o GGTPase which is enriched at the luminal surface. The presen study extends these findings to show that in the rat paroti gland, this enzyme is also present on secretion granule mem branes as well. Our data show also that Na^+/K^+-ATPase (K^+ NPPase), another plasma membrane enzyme, which in othe polarized epithelia is found to reside exclusively at the baso lateral membrane surface, is absent from these same secretion granule membranes. Furthermore, the plasmalemmal isolation scheme presented in this report is able to distinguish a n 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^+-A Pase activity is associated with the pellet. Electron microscop ob servations of the latter fraction, which reveal fragmente cel lular membranes closely apposed to extensive sheets of basa lamina (data not shown), suggest that the resuspended p let is enriched in basolateral surface.

Taken together, we feel that these data strongly suppo the hypothesis that the plasmalemmal localization of GGTI se is primarily restricted to the apical domain in unstimulat l ra parotid acinar cells, and that Na^+/K^+-ATPase is prim ily basolateral plasma membrane marker. We are now pre ring to use immunocytochemical techniques to directly confir the above hypothesis.

The preliminary biochemical evidence presented in t s re port points to a compositional overlap between the sicn partner membranes involved in exocytosis in the rat p oti gland. This conclusion contrasts with that of others wh nav stressed the uniqueness of these two membrane types in the systems (nerve terminal [65]; adrenal medulla, [66]; sea i chin egg [67]). Several points are pertinent in evaluating this fer ence. First, high levels of organelle cross-contamination th isolated fractions may bias the interpretation of data Thi appears unlikely for our plasmalemmmal preparation sin th hyposmotic homogenization procedure is expected to l d to the removal of the contents and membrane fragments of sed secretion granules into the supernatant fractions S_4 a S Second, secretion granule membrane fractions have long een noted for their contamination by residual secretory pr eir (48, 68). Consequently, enzymatic activities, antigens, or oly peptides viewed as SDS gel bands that are associated selec vely with granule membranes and not with the plasmalemma uns be rigorously shown to be bona fide membrane constit nt before being rated as unique. Third, uniqueness and over p o membrane constituents are not of necessity mutually excl ive and the extent of each may vary between cell types. Our r ult support the compositional overlap of GGTPase in pa ati secretion granule and plasma membranes; however, the re or ably small amount of activity and large surface area of secr ion granule membranes, suggest that the GGTPase specific ac vity of these membranes is likely to be lower than that of th ce surface. Therefore, the overlap of GGTPase between th pa rotid fusion partner membranes may be qualitative bu nc quantitative.

The extent and generality of compositional overlap bet een the two membranes under investigation may have broad sig nificance with regard to luminal membrane biogenesis an

rnover. GGTPase may prove to be a useful probe in parotid inar 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 microsmal 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.

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