

EXOCYTOSIS IN SECRETORY CELLS OF RAT LACRIMAL GLAND

Peroxidase Release from Lobules and Isolated Cells upon Cholinergic Stimulation

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ABSTRACT

Release of peroxidase from secretory cells of rat lacrimal gland upon cholinergic stimulation was studied *in vitro* with single lobules and isolated cells (lacrimocytes). Isolated lobules, kept in Eagle's medium, remain structurally intact and reaction product of peroxidase is confined to cisternae of rough endoplasmic reticulum, elements of the Golgi apparatus, and all secretory granules. Morphologically, exocytosis occurs by membrane fusion and discharge of granule content. The highest rate of peroxidase released from lobules is observed at 10^{-4} M carbamylcholine. The specific activity of peroxidase released into the medium is fourfold higher as compared to the lobules. Release of peroxidase is suppressed by atropine when added before or after the addition of carbamylcholine. At 4°C , no peroxidase release occurs upon cholinergic stimulation. The exocytotic release of peroxidase is dependent on energy supply, as indicated by substantial inhibition (at 37°C) under anoxic conditions or in the presence of dinitrophenol, KCN, or carboxyatractyloside. Furthermore, the process is sensitive to colchicine and vinblastine. Isolated lacrimocytes, consisting of 95% secretory acinar cells, are prepared by digestion with collagenase, hyaluronidase, and trypsin. They retain the characteristic polarity of secretory cells *in situ*, and localization of peroxidase is the same as in lobules. Since isolated lacrimocytes respond to cholinergic stimulation in the same way as lobules, the receptors are not damaged by the isolation procedure and appear to be associated directly with the exocrine cell. Oxygen uptake by isolated lacrimocytes is about $14 \text{ nmol O}_2 \times \text{min}^{-1} \times 10^{-6} \text{ cells}$; it is about doubled by uncoupling with dinitrophenol. Oxygen uptake rises by 20–30% above the resting rate upon cholinergic stimulation. This additional uptake is suppressed by atropine or by added cholinesterase, indicating that continuous receptor occupancy may be required for the energy demand by exocytosis. On the basis of the specific activity of peroxidase in the medium, the energy demand resulting from cholinergic stimulation is estimated to be $0.08 \mu\text{mol ATP}$ (or energy-rich phosphate bonds) per microgram of protein released from the lacrimocytes.

Secretory cells of rat lacrimal gland contain peroxidase (28) which is localized in all membrane-bounded cellular compartments known to be involved in transport and discharge of exportable proteins (18). Lacrimal gland peroxidase is released into the lacrimal fluid (18) upon cholinergic stimulation. Peroxidase can be visualized cytochemically with the 3,3'-diaminobenzidine (DAB) technique (14) and quantitated with a colorimetric assay using DAB as hydrogen donor (15). Recently, optimal conditions for the cytochemical demonstration of lacrimal gland peroxidase have been determined (16). The enzyme can be differentiated cytochemically from catalase which is localized in peroxisomes of secretory cells and is not secreted into the lacrimal fluid (16). Peroxidase, therefore, is a marker enzyme well suited for the study of protein export in this system.

In this study, we present light and electron microscope observations on the localization of lacrimal gland peroxidase in two types of *in vitro* systems, (a) single lobules and (b) isolated secretory cells (lacrimocytes), and describe functional characteristics of discharge of this peroxidase upon cholinergic stimulation. Furthermore, the effect of various inhibitors on secretion and the oxygen consumption during discharge are studied. These experiments provide information on the localization of peroxidase in the *in vitro* systems of single lobules and isolated cells and on the kinetics, the energy requirement, and the mode of secretion of lacrimal gland peroxidase. In addition, they can answer the question of whether or not cholinergic receptors are associated directly with the exocrine cells of rat lacrimal gland.

MATERIALS AND METHODS

Animals

Male albino rats (Wistar colony of the Department of Cell Biology) weighing 150–200 g, kept on a normal laboratory diet (Altromin) with water *ad libitum*, were used.

Materials

3,3'-diaminobenzidine tetrahydrochloride (DAB), DNA from calf thymus, Type I, vinblastine sulfate, colchicine, and carbamylcholine chloride were obtained from Sigma Chemical Company, German Branch, Munich, Germany; collagenase (125 U/mg), soybean trypsin inhibitor (1 mg inhibits 1.73 mg trypsin), and hyaluronidase (from bovine testes, lyophilized, salt-free, 3,000 U.S.P. U/mg) were obtained from Worthington

Biochemical Corp., Freehold, N. J.; trypsin (from bovine pancreas, lyophilized, salt-free, 9,000 U/mg), acetylcholine esterase (from *Electrophorus electricus*), and carboxyatractyloside were purchased from Boehringer, GmbH, Mannheim, Germany; Eagle's Basal Medium with Earle's Salts was obtained from Flow Laboratories GmbH, Bonn, Germany; atropine sulfate, gelatin, hydrogen peroxide (30%), acetylcholine chloride, and glutaraldehyde were purchased from E. Merck, Darmstadt, Germany. Morpholinopropane sulfonic acid was purchased from Serva, Heidelberg, Germany; Leitsilber for mounting of specimens for scanning electron microscopy was obtained from Emetron, Hanau, Germany. All chemicals were of analytical grade.

Methods

PREPARATIONS OF SEPARATED LOBULES: Rats were anesthetized with chloralhydrate (50 mg intraperitoneally [i.p.]) and perfused through the left ventricle (after ligation of the aorta descendens and incision of the right atrium) for 5 min at a continuous hydrostatic pressure of 120 mm Hg with Krebs-Ringer solution (KRB) equilibrated by gassing with 95% O₂ and 5% CO₂ at room temperature in order to remove erythrocytes and leukocytes from blood vessels; both cell types contain peroxidase and catalase which would interfere with the peroxidase assay in tissue homogenates and in the secretion. Lacrimal glands were excised and disintegrated by injection of oxygenated Eagle's medium into the interstitial tissue. Small lobules 2–3 mm in diameter became visible and were dissected, according to the procedure described by Castle et al. (8), and collected in Eagle's medium containing 10 mM D-glucose and 1.8 mM Ca⁺⁺ at 4°C. Usually, lobules from one gland (ca. 80 mg wet weight) were used for two to four incubation experiments.

INCUBATION FOR RELEASE OF PEROXIDASE FROM LOBULES: 25-ml siliconized Erlenmeyer flasks were used in all incubation experiments. Dissected lobules were immersed in 10 ml of Eagle's medium and gassed continuously with 95% O₂ and 5% CO₂. For study of the release of peroxidase from unstimulated lobules into the surrounding medium, 0.5 ml of the medium was removed at various time points and centrifuged for 5 min in a Beckman 152 Microfuge (Beckman Instruments, Inc., Palo Alto, Calif.) in order to remove particulate matter. Peroxidase activity in the samples was measured for up to 2 h after preparation of the lobules and the morphological integrity of the lobules was monitored by light and electron microscopy. Similarly, peroxidase activity in the various samples was determined after stimulation of secretion with various concentrations of carbamylcholine ranging from 0.1 μM to 1 mM. At optimal concentration of carbamylcholine (100 μM, see Results), discharge of peroxidase was studied after the application of various metabolic inhibitors such as dinitrophenol, KCN, atractyloside, gassing with nitrogen

(95% N₂ and 5% CO₂), atropine as a specific inhibitor for carbamylcholine action, and alkaloids (colchicine and vinblastine) known to inhibit secretion in a variety of secretory tissues (34). In addition, discharge of peroxidase was measured at various temperatures ranging from +4°C up to +37°C during stimulation with optimal concentrations of carbamylcholine. Because of the sensitivity of the pH value of Eagle's medium to temperature changes, the pH was adjusted with 0.1 N NaOH separately for each temperature. In some experiments, carbamylcholine was replaced by acetylcholine.

Lobules from six glands were prepared as described above, suspended in 20 ml Eagle's medium, and stimulated with 100 μM carbamylcholine at 37°C for 1 h; they were then separated from the medium by centrifugation and homogenized in 20 ml distilled water with an Ultra Turrax TP 18/10 (Janke and Kunkel KG., Staufen, Germany) for 5 min at 4°C. Protein, DNA, and peroxidase activity in the medium and in the homogenate were determined.

PREPARATION OF ISOLATED LACRIMAL GLAND CELLS (TABLE I): Intact lobules were prepared from perfused rat lacrimal glands as described above and incubated for dissociation in a modification of the media described by Amsterdam and Jamieson (1). For dissociation, a medium containing collagenase, hyaluronidase, and trypsin was used. Trypsin was added to the medium because the mixture of a crude collagenase and hyaluronidase did not provide sufficient tryptic activities, so that isolation was incomplete. Lobules from the glands of two animals were usually immersed in 20 ml of dissociation medium in a 25-ml Erlenmeyer flask and constantly gassed with 95% O₂ and 5% CO₂ in a shaking water

bath at 37°C ± 0.2 at 120 movements per minute. The enzymatic digestion was interrupted for 10 min by chelation of divalent cations with 2 mM EDTA followed by a wash with Eagle's medium containing 1.8 mM Ca⁺⁺, 0.8 mM Mg⁺⁺, and 10 mM glucose. After this wash, by which EDTA is removed and divalent cations (Ca⁺⁺, Mg⁺⁺) are added, the lobules were placed again in freshly prepared dissociation medium containing collagenase, hyaluronidase, and trypsin. Usually, after 30–45 min the lobules began to disintegrate. At this stage, the dissociation was completed by pipetting followed by filtration of the dispersed cells through a 25-μm mesh siliconized steel gauze and by three washes in Eagle's medium without enzymes. The cells were resuspended in Eagle's medium containing 1.5% gelatin, 2.5 mM Ca⁺⁺, 0.8 mM Mg⁺⁺, 10 mM D-glucose, and 0.1 mg soybean trypsin inhibitor per milliliter. With this technique, a suspension of single cells was obtained, consisting of ~80% secretory cells (lacrimocytes), ~10% duct cells, and ~10% myoepithelial and endothelial cells. The amount of lacrimocytes was improved to 95% by centrifugation of the cell suspension for 5 min at 50 g (800 rpm), which leads to sedimentation of mainly the larger lacrimocytes, whereas the majority of the endothelial cells, duct cells, and myoepithelial cells remain in the supernate. Due to the perfusion of the lacrimal gland before dissociation, red or white blood cells are excluded from the cell suspension. The dissociation was monitored in a Zeiss Ultraphot III equipped with Nomarski optics. The number of cells was counted in a Zeiss-Thoma hemocytometer, and cell viability was estimated with the trypan blue exclusion test (31).

DISCHARGE OF PEROXIDASE FROM ISOLATED

TABLE I
Preparation of Isolated Cells from Rat Lacrimal Gland (Lacrimocytes)

Step	Medium	Time
(1) Enzymatic digestion of isolated lobules	Eagle's medium with 1.8 mM Ca ⁺⁺ , 0.8 mM Mg ⁺⁺ , and 10 mM glucose, containing collagenase 100 U/ml, hyaluronidase 400 U/ml, trypsin 9,000 U/ml	15 min
(2) EDTA treatment	Eagle's medium without Ca ⁺⁺ or Mg ⁺⁺ containing 2 mM EDTA	2 × 5 min
(3) Washes	Eagle's medium with 1.8 mM Ca ⁺⁺ , 0.8 mM Mg ⁺⁺ , and 10 mM glucose	2 × 2 min
(4) Enzymatic digestion	Same medium as in step 1	30–45 min
(5) Pipetting	Five gentle strokes with a 5-ml siliconized glass pipette	5 min
(6) Filtration through 25-μm mesh siliconized steel gauze		5 min
(7) Washes	Eagle's medium with 2.5 mM Ca ⁺⁺ , 0.8 mM Mg ⁺⁺ , 10 mM glucose, 1.5% gelatin	3 × 5 min
(8) Final medium	Eagle's medium with 2.5 mM Ca ⁺⁺ , 0.8 mM Mg ⁺⁺ , 10 mM glucose, 1.5% gelatin, 0.1 mg/ml trypsin inhibitor	1–5 h

LACRIMAL GLAND CELLS: A 10-ml suspension of freshly isolated cells ($\sim 2 \times 10^6$ cells) was transferred into a siliconized 25-ml Erlenmeyer flask and continuously gassed with 95% O₂ and 5% CO₂. Discharge of peroxidase from isolated cells without and after cholinergic stimulation was determined as described for dissociated lobules. At different time points, 0.5 ml of the medium (containing the suspended cells) was removed and centrifuged in a Beckman 152 Microfuge. The supernate was used for the assay of the discharged peroxidase. The cellular pellet was resuspended in 1 ml of distilled water and homogenized for 2 min at 4°C by sonication with a Sonipen TM homogenizing rod (Technic International, Bergenfield, N. J.). Peroxidase activity was determined, and secreted peroxidase was related to total activity in the homogenate.

DETERMINATION OF ENZYME ACTIVITIES: Peroxidase activity in samples of the incubation medium and in tissue homogenates was determined with the colorimetric DAB-oxidase assay (15) modified for lacrimal gland peroxidase (16). The assay mixture contained 10^{-2} M DAB in 0.1 M Tris-HCl buffer at pH 7.0. Usually, 0.85 ml of this solution was mixed with 0.05 ml of the sample, and the reaction was started by the addition of 0.1 ml 10^{-2} M H₂O₂ to give a final concentration of 10^{-3} M H₂O₂. Absorbance was recorded at 460 nm at 25°C, and the results were expressed as units (U) with $E = 3.16 \text{ mM}^{-1} \text{ cm}^{-1}$ (15). Catalase activity in the secretion fluid and in homogenates was measured with the method of Lück (25). Protein in the secretion fluids and the homogenates was estimated with the Lowry technique (24). DNA content in the homogenates was determined according to Burton (7) with calf thymus DNA as a standard.

CONTINUOUS MEASUREMENT OF OXYGEN UPTAKE OF ISOLATED LACRIMOCYTES: Oxygen uptake of isolated rat lacrimal gland cells was determined with a Clark-type electrode in a 1.2-ml lucite chamber maintained at 37°C. The cells were stirred magnetically, and additions were made with Hamilton syringes. Two signals were recorded simultaneously, one proportional to oxygen concentration ([O₂]) in the chamber, the other proportional to oxygen uptake ($d[\text{O}_2]/dt$) as obtained from a special differentiator circuit. The oxygen electrode and the electronic circuits were designed and constructed by the Electronics Department, Sonderforschungsbereich 51, Munich.

Isolated lacrimocytes from a freshly prepared stock solution in Eagle's medium equilibrated with 95% O₂ and 5% CO₂ were placed into the chamber and diluted approximately 1.5-fold with a buffer containing morpholinopropane sulfonic acid (10 mM), NaCl (154 mM), KCl (6.2 mM), pH 7.4, to give a cell concentration of about 0.5×10^6 cells per ml. After each incubation, the cells were counted in a Thoma-Zeiss hemocytometer. Oxygen uptake, as recorded continuously from the differentiator output, was expressed as $\text{nmol O}_2 \times \text{min}^{-1} \times 10^{-6}$ cells. No significant differences in oxygen uptake

were detected at oxygen saturation or at lower O₂ concentrations down to 0.1 mM. After an experiment with atropine, the chamber was "washed" with lacrimocytes in order to remove efficiently any residual atropine from the chamber surface.

ELECTRON MICROSCOPY AND CYTOCHEMISTRY: Tissue lobules were fixed in 3% glutaraldehyde for 2 h (for morphological studies) or in 1.25% glutaraldehyde for 60 min (for optimal visualization of peroxidase) (16) in 0.1 M Na cacodylate buffer at pH 7.4. Isolated cells were fixed in suspension with the same concentrations of glutaraldehyde and pelleted by centrifugation for 5 min in a Beckman 152 Microfuge. Tissue lobules and pellets (fixed in 1.25% glutaraldehyde) were cut with a razor blade under a dissecting microscope into thin slices, rinsed briefly in 0.15 M Na cacodylate buffer, pH 7.4, immersed at room temperature and in darkness in 10^{-2} M DAB in 0.1 M Tris-HCl buffer, pH 7.0, for 1 h, and incubated for 2 h at room temperature in the complete medium containing 10^{-2} M DAB and 10^{-3} M H₂O₂ in 0.1 M Tris-HCl buffer, pH 7.0. After a brief rinse in 0.15 M Na cacodylate buffer, the slices were postfixed in 2% unbuffered OsO₄ for 1 h at 4°C, dehydrated, and embedded in Epon 812 (26). Tissue lobules and pellets (fixed in 3% glutaraldehyde) were postfixed in 2% unbuffered OsO₄ as described above, kept for 1 h at room temperature in 0.5% uranyl acetate (12), and embedded in Epon. 1- μm thick sections were examined unstained or stained with toluidine blue-pyronine in a Zeiss Ultraphot III photomicroscope. Ultrathin sections were cut on an LKB-Ultratome III (LKB Produkter, Stockholm, Sweden) with a diamond knife and observed unstained or stained with uranyl acetate and lead cit (35) in a Siemens Elmiskop I.

PREPARATION OF LOBULES AND ISOLATED CELLS FOR SCANNING ELECTRON MICROSCOPY: Lobules and isolated cells from rat lacrimal gland were prepared and fixed in 3% glutaraldehyde and 2% OsO₄ as described above, dehydrated in a graded series of acetone, and dried in a Sorvall Critical Point Drying System (DuPont Instruments, Sorvall Operations, Newtown, Conn.) with oil-free liquid CO₂ (33). Lobules were mounted on specimen holders with Leitsilber, whereas isolated cells were spread on pieces of cover slips which were mounted with Leitsilber on specimen holders. In one experiment, lobules at various stages of enzymatic dissociation were prepared. All specimens were coated with gold in a "Sputter Coater" (LWU, Eching, Germany) and examined in a Cambridge Stereoscan Mark II electron microscope operated at 20 kV.

RESULTS

In Vitro System of Lacrimal Gland Lobules

MORPHOLOGY: The structure of single lobules appears intact even after 5 h of incubation in

Eagle's medium continuously gassed with 95% O₂ and 5% CO₂ (Fig. 1). Reaction product of peroxidase is localized in all cisternae of the rough endoplasmic reticulum, in elements of the Golgi apparatus, and in secretory granules (Fig. 2) as described previously for intact tissue (18). After 10 min of incubation in Eagle's medium in the presence of 10⁻⁴ M carbamylcholine, large vacuoles appear in the apical portion of acinar cells (Fig. 3*b*) which are absent in unstimulated control lobules (Fig. 3*a*). The membranes of secretory granules appear to fuse with the apical plasma membrane (Fig. 4*a*) and to rupture in the region of fusion (Fig. 4*b*) upon cholinergic stimulation.

DISCHARGE OF PEROXIDASE FROM SINGLE LOBULES: To follow the kinetics of peroxidase discharge upon stimulation of lacrimal gland lobules, the appearance of peroxidase and of protein in the medium was followed with time and was related to the total protein and total peroxidase contents of the lobules. In addition, the effect of cholinergic stimulation on catalase was measured. The results of two experiments are summarized in Table II. About 7–10% of the total tissue protein is discharged within 1 h of stimulation. This amount of protein corresponds to 30–35% of total peroxidase. Catalase remains within the tissue and is not released into the medium.

The dose response of peroxidase discharge from single lobules of rat lacrimal gland to carbamylcholine is shown in Fig. 5. Discharge of peroxidase was determined after 30 min of stimulation. Optimal concentration was at 10⁻⁴ M carbamylcholine and half-maximal stimulation at 10⁻⁵ M. The discharge of peroxidase started immediately upon addition of 10⁻⁴ M carbamylcholine, whereas without stimulation the release of peroxidase remained very low and did not exceed 5% of the total secretion. Stimulated discharge of peroxidase increased with increasing temperature and was almost completely inhibited at 4°C. Since the activity of lacrimal gland peroxidase decreased at higher temperature (18), the effect of temperature on stimulated discharge was studied up to 37°C only. In the presence of 10⁻⁴ M carbamylcholine, single lobules could be stored at 4°C with negligible secretion for at least 1 h. After this time, discharge of peroxidase could be initiated by increasing the temperature to 37°C. Addition of atropine (10⁻⁴ M) 20 min after the addition of carbamylcholine markedly inhibited discharge of peroxidase (Fig. 6). Inhibitory effects were also

observed with 10⁻⁴ M KCN, which suppressed carbamylcholine-induced release of peroxidase from lobules when added before or 20 min after stimulation (Fig. 6). Carbamylcholine-induced release is almost completely inhibited by equilibration of lobules with 95% N₂ and 5% CO₂ (Table III). The effects of other metabolic inhibitors such as carboxyatractyloside (10⁻⁷ M) and dinitrophenol (10⁻⁴ M) are included in Table III. Colchicine and vinblastine are known to inhibit secretion of exportable proteins in a variety of secretory cells (34). The effect of various concentrations of both alkaloids on stimulated discharge of peroxidase from single lobules is also shown in Table III.

Isolated Lacrimocytes

MORPHOLOGICAL AND CYTOCHEMICAL OBSERVATIONS: The acini of isolated lobules are covered by a basement membrane which obscures details of the cells (Fig. 7). After its removal by enzymatic digestion, the surface of individual cells becomes visible (Fig. 8). Dispersion of the cells by gentle pipetting and washing yields a suspension of isolated lacrimocytes (Fig. 9) of roughly spherical shape (*inset*, Fig. 9). Cell viability as estimated with the trypan blue exclusion test (31) is more than 95%. On the basis of DNA in tissue and isolated cells, the yield of cells after isolation is 60–65%. Fine structure and morphological polarity are maintained in isolated lacrimocytes after digestion and release from the lobule (Fig. 10); the secretory granules remain in the apical portion of the cytoplasm, and no leakage of peroxidase from membrane-bounded compartments is observed.

FUNCTIONAL CHARACTERISTICS: To assess the functional integrity of the isolated lacrimocytes, release of peroxidase and oxygen uptake were studied. After 60 min of incubation at 37°C in the presence of 10⁻⁴ M carbamylcholine, 450 mU of peroxidase per 10⁶ cells (corresponding to about 40% of total peroxidase in cells) is released into the medium, whereas in the presence of 10⁻⁴ M atropine less than 10% of total peroxidase is discharged (Fig. 11). Thus, cholinergic stimulation is effective in isolated lacrimocytes, leading to an approximately fivefold increase in the rate of release of the marker enzyme, peroxidase. These observations on isolated cells demonstrate that the cholinergic receptors are associated with the lacrimocytes.

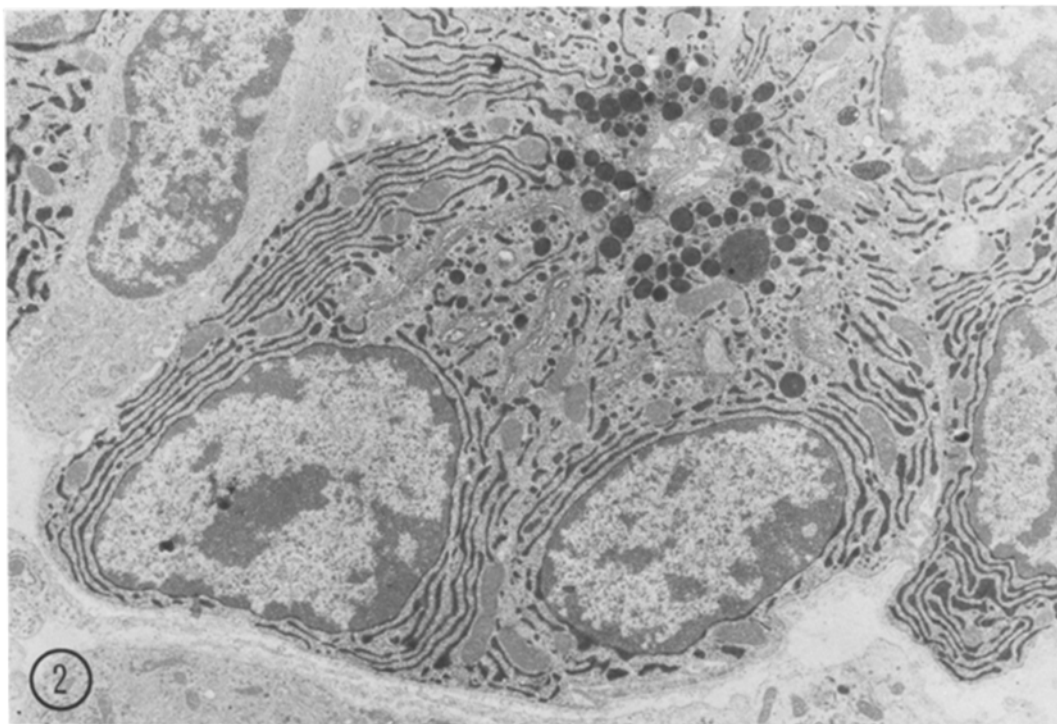
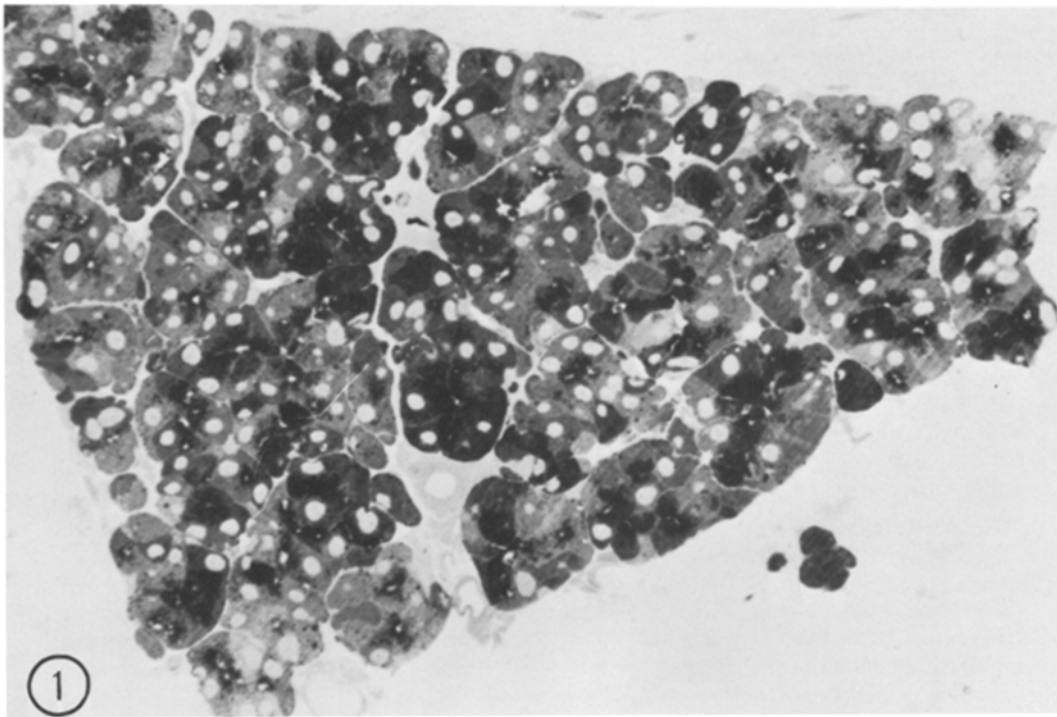


FIGURE 1 1- μ m thick unstained Epon section through a lobule from rat lacrimal gland after incubation for 5 h in Eagle's medium and continuous gassing with 95% O₂ and 5% CO₂. Peroxidase reaction. \times 300.

FIGURE 2 Part of an acinus in a lobule from rat lacrimal gland, kept in vitro for 1 h in Eagle's medium. Reaction product of peroxidase is visible in all cisternae of the rough endoplasmic reticulum, in elements of the Golgi apparatus, and in all secretory granules. Uranyl and lead stained. \times 7,600.

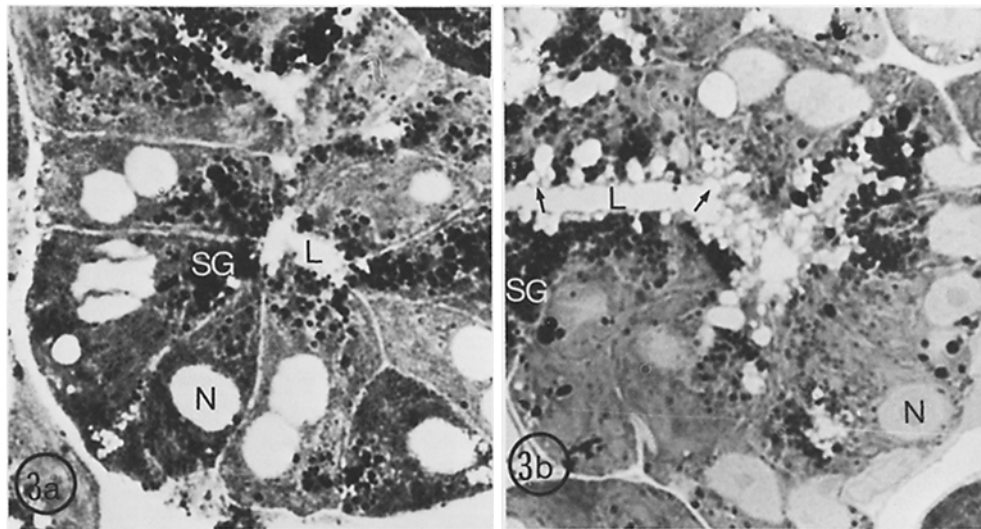


FIGURE 3 *a* and *b* 1- μ m unstained Epon sections from lobules after incubation in Eagle's medium and continuous gassing with 95% O₂ and 5% CO₂ without stimulation (Fig. 3 *a*) and after stimulation (Fig. 3 *b*). Reaction product of peroxidase is present in the basal cytoplasm of acinar cells and in secretory granules (SG) located in the apical portion. After 10 min of stimulation with 10⁻⁴ M carbamylcholine (Fig. 3 *b*), large vacuoles appear at the apical portion of secretory cells. Some of these vacuoles (arrows) are continuous with the acinar lumen (L). N, nuclei. \times 1,250.

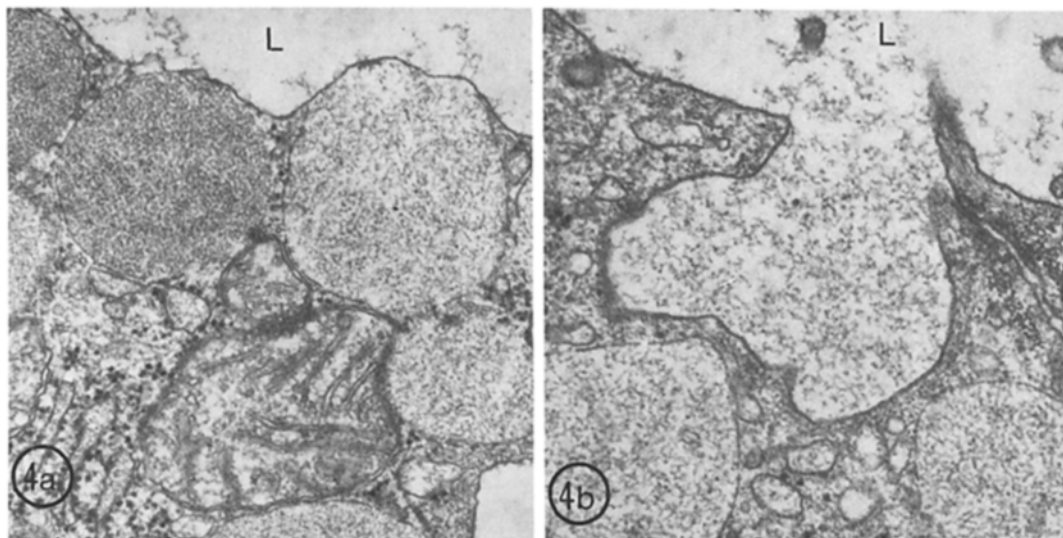


FIGURE 4 *a* and *b* Apical fields of acinar cells from lobules incubated for 10 min in Eagle's medium in the presence of 10⁻⁴ M carbamylcholine. During cholinergic stimulation, membranes of secretory granules appear in close association with the luminal plasma membrane. Both membranes fuse (Fig. 4 *a*) and rupture in the region of fusion (Fig. 4 *b*) so that the granule content is discharged into the lumen (L). Fig. 4 *a*: \times 24,000. Fig. 4 *b*: \times 32,500.

Further information on the functional integrity of the cells is obtained from measurements of oxygen uptake. Oxygen uptake at 37°C is about 14 nmol O₂ per min per 10⁶ lacrimocytes (Figs.

13, 14). This basal rate is reached within about 5–7 min after transfer into the measuring chamber. Upon addition of acetylcholine (Fig. 12) or carbamylcholine (Figs. 13, 14), an additional oxygen

TABLE II
Discharge of Peroxidase and Catalase after 1 h of Cholinergic Stimulation of Isolated Lobules from Rat Lacrimal Gland (Results from 2 Separate Experiments, A and B)

Contents in	Protein		DNA mg	Peroxidase*			Catalase† U	
	mg	percent of total		specific activity		percent of total		
				U/mg protein	U			
Lobules	A	28.6	100	1.2	14.2	406	100	160
	B	57.0	100	2.1	14.0	800	100	280
Medium	A	2.8	10	0	51.4	144	35	0
	B	4.0	7	0	60.0	240	30	0

Isolated lobules from four glands (A) or eight glands (B) were kept in Eagle's medium at 37°C equilibrated with 95% O₂ and 5% CO₂. Upon stimulation with carbamylcholine (10⁻⁴ M), about 7-10% of the total protein within the secretory cells of rat lacrimal gland is released within 1 h into the surrounding medium. This amount of discharged protein corresponds to about 30-35% of the total peroxidase within the lobules. Catalase is not released into the medium. Note the about fourfold higher specific activity of peroxidase in the medium (U/mg protein) as compared with values in the lobules.

* Release of peroxidase from lobules into the medium in the absence of carbamylcholine is 1.5-2% of total peroxidase.

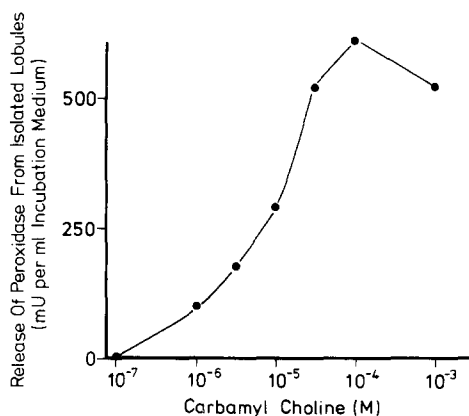


FIGURE 5 Dose response curve for release of peroxidase from lobules isolated from rat lacrimal gland after 30 min of stimulation with various concentrations of carbamylcholine. Half-maximal stimulation is reached at 10⁻⁵ M carbamylcholine.

uptake of 25-30% ensues. The higher steady-state rate of oxygen uptake depends on the occupancy of receptor sites by the cholinergic effector, since enzymatic cleavage of acetylcholine by choline esterase leads to a prompt return of oxygen uptake to the basal rate (Fig. 12). When choline esterase is added in the absence of acetylcholine, there is

no change in oxygen uptake. Conversely, prior occupancy of receptor sites by atropine suppresses additional oxygen uptake upon cholinergic stimulation (Fig. 13).

The respiratory chain in isolated lacrimocytes is controlled by the availability of phosphate acceptor. This is demonstrated by the ability of an uncoupler, 2,4-dinitrophenol, to stimulate oxygen uptake by about 100% (Figs. 12-14). Interestingly, a further increase of oxygen uptake cannot be provoked by carbamylcholine in the presence of the uncoupler (Fig. 14).

DISCUSSION

The findings show that peroxidase is released from secretory cells of rat lacrimal gland upon stimulation of cholinergic receptors which are associated directly with the secretory cells. Morphologically, this process is characterized by exocytosis and discharge of granule content into the acinar lumen. Biochemically, release of peroxidase requires energy since it is abolished by inhibition of oxidative phosphorylation and since oxygen uptake increases upon cholinergic stimulation. Furthermore, discharge of peroxidase is strongly inhibited by low temperatures and by colchicine and vinblastine.

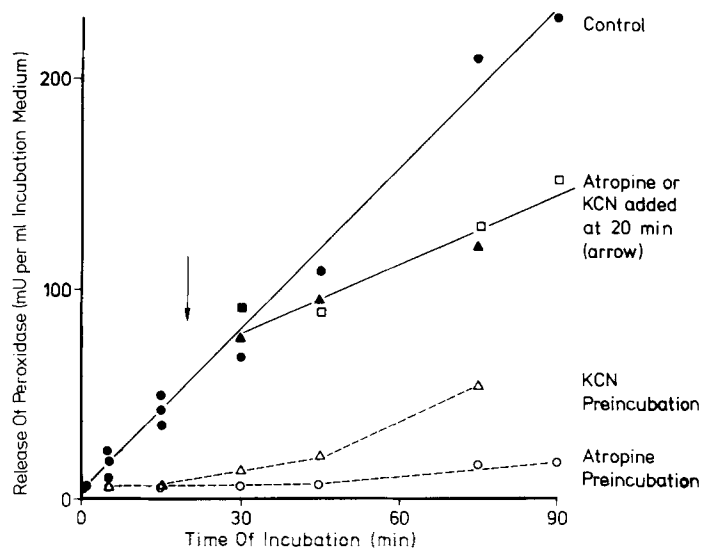


FIGURE 6 Effect of atropine and KCN on release of peroxidase from single lobules of rat lacrimal gland upon cholinergic stimulation. Atropine (10^{-4} M) (\square — \square) and KCN (10^{-4} M) (\triangle — \triangle) given 5 min before stimulation with 10^{-4} M carbamylcholine (at 0 time) inhibit stimulated peroxidase discharge. Addition of atropine (\square — \square) and KCN (\triangle — \triangle) 20 min after the addition of carbamylcholine also markedly inhibits release of peroxidase, as compared to stimulated discharge in the absence of such inhibitors (\bullet — \bullet).

TABLE III
Inhibition of Release of Peroxidase from Isolated Lobules of Rat Lacrimal Gland

Additions	Inhibition	
	M	%
Nitrogen (95% N ₂ , 5% CO ₂)		90
Dinitrophenol	10^{-4}	67
KCN	10^{-4}	80
Carboxyatractyloside	10^{-7}	80
Colchicine	10^{-6}	35
	10^{-4}	91
Vinblastine	10^{-6}	51
	10^{-4}	90

Isolated lobules were incubated for 30 min with 10^{-4} M carbamylcholine and in the presence of inhibitors as indicated. Peroxidase activity in the medium was determined in both cases and inhibition expressed in percent of control.

Peroxidase Release from Lobules upon Cholinergic Stimulation

Morphologically, stimulated release of granule content in rat lacrimal gland is in agreement with the current concept of exocytosis by membrane fusion described in various secretory glands, especially in the exocrine pancreas of the guinea pig

(for a recent review, see reference 30). An alternative hypothesis for release of exportable proteins has been proposed by Rothman (37) who suggested an equilibrium or membrane transport model. According to this model, exportable proteins move bidirectionally through specialized membranes so that they can be in equilibrium with each other in the various compartments. This hypothesis postulates the direct release of secretory proteins from the cytoplasm into the acinar lumen through the apical plasma membrane. In rat lacrimal gland, this mechanism has not been observed. Peroxidase reaction product is confined to cisternae of the rough endoplasmic reticulum, to elements of the Golgi apparatus, and to secretory granules (18) and does not occur outside these membrane-bounded compartments in the cytoplasm.

Cytochemical localization and release of peroxidase has been described in rat parotid gland (17) where, upon cholinergic stimulation *in vivo*, peroxidase can be visualized in acinar lumina and secretory ducts. In isolated lobules of rat lacrimal gland stimulated *in vitro*, however, reaction product of peroxidase was rarely visible within the lumina. This could be due to the more direct communication between the lumina and the sur-

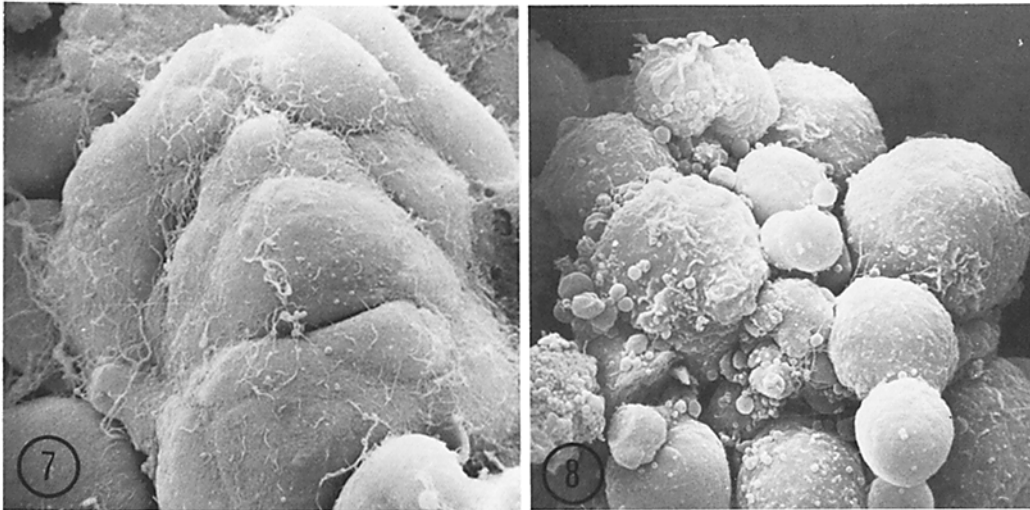


FIGURE 7 Scanning electron micrograph of parts of a single lobule from rat lacrimal gland before treatment with enzymes for isolation of secretory cells. Acinar cells are covered by a basement membrane which obscures the fine structure of cell surfaces. Thin fibers, presumably collagen fibers of the interacinar space, adhere to the basement membrane. $\times 3,200$.

FIGURE 8 Scanning electron micrograph of part of a lobule after 20 min of treatment with collagenase, hyaluronidase, and trypsin. The basement membrane is removed, so that details of acinar cell surfaces become visible. Some acinar cells are rounded up. $\times 1,200$.

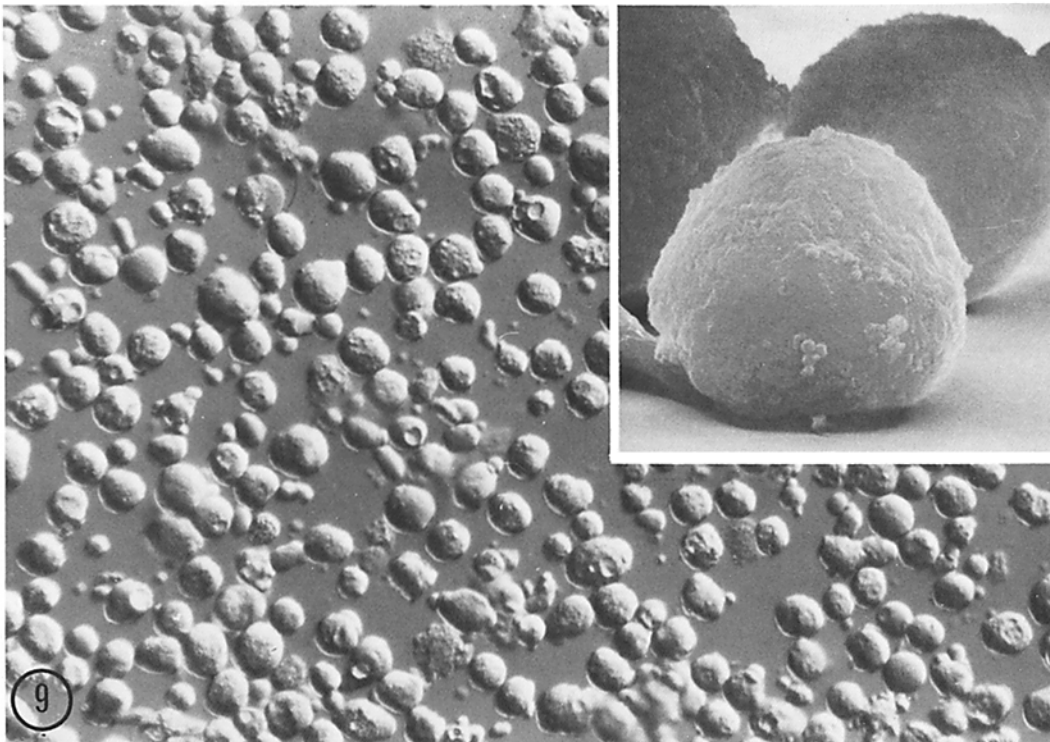


FIGURE 9 Survey picture of isolated cells from rat lacrimal gland. Interference phase contrast (Nomarski). $\times 290$. *Inset*: scanning electron micrograph of isolated spherical cells. $\times 2,800$.

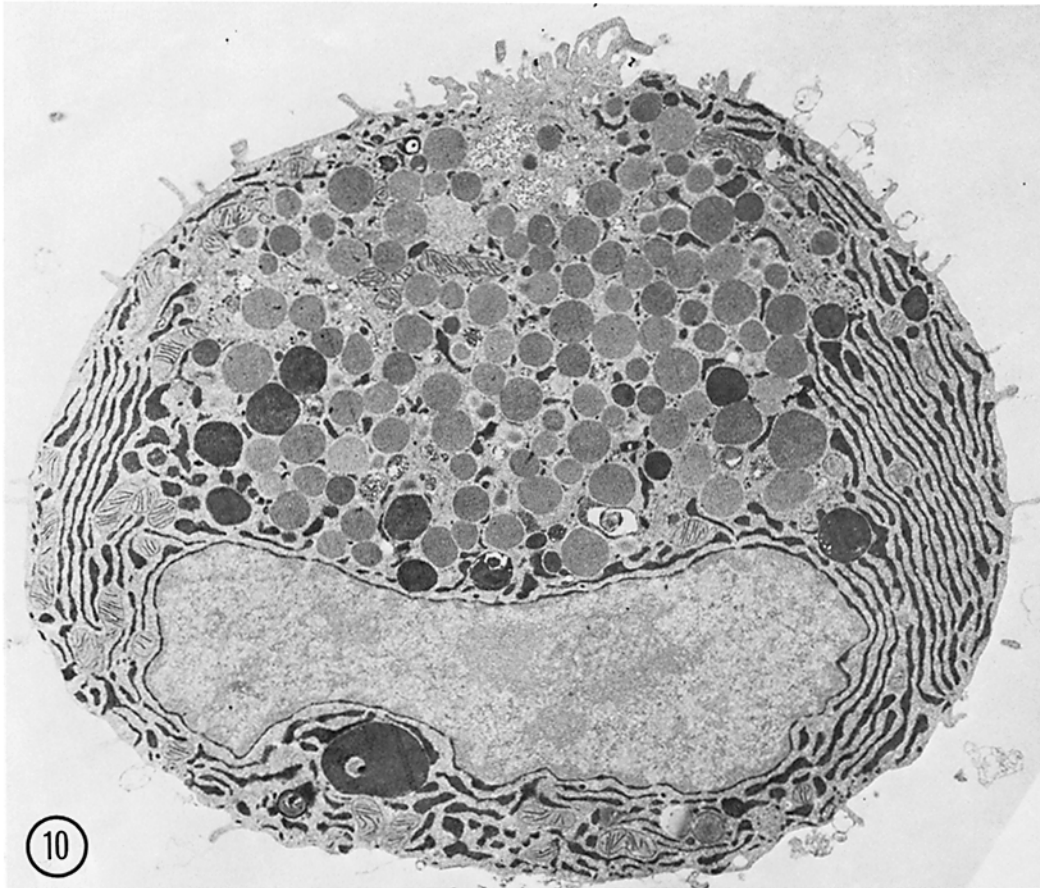


FIGURE 10 Isolated secretory cell of rat lacrimal gland reacted for peroxidase. The polar structure of cells *in situ* is preserved. The apical portion of the plasma membrane bears numerous microvilli. $\times 7,300$.

rounding medium in which the lobules are suspended during incubation. Hence, peroxidase becomes detectable in this medium shortly after stimulation.

The specificity of cholinergic stimulation is demonstrated by the experiments with atropine as inhibitor. Atropine not only inhibits release when present before cholinergic stimulation, but also when release of peroxidase from lobules has been proceeding for 20 min. From this experiment, we conclude that release of peroxidase can be stopped specifically with atropine and that exocytosis proceeds only as long as receptors are occupied by acetylcholine or carbamylcholine. Strong inhibition was also noted by lowering the temperature and by addition of cholchicine or vinblastine to the incubation medium (see Table III). Colchicine has a strong affinity for tubulin (32, 39) and appears

to inhibit the polymerization of tubulin into microtubules (4). Furthermore, low temperature leads to depolymerization of microtubules. Microtubules are believed to function in directing the movement of cellular components (29) and to be involved in the secretory transport (38). The effect of colchicine on the discharge of peroxidase is in agreement with this concept of the function of microtubules. However, in the lacrimal gland, as well as in other tissues (34), direct evidence for the involvement of microtubules is still lacking. In this context, it may be mentioned that somatostatin, a tetradecapeptide hormone known to inhibit the release of growth hormone (5) and insulin and glucagon (22), was also found to inhibit the release of peroxidase from single lobules of rat lacrimal gland (our unpublished results).

Various metabolic inhibitors affect the stimu-

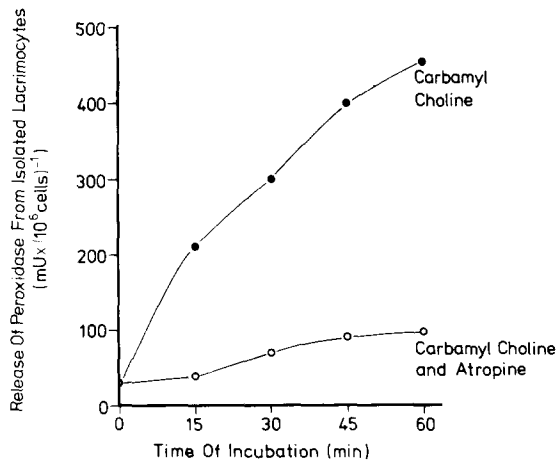


FIGURE 11 Release of peroxidase from isolated cells of rat lacrimal gland (lacrimocytes) upon stimulation with 10^{-4} M carbamylcholine (●—●) and in the presence of 10^{-4} M atropine (○—○).

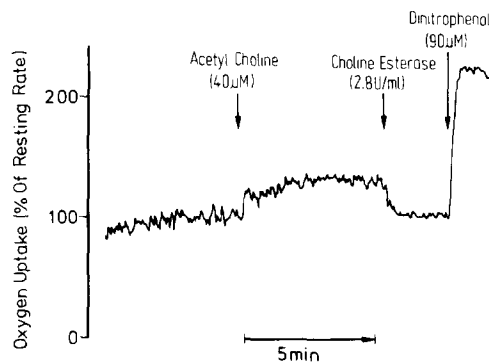


FIGURE 12 Oxygen uptake ($d[O_2]/dt$) in isolated lacrimocytes measured with a Clark-type electrode. Resting rate (100%) is about $14 \text{ nmol } O_2 \times \text{min}^{-1} \times 10^{-6} \text{ cells}$ (compare with Figs. 13 and 14). Upon cholinergic stimulation with $40 \mu\text{M}$ acetylcholine, oxygen uptake rises by 30% above resting rate. Addition of acetylcholine esterase (2.8 U/ml) causes a rapid return to the basal rate.

lated release of peroxidase from lacrimal gland lobules. The inhibition ranges from 60 to 90% with 10^{-4} M dinitrophenol, 10^{-7} M carboxyatractyloside, 10^{-4} M KCN, and under anoxic conditions, consistent with findings on tissue slices from guinea pig pancreas (19, 21, 30). Thus, the exocytotic release of peroxidase requires energy. Since secretion can be suppressed by the addition of KCN subsequent to the initiation by carbamylcholine, cell metabolism has to provide energy continuously during release of peroxidase. At the mo-

ment, it cannot be determined with sufficient accuracy to what extent the lacrimocyte can utilize energy derived from glycolysis rather than from the mitochondrial respiratory chain. However, the data indicate that such a contribution is relatively small.

Isolation and Functional Characterization of Lacrimocytes

Viable single cells have been successfully isolated from a variety of tissues, such as liver (3),

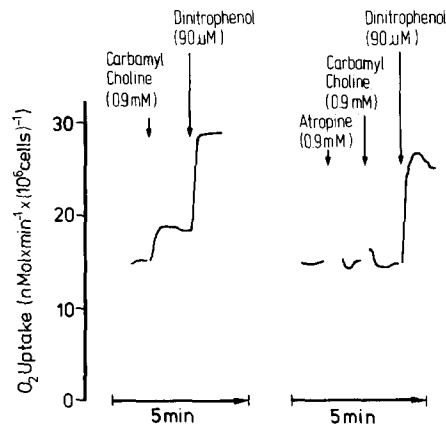


FIGURE 13 Oxygen uptake in isolated lacrimocytes. The increase of oxygen uptake upon stimulation with 0.9 mM carbamylcholine (left curve) is inhibited by prior addition of atropine (right curve). Dinitrophenol ($90 \mu\text{M}$) increases oxygen uptake by 100% above the resting rate.

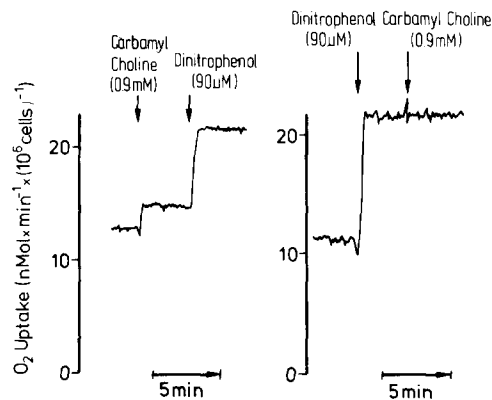


FIGURE 14 Oxygen uptake in isolated lacrimocytes. Upon addition of 10^{-4} M carbamylcholine, oxygen uptake rises by 25–30% above the resting rate (left curve). When dinitrophenol is given before addition of carbamylcholine, this effect is not observed (right curve).

thyroid gland (27), anterior pituitary gland (20), parotid gland (2), and exocrine pancreas (1). In the present study on secretory cells of rat lacrimal gland (lacrimocytes), the isolation procedure is based largely on the method described for dissociation of guinea pig pancreas (1).

Morphologically, isolated lacrimocytes lose their pyramidal shape and appear more spherical. Similar changes have been observed in dispersed cells from exocrine pancreas (1). The characteristic polarity of lacrimocytes, however, is retained in most of the dispersed cells for 5 h after isolation. Longer incubation periods cause reassociation of isolated lacrimocytes. The structure of such newly formed contacts between lacrimocytes is not yet known. When isolated hepatocytes reassociate, newly formed tight junctions and desmosomes appear (11).

Isolated lacrimocytes respond to cholinergic stimulation, suggesting that receptors are associated directly with these cells. Since the response is observed for several hours immediately after isolation, the isolation procedure does not appear to damage the cholinergic receptors on the cell surface. This is in contrast to the observations of Hopkins and Farquhar (20) on dispersed cells of anterior pituitary gland which fail to respond to secretagogues immediately after tissue dissociation and which require ~15 h for regeneration of receptor activity.

Oxygen uptake of isolated lacrimocytes rises 20–30% above the resting rate upon cholinergic stimulation, which is in agreement with early manometric measurements made by Deutsch and Raper (10) on cat submaxillary gland and by various authors on tissue slices of exocrine pancreas (9, 13). The resting rate was about $14 \text{ nmol O}_2 \times \text{min}^{-1} \times 10^{-6} \text{ cells}$. This is comparable to resting rates of oxygen uptake by isolated hepatocytes (23) and gastric parietal cells (36) which were 18 and $12 \text{ nmol} \times \text{min}^{-1} \times 10^{-6} \text{ cells}$, respectively. The effects of various inhibitors suggest that increased oxygen uptake by lacrimocytes depends upon the continuous presence of acetyl- (or carbamyl-) choline on the binding sites of cholinergic receptors as well as on energy supply, largely by oxidative phosphorylation. Since rapid information is obtained from polarographic measurements, O_2 uptake is a suitable criterion for cell viability. In this respect, the uncoupler control ratio (about 2 in our experiments) or, as a more specific parameter, the cholinergic stimulus-dependent O_2 uptake will be useful.

In a first approximation, we attribute the additional oxygen uptake upon cholinergic stimulation to the activity of the mitochondrial respiratory chain. However, the possibility must be left open that part of the additional oxygen is not utilized for ATP synthesis but rather for production of H_2O_2 , as is the case with polymorphonuclear leukocytes during the process of phagocytosis (6). So far, evidence for H_2O_2 production by lacrimocytes upon cholinergic stimulation is not available. Also, the identification of hydrogen donors for peroxidase and the determination of H_2O_2 concentration in the lacrimal fluid have not been performed.

Energy Demand upon Cholinergic Stimulation

The data obtained from measurements of peroxidase release and oxygen uptake upon cholinergic stimulation allow an estimate of energy requirements for exocytosis and concomitant processes such as water and electrolyte transport. About $0.08 \mu\text{mol}$ of ATP (or, more generally, energy-rich phosphate) is expended during exocytotic release of $1 \mu\text{g}$ of protein from the lacrimocyte.

The calculation is as follows. Release of peroxidase is $12 \text{ mU} \times \text{min}^{-1} \times 10^{-6} \text{ cells}$ (Fig. 11), concomitant with an additional oxygen uptake of about $3 \text{ nmol O}_2 \times \text{min}^{-1} \times 10^{-6} \text{ cells}$ (Figs. 12–14). As shown in Table II, the specific activity of peroxidase released is about 55 U per mg protein. If one assumes that all of the extracellular protein found upon cholinergic stimulation is due to the exocytotic process, it follows that the observed additional oxygen uptake is about 14 nmol O_2 expended per microgram of protein released. This corresponds to $0.08 \mu\text{mol}$ energy-rich phosphate bonds expended per microgram of exocytotically released protein, when one assumes that the P/O ratio is 3 and that additional O_2 uptake is entirely due to increased flow through the mitochondrial respiratory chain.

The energy expended upon cholinergic stimulation is of the order of that required for the synthesis of the exported protein (with the utilization of about 4 mol energy-rich phosphate bonds per mole peptide linkage formed, this would be about $0.04 \mu\text{mol}$ energy-rich phosphate bonds per microgram protein). This rough estimate shows that the cholinergic response of the cell requires considerable energy expenditure.

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