

Isolated Secretion Granules from Parotid Glands of Chronically Stimulated Rats Possess an Alkaline Internal pH and Inward-directed H⁺ Pump Activity

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Abstract. Secretion granules have been isolated from the parotid glands of rats that have been chronically stimulated with the β -adrenergic agonist, isoproterenol. These granules are of interest because they package a quantitatively different set of secretory proteins in comparison with granules from the normal gland. Polypeptides enriched in proline, glycine, and glutamine, which are known to have pI's >10, replace α -amylase (pI's = 6.8) as the principal content species. The internal pH of granules from the treated rats ranges from 7.8 in a potassium sulfate medium to 6.9 in a choline chloride medium. The increased pH over that of normal parotid granules (\sim 6.8) appears to reflect the change in composition of the secretory content. Whereas normal mature parotid granules have practically negligible levels of H⁺ pumping ATPase activity (Arvan, P., G. Rudnick, and J. D. Castle, 1985,

J. Biol. Chem., 260, 14945–14952) the isolated granules from isoproterenol-treated rats undergo a time-dependent internal acidification (\sim 0.2 pH unit) that requires the presence of ATP and is abolished by an H⁺ ionophore. Additionally, an inside-positive granule transmembrane potential develops after ATP addition that depends upon ATP hydrolysis. Two independent methods have been used that exclude the possibility that contaminating organelles are the source of the H⁺-ATPase activity. Together these data provide clear evidence for the presence of an H⁺ pump in the membranes of parotid granules from chronically stimulated rats. However, despite the presence of H⁺-pump activity, fluorescence microscopy with the weak base, acridine orange, reveals that the intragranular pH in live cells is greater than that of the cytoplasm.

IN contrast to the peptide-containing secretory vesicles of neural and endocrine cells (29, 39), mature exocrine granules of the rat parotid gland do not have an acidic internal pH (7) and exhibit almost no inward-directed H⁺ pump activity (8). However, both exocrine and endocrine granules originate within compartments located at the trans aspect of the Golgi complex (15, 23), and several recent studies provide reason to suspect that this stage of the secretory pathway (Golgi/post-Golgi) may include a compartment that possesses H⁺-ATPase activity at levels sufficient to cause internal acidification. First, membrane vesicles derived from the secretion granule-like fractions of hepatocyte Golgi complexes undergo an ATP-dependent internal acidification that is inhibitable by the sulfhydryl-reactive reagents *N*-ethyl maleimide and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (Nbd-Cl;¹ reference 22). Second, the addition of chloroquine (a membrane-permeating weak base that elevates the

pH of acidic intracellular compartments [40]) to cultured pituitary tumor cells causes a diversion of nascent glycoproteins from normal storage in granules into an intracellular pathway leading to constitutive discharge (37). Third, vacuoles located at the *trans*-face of the Golgi complex in fibroblasts and cultured hepatoma cells accumulate similar weak bases that can be visualized by electron microscopy (2, 45). Fourth, certain enzymes involved in Golgi/post-Golgi processing of secretory polypeptide precursors (20, 21, 27, 31, 33) exhibit acidic pH optima with only low activity levels at neutral pH. Finally, in exocrine pancreatic cells, condensing vacuoles, but not mature granules, accumulate the biogenic amine, serotonin (48) by a process that in other cell types is known to be driven by ATP-dependent H⁺ pumping.

In parallel with segregation, packaging, and storage of secretory proteins, the course of exocrine granule formation in the parotid acinar cell involves the progressive disappearance (by removal or suppression) of selected Golgi activities (such as nucleoside diphosphatase [41], acid phosphatase [23], and galactosyl transferase [11]) with retention (or emergence) of other enzymatic activities necessary for granule functions. Thus, the nearly undetectable levels of H⁺-ATPase in mature parotid granules may represent sorting or sup-

1. *Abbreviations used in this paper:* $\Delta\psi$, transmembrane electrical potential; Δ pH, pH difference across the granule membrane ($\text{pH}_{\text{in}}-\text{pH}_{\text{out}}$); AMP-PNP, Mg²⁺- β , -imidoadenosine-5'-triphosphate; AO, acridine orange; CCCP, carbonyl cyanide *m*-chloromethoxyphenyl hydrazone; MOPS, morpholino propane sulfonic acid; Nbd-Cl, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole.

pression that proceeds in the absence of any sustained role for H⁺ pumping in normal storage of (parotid) exocrine secretory proteins, whereas the presence of H⁺-ATPase activity in granules that accumulate biogenic amines reflects a sustained role in intragranular packaging and storage in certain other cell types.

In the present study it was our intention to perturb parotid secretory composition in an attempt to evaluate parameters which could influence granule packaging, including H⁺-ATPase activity. To achieve this goal, we have altered the transcriptional program of parotid cells by chronic stimulation (in vivo) with the β -adrenergic agonist, isoproterenol (52). The major phenotypic effects of this treatment on the acinar cell are the increased granule size and number (10) and the dramatic alteration of salivary composition such that the normal spectrum of secretory proteins is largely replaced by a family of highly basic species (38).

We have isolated a highly purified fraction of granules from chronically stimulated parotid tissue and have confirmed that the content of stored proteins is markedly different from that found in normal granules. Further, we have found that these granules (both in vitro and in situ) exhibit an alkaline rather than an acidic internal pH, yet they contain inward-directed H⁺-translocating ATPase activity. By contrast, a different activity found in the Golgi/post-Golgi region (galactosyl transferase, [43]) remains efficiently excluded from the granule compartment. These findings raise the interesting possibility that under selected conditions, the H⁺-ATPase activity may be purposefully retained in the exocrine storage compartment.

A portion of the studies described herein have appeared in the form of an abstract (6).

Materials and Methods

Chronic β -Adrenergic Stimulation of Rats

Intraperitoneal injections of d,l-isoproterenol (30 mg/kg/d in 0.15 M NaCl [0.3 ml]) were administered to male Sprague-Dawley rats (150–225 g) for 10 successive days to achieve gland enlargement (46). Injections were given between 10 am and 2 pm. After the last injection, animals were starved 16–22 h before sacrifice.

Isolation of Parotid Granules from Isoproterenol-treated Rats

All steps for processing parotid tissue were carried out at 4°C. Rats were killed by cardiac incision under ether anesthesia. The enlarged parotid glands of two or three rats were excised, cleaned of connective tissue, minced thoroughly with razor blades, and gently homogenized (with four strokes in a cylindrical glass, teflon pestle homogenizer at 1300 rpm) in 33 ml (~15% wt/vol) of ice cold sucrose (0.3 M), 4-morpholino propane sulfonic acid (MOPS) (2 mM [pH 6.9]), and MgCl₂ (0.2 mM). The resulting suspension was then spun at 300 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 supernatant was pooled with the first, adjusted to 1.2 mM EDTA, filtered through one layer of nylon screen (20- μ m mesh), and dispersed with three gentle strokes in a Dounce homogenizer (tight pestle). This "homogenate" (nuclei removed) forms the basis for recoveries calculated for enzymes assayed.

The homogenate was mixed with 2 vol of buffered Percoll medium: 0.3 M sucrose, 86% Percoll, 1 mM EDTA, and 2 mM MOPS, pH 6.9 (Percoll contributes <12 mosM to the osmolality of the diluted homogenate). This mixture was loaded into eight polycarbonate centrifuge tubes containing a 2-ml cushion of buffered Percoll medium and spun in a rotor (model 60 Ti; Beckman Instruments, Inc., Fullerton, CA) at 15,000 rpm for 30 min.

Granules were banded in the lower (denser) quarter of the self-formed gradients; most other organelles were near the top. The collected granule band was mixed, reloaded into two polycarbonate centrifuge tubes, and spun again in the 60 Ti rotor at 25,000 rpm for 30 min. Granules were well separated from a faint underlayer containing few erythrocytes and nuclei and an overlying layer mostly comprising mitochondria. These layers were saved along with the residual gradient fluid for assays. The granule band was collected and diluted at least fivefold with a solution containing 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 ~2300 g for 30 min. A significant number of intact granules remained in the final supernate under these conditions (chosen to minimize the sedimentation of Percoll). The final pellets were white and thus similar in gross appearance to those from normal glands.

Biochemical Analyses

Enzymatic activities of α -amylase; cytochrome c oxidase, γ -glutamyl transferase, β -N-acetyl glucosaminidase, and UDP-galactosyl transferase were determined as described previously (5). Protein was assayed with fluorescamine as described by Udenfriend et al. (49) using BSA as standard.

For amino acid analysis, parotid granule content proteins and 10 nmol norleucine (used as an internal standard) were hydrolyzed for 20 h at 110°C in 6 N HCl. Amino acids were resolved and quantitated using a Dionex D-500 analyzer (Durrum Instruments Co., Sunnyvale, CA).

SDS PAGE of secretory polypeptides (reduced with 2-mercaptoethanol) was carried out on 10–15% (wt/vol) polyacrylamide linear gradients using the Laemmli discontinuous buffer system (32). After electrophoresis, gels were fixed and stained in 0.04% Coomassie Brilliant Blue in 25% isopropanol plus 10% acetic acid (19) and destained in 10% acetic acid. Omission of isopropanol from destaining solutions aids in retaining proline-rich secretory proteins in the stained polypeptide profile.

Microscopy, Immunolocalization, and Autoradiography

In preparing samples for routine observation by light and electron microscopy, parotid tissue and granule suspensions were fixed for ≥ 3 h at 4°C in 3% (wt/vol) glutaraldehyde and 1% (wt/vol) formaldehyde in sodium cacodylate (or phosphate) buffer (pH 7.2). Granule samples were pelleted by centrifugation (3–5 min, ~3,000 g) after aldehyde fixation and all specimens were then postfixed in OsO₄, stained with uranyl acetate, dehydrated, and embedded (in either Epon 812 or Spurr's resin) as described previously (16). Methylene blue-stained 0.5- μ m sections were examined using a Zeiss photomicroscope whereas ~60-nm sections (stained with uranyl acetate and lead citrate) were viewed on a Philips 300 electron microscope.

Immunolocalization of parotid secretory proteins was carried out by indirect immunofluorescence on tissue that had been fixed in phosphate-buffered 3% formaldehyde and 0.05% glutaraldehyde, frozen, cryosectioned (5 μ m), and permeabilized with 0.3% (wt/vol) Triton X-100 (18). Tissue sections were incubated with rabbit antisera prepared to either purified α -amylase or basic proline-rich proteins and stained subsequently using rhodamine-conjugated goat anti-rabbit IgG.

In preparation for acridine orange (AO) fluorescence microscopy, the parotid glands of chronically stimulated rats were dispersed into a mixture of small cell clumps, acini, and individual cells, using collagenase digestion and mild mechanical shear by repeated pipetting through a series of siliconized glass pipettes of progressively decreasing diameter (1.0–0.4 mm), followed by sieving through 200- μ m nylon screen (25). The dispersion medium consisted of 10 ml Eagle's modified minimal essential medium containing ~0.4 U collagenase (see Materials below), 0.1% BSA and 0.01% soy bean trypsin inhibitor, 15 mM Hepes-NaOH [pH 7.4] and was continuously oxygenated with 100% O₂ at 37°C.

To collect cell populations containing mast cells, the same medium without collagenase was used for lavage of the rat peritoneal cavity followed by gravity sedimentation at 0°C. Cells of both types were incubated with 5 μ M AO and examined under the microscope within 5–10 min after exposure to the pH probe. Specimens were photographed using both phase illumination (with the condenser diaphragm slightly offset to improve resolution of intracellular organelles) and epifluorescence with a fluorescein filter.

In autoradiographic studies, granule suspensions were incubated with ~2 μ Ci [³H]methylamine (under conditions identical to that described for biophysical measurements of internal pH, see below) and then were fixed for 60 min at 0°C by the addition of one-seventh volume of 46.5% glutaraldehyde (final concentration, 6.6%) containing 155 mM lithium phosphate

buffer (selected for minimal permeability; [pH 7.0]) (final concentration, 22 mM) and tracer amounts of radioactive methylamine to maintain the extragranular methylamine concentration as a constant. Granules were then sedimented by centrifugation (2 min, ~3,000 g) and fixation was continued overnight at 4°C in a fresh solution of 6.6% glutaraldehyde and 22 mM lithium phosphate (pH 7.0). Subsequently, the pellets were postfixed in OsO₄, dehydrated, and embedded in the usual manner (16). Autoradiography was performed on ~100-nm sections of embedded granule pellets (12). Quantitation of autoradiographic grain distributions was carried out on uniform-magnification electron micrographs that were representative of the top, middle, and bottom of pellets (50). Stereologic measurement of the volume fraction of granules in these preparations was made using point-count analysis on a quadratic lattice (50).

Measurement of Internal Aqueous Volume and Internal pH of Isolated Granules

These determinations were performed as described for parotid granules from normal rats (7); [¹⁴C]sucrose (marker of the excluded H₂O volume of granule pellets) was added (1% of total volume) just before termination of incubation by centrifugation. Both [¹⁴C]methylamine and [³H]acetate were used as probes of ΔpH; all tracers were used at the concentrations described previously (7). The equilibrium distributions of these probes were calculated with the aid of parallel measurements of intragranular aqueous space (44). Unless otherwise indicated, granules were incubated with either Li₂SO₄ or MgSO₄/Na₂ATP such that both sets of samples were maintained at equal osmolality.

Measurement of Transmembrane Potential in Isolated Granules

Effects of ATP on Δψ were determined using the equilibrium distribution of tracer amounts of ⁸⁶Rb⁺ in the presence of valinomycin (44). In previous studies (8, 24), good agreement was observed between measurements of inside-positive Δψ using ⁸⁶Rb⁺ exclusion and S⁴CN⁻ accumulation; however, the ⁸⁶Rb⁺ method was chosen in the present study to avoid background binding of the probe (encountered with S⁴CN⁻). Valinomycin (final concentration, 10 μM) was added in absolute ethanol (<0.5% contribution to sample volume). All incubations were carried out at 25°C in parallel with measurements of intragranular aqueous space. Membrane potential values were calculated using the out-in concentration ratios of radioactive cation according to the Nernst equation (44).

Materials

Efrapeptin was the gift of Dr. R. L. Hamill, Lilly Research Laboratories, Indianapolis, IN. CCCP was obtained from Calbiochem-Behring (Corp., La Jolla, CA); valinomycin, Nbd-Cl, ATP, GTP, AMP-PNP, d,l-isoproterenol and stock chemicals were from Sigma Chemical Co. (St. Louis, MO). Collagenase (*Clostridium histolyticum*, 0.15 Boehringer U/mg) was from Boehringer Mannheim Diagnostics, Inc. (Houston, TX). AO was from Eastman Chemical Products Inc. (Kingsport, TN). Photographic emulsion for autoradiography was obtained from Ilford Ltd., Essex, UK. [³H]water, NaS⁴CN, [³H]acetate, [¹⁴C]methylamine were from New England Nuclear (Boston, MA); [¹⁴C]sucrose was from ICN Radiochemicals, Div. ICN Biomedicals Inc. (Irvine, CA); and ⁸⁶RbCl was from Amersham Corp., Arlington Heights, IL. Sodium vanadate was the kind gift of Dr. Gary Rudnick (Department of Pharmacology, Yale Medical School, New Haven, CT) as was [³H]methylamine (also obtainable from Amersham Corp.). Antisera prepared in rabbits against purified α-amylase and against proline-rich proteins (both from the rat parotid) were the kind gifts of Dr. Richard S. Cameron (Department of Cell Biology, Yale Medical School).

Results

Purity and Recovery of Secretion Granules Obtained from Parotid Glands of Isoproterenol-treated Rats

Although interest in the enlarged secretion granules of parotid tissue from isoproterenol-treated rats has been longstanding (9, 10, 47), these granules have not been isolated previously. To obtain a representative population of granules that would be suitable for biophysical studies, we developed

a method of isolation using isoosmotic media (see Materials and Methods). Initial processing steps used to prepare the homogenate have been modified from those described previously for normal tissue (7, 11) because the tissue from treated animals requires less vigorous homogenization to achieve disruption and because the enlarged granules sediment more readily. Major granule purification is obtained by Percoll density gradient centrifugation, similar to an approach used recently to purify adrenal chromaffin granules (13).

The purity and recovery of granules has been evaluated morphologically and by analysis of marker enzymes. Fig. 1, *a* and *b* presents comparative light micrographs of parotid tissue from normal and treated rats, emphasizing that chronic isoproterenol treatment induces an increase in the size and number of granules. Immunofluorescence micrographs showing the localization of α-amylase (Fig. 1 *c*) and proline-rich proteins (Fig. 1 *d*) from treated tissue reveal a uniform distribution of these secretory polypeptides among the acinar cells and their granules. This suggests the absence of major compositionally distinct subpopulations. Electron microscopic observation (Fig. 1 *e*) of the granule fraction from the treated rats reveals that the secretory granules have been purified extensively. Further, the diameters of isolated granules (1.4–2.0 μm) are the same as that observed in situ (Castle, J. D., unpublished observations, and reference 10), suggesting that the fraction is representative of the total granule population.

The distribution of marker enzyme activities during granule purification is shown in Table I. α-Amylase, a secretory granule marker, is recovered at >20% of the total homogenate activity (which represents ≥50% of the granules that remained intact after homogenization). It is important to note that the parotid acinar cells of normal fasted rats can be considered to be unusually enriched in storage granules, even before fractionation (~31% of the cell volume is occupied by granules [10]). Chronic isoproterenol treatment results in a further enrichment in these structures (~66% of the cell volume occupied by granules) and the volume fraction of other organelles is substantially reduced (10). Thus, the 3.5-fold purification of these granules measured biochemically (as an increase in the relative specific activity of amylase, Table I) indicates substantial purity and is in the same range as values reported previously for other highly purified exocrine granule preparations (11). In contrast, the measurements of β-N-acetylglucosaminidase and cytochrome c oxidase indicate that the specific activities of these lysosomal and mitochondrial markers are, respectively, 4.5- and 9.5-fold lower than those of the homogenate. In the case of UDP-galactosyl transferase, the relative specific activity declines 10-fold (Table I), signifying that selected trans-Golgi activities are still effectively excluded from the granule compartment in the chronically stimulated tissue.

The common granule and plasma membrane marker, γ-glutamyl transferase (4) increases approximately fourfold in activity per wet weight of tissue after 10 d of isoproterenol treatment. More than 20% of the activity present in the Percoll density gradient is associated with the granule band and is likely to be associated with granule membranes since plasmalemmal elements are not detected morphologically in the granule fraction. No attempt was made to quantitate the recovery of elements of the endoplasmic reticulum (observed to be present at very low levels by electron microscopy).

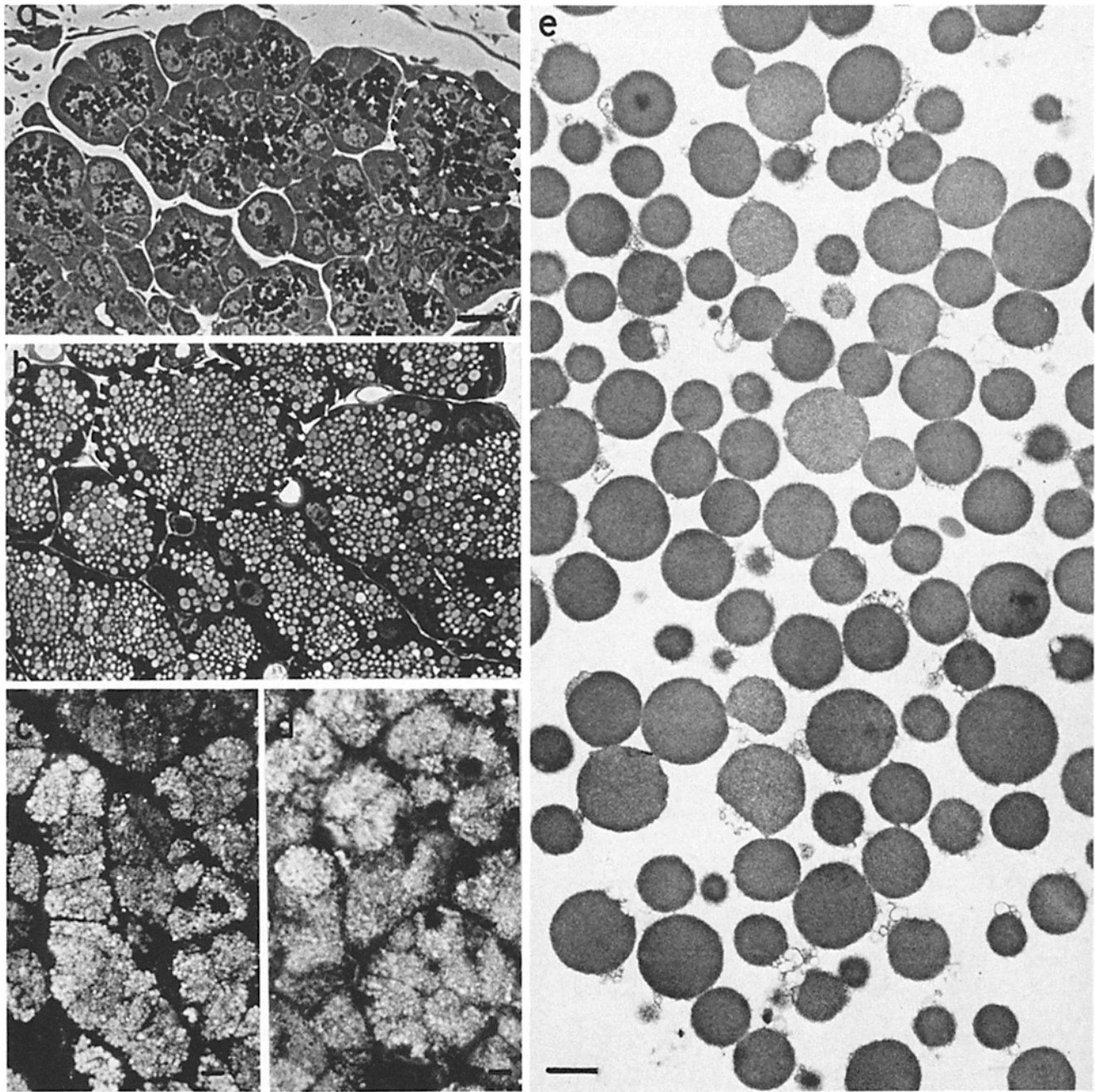


Figure 1. Morphology of parotid granules in situ and in vitro: effect of chronic isoproterenol treatment. Light micrograph of normal parotid tissue (a) in which darkly stained secretion granules ($\sim 1 \mu\text{m}$ diameter) occupy $\sim 25\%$ of the acinar cell volume. The discontinuous line delineates the profile of one acinus. Bar, $10 \mu\text{m}$. Light micrograph of parotid tissue from a rat that had received 10 daily injections of isoproterenol (b). Enlarged acini (example is outlined) contain greatly enlarged acinar cells in which the basal cytoplasm is intensely stained and pale-staining secretion granules ($\sim 1.7 \mu\text{m}$ diameter) fill $>50\%$ of the cell volume. Bar, $10 \mu\text{m}$. Immunolocalization of α -amylase (c) and basic proline-rich proteins (d) in the parotid gland of isoproterenol-treated rats. Cryosections were reacted with antibodies as described in Materials and Methods. Note the similar granule staining pattern for both secretory species. Bars, $10 \mu\text{m}$. Low power electron micrograph of the secretion granule fraction from isoproterenol-treated rats (e). Variations in density of individual granules probably reflect the variable preservation of secretory species by fixation. Bar, $1 \mu\text{m}$.

Isoproterenol Induces Changes in Parotid Granule Content Polypeptides; Effects on Chemically Assayable Protein

Repeated isoproterenol injections cause a profound change in the relative quantities of the secretory polypeptides found in rat parotid saliva (1, 9, 36, 38). Specifically, the levels of

amylase, DNase, and RNase decline in comparison to a family of more than six proteins ($\text{pI}'s >10$) that are highly enriched in proline, glutamine, and glycine. These basic proline-rich proteins increase from $<2\%$ to more than two-thirds of total parotid secretory protein during a 10-d isoproterenol treatment (38). Fig. 2 illustrates this change in

Table I. Distribution of Protein and Enzyme Activities in Preparation of Parotid Secretory Granules from Chronically Stimulated Rats

		Protein	α -Amylase (granules)	γ -Glutamyl transferase (granules + plasma membrane)	β -N-Acetyl glucosaminidase (lysosomes)	Cytochrome c oxidase (mitochondria)	UDP-Galactosyl transferase (Golgi)
Homogenate	Recovery	100	100	100	100	100	100
	R.S.A.	1.0	1.0	1.0	1.0	1.0	1.0
Remaining gradient	Recovery	88.4%	56.3%	80.9%	90.6%	98.4%	108%
	R.S.A.	—	0.637	0.916	1.03	1.11	1.23
Granule band	Recovery	13.1%	42.8%	22.4%	6.42%	1.69%	2.08%
	R.S.A.	—	3.25	1.70	0.488	0.128	0.158
Final supernate	Recovery	7.20%	22.8%	13.9%	5.08%	1.28%	1.93%
	R.S.A.	—	3.17	1.93	0.705	0.179	0.268
Granule pellet	Recovery	5.83%	20.4%	14.0%	1.32%	0.611%	0.577%
	R.S.A.	—	3.50	2.40	0.226	0.105	0.099
Total recovery		101%	99.5%	109%	97.0%	100%	110%

The activities of enzymes (which serve as markers of the organelles listed in parentheses) were measured in four preparations of granules (mean values are shown). Total recovery represents the summed fraction activities homogenate activity. Homogenate activities were: α -amylase 49,300 μ mol/min; γ -glutamyl transferase, 20.8 μ mol/min; β -N-acetyl glucosaminidase, 4.35 μ mol/min; cytochrome c oxidase, 110 U (proportional to the first-order rate constant); UDP-Galactosyl transferase, 2.1 nmol/min. Relative specific activities (R.S.A.) refer to specific activities in the fraction with respect to the homogenate.

granule content polypeptides (emphasizing a spectrum of pink-staining basic proline-rich proteins).

Because basic proline-rich proteins contain little or no tyrosine and lower amounts of lysine in relation to other secretory proteins (38), we questioned the applicability of conventional protein assays (34, 49) for comparing amounts of protein (used to normalize the internal aqueous space measurement) between granules from normal and amplified tissues. Table II shows the absolute and relative amino acid contents for granule lysates from normal and 10-d injected rats. In each case the analyses were conducted on amounts which by the fluorescamine assay (49) were equivalent to 25 μ g of a serum albumin standard. Evidently, the sample from the chronically stimulated rats has a total amino acid content nearly 2.5-fold greater than that of the control. Increases in the amounts of only three amino acids (proline, glutamine, and glycine) account for >95% of this discrepancy and emphasize the relative prominence of basic proline-rich proteins as secretory proteins in the amplified tissue.

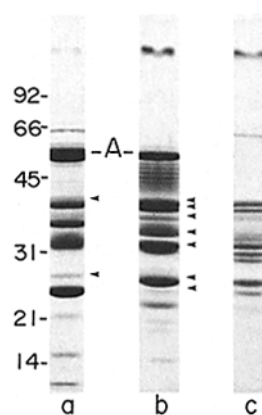


Figure 2. Gel electrophoretograms for parotid fractions from normal (a) and 10-d isoproterenol-treated (b) tissue. (c) The profile for basic proline-rich proteins reconstituted from six purified fractions (38); the proportion of individual species do not reflect the relative levels found in granules, however, their uniform low retention of Coomassie Blue imparts a metachromatic (pink) staining that provides a qualitative marker for chemically similar species in a and b. Although the assayed protein loads in a and b were each \sim 50 μ g, the actual amount in b is much higher (Table II). The shift in polypeptide

composition to yield a secretory spectrum highly enriched in basic proline-rich proteins (arrowheads) and with decreased amylase (A) content as a result of isoproterenol treatment is evident. In addition, a family of closely spaced bands of unknown identity and extending between apparent M_r 43–55 K appears in treated samples.

Internal Aqueous Volume and pH of Isolated Granules from Chronically Stimulated Parotid Glands

Intragranular aqueous space was measured for twelve separate preparations of parotid granules suspended in 300–350 mosM media. The mean internal space observed was 3.6 μ l/mg protein with individual determinations ranging from 3.1 to 4.2 μ l/mg. The variation depends on the compo-

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

	Normal		Isoproterenol	
	Absolute	Amino acid	(10 d) Absolute	Amino acid
	nmol	%	nmol	%
ASX	26.0	11.7	30.6	5.6
THR*	8.6	3.9	4.0	0.7
SER†	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§	222.8	100	546.4	100

Granules were lysed by successive freeze-thaw, hypoosmotic shock (by aqueous dilution), and brief sonication. After centrifugation (172,000 g min), samples of the supernatant fluid equivalent to 25 μ g BSA by fluorescamine assay were hydrolyzed for 20 h in 6 N HCl with 10 nmol norleucine as an internal standard; they were then analyzed for amino acid content.

*† Analyses were corrected for the destruction of threonine and serine (5 and 10%, respectively).

§ Tryptophan and cysteine are not quantitated, but each is nearly absent from proline-rich proteins of the rat parotid gland (38).

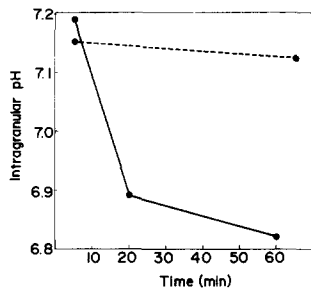


Figure 3. Time course of ATP-dependent acidification in parotid granules from isoproterenol-treated rats. Granule samples were incubated \pm 10 mM ATP in a medium (\sim 350 mosM) containing sucrose, 100 mM KCl, and 50 mM MOPS-NaOH (pH 7.10). External pH changed \leq 0.03 U throughout incubation. Intragranular pH was determined using [14 C]methyl-

amine distribution. Broken line, control. Solid line, plus Mg-ATP. Samples were incubated in duplicate; the difference between duplicates was \leq 10%. In different preparations, the magnitude of the ATP-dependent acidification was always $<$ 0.4 pH unit and $>$ 0.1 pH unit.

sition of the medium, with internal volumes being generally larger in media containing KCl rather than sucrose. If the protein values are "corrected" for underdetection of granule protein content (according to the results presented in Table II) then the mean internal aqueous space reduces to \sim 1.5 μ l/mg protein equivalent.

Intragranular pH was measured initially in a sucrose-containing medium using three separate isotope distribution procedures (Table III A). Two of the measurements rely on the equilibrium distribution of a weak base ([14 C]methylamine) or a weak acid ([3 H]acetate) (44). The third measurement is based on the equilibrium distribution of $^{86}\text{Rb}^+$ (a probe of transmembrane potential) under conditions² where ΔpH is equal in magnitude but opposite in direction to $\Delta\psi$. A nearly identical internal pH \geq 7.7 is obtained by all three procedures. This value is considerably higher than that reported for normal parotid granules ($\text{pH}_{\text{in}} \cong 6.8$ [7]) and all other types of secretion granules studied to date.

Although pH_{in} is not affected by reducing the ionic strength of the medium (when the buffer concentration is decreased 10-fold), ionic composition has a notable effect (Table III B). Adding KCl or choline chloride causes a considerable decrease in pH_{in} , whereas the presence of K_2SO_4 causes an increase above the value obtained in buffered sucrose. Apparently the parotid granules of isoproterenol treated rats exhibit increased ionic permeabilities or decreased internal buffer capacity (or both) as compared with parotid granules from untreated rats (7).

Detection of H^+ Pump Activity in Parotid Granules of Chronically Stimulated Rats

Isolated granules from the treated rats were tested for their ability to translocate H^+ into the granule interior in an ATP-dependent fashion. The generation of both ΔpH and $\Delta\psi$ were detected.

Effect of ATP on Intragranular pH

In each of 10 granule preparations, addition of ATP resulted in measurable acidification of the intragranular space. Typically, ATP-dependent intragranular acidification of \sim 0.2 pH units is observed (with variability depending on the condi-

2. As in our previous studies with $^{86}\text{Rb}^+$ in the presence of valinomycin (3), the ability to measure ΔpH with a probe of $\Delta\psi$ requires the presence of the proton ionophore CCCP to insure that H^+ is in equilibrium across the membrane (i.e., H^+ electrochemical potential, 0).

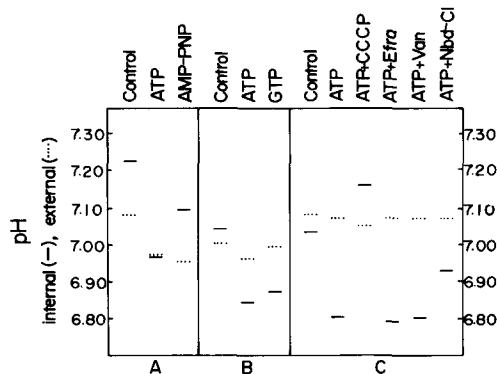


Figure 4. Properties of ATP-driven acidification in parotid granules from isoproterenol-treated rats. Isolated granules from three different preparations were incubated either 30 (A) or 20 min (B and C) at 25°C with either [3 H]acetate (A) or [14 C]methylamine (B and C) as probes of ΔpH . Each value of external pH (dotted lines, measured with a pH electrode) and internal pH (solid lines, calculated from the distribution of radioactive probe) is the mean of duplicate determinations (differences between duplicates were \leq 10%). Incubation media, adjusted in all cases to \sim 350 mosM with sucrose contained: (A) 5.0 mM MOPS-NaOH, 25 mM KCl, 45 mM K_2SO_4 , and (when used) 10 mM Mg-ATP or Mg-AMP-PNP; (B) 50 mM MOPS-NaOH, 100 mM KCl, and (when used) 10 mM Mg-ATP or Mg-GTP; (C) 50 mM MOPS-NaOH, 100 mM KCl, and (when used) 10 mM Mg-ATP, 10 μ M CCCP, 1 μ M efrapentin, 50 μ M sodium vanadate, 20 μ M Nbd-Cl. ATPase inhibitors were incubated with granules for 10 min before ATP addition.

tions used). As reported with other granule fractions (14), the conditions favoring a large acidification (high levels of chloride in the medium) tended to result in a greater degree of granule lysis and for this reason, such conditions were not employed routinely.

Fig. 3 shows that in contrast to control samples (no ATP), samples containing 10 mM ATP (without an ATP-regenerating system) exhibit a \sim 0.3-pH unit acidification in 20 min, with an additional \sim 0.07-pH unit decrease at 1 h (and without further acidification up to 2 h, not shown). Similar acidification is observed using 1 mM exogenous ATP, however, a systematic analysis of the ATP-concentration dependence of granule acidification has not yet been made. Fig. 4 illustrates other properties of internal acidification, showing both pH_{in} and pH_{out} after a period of incubation. The pH of the external medium tends to be more constant in 50 mM MOPS buffer (Fig. 4, B and C) than in 5 mM MOPS (Fig. 4 A). Of particular importance is the observation that unlike ATP, addition of AMP-PNP (a nonhydrolyzable ATP analog, Fig. 4 A) does not result in an increase in ΔpH , thus serving to exclude possible effects of ATP that are independent of ATP hydrolysis.³ By contrast, GTP (Fig. 4 B) appears to promote intragranular acidification (possibly reflecting the presence of a nucleoside diphosphokinase activity [17]).

The effects of various ATPase inhibitors and uncouplers were examined (Fig. 4 C). Efrapentin, at a dose that inhibits $>$ 90% of parotid mitochondrial ATPase (8), fails to influence the ATP-dependent acidification of these granules. Sodium vanadate, which inhibits ATPases that proceed through a

3. Although a pH_{in} lower than that of the control granules is seen upon addition of AMP-PNP in Fig. 4 A, note that it parallels a decrease in medium pH. Other experiments (not shown) confirmed that AMP-PNP is not hydrolyzed by parotid granules.

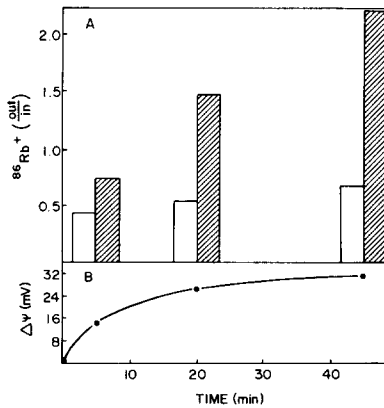


Figure 5. An inside-positive membrane potential is generated that depends on ATP hydrolysis. Granule samples were incubated (in duplicate; difference between duplicates $\leq 10\%$; mean values are shown) for the times indicated in 3 mM Mg-AMP-PNP (open bars) or 3 mM Mg-ATP (hatched areas). (A) The exclusion of $^{86}\text{Rb}^+$ with increasing inside-positive potential was followed at various times after nucleotide addition. This technique has been used to advantage because of its high sensitivity resulting from both the negligible levels of background binding of radioactive probe, and the unusually large internal volumes ($\sim 50\%$ total pellet volume) measured by sedimentation. In each case, the medium contained 0.1 μCi $^{86}\text{Rb}^+$, 10 μM valinomycin, 0.3 M sucrose, and 5 mM MOPS-NaOH (pH 7.26 ± 0.03 in all samples). (B) The data in A was used to calculate the differences in transmembrane potential between AMP-PNP and ATP-containing samples (in millivolts); this difference value is reported as $\Delta\psi$. Results are representative of two experiments.

phosphorylated enzyme intermediate (35) also is ineffective. By contrast, a partial (and, for unknown reasons, variable) inhibition of intragranular acidification is obtained when granules are exposed to Nbd-Cl, a compound that inhibits the H^+ pumps of chromaffin and platelet granules (17). Finally, addition of CCCP abolishes completely the ATP-driven acidification; this effect rules out the possibility that the observed acidification is due to passive H^+ movement.⁴

Effect of ATP on Transmembrane Potential

Experiments were undertaken to check for inside-positive changes in $\Delta\psi$ that depended on ATP hydrolysis as observed in acidic organelles known to contain electrogenic H^+ pumps (17, 24, 26, 30). For this purpose, effects of AMP-PNP and ATP were studied in parallel. $\Delta\psi$ was measured using the equilibrium distribution of $^{86}\text{Rb}^+$ in the presence of valinomycin (8, 24, 44). Results with AMP-PNP (over a 45-min time period, Fig. 5 A) demonstrate a slow but progressive exclusion of the positively charged probe, consistent with a gradual shift of $\Delta\psi$ to a more inside-positive value. Although the reason for this shift in the baseline Δ is not established, it may be explained by an H^+ -diffusion potential since the conditions required to measure $\Delta\psi$ (non-ionic medium [pH 7.2]) result in an intragranular pH of 7.7–7.8 (Table III A), which favors inward-directed H^+ diffusion.⁵

4. Note in Fig. 4 c that the addition of CCCP actually results in an elevated intragranular pH above control values, consistent with the presence of net fixed-positive charges in the granule interior.

5. It is unlikely that the baseline $^{86}\text{Rb}^+$ exclusion seen in Fig. 5 represents H^+ pumping driven by endogenous ATP because the ATP concentration

Table III. Internal pH of Parotid Granules from Rats Chronically Stimulated with Isoproterenol

A. Measurement using three different ion distribution techniques		
pH Probe	pH _{out}	pH _{in}
[¹⁴ C]Methylamine	7.17	7.72
[³ H]Acetate	7.13	7.70
⁸⁶ Rb ⁺ /valinomycin + CCCP	7.10	7.74
B. Effect of composition of the external medium		
Medium	pH _{out}	pH _{in}
Sucrose	7.17	7.72
KCl, 75 mM	7.15	7.14
K ₂ SO ₄ , 50 mM	7.15	7.83
Choline Cl, 100 mM	7.06	6.91

Granules were incubated for 30 min at 25°C in 350 mosM media. In A, samples contained sucrose and 50 mM MOPS-NaOH. In B, sucrose was replaced isoosmotically by the specified concentrations of electrolytes and [¹⁴C]methylamine was used as pH probe. Values are the mean of duplicate samples. Valinomycin, CCCP were 10 μM .

From the first time point measured, the presence of ATP results in an increase in $\Delta\psi$ over that observed in AMP-PNP-containing samples. This difference is ~ 14 mV at 5 min and progresses to ~ 31 mV by 45 min (Fig. 5 B). Despite the shift in baseline (in the presence of AMP-PNP) we take these data to indicate that parotid granules from the treated rats are capable of generating an inside-positive membrane potential which depends on ATP hydrolysis. This potential is less than that seen for chromaffin granules (50–70 mV at 30–40 min; 8, 26, 30), but much greater than that seen in normal parotid granules (~ 2 mV at 30 min using the $^{86}\text{Rb}^+$ method; 3, 8).

Contaminating Organelles Contribute Minimally to ATP-Dependent Acidification of the Granule Fraction

Two approaches were used to exclude the possibility that the H^+ pump activity described above might occur within vesicular contaminants rather than in the granules. First, from a series of representative electron micrographs, we measured the volume fraction (50) occupied by contaminants of the granule preparation in order to predict the extent of acidification expected of such structures as the exclusive source of H^+ pump activity. In three independent experiments, the internal volume of nongranule structures (which consist partly of organelle contaminants and partly of the membranes of damaged granules) averaged only 2.3% of the total internal volume (see Table IVA). Consequently, a measured acidification of 0.2 pH units, if ascribed entirely to contaminants, would require a selective acidification in these structures of >8 pH units. Such a magnitude seems extremely unlikely for vesicles consisting of biological membrane studied in vitro.

In a second approach we sought to establish directly the relative contributions of granules and organelle contaminants to the internal [³H]methylamine content measured in biophysical experiments using electron microscope level autoradiography to detect [³H]methylamine in specimens fixed after incubation. We reasoned that if contaminants were responsible for weak base accumulation in pH measurements, they would contain the probe at a level that is disproportionately large compared with their contribution to to-

within these granules has been measured at <10 μM (Castle, J. D., unpublished observations).

Table IV. Stereologic Analysis of the Granule Fraction and Measurements of [³H]Methylamine Distribution

A. Stereologic measurements		
Fractional contribution of granule volume to that of all vesicular structures		0.977 ± 0.015
B. Autoradiographic measurements		
		%
Grains over granules	-ATP	91 ± 8.5
	+ATP	93 ± 1.4
Grains over nongranular structures	-ATP	2.0 ± 0.1
	+ATP	2.5 ± 0.7

(A) Three separate preparations of granules from chronically stimulated rat parotid tissue were prepared for electron microscopy as described in Materials and Methods. Using the point-count procedure described in reference 50, the internal volume of granules (mean diameter ~1.5 μm) was measured for a total of 503 granule profiles taken from representative photographs of the top, middle, and bottom of pellets. The remaining structures were similarly counted, and mean values ± SD were determined. In general, most of the contaminant structures were found in the top regions of pellets, which make a minimal contribution to the biophysical measurements (44). (B) Results are shown for two preparations of granules in which equal-sized samples of granules were incubated with [³H]methylamine in a medium adjusted to 350 mosM with sucrose and containing 100 mM choline chloride and 7 mM MOPS-NaOH (pH 7.1) for 20 min at 25°C. The samples were then processed in parallel for autoradiographic measurements as described in Materials and Methods. Grains overlying any portion of granule profiles were counted as granule-associated, with a similar measure for nongranule structures. Using this method, ~6% of grains were found to lie over organelle-free background. Mean values ± SD are shown.

tal internal volume. Further, in the event that the retention of internal label were complete, measurement of autoradiographic grain density (in the presence and absence of ATP) might identify directly the acidifying structures.

Quantitative measurements performed on a series of electron micrographs (Table IV B) indicate that only 2–3% of the grains occur over nongranule structures, a value that is not affected by the addition of ATP. By contrast, the vast majority of grains are over granules in the absence or presence of ATP, and the granule portion of total labeling is similar to the granule portion of total volume (Table IV). Although we did observe an increase in the grain density over granules in the presence of ATP (of ~1.3-fold in two separate experiments, which would correspond to a ΔpH of ~0.13 units) we are unable to conclude that we have measured granule acidification directly with this procedure, because the increase is not statistically significant and retention of internal label during processing was only ~75%. Nevertheless, the present data suggest that internal labeling is proportional to the fractional contribution to total internal volume and thus augment the case against contaminants of the granule fraction as a major source of acidification activity. From these morphological considerations, we find the support for H⁺-ATPase activity in the membranes of parotid granules from isoproterenol-treated rats to be compelling.

Acridine Orange Fluorescence Microscopy

The novel finding of storage granules whose internal pH is alkaline under most ionic conditions yet which possess inward-directed H⁺ translocase activity, raises the interesting question about the intragranular pH in living cells where millimolar levels of ATP are maintained and ionic conductances are carefully regulated. Initially we attempted to check for exclusion or concentration of a weak base probe detectable by immunocytochemistry (2). Unfortunately, the

results were inconclusive due to difficulties in preserving the internal structure of these unusual granules (this occurred largely during prefixation in the absence of glutaraldehyde before the immunoperoxidase reaction).

To avoid the preservation problem, we used a different weak base probe, AO, to examine live cells from dispersed parotid acini by fluorescence microscopy. The fluorescence emission of AO is concentration-dependent; it emits a green color at low concentrations and an orange color (reflecting oligomer formation) at higher concentrations when viewed with a fluorescein filter. In each of three preparations, a reproducible pattern of fluorescence was obtained, namely, that granules visible by phase microscopy appear to exclude the fluorescent probe in comparison with the surrounding cytoplasm (Fig. 6). Nuclei fluoresce brightly (AO also intercalates into the DNA), and the surrounding cytoplasm exhibits a weak green staining. In most cases the apical (granule-rich) pole of the cell is even less fluorescent; orange-stained granules are never seen. In favorable planes of focus through these large, three-dimensional cells, a honeycomb-like pattern corresponding to green-stained cytoplasm surrounding unstained granules can be appreciated (Fig. 6 B). By contrast, rat peritoneal mast cells (known to contain granules with an acidic interior, [29]) when viewed under identical conditions (Fig. 6, C and D) characteristically show a cytoplasm replete with orange-staining granules, demonstrating intragranular concentration of the weak base. Thus, although AO fluorescence microscopy does not yield a precise measurement, it suggests that the pH in situ of the granules of the chronically stimulated rat parotid is higher than that of the cytosol.

Discussion

We have shown previously that mature exocrine granules from the rat parotid have an internal pH slightly below neutrality (7) and exhibit almost no inward-directed H⁺ pump activity (8). In the present studies, we have employed chronic β-adrenergic stimulation of the same tissue to cause a change in transcription (52), which results in a quantitative alteration in the spectrum of granule-content polypeptides (Fig. 2) as well as morphological changes in the granule population (Fig. 1, b–d). We believe that the biophysical findings presented in this report result primarily from this change in granule composition.

The passive distribution of H⁺ is such that granules isolated from chronically stimulated rats have an internal pH of ~7.7 when measured in a pH ~7.1 sucrose-containing medium (Table IIIA). Evidently, these granules possess net fixed-positive internal charges, consistent with their observed enrichment in basic proteins (Table II). These data lend support to the notion that intragranular pH is influenced to a large degree by content molecules (analogous to the influence of fixed-negative internal charges in chromaffin granules, [39]). In contrast to normal parotid or chromaffin granules, however, the internal pH of these unusual granules varies with external ionic conditions (Table III B), suggesting that membrane ionic permeability is relatively large with respect to internal buffer capacity.

ATP addition to the isolated granules from isoproterenol-treated rats results in the generation of an internal acidification (Fig. 3) that is abolished by the proton ionophore CCCP

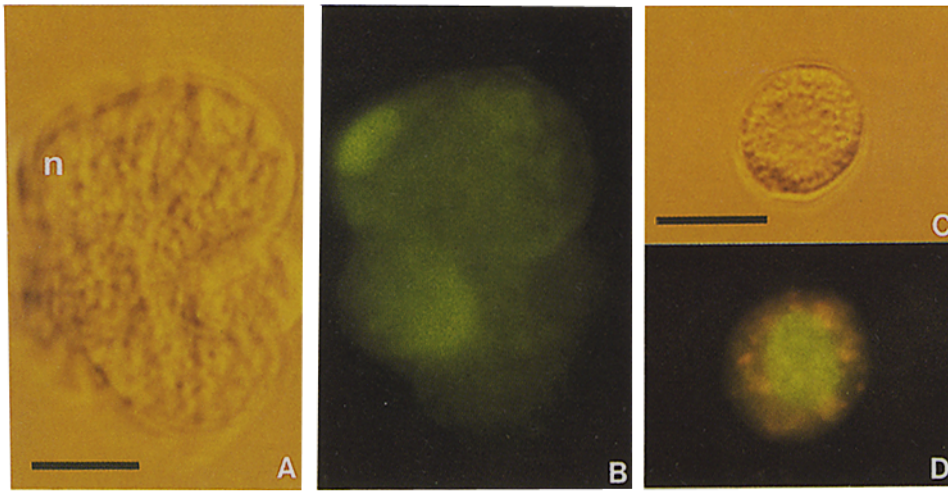


Figure 6. AO staining of dispersed parotid acini from stimulated rats, and from mast cells. Paired phase and fluorescence micrographs were taken within 5–10 min of addition of 5 μM AO as described in Materials and Methods. In all cases parotid acinar cells (*A* and *B*) exhibit bright nuclear staining surrounded by fainter yellow-green or green cytoplasmic staining. By contrast, the apical granule population is unstained and in favorable planes of focus (*B*) green-stained cytoplasm outlining individual granules is seen. For orientation, a nucleus (*n*) is marked in *A*, corresponding to the bright green staining (*upper left*) of *B*. Examination

of rat peritoneal mast cells under identical conditions (*C* and *D*) shows that they exhibit punctate orange AO staining of their acidic granules surrounding a central nucleus. Bars, 10 μm . Doses of AO $>5 \mu\text{M}$ resulted in no change in the unstained appearance of the internal populations of parotid granules but caused the punctate orange staining of mast cell granules to become obscured within the dense red-orange granule population.

(Fig. 4 *C*), and the generation of an inside-positive $\Delta\psi$ that depends upon ATP hydrolysis (Fig. 5). Other studies (not shown) reveal an ATP hydrolase activity associated with this granule fraction that, as in the case of chromaffin granules (but not in a fraction containing mature parotid granules, [8]), is inhibited by 20 μM Nbd-Cl (which also partially inhibits acidification, see Fig. 4 *C*). Further experimentation will be needed to test whether inhibition of both ATP hydrolase and H^+ pump activities are mediated through the same protein(s).

Several lines of evidence serve to exclude the possibility that contaminants in the granule fraction are a major source of the H^+ -ATPase activity. The lysosomal contribution to H^+ -ATPase activity must be very small, because the level of lysosomal contamination (as judged by the relative specific activity of β -N-acetylglucosaminidase, see Table I) is approximately threefold lower than that obtained with normal parotid granule fractions (7) in which reliable acidification could not be demonstrated (8). The lack of inhibition of acidification by efrapeptin and vanadate (Fig. 4 *C*) suggests that contributions by mitochondrial and other selected H^+ -ATPases are also negligible. Stereologic measurements made on the fraction (Table IV *A*) suggest that the internal volume contribution of nongranule structures to this fraction is not sufficient to account for the magnitude of the observed acidification. Finally, [^3H]methylamine autoradiography of the granule fraction, despite practical limitations, reveals a preponderance of granule-associated autoradiographic grains rather than a localized grain density over nongranule structures (Table IV *B*). These data, taken together, argue strongly in favor of the interpretation that acidification is a granule membrane activity.

In recent investigations, we have begun to address what biochemical features provide the characteristic properties and function of secretion granules⁶ (11). Studies performed herein show that it is possible to make major changes in the

internal composition of these structures without compromising their storage capabilities.⁷ In fact, when the underdetection of granule content protein (Table II) is taken into account, the internal packaging (as judged by intragranular aqueous space measurements) of parotid granules from isoproterenol-treated rats is equal to that found in normal rats (7). Such flexibility in packaging diverse content molecules (such as those with high isoelectric points) suggests the presence of adaptive mechanisms designed to meet a range of internal storage requirements. This machinery may normally reside in the membranes associated with the trans-Golgi where concentration usually begins. We have hypothesized elsewhere⁸ that the acidifying ATPase could be a part of this machinery, and it now seems reasonable to propose that the finding of H^+ pumping in parotid granules from isoproterenol-treated rats signifies a selective and purposeful retention of this activity (and perhaps others) for a function in this exocrine storage compartment. By contrast, the Golgi activity, galactosyl transferase (43), remains efficiently excluded from the granules (Table I), suggesting that it continues to play no important role in granule function per se.

Since it is presumed that the H^+ -ATPase is operant in the intracellular milieu, our results with acridine orange in situ (Fig. 6) make a distinction between a compartment which can acidify and one which is acidic. If the presence of H^+ pump activity represents a compensatory response for the augmented presence of alkaline secretory proteins, then this compensation is simply of insufficient magnitude to effect an accumulation of AO. High intragranular buffering or other regulated ion conductances could account for this observation. However, it should be pointed out that an elevated intragranular pH does not preclude a role for H^+ pump activity in facilitating (or maintaining) the packaging of secretory content. Indeed, such a role may well be manifest in the

7. In other studies (reference 42; Castle, J. D., unpublished observations) it has also been found that discharge of parotid granules can proceed normally under the present conditions of chronic isoproterenol stimulation.

8. Arvan, P., R. S. Cameron, and J. D. Castle, manuscript submitted.

6. Cameron, R. S., P. L. Cameron, and J. D. Castle, manuscript submitted.

generation of the $\Delta\psi$ component of $\Delta\mu_{H^+}$ by an electrogenic H^+ -ATPase, as recently postulated (3).

In other experiments (not reported herein), a direct comparison of parotid granules from uninjected rats and rats injected 3, 6, and 10 d with isoproterenol showed progressive alterations in their content composition, their internal (uncorrected) volume, their internal pH, and their ability to acidify upon ATP addition. This information, coupled with recently developed procedures for obtaining subfractions of normal parotid granules that contain content of different posttranslational age (28, 51) underscores the unusual potential of this system for exploring mechanisms of exocrine granule formation, packaging, and storage.

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