

FUNCTIONAL AND MORPHOLOGICAL CHARACTERIZATION OF ISOLATED BOVINE ADRENAL MEDULLARY CELLS

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ABSTRACT

Single bovine adrenal medullary cells have been obtained by retrograde perfusion of adrenal medullae with a solution of 0.05% collagenase in Ca^{++} -free Krebs Henseleit buffer. Chromaffin cells were obtained in high yield (5×10^6 cells/g medulla), and more than 95% of these were viable as shown by exclusion of trypan blue. The isolated cells were capable of respiring at a linear rate for a minimum of 120 min.

Ultrastructural examination revealed that the cells were morphologically intact, and two distinct types of adrenal medullary cells were identified, on the basis of the morphology of their electron-dense vesicles, as (a) adrenaline-containing and (b) noradrenaline-containing cells.

Biochemical analysis showed that the cells contained catecholamines and dopamine- β -hydroxylase (DBH). The cells released catecholamines and DBH in response to acetylcholine (ACh), and this release was accompanied by changes in the vesicular and surface membranes observed at the ultrastructural level. The time-course of ACh-stimulated catecholamine and DBH release, and the dependence of this release on the concentration of ACh and extracellular Ca^{++} have been investigated. The isolated cells were pharmacologically sensitive to the action of the cholinergic blocking agents, atropine and hexamethonium.

KEY WORDS exocytosis · secretion · catecholamines · isolated cells · adrenal medullary cells

Secretion from endocrine glands, in response to physiological stimuli, occurs by selective release of stored hormones. In the adrenal gland the hormones of the adrenal medulla (the catecholamines [CA's]) are stored in membrane-limited vesicles (chromaffin granules). The release of CA's from the adrenal medulla is thought to

occur by exocytosis, a calcium-dependent process that involves fusion of the vesicle membrane with the plasma membrane of the medullary cell and selective release of the vesicular contents directly into the extracellular space.

In support of this hypothesis, exocytotic vesicle profiles have been observed at the ultrastructural level in the adrenal medulla by transmission (2, 13, 22, 39) and freeze-etch (38) electron microscopy. Additional support for release of CA's by exocytosis comes from biochemical studies which

show that the CA's are secreted together with the other soluble components of the adrenal medullary vesicles (nucleotides, soluble dopamine- β -hydroxylase (DBH), and the soluble proteins), but that the vesicle membrane components (membrane bound DBH and lipids) and cytoplasmic constituents of the adrenal medullary cells (lactate dehydrogenase, tyrosine hydroxylase, and phenylethanolamine-*N*-methyltransferase) are not released (for reviews, see references 14 and 41). These experiments have been performed mostly on intact perfused bovine adrenal glands and subfractions of the medullae. One limitation of the perfusion technique is that the released vesicle constituents (particularly proteins) are subject to diffusion barriers present in the extracellular tissue space before they can be collected and analyzed.

A more direct approach to the study of the mechanism of release of the vesicle contents from the adrenal medulla is afforded by the use of isolated adrenal medullary cells (19, 25, 28). This preparation has the advantage that release of the vesicle contents occurs directly into the extracellular bathing solution, so that components released after stimulation of the cells can be collected immediately and analyzed, and membrane events associated with exocytosis can be observed more directly.

In the present paper, single adrenal medullary cells have been isolated by retrograde perfusion of bovine adrenal medullae with a solution containing collagenase. These cells have been used in functional studies to examine the mechanism of release of the vesicle contents after appropriate pharmacological stimulation. Since most of the earlier biochemical evidence for exocytosis has been obtained from experiments using bovine adrenal glands, isolated cells were prepared from bovine adrenal medullae so as to allow easy comparison of our results with those using perfused glands (35, 36).

MATERIALS AND METHODS

Materials

Collagenase (Type I) was obtained from Sigma Chemical Co. (St. Louis, Mo.). Trypan blue was obtained from Commonwealth Serum Laboratories (Melbourne, Australia) and hyamine hydroxide was obtained from Packard Instrument Co., Inc., Downers Grove, Ill. [14 C]glucose was obtained from International Biophysics, Div. of Berkeley Controls (Irvine, Calif.). Fluores-

camine ("Fluorim") was obtained from Hoffman-La Roche (Basel, Switzerland).

Cell media were made with reagent grade chemicals and sterile distilled deionized water. The cells were prepared using Ca $^{++}$ -free Krebs-Henseleit solution buffered to pH 6.7-6.9 with 95% O $_2$ /5% CO $_2$. This will be referred to throughout this paper as Ca $^{++}$ -free Krebs buffer. Bovine serum albumin (BSA) was dialyzed at a concentration of 10.0 mg/ml against two changes of Ca $^{++}$ -free Krebs buffer for two 8-h periods at 4°C. Where included in cell media, BSA was used at 5.0 mg/ml and Ca $^{++}$ at 2.2 mM, unless otherwise stated. Plastic equipment was used wherever the cells were in contact, except for large glass centrifuged tubes which were siliconized with Siliclad (Clay Adams, Inc., Div. of Becton, Dickinson & Co., Parsippany, N. J.). Nylon mesh (Nytal T5, pore size 250 μ m) used for filtering the cells was obtained from Collie and Sons (Melbourne, Australia).

Methods

PREPARATION OF ISOLATED ADRENAL MEDULLARY CELLS

Bovine adrenal glands were obtained fresh from the local slaughter house where they were excised within 5 min of death and freed from adhering fat. Slits were made in the cortex with a scalpel blade, and the gland was perfused through the central lobular vein with 10 ml of Ca $^{++}$ -free Krebs buffer. The glands were transported back to the laboratory within 20 min, where the cortex was carefully dissected away from the adrenal medullary tissue. Careful dissection of the gland was necessary to ensure that the vascular system of the medulla remained relatively intact. This resulted in a thorough, even perfusion of the tissue, giving a maximal yield of cells.

Isolated adrenal medullary cells were produced essentially by the method described for the isolation of rat liver parenchymal cells by Berry and Friend (4) with the exception that (a) hyaluronidase was not included in the perfusion solution and (b) Ca $^{++}$ -free Krebs buffer was used in preference to Ca $^{++}$ - and Mg $^{++}$ -free Hanks' buffer.

The adrenal vein, which was left intact during dissection, was cannulated to enable the adrenal medulla to be perfused in a retrograde manner for 10 min with 15.0 ml of Ca $^{++}$ -free Krebs buffer. Buffer was recirculated through the organ at 3.0 ml/min, by means of a peristaltic pump. The incubation chamber and circulating buffer solution were maintained at 37°C (Fig. 1).

The initial perfusate containing mainly erythrocytes and plasma was discarded and replaced with 15.0 ml of fresh buffer containing 0.05% collagenase. Perfusion was continued for 30 min by which time the consistency of the remaining medullary tissue was flaccid. The tissue was removed from the perfusion apparatus and minced finely with a pair of sharp scissors. A further 5.0 ml of

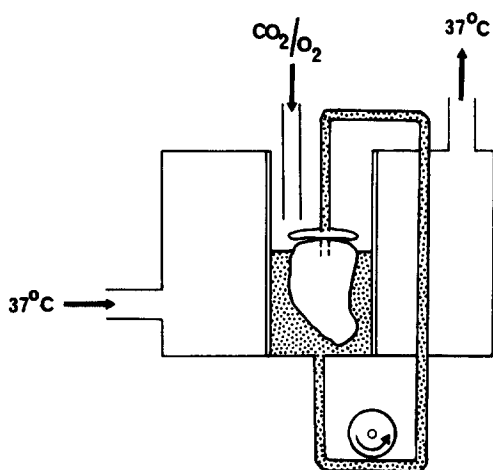


FIGURE 1 Apparatus used for the perfusion of an adrenal medulla with a solution containing collagenase. The enzyme solution is recirculated by means of a peristaltic pump and the incubation chamber is maintained at 37°C by water pumped around the chamber from a heated water bath.

enzyme solution was added, and the chopped medulla, perfusate and enzyme solution were placed in a plastic flask and incubated at 37°C for 15 min with shaking (140 oscillations per min). The flask was flushed with 95% O₂/5% CO₂ throughout the incubation.

After incubation the mixture was drawn up and down several times through a series of three plastic pipettes of decreasing bore diameter (5, 3, and 2 mm) and then filtered twice through nylon mesh to remove undigested material, yielding a final crude suspension of medullary cells, "the dispersion."

This preparation was chilled on ice, then centrifuged at 50 g for 2.0 min in an International PR2 refrigerated centrifuge. (International Equipment Co., Boston, Mass.) The supernate was removed, and the cell pellet was gently suspended in 10.0 ml of cold Ca⁺⁺-free Krebs buffer. Resuspension of cells was done using an automatic pipette with a fine plastic tip. The suspension was centrifuged and resuspended in this manner twice more to give a final preparation of isolated medullary cells (Fig. 2).

Cells were counted in a hemacytometer, and viability was estimated by mixing the cell suspension with an equal vol of 0.4% trypan blue in Ca⁺⁺-free Krebs buffer.

In order to increase the number of cells obtained, a perfusion apparatus was designed and built to allow the simultaneous perfusion of three medullae; thus, in most experiments isolated adrenal medullary cells from three glands were used.

Protein was measured with the fluorescamine assay of Böhlen et al. (6) with BSA as a standard. Samples were precipitated with 0.4 M perchloric acid (PCA) and washed twice with PCA to remove CA's. The resultant supernates were used for the measurements of CA's.

CA's were measured by the fluorimetric trihydroxyindole method of Crout (10). All samples were simultaneously assayed at two pH values to permit estimation of both noradrenaline and adrenaline. Internal standards were routinely included. Average percentage recovery was 96.3% for noradrenaline and 100% for adrenaline, calculated over 13 assays.

DBH activity was assayed by the colorimetric method of Nagatsu and Udenfriend (30). All samples were dialyzed against 0.2 M sodium acetate pH 5.0 overnight before assaying for DBH. Lactate dehydrogenase activity was assayed by the method of Neilands (32).

Adrenal medullae homogenates were prepared by disrupting finely chopped adrenal medullae pieces, in 5 vol of cold 0.3 M sucrose with an Omnimix homogenizer, operated at 1,000 rpm for 30 s (DuPont Instruments, Sorvall Operations, Newtown, Conn.).

ELECTRON MICROSCOPY

The fixation of adrenal medullary cells for electron microscopy was carried out essentially as described by Benedeczy and Smith (2). All steps in the procedure were performed at 0°–4°C. A 1-ml aliquot of the cell suspension was centrifuged in a Beckman microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), and the cell pellet was resuspended in 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 and left for a minimum of 2 h. The fixed cells were pelleted in the microfuge and then washed for 10 min in 0.1 M phosphate buffer pH 7.4 containing 7.5% (wt/vol) sucrose, followed by postfixation for 2 h in 2% (wt/vol) osmium tetroxide in 0.1 M phosphate buffer pH 7.4 containing 7.5% (wt/vol) sucrose.

After postfixation, the cells were washed in 0.1 M phosphate buffer pH 7.4 to remove residual osmium, then stained for 2 h with 1.5% uranyl acetate in 0.5 M maleate buffer pH 5.1.

The cells were dehydrated in a graded series of ethanol, then brought to room temperature, and embedded in Spurr's (42) epoxy resin. The blocks were sectioned on an LKB Ultratome (LKB Instruments, Inc., Rockville, Md.) and silver-gold sections were collected on 100-mesh copper grids. The sections were stained with uranyl acetate and alkaline lead citrate and examined in a Hitachi HU-IIA electron microscope.

RESPIRATORY ACTIVITY OF ISOLATED CELLS

The respiratory activities of the initial dispersion and the final purified cell fraction were determined by measuring the rate of conversion of uniformly labeled [¹⁴C]glucose to labeled CO₂. The incubations were performed in siliconized 25-ml Erlenmeyer flasks which contained a center well at the base. Into the outer compartment of each flask was placed a 1-ml aliquot of the cell suspension (containing approx. 1 × 10⁶ cells), to which was added 10 μl of [¹⁴C]glucose (50 μCi/ml) and 10 μl of [¹²C]glucose (600 mM). The flasks were

flushed with 95% O₂ and 5% CO₂, sealed with rubber stoppers, and incubated with shaking (100 oscillations per min) at 37°C. The incubation was stopped after 0, 30, 60, 90, and 120 min by placing the flasks on ice and injecting 0.35 ml of hyamine hydroxide into the center-well followed by 0.2 ml of 20% H₂SO₄ into the outer compartment of each flask.

The flasks were transferred to a water bath and incubated at 30°C with shaking (80 oscillations/min) for 30 min to allow hyamine hydroxide to trap CO₂ evolved from the disrupted cells. The flasks were then returned to ice, and the contents of the center wells were quantitatively transferred to counting vials containing 10 ml of toluene scintillation fluid. The level of radioactivity was determined with a Philips liquid scintillation counter (Philips Electronic Instruments, Inc., Mount Vernon, N. Y.).

COMPARISON OF CELL MEDIA

The effects of the inclusion of BSA and Ca⁺⁺ in the media used for cell purification were examined. The initial dispersion from one preparation was divided into four aliquots and centrifuged. Each of the four cell pellets was resuspended in a separate medium being (a) Ca⁺⁺-free Krebs buffer containing both Ca⁺⁺ (2.2 mM) and BSA (5.0 mg/ml), (b) buffer containing BSA only, (c) buffer containing Ca⁺⁺ only, and (d) buffer only. The cell suspensions were then purified as described and resuspended after each centrifugation in 2.5 ml of their respective media. All operations were performed at 4°C. After purification, a sample of each cell preparation was taken for cell counts and catecholamine (CA) assay.

EFFECTS OF PREINCUBATION

During the isolation procedure, the cells experienced an extracellular Ca⁺⁺ depletion for approx. 2 h. Preliminary studies showed that these cells were relatively unresponsive to acetylcholine (ACh). In order to check for recovery of ACh sensitivity, an investigation was made into the effects of preincubation of the isolated cells in the presence of Ca⁺⁺.

The dispersion was divided into two fractions and purified in Krebs buffer containing BSA and Ca⁺⁺ or BSA only. Half of each cell suspension was kept on ice for 2 h while the other half was incubated at 37°C for the same period. All fractions were chilled and then incubated at 37°C for 10 min either in the presence or absence of ACh. After incubation, the cell suspensions were chilled on ice and the supernates were removed after centrifugation for CA determination.

FUNCTIONAL STUDIES

The characteristics of CA secretion by the prepared cells were investigated by incubating cells in the presence of their physiological stimulus, ACh, and measuring the CA released into the suspending medium. All cells were purified in medium containing BSA and Ca⁺⁺, incubated at 37°C for 2 h, and kept on ice before use. All cells

were allowed to equilibrate to 37°C for 10 min and aliquots of 1 ml containing 1–3 × 10⁶ cells were used for incubations. After incubation, the cells were chilled on ice and centrifuged at 50 g for 2 min at 4°C. The catecholamine content of the supernates was determined as described above.

TIME-COURSE: The time-course of ACh-stimulated CA secretion was examined by measuring the CA and DBH released by the isolated chromaffin cells, in the presence of ACh (10⁻⁴ M) and Ca⁺⁺ (2.2 mM). After temperature equilibration, the cells were incubated at 37°C for periods of 1, 3, 5, and 10, and 15 min. A series of control incubations were performed in the absence of secretagogue.

DOSE RESPONSE: Cells were incubated at 37°C for 5 min, with varying concentrations of ACh (0, 10⁻⁷, 10⁻⁶, 10⁻⁵, 2.5 × 10⁻⁵, 5.0 × 10⁻⁵, and 7.5 × 10⁻⁵ M) at a fixed Ca⁺⁺ concentration (2.2 mM).

CALCIUM DEPENDENCE: The dependence of ACh-stimulated CA secretion on extracellular Ca⁺⁺ was examined by incubating the cells with ACh (5 × 10⁻⁵ M) in varying concentrations of Ca⁺⁺ (0, 1, 2, 3, and 4 mM) at 37°C for 5 min.

PHARMACOLOGICAL INHIBITION: The pharmacological responsiveness of the isolated adrenal cells was assessed by incubating the cells in the presence of ACh (5 × 10⁻⁵ M) together with (a) atropine (b) hexamethonium, or with (c) both atropine and hexamethonium, at concentrations of 5 × 10⁻⁵ M.

RESULTS

Cell Isolation and Viability

The isolation technique described above, yielded approx. 5 × 10⁶ purified cells per gram of perfused medulla. Medullae were perfused simultaneously, and approx. 24 × 10⁶ cells were isolated in each preparation.

Examination of the dispersion under the light microscope (Fig. 2a and b) showed that this fraction contained isolated medullary cells of about 20 μm diameter, a large number of erythrocytes, and some other small cells (approx. 7 μm diameter). The number of medullary cells present in the dispersion was in the order of 12 × 10⁶ cells/g of perfused medulla.

The purified cell preparation under the light microscope (Fig. 2c and d) was seen to contain a homogeneous population of single cells, with very little contamination. The cells appeared intact, spherical, and under phase-contrast microscopy, lightly granular with a visible nucleus. Greater than 97% of the cells appeared to be viable by exclusion of trypan blue. Between 30% and 60% of the cells in the dispersion were recovered in the purified cell fraction, with a mean of 40% calculated over 27 preparations.

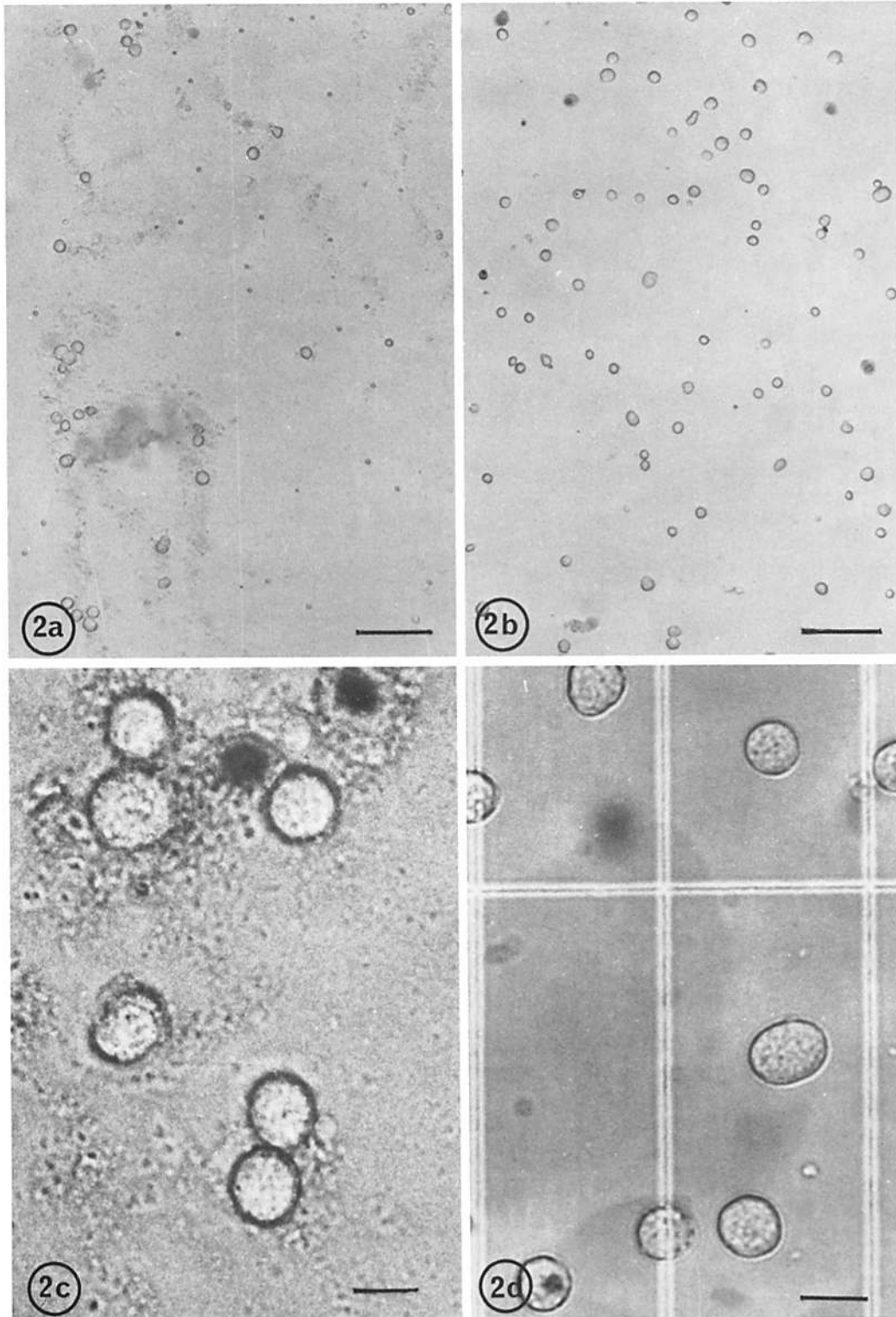


FIGURE 2 Light micrograph of isolated adrenal medullary cells prepared by the retrograde perfusion technique and mixed with trypan blue. (a) The initial cell dispersion. Note the presence of many "small" cells in addition to the large adrenal chromaffin cells. Some undigested cellular material is also present. $\times 110$. (b) The purified cell fraction which consists principally of adrenal chromaffin cells, very few small cells or cellular debris are present. $\times 110$. (a and b) Bars, $100\ \mu\text{m}$. (c) Higher power light micrograph of the adrenal medullary cells in the dispersion, and (d) in the purified cell fractions. (c and d) $\times 450$. Bars, $20\ \mu\text{m}$.

Cellular Respiratory Activity

The rate of respiration of the isolated medullary cells is shown in Fig. 3. The respiration rate for cells in the dispersion, and for cells in the purified fraction suspended in either the presence or absence of BSA was linear for 120 min, the longest period for which cells were incubated. Cells in the purified fraction respired at approximately the same rate as those in the dispersion, and this rate was only marginally altered by the inclusion of BSA into the suspending medium.

Biochemical Analysis of Isolated Cell Fractions

Assays performed on the chromaffin cells iso-

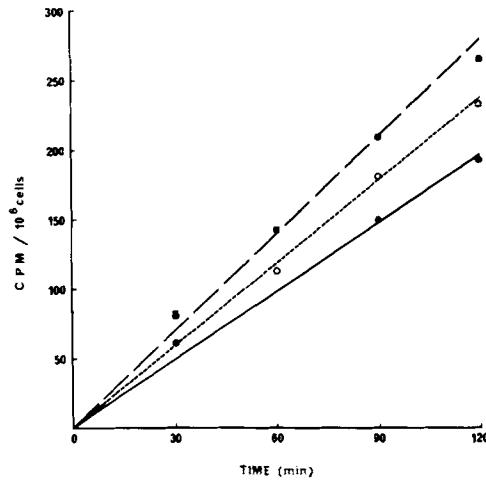


FIGURE 3 Cellular respiration. Conversion of [¹⁴C]glucose to ¹⁴CO₂ by: (■) cells in the dispersion; (O) cells purified in the absence of BSA; and (●) cells purified in the presence of BSA.

lated in the presence of Ca⁺⁺-free Krebs buffer (Table I), show that the cytoplasmic enzyme marker, lactate dehydrogenase, is enriched in the purified cell fraction compared with the dispersion. The chromaffin vesicle marker, DBH, however, is enriched only slightly while CA's are less concentrated in the purified cell fraction. This partial depletion of CA's, and to a lesser extent DBH, from the cells during isolation is also reflected in the lower values for percentage recovery of CA (10%) and DBH (14%) when compared to that of lactate dehydrogenase (20%) and intact cells (24%).

The above assays were also performed on a homogenate of adrenal medullae. The specific activities of CA's and lactate dehydrogenase are decreased in the dispersion with respect to the adrenal medullae homogenate, whereas the specific activity of DBH is greater in the dispersion.

CA Assay of Cell Isolation Fractions

CA assays performed on homogenates of fresh adrenal medullae showed that the total CA content of the whole medulla was 9.3 mg CA/g tissue, with adrenaline and noradrenaline present in the ratio 1.9:1. The initial dispersion was found to contain 86% of the CA content of fresh tissue i.e., 8.0 mg CA/g perfused medulla, with adrenaline and noradrenaline present in the same relative amounts as in whole tissue, i.e., 1.9:1. This ratio was, however, decreased to 1.6:1 in the purified cell fraction indicating a selective loss of adrenaline from the cells during isolation in Ca⁺⁺-free buffer. The purified cell fraction contained mean CA content of 18.6 ± 8.2 pg CA/cell (0.057 ± 0.025 pmol/cell) calculated over 14 preparations.

TABLE I

Biochemical Analysis of Homogenates and Isolated Cell Fractions Obtained from Bovine Adrenal Medullae

	Cell count	Protein mg	Catecholamine		Dopamine-β-hydroxylase		Lactate Dehydrogenase	
			Total amount μmol	Concn nmol/mg protein	Total activity μmol octopamine formed/h	Sp act μmol octopamine formed/h/mg protein	Total activity μmol NAD reduced/min	Sp act nmol NAD reduced/min/mg protein
Homogenate adrenal medulla	—	40.3 ± 4.2	34.2 ± 3.3	852 ± 12	54.3 ± 2.2	1.38 ± 0.21	6.5 ± 0.4	168 ± 21
Initial dispersion	24 × 10 ⁶	50.0	22.5	450	112.7	2.25	1.11	22.2
Purified cell fraction	6 × 10 ⁶	6.5	2.34	360	16.1	2.47	0.22	34.0
Recovery in purified cell fraction (%)	24.0	13.0	10.4		14.3		19.8	

Figures for the homogenate represent the average content per adrenal medulla as determined by analysis of three separate homogenates of 20 medullae each. The results for the initial dispersion and the purified cell fraction are from a typical cell isolation experiment.

Ultrastructure

UNSTIMULATED CELLS

Electron microscopy of the final cell fraction showed that it was composed of intact cells that contained large numbers of vesicles with electron-dense contents (diameter 130–260 nm) within their cytoplasm. The cells frequently exhibited polarity with the vesicles distributed toward one end of the cells and the nucleus and other cell organelles located toward the other end.

Two cell types were evident: (a) those containing vesicles with electron-dense granular contents that showed some degree of contraction from the vesicle membrane giving the cell a vacuolated appearance (Fig. 4) and (b) those containing somewhat less electron-dense granules surrounded closely by the vesicle membrane (Fig. 5). The vesicles of cell type (a) contained round, dense granules often showing irregularities in density giving a "mottled" or "target" appearance. The vesicles of cell type (b) on the other hand were usually larger, elongated, and more irregular in shape with the granules showing a more uniform density. The dense, contracted appearance of the granules of cell type (a) is thought to reflect the intensity of the condensation reaction between the CA and glutaraldehyde and the greater osmiophilic nature of noradrenaline (9); thus, the cells of type (a) probably contain noradrenaline while those of type (b) probably contain adrenaline.

The plasma membranes of the isolated cells were intact and in some places gave rise to invaginations and in others to pseudopodia (Figs. 4, 5, and 6). The nuclei were often irregular in shape showing deep invaginations and were (apart from the margin) typically poor in chromatin. Elongated mitochondria with lamellar cristae were frequently found in the apical perinuclear region along with the Golgi apparatus and short tubules of rough and smooth endoplasmic reticulum. Pockets of free ribosomes were numerous throughout the cytoplasm, but lysosomes were rarely observed as is the case in intact tissue (2).

In some places, the plasma membrane was directly exposed to the external environment (Fig. 6a), but at others a thin layer of basement membrane could be observed (Fig. 6b and c) still attached to the plasma membrane. In a few cells, evidence of tight contacts having existed between two cells was given by the close apposition of plasma membrane between two cells (Fig. 7b)

facilitated by finger like protrusions from one cell that partially enveloped an area of cytoplasm of the other (Fig. 7a).

Cilia were occasionally observed (Fig. 7c) projecting from the adrenal medullary cells into the extracellular space. The base of the cilium was located well within the cytoplasm of the cell while the membrane surrounding the cilium was continuous with the plasma membrane. Similar observations have been made in electron micrographs of whole adrenal medullary tissue from adult hamster (1, 11), rat (7), mouse (20), prenatal rat (33), and chicken (20). The function of this structure in the adrenal medulla is not known.

STIMULATED CELLS

Cells stimulated with ACh (10^{-4} M) for 10 min in the presence of Ca^{++} (2.2 mM) showed signs of secretory activity. This was evidenced by the close apposition of chromaffin vesicles to the plasma membrane (Fig. 8), the fusion of vesicles located near the plasma membrane into larger membranous profiles containing electron-dense chromaffin material (Fig. 8b), the occasional fusion of vesicle and plasma membranes to give an exocytotic profile (Fig. 8b, d, and 9a), and multiple infoldings or depressions in the plasma membranes (Fig. 8c; Fig. 9a and b). Other evidence of secretory activity in these cells included the increased numbers of agranular vesicles of the size of chromaffin vesicles adjacent to the plasma membrane (Fig. 9a) which in some cases appear to have fused together during release of their contents (Figs. 8b and 9b), and the presence of large areas of small membranous vesicles or tubules (Fig. 8a and b).

Comparison of Cell Media

The CA content of medullary cells purified and suspended in different media is shown in Fig. 10. The cells suspended in Krebs buffer containing BSA and Ca^{++} have between two and three times the total CA content of cells suspended in buffer lacking either Ca^{++} , BSA, or both. Thus, it appears that the omission of these constituents from the cell medium resulted in a cellular depletion of CA's, particularly noradrenaline.

CA Secretion

The effect of preincubation of the isolated chromaffin cells in the presence of Ca^{++} , before ACh stimulation, is seen by the comparison of Fig. 11a and b.

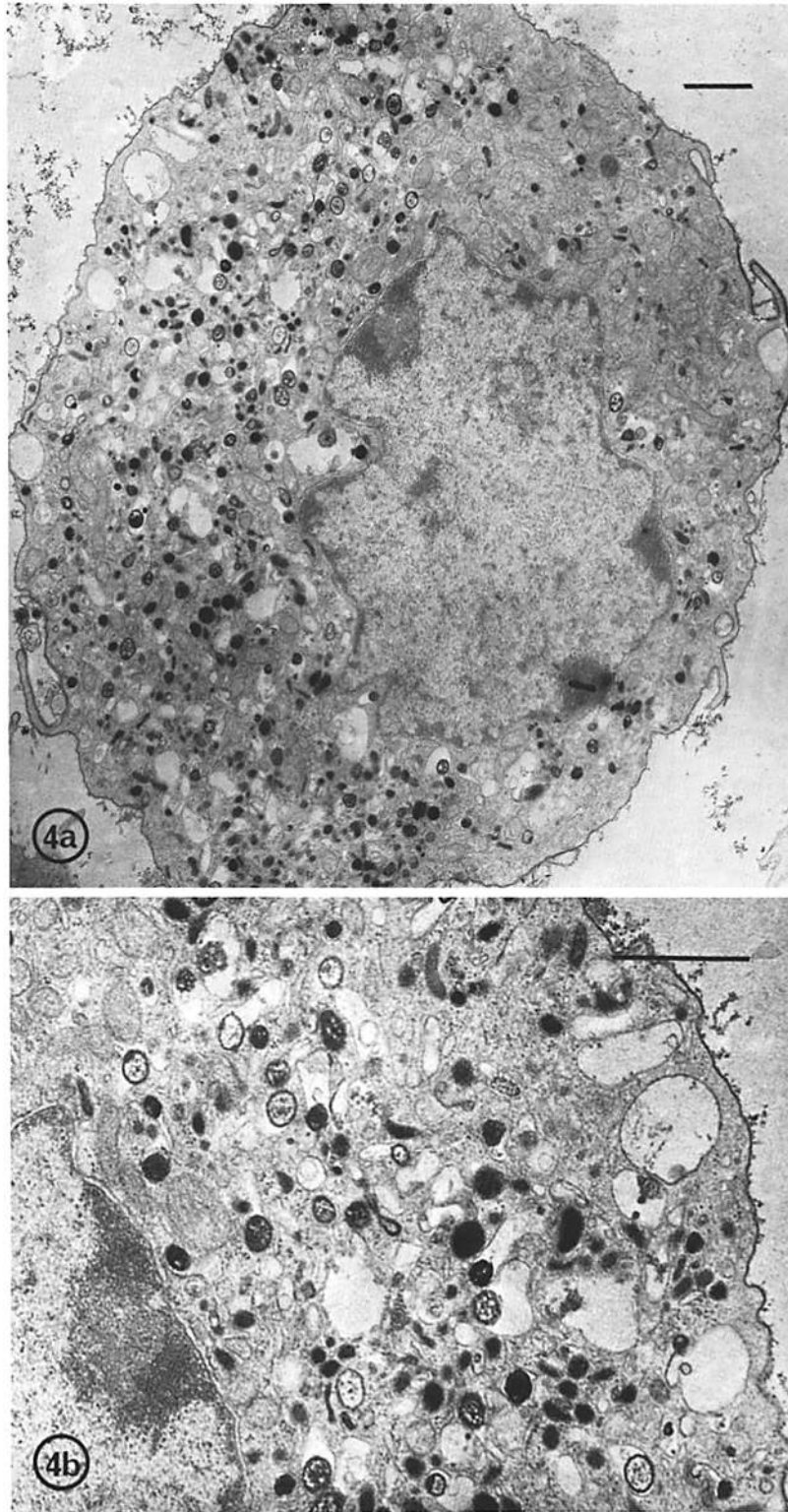


FIGURE 4 Isolated adrenal medullary cell containing electron-dense (noradrenaline) vesicles. (a) \times 9,000. (b) \times 19,000. Bar, 1 μ m.

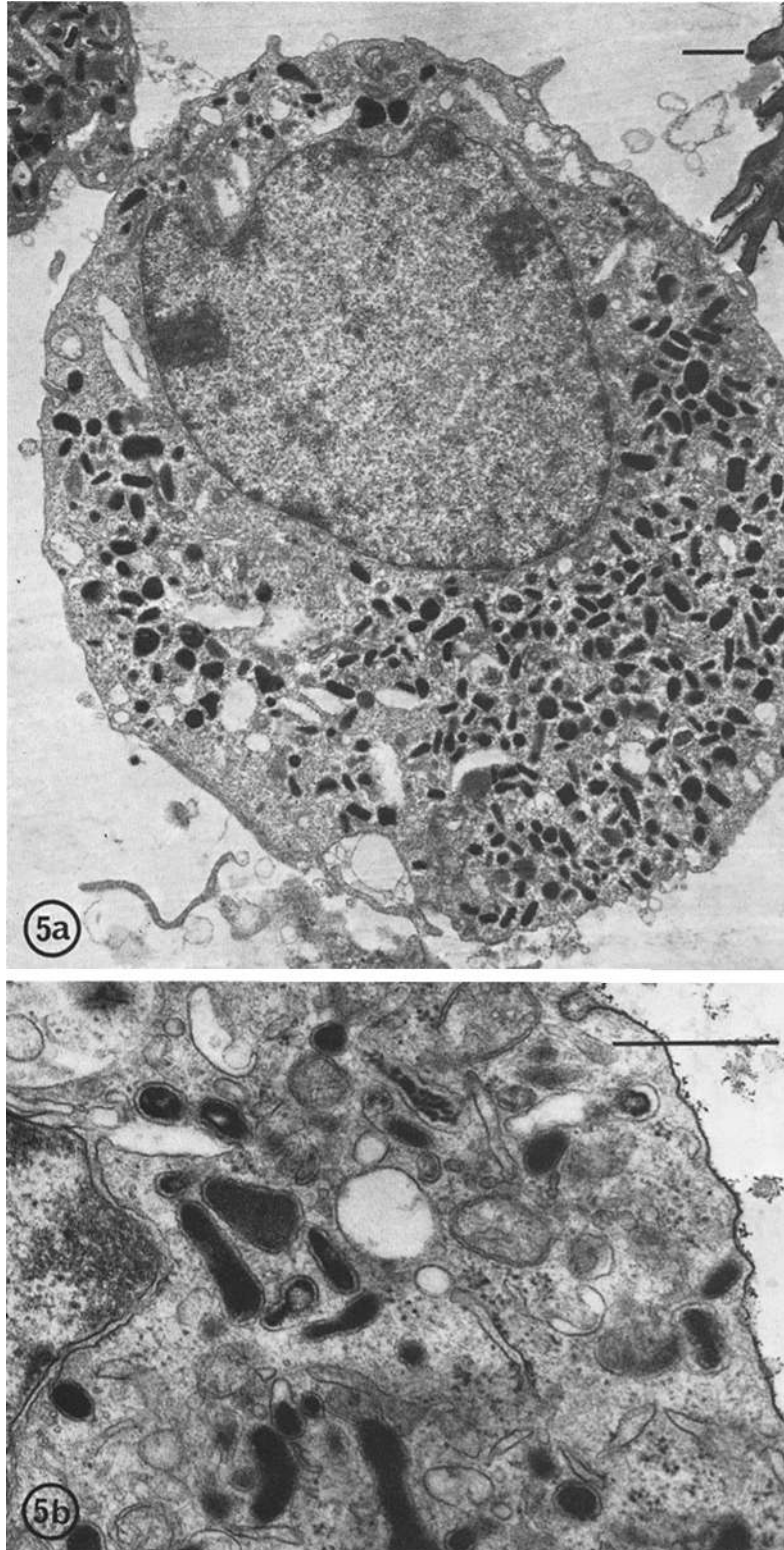


FIGURE 5 Isolated adrenal medullary cell containing moderately electron-dense (adrenaline) vesicles
(a) $\times 8,000$. Bar, $1 \mu\text{m}$. (b) $\times 43,000$. Bar, $0.5 \mu\text{m}$.

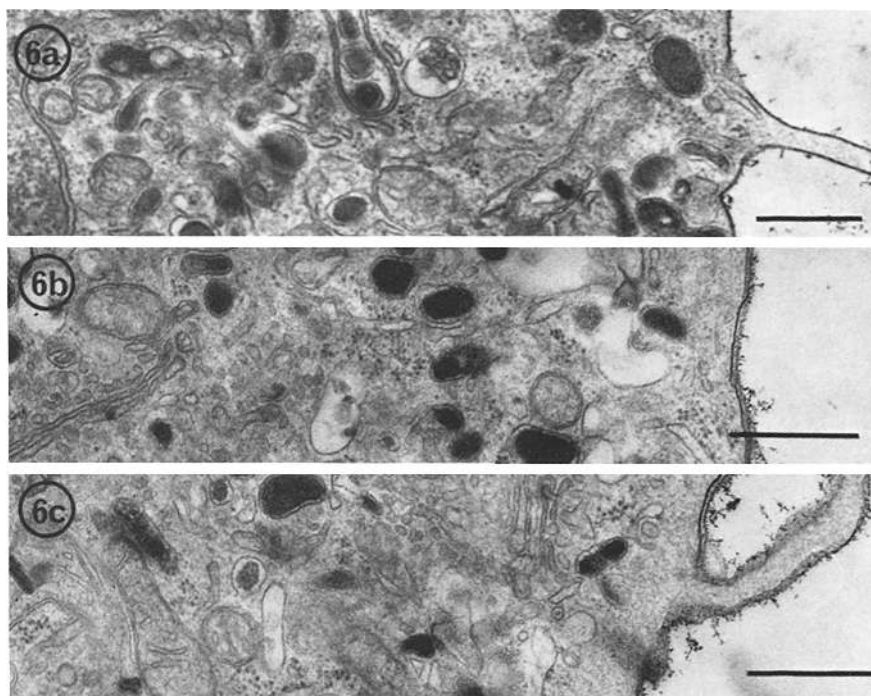


FIGURE 6 The plasma membranes of the isolated cells often form pseudopodia and in some places are covered with a basement membrane. (a) $\times 29,000$. (b and c) $\times 34,000$. Bar, $0.5 \mu\text{m}$.

Cells which had been kept at 0° – 4°C after isolation (Fig. 11a) did not release CA in response to ACh; however, cells which had been preincubated at 37°C (Fig. 11b) did release CA's in the presence of ACh, and this secretory response was dependent on the presence of extracellular Ca^{++} . ACh induced a two- to three-fold increase in the CA's secreted over that released in the absence of secretagogue, and this increase represented 5–10% of the total cellular CA content of the cells.

In view of the above results, all cells which were used for subsequent release studies were prepared in the presence of Ca^{++} and BSA and then preincubated at 37°C . Cells prepared this way released both CA's and DBH in response to ACh (Fig. 12). These two vesicles components were released in parallel with each other over a period of 15 min (Fig. 12) with a coefficient of correlation of 0.992 (Fig. 13).

The ACh-stimulated release of CA's was dependent on the extracellular concentration of the secretagogue (Fig. 14) and was shown to increase with increasing concentrations of ACh. Maximal stimulation was achieved at a ACh concentration

of $7.5 \times 10^{-5} \text{ M}$ with no additional CA release occurring at higher concentrations. Half maximal release was obtained at 10^{-5} M and CA release could be detected at ACh concentrations as low as 10^{-7} M .

CA release from the isolated chromaffin cells was also dependent upon the extracellular Ca^{++} concentration (Fig. 15). Secretion increased with increasing Ca^{++} concentration, and was 90% of maximum at a concentration of 2.0 mM, levelling off to maximal secretion at 4.0 mM.

The pharmacological blockers, atropine, and hexamethonium, were effective in blocking the ACh-evoked release of CA's from the cells (Table II). Separately, the blocking agents inhibited secretion by approx. 60%, but when present together, the action of ACh was inhibited by 84%.

DISCUSSION

Early attempts to isolate single cells from tissues by mechanical means (5) have been superseded by the use of enzymatic methods. To date, there have been no published attempts to isolate single cells from the adrenal medulla, but there have been several reports of the isolation of cells from

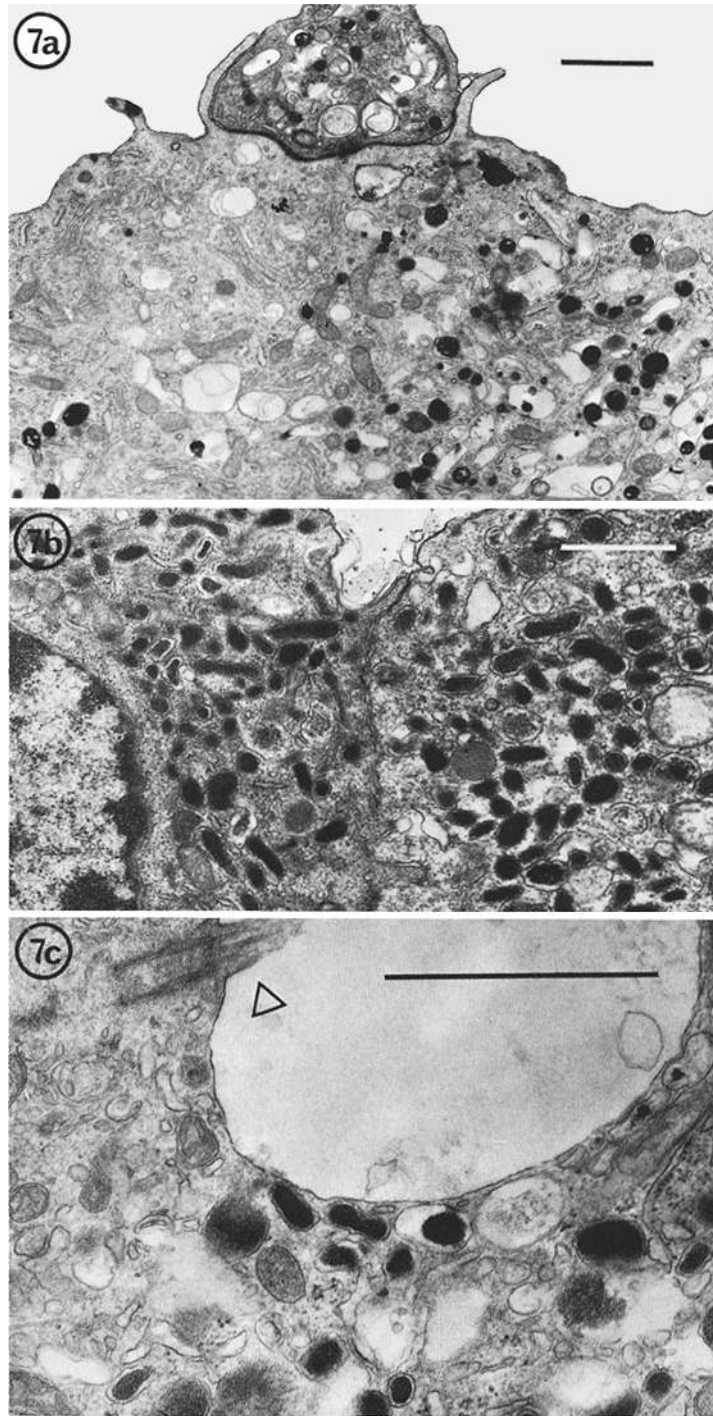


FIGURE 7 Isolated adrenal medullary cells showing: (*a* and *b*) tight contacts and (*c*) cilia (arrow). (*a*) $\times 12,000$. (*b*) $\times 15,000$. (*c*) $\times 36,000$. Bar, $1.0 \mu\text{m}$.

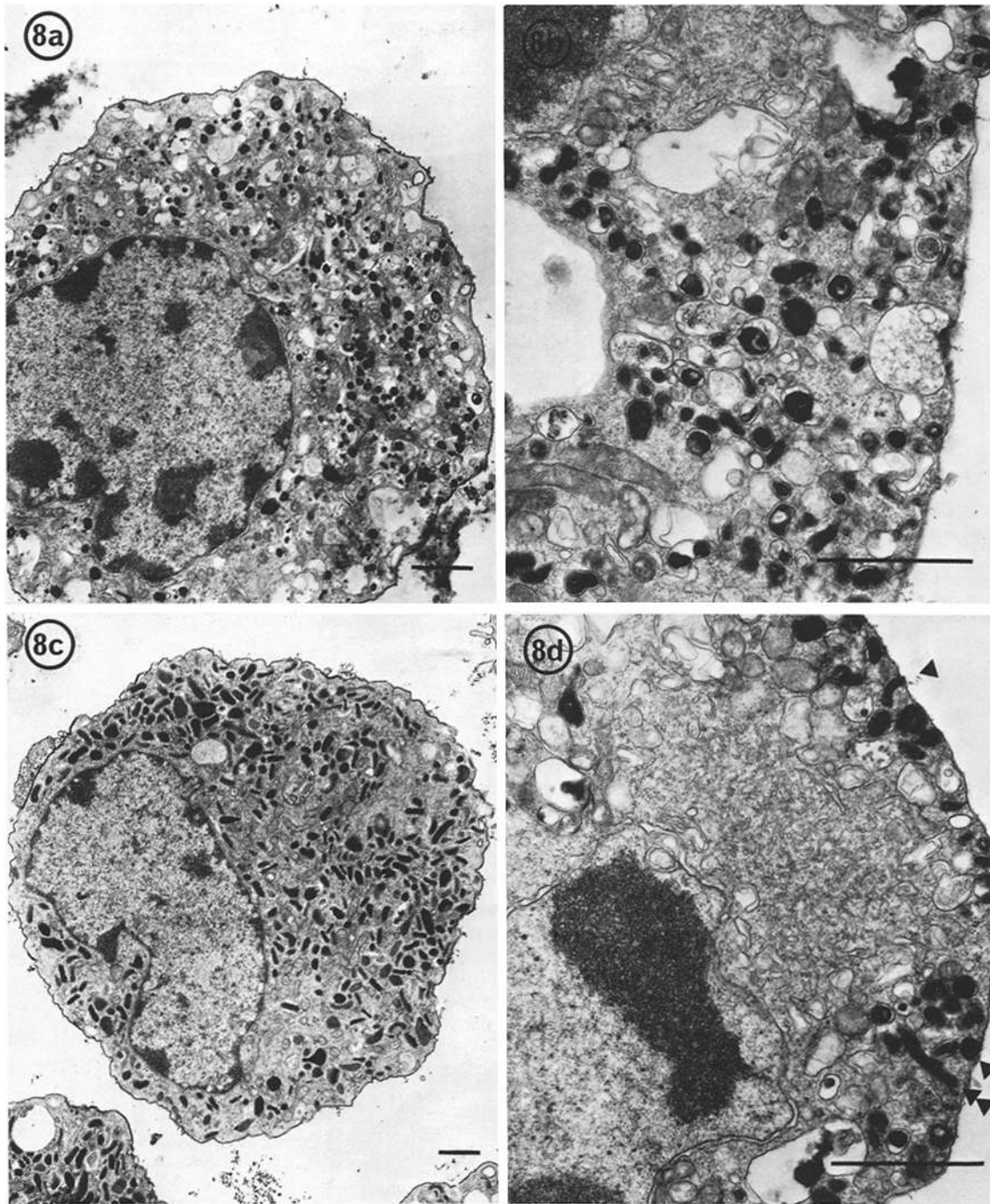


FIGURE 8 Isolated cells which have been stimulated with ACh. CA-containing vesicles can be seen fused to the plasma membrane (arrow) and occasionally appear fused together (double arrow). (a) $\times 9,000$. (b and d) $\times 23,000$. (c) $\times 6,000$. Bars, $1 \mu\text{m}$.

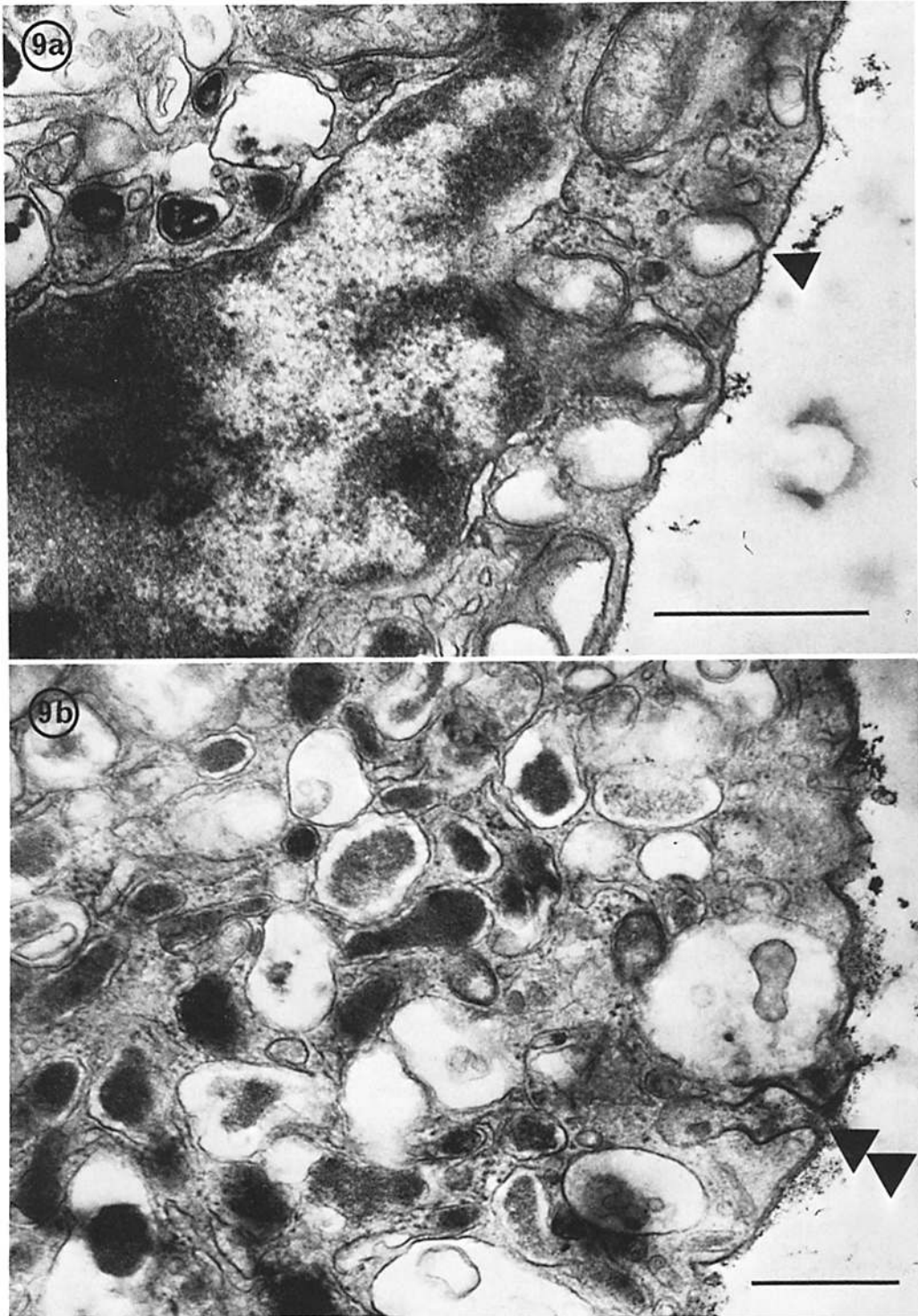


FIGURE 9 Empty membrane profiles are observed in the cytoplasm of the isolated cells after stimulation with ACh. Some of these appear continuous with the plasma membrane (arrow), while others appear fused together (double arrow). (a) $\times 65,000$. (b) $\times 45,000$. Bars, $0.5 \mu\text{m}$.

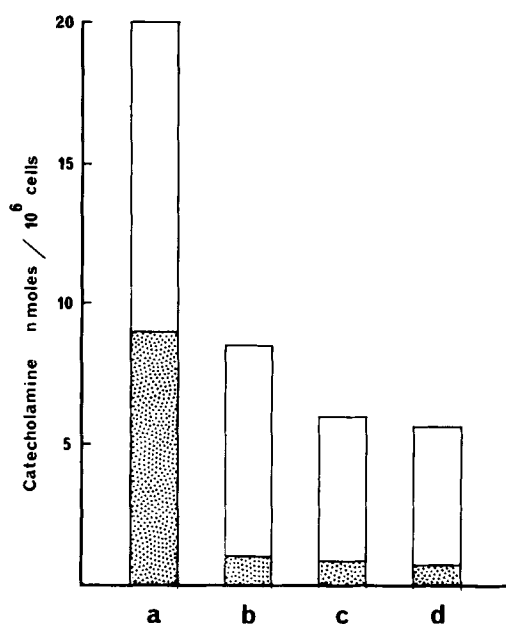


FIGURE 10 Comparison of cell media. CA content of isolated adrenal medullary cells purified in Krebs buffer containing: (a) BSA and Ca^{++} ; (b) BSA only; (c) Ca^{++} only; and (d) neither BSA nor Ca^{++} . The cross-hatched area represents the contribution of non-adrenaline to total cellular CA.

the whole adrenal gland. In these studies, cells were obtained by incubating pieces of adrenal gland with trypsin (31, 43), collagenase (25, 27, 34), collagenase with DNase (18, 24), and a combination of five enzymes (23).

By adapting the enzymatic perfusion method of Berry and Friend (4), we were able to isolate adrenal medullary cells for use in studies on the mechanism of CA release. The viability of the cells isolated by the present method is indicated by the absence of trypan blue staining and the ability of the cells both in the dispersion and purified cell fraction to respire linearly for a minimum of 2 h. The respiratory rate of the adrenal medullary cells in the dispersion and final cell preparation is approximately the same, allowing for the fact that the slightly higher rate of respiration of cells in the dispersion is probably due to the presence in this fraction of cell types other than chromaffin cells.

The cells, prepared in Ca^{++} -free Krebs buffer, are partially depleted of their CA and DBH content, as shown by analysis of these components in the dispersion and purified cell fractions (Table I). However, it was found that by (a) purifying

the cells in the dispersion in the presence of Ca^{++} and BSA and by (b) incubating the cells, after isolation, for 2 h at 37°C in the presence of Ca^{++} and BSA, the CA content of the cells was greatly increased (Fig. 10) and the cells could respond (by secretion of CA's) to the addition of ACh (Fig. 11). Thus, the period during which the cells were exposed to a calcium-deficient medium was minimized, and the cells had been equilibrated with Ca^{++} , in the presence of Mg^{++} , before subsequent release studies were performed. This pre-equilibration of the cells with Ca^{++} was necessary before CA release experiments could be performed, since Ca^{++} is required for the maintenance of the impermeable state of the plasma membrane and since addition of Ca^{++} to adrenal

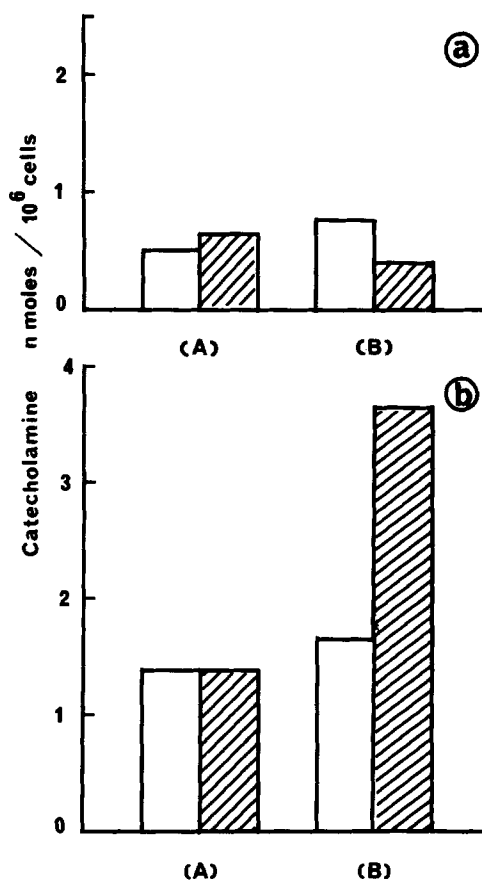


FIGURE 11 Effect of preincubation. Comparison of CA secretion by isolated chromaffin cells preincubated for 2 h at: (a) $0^\circ\text{-}4^\circ\text{C}$ and (b) 37°C . (A) Cells incubated in the absence of ACh for 10 min. (B) Cells incubated in the presence of ACh (10^{-4}M) for 10 min. The cross-hatched areas represent the CA secreted in the presence of Ca^{++} .

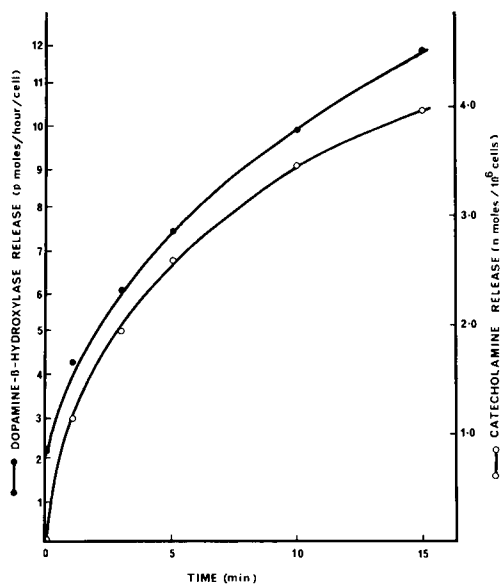


FIGURE 12 Time-course of CA and D β H secretion by isolated adrenal medullary cells. Cells were incubated at 37°C in the presence of ACh (10^{-4} M) and Ca^{++} for the times indicated. CA and D β H release in the absence of ACh was constant over the 15-min period and represented less than 13% of the stimulated release of any time over the period studied. The above values represent release in the presence of ACh, after subtraction of the control values in the absence of ACh.

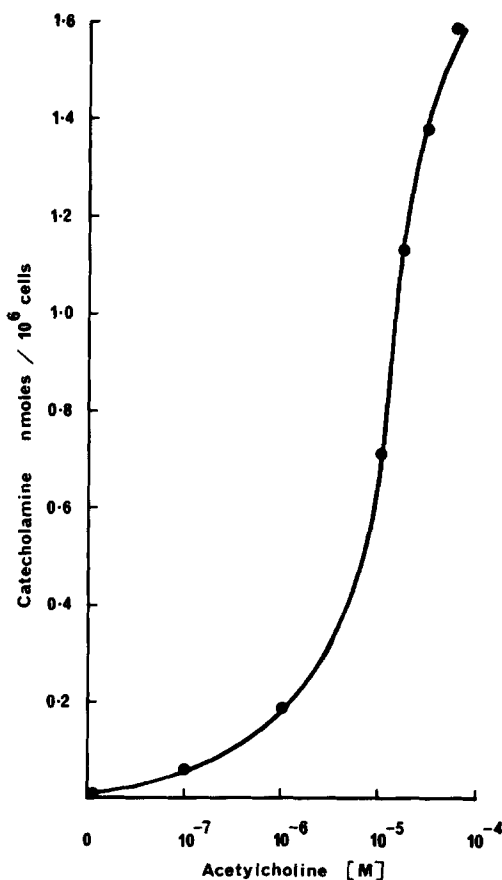


FIGURE 14 CA secretion by isolated adrenal cells in response to varying ACh concentrations. Cells were incubated in the presence of Ca^{++} (2.2 mM).

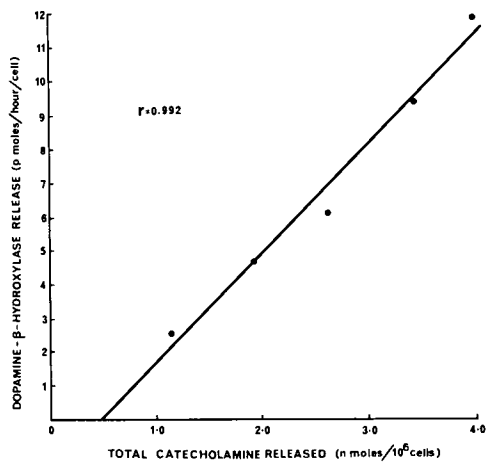


FIGURE 13 Correlation between the amounts of D β H and CA released from isolated chromaffin cells upon stimulation in vitro with ACh (10^{-4} M). The correlation coefficient $r = 0.992$; bandwidth of $r = 0.008$; and number of pairs $n = 5$.

glands, previously exposed to a Ca^{++} -deficient medium, results in a spontaneous release of CA's (17).

The integrity of these cells has been confirmed at the ultrastructural level. The cell membranes are intact, and numerous CA-containing chromaffin vesicles can be seen in the cytoplasm. The two types of chromaffin vesicles observed within the adrenal medullary cells are thought to represent the noradrenaline-containing vesicles (electron-dense) and the adrenaline-containing vesicles (moderately electron-dense) (21). The observed predominance of adrenaline-containing cells in the isolated cell preparation is consistent with the measured ratio of adrenaline:noradrenaline in the purified cell fraction, i.e., 1.6:1. The size range of the vesicles (130–260 nm) seen in the isolated cells is in close agreement with that reported for

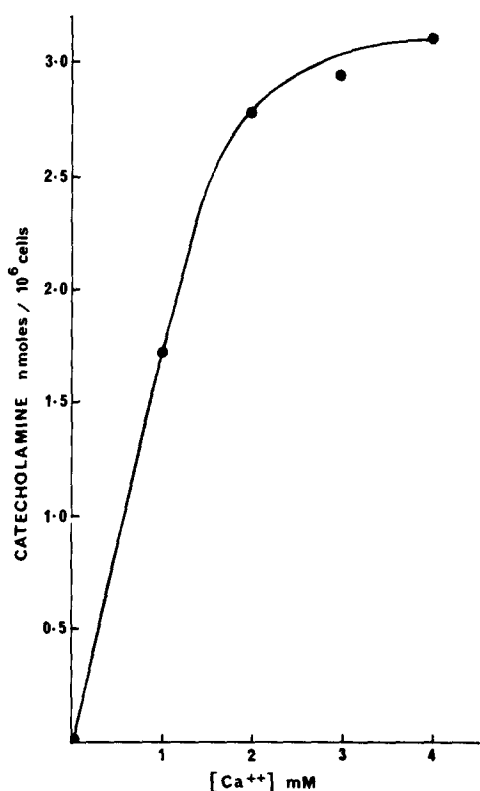


FIGURE 15 Dependence of catecholamine secretion on extracellular Ca^{++} concentration in the presence of 5×10^{-5} ACh.

TABLE II
Catecholamine Secretion in the Presence of
Cholinergic Blocking Agents

Additions	Catecholamine Secretion	
	nmol/10 ⁶ cells	Inhibition %
Acetylcholine (5×10^{-5} M)	1.32	
Acetylcholine + Atropine (5×10^{-5} M)	0.55	58
Acetylcholine + Hexamethonium (5×10^{-5} M)	0.58	56
Acetylcholine + Atropine (5×10^{-5} M) + Hexamethonium (5×10^{-5} M)	0.21	84

chromaffin vesicles in sections of bovine adrenal medullary tissue (8). Some of the vesicles, especially the noradrenaline-containing vesicles, are depleted of part of their electron-dense contents, which is consistent with biochemical assays indicating some loss of cellular CA during the isolation procedure.

Isolated adrenal medullary cells were obtained previously by Hochman and Perlman (25) after incubating sections of whole guinea-pig adrenal gland with collagenase. They reported a yield of 20×10^6 cells/g whole adrenal gland; however, their method of isolation required the incubation of adrenal sections in higher concentrations of collagenase (0.05–0.2%) and for extended periods (up to 2.5 h). The range in CA content of these cells was 10–105 nmol/10⁶ cells with a mean of 36 nmol of CA/10⁶ cells. By comparison, the cells isolated here from bovine adrenal medullae contain 57 ± 25 nmol of CA's/10⁶ cells.

Catecholamine Secretion

The mechanism by which the isolated adrenal medullary cells secrete CA's is similar in many ways to the release of CA's by the intact gland: (a) Release is stimulated by low levels of ACh and increases with the concentration of the secretagogue (Fig. 14); (b) it is dependent on the presence of Ca^{++} and increases linearly with increasing concentration of Ca^{++} , up to approx. 2 mM (Fig. 15); (c) it can be inhibited by atropine and hexamethonium (Table II); and (d) there is no detectable release of the cytoplasmic enzyme lactate dehydrogenase (our unpublished observations).

The cells are extremely sensitive to stimulation by extracellular ACh in the presence of Ca^{++} . We were able to detect CA secretion (above control values) at levels of ACh as low as 10^{-7} M, with a maximal response at 7.5×10^{-5} M. By comparison, the guinea pig adrenal cells prepared by Hochman and Perlman (25) required ACh levels above 10^{-6} M before a response was detectable and maximal secretion was observed at 10^{-3} M. The response of chromaffin cells from both sources to varying concentrations of Ca^{++} is similar in that the response begins to plateau at 2.0 mM Ca^{++} . The levels of both ACh and Ca^{++} which are required for a near maximal response by the isolated cells are in close agreement with those used in perfused adrenal gland experiments (for review, see reference 44). The increments in CA release from the cells in response to ACh (Figs. 11 and 12) are, however, less than those observed for intact glands (36), which may reflect some damage to the cells during their preparation.

The ACh-stimulated release of CA's from the isolated cells is paralleled by the release of DBH over a time period of 15 min (Figs. 12 and 13),

and the CA:DBH ratio of these two components (2.9 nmol CA:μmol octopamine formed/h) closely approximates the ratio in a soluble lysate of chromaffin vesicles (2.7 nmol CA:μmol octopamine formed/h) which was prepared from a homogenate of adrenal medulla by the method of Smith and Winkler (40). The value for the coefficient of correlation ($r = 0.99$) compares well with that obtained for the content of CA and DBH in purified storage vesicles ($r = 0.89$) (37). The concomitant release of CA's, DBH, and other soluble vesicle components, in the same relative amounts as those present in a chromaffin vesicle fraction, has also been observed in the perfused adrenal gland (for review, see reference 26). These results are consistent with the hypothesis that secretion occurs by the release of the entire soluble vesicle contents directly into the extracellular space.

In support of the biochemical results showing release of CA's by ACh in the presence of Ca^{++} , ultrastructural changes which were indicative of secretory activity were observed in the isolated cells. The most striking observation in electron micrographs of stimulated cells was the presence of numerous vesicles which were fused with the plasma membrane of the stimulated cells. Similar exocytotic profiles have been observed in sections of intact adrenal medulla from hamster (3, 13, 15, 22, 38), cat (39), rat (7), and rabbit (12), and an increase in the number of these profiles after stimulation has also been noted after treatment of cat adrenal medulla with insulin (39). These authors also observed the appearance of many empty membrane sacs similar to those which appeared after stimulation of the isolated adrenal medullary cells with ACh. It is thought that these represent the membranes of vesicles which have released their soluble contents, and may correspond to a fraction containing empty membrane structures isolated from stimulated adrenal glands (29).

Partial inhibition of ACh-stimulated secretion by the isolated cells occurs on the addition of either atropine or hexamethonium, but for near-complete inhibition, both cholinergic blocking agents are required together (Table II). Hochman and Perlman (25) obtained similar levels of inhibition of total catecholamine release with atropine (10^{-4}) and hexamethonium (10^{-4} M) on isolated adrenal medullary cells. Although these authors did not report separate values for adrenaline and noradrenaline release, we observed no change in the ratio of release of the two catecholamines

from the isolated bovine chromaffin cells during incubation with the blockers. Thus, it appears from these results that release of adrenaline and noradrenaline is mediated by both nicotinic and muscarinic receptors. However, atropine, a "muscarinic antagonist," has also been shown to react with nicotinic receptors (45). This result (together with the observation that muscarinic agonist, pilocarpine, did not stimulate release in the concentration range of 10^{-5} to 3×10^{-3} M) lead the authors to the conclusion that ACh-induced CA release from the bovine adrenal medulla is mediated entirely by nicotinic receptors. The distribution of nicotinic and muscarinic receptors in the adrenal glands from other species differs markedly (see reference 45); for example, the cat adrenal gland is thought to contain both types of receptors (16).

In conclusion, the results obtained with isolated adrenal medullary cells parallel those obtained with perfused adrenal gland and therefore lend support to the idea that release of catecholamines from the adrenal medulla occurs by an exocytotic process. The cells as prepared here are morphologically intact, biochemically viable, and pharmacologically responsive. Therefore, such isolated adrenal medullary cell preparations should allow the isolation of purified chromaffin-cell plasma membranes for comparison with chromaffin granule membranes and thus should be a useful model system for studying membrane events associated with exocytosis.

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