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α-ADRENERGIC, β-ADRENERGIC AND CHOLINERGIC MECHANISMS FOR AMYLASE SECRETION BY RAT PAROTID GLAND *IN VITRO*

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SUMMARY

1. Rat parotid gland slices, incubated in a balanced, buffered salt solution, were found to be physiologically stable for up to 2 hr with respect to O_2 consumption, water content, extracellular space and cation content.

2. The slices could be stimulated to secrete amylase by activation of α-adrenergic, β-adrenergic or muscarinic cholinergic receptors.

3. The secretion elicited through all three receptors appeared to involve exocytosis as revealed by electron microscopy.

4. The β-agonist, isoprenaline, increased tissue content of cyclic adenosine 3′,5′-monophosphate (cyclic AMP); α-adrenergic and cholinergic agents had no effect on the level of this cyclic nucleotide.

5. Secretion via cholinergic or α-adrenergic mechanisms required extracellular calcium; the β-adrenergic mechanism did not.

6. It was concluded that stimulation of rat parotid cells activates distinctly separate pathways leading ultimately to exocytosis, one pathway involving cyclic AMP, and the other, external Ca^{2+} ion.

INTRODUCTION

Pieces or slices of rat parotid gland, incubated *in vitro*, respond to catecholamines by releasing α-amylase (Bdolah, Ben-Zvi & Schramm, 1964). The process appears to involve classical β-receptor activation resulting in an increase in the cellular content of cyclic AMP (Bdolah & Schramm, 1965), which in some manner triggers exocytosis (Amsterdam, Ohad & Schramm, 1969). Whereas long-time depletion experiments suggest a semiquantitative dependence on Ca (Selinger & Naim, 1970), the classical dependence on extracellular Ca characteristic of many other secretory systems (Rubin, 1970) is not evident (Batzri & Selinger, 1973).

Some evidence has appeared that cholinergic receptors mediate amylase release by a Ca-dependent mechanism (Ishida, Miki & Yoshida, 1971; Rossignol, Herman, Chambaut & Keryer, 1974) not involving cyclic AMP (Sherman & Putney, 1975). In addition, Butcher, Goldman & Nemerovski (1975) observed amylase secretion induced by phenylephrine (an α-agonist) which was only partially blocked by the β-blocker, propranolol. Butcher (1975) has also mentioned unpublished observations of Ca dependence for the α-adrenergic effect. Finally, Maurs, Herman, Busson, Ovtracht & Rossignol (1974) observed a 'slight' effect of phentolamine (an α-blocker) on norepinephrine-induced protein discharge. Despite the above observations, the existence of distinct cholinergic or α-adrenergic mechanisms for protein discharge has failed to gain general acceptance (for recent reviews see Schramm & Selinger, 1974, 1975; Berridge, 1975). This may be due, in part, to an earlier report suggesting that release of amylase by other than adrenergic agents might be due to release of endogenous catecholamines (Schramm, 1968). Also (as we show below), cholinergic and α-adrenergic agents are incapable of inducing amylase discharge at rates comparable to those obtained *via* the β-adrenergic mechanisms, leading some to doubt, perhaps, the physiological significance of those pathways.

In the rat, parotid saliva produced by *in vivo* post-ganglionic parasympathetic nerve stimulation contains α-amylase (Schneyer & Hall, 1965). Thus, a cholinergic mechanism for amylase release should be demonstrable in an *in vitro* preparation. The observation of one type of secretion or another might well depend on the physiologic status of the *in vitro* preparation used. Previously, however, no such data have been given. In this report, we present data that suggest that our *in vitro* parotid gland preparation is quite stable for up to 2 hr of incubation with respect to O_2 consumption, ion and water contents, and extracellular space. In addition, we find distinct α-adrenergic, β-adrenergic, and cholinergic mechanisms for amylase discharge. Finally, we have characterized these mechanisms with respect to cellular ultrastructure, and involvement of Ca and cyclic AMP. A preliminary report of some of these findings has appeared previously (Sherman & Putney, 1975).

METHODS

Male Wistar rats weighing between 125 and 200 g (38–45 days old, approximately) were fasted (with water *ad lib.*) for 16–24 hr before use. Rats exceeding 250 g generally yielded parotid tissue which tended to lose amylase spontaneously during incubation and responded poorly to the stimuli used.

The rats were anaesthetized with Na pentobarbitone (50 mg/kg, I.P.) and the superficial portions of the parotid gland (*ca.* 100 mg/gland) were removed by blunt

dissection. Immediately following dissection, each gland was divided approximately in half with a Stadie–Riggs microtome (slice thickness *ca.* 0·5 mm) and placed in a basket of nylon net (bridal veil) and glass. Four baskets, each containing one slice of approximately 50 mg tissue, were immersed in 5 ml. physiological salt solution (Krebs–Ringer–Tris, KRT) of the following composition (mM): NaCl, 120·0; KCl, 5·0; $MgCl_2$, 1·2; $CaCl_2$, 3·0; β-hydroxybutyrate Na, 5·0; Tris(hydroxymethyl)-aminomethane, 20·0; and buffered with HCl to pH of 7·40 at 37 °C. The KRT was continuously gassed with 100 % O_2 by means of small polyethylene tubing attached to the tissue basket.

Secretion of α-amylase was measured as follows. Tissues were permitted to equilibrate in KRT for 20–30 min. Immediately after slicing, tissues release large quantities of α-amylase into the incubation media, presumably from cells which had been damaged by the dissection or slicing. After the 20–30 min preincubation, the slices were transferred through three 5 ml. volumes of KRT for 2, 10 and 10 min. The 2 min incubation was utilized as a rinse of the preincubation medium. Amylase activity in the first 10 min incubation volume was measured to determine the resting rate of enzyme secretion, and the activity in the second 10 min incubation volume, to which the appropriate agent was added, was assumed to be a measure of the sum of resting and stimulated rates. Thus, the net secretory rates reported represent differences between these two measurements.

Amylase activity in the incubation media was assayed by the method of Bernfield (1955). Rates were calculated as units of activity secreted per gram (wet weight) per minute. Others have reported that superior statistics are obtained by assaying a homogenate of the tissue and calculating the % total α-amylase released, but we did not find this to be the case. The mean ratios of standard errors to means ($\pm$ 1 S.E.) were, for %/min, $0{\cdot}40 \pm 0{\cdot}06$; for μ./g.min, $0{\cdot}33 \pm 0{\cdot}06$ (n = 24 groups). The two methods were thus not significantly different from one another; for convenience the latter was chosen. For comparison with earlier results by others, the ratio of values in μ./g.min to %/min was 256 ± 20 (n = 24). Wet weights were determined by lightly blotting the slices on filter paper (moistened with KRT) and rapidly weighing on a torsion balance. For experiments in which tissue amylase was of interest the tissues were homogenized in distilled water, aliquots taken for the enzyme assay, and an aliquot transferred to a tared fused-quartz crucible for determination of cations. In the other experiments, the whole tissue was transferred to the tared crucible. In both cases, the crucibles were dried overnight at 105 °C and reweighed to obtain dry weights from which subsequent calculations of tissue water content could be made. The dried residues were ashed at 500° C for 12–18 hr. The ash was dissolved in 0·1 N-HCl containing 10 mM-$SrCl_2$ and assayed for Na, K, Mg and Ca with a Perkin–Elmer Model 403 atomic absorption spectrophotometer.

Distribution of ^{14}C-labelled compounds was determined by incubating slices in the presence of the appropriate ^{14}C-compound, dipping the slice quickly in a large volume of non-radioactive KRT, blotting and weighing. The slices were solubilized in 1·0 N-NaOH and counted for ^{14}C-content in a scintillation counter. Efflux of radiolabelled sucrose was determined by incubating the tissue in [^{14}C]sucrose, removing the tissue from the basket with fine tipped forceps, quickly dipping the slice in a large volume of KRT, and transferring the slice to a fresh holder. The tissue was sequentially incubated for fixed intervals in separate 5 ml. volumes of non-radioactive KRT, and finally made soluble and counted as before. Counts from the wash volumes and final tissue extract were used to calculate the % total radio-activity remaining in the tissue at the end of each wash interval. Such data plotted semilogarithmically (desaturation or efflux curves) can be used to follow the exponential decline of tissue radioactivity characteristic of apparent first-order efflux

(Shanes & Bianchi, 1959, 1960). Extrapolation of the linear portions of such curves to zero time has been used to estimate the penetration of isotope into more slowly exchanging compartments (Bianchi & Shanes, 1959).

Cyclic AMP was measured by the radioimmunoassay technique of Steiner, Kipnis, Utiger & Parker (1969). At the end of the prescribed time of incubation, the slices were quickly frozen by clamping between two steel blocks pre-cooled in liquid nitrogen. The frozen tissues were rapidly homogenized in ice-cold 7 % trichloroacetic acid with a Willem's Polytron. After centrifugation, the trichloroacetic acid was extracted from the supernate by shaking three times with three volumes of diethyl ether previously saturated with water. The trichloroacetic acid insoluble pellets were solubilized in 1·0 N-NaOH and assayed for protein by the method of Lowry, Rosebrough, Farr & Randall (1951).

O_2 consumption was measured with an oxygen electrode and standard polarographic techniques (Davies, 1962).

Tissue slices for electron microscopy were initially prepared and treated in the same manner as described for the measurement of amylase secretion and stabilized in KRT for 20 min. Slices were removed before, and at 5 and 15 min after, the addition of carbachol, isoprenaline or phenylephrine (10^{-5} M) to the tissue bath. Immediately upon removal, the slices were swirled in cold neutralized 2 % glutaraldehyde containing 120 mM-NaCl, 5 mM-KCl, 1 mM-$CaCl_2$, and 20 mM Tris-(hydroxymethyl)aminomethane, pH 7·7 at 4° C. After a fixation of 2 hr at 4° C, the tissues were washed in cold Tris buffer containing 200 mM sucrose (three changes of 15 min each) and then in cold 0·1 M Sorenson's phosphate buffer (two changes of 15 min each) containing 250 mM sucrose and buffered to pH 7·4 at 22° C. The tissues were post-fixed for 2 hr in cold 2 % osmium tetroxide in Sorenson's phosphate buffer containing 500 mM sucrose. The sucrose added to the above solutions served to maintain constant osmolarity throughout fixation (*ca.* 450 m-osmole/l.). After a final wash in the phosphate buffer, the tissues were washed in 20 % ethanol (two washes of 15 min each) and then stained *en bloc* in 2 % aqueous uranyl acetate for 15 min (Hayat, 1970). The tissue pieces were subsequently dehydrated through increasing concentrations of ethanol and embedded in Spurr epoxy resin (Spurr, 1969). Thin (silver) sections were cut from 5–10 blocks of each sample with glass knives on a Porter-Blum MT-1 ultramicrotome, stained with Reynold's lead citrate for 5 min, and examined with a Philips EM 300 electron microscope at an accelerating voltage of 60 kV.

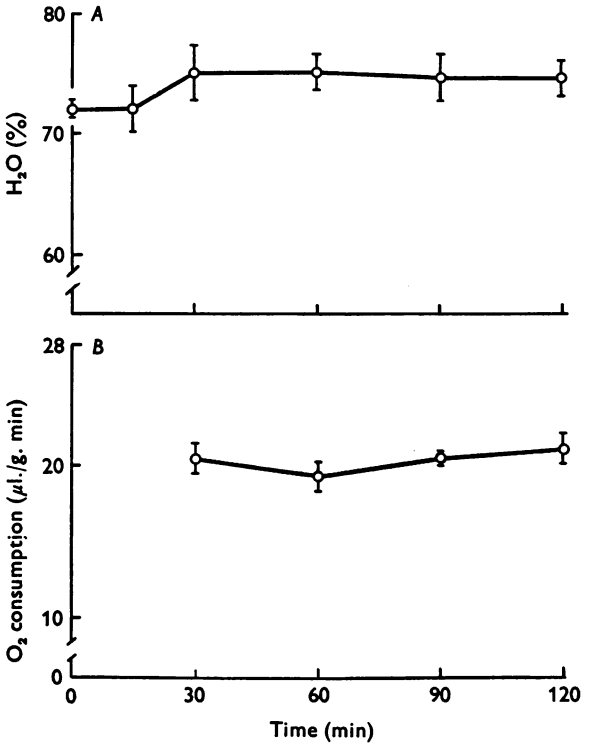
Radiolabelled materials were obtained from ICN Pharmaceuticals, Inc., Isotope and Nuclear Division, Cleveland, Ohio. Ethyleneglycol bis(aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) was obtained from Eastman Kodak Co., Rochester, New York and was neutralized with Tris(hydroxymethyl)aminomethane before use. $LaCl_3$ was obtained from Fisher Chemical Co., Fair Lawn, New Jersey. Carbachol, L-isoprenaline, D,L-propranolol, and atropine were obtained from Sigma Chemical Co., St Louis, Missouri. The Sterling-Winthrop Research Institute, Rensselaer, New York kindly donated phenylephrine HCl, and phentolamine HCl was a gift from the Ciba-Geigy Corp., Summit, New Jersey. An 8 % glutaraldehyde solution was purchased from Electron Microscopy Sciences, Fort Washington, Pennsylvania, and a 4 % aqueous solution of osmium tetroxide was obtained from Polysciences, Inc., Warrington, Pennsylvania.

Summarized data are generally represented by the arithmetic mean; dispersions are given as the standard error of the mean. For statistical comparisons between two groups, Student's *t* test, for paired data when appropriate, was used. The analysis of variance was used when three or more groups were compared. The critical probability for rejection of null hypotheses was 5 % throughout.

RESULTS

Stability of the in vitro preparation

Water content. The water content of parotid gland slices does not change significantly during 2 hr of incubation in KRT (Text-fig. 1*A*). This, together with the apparent stabilities of the extracellular space and ion contents (see below), suggests that the preparation is well balanced



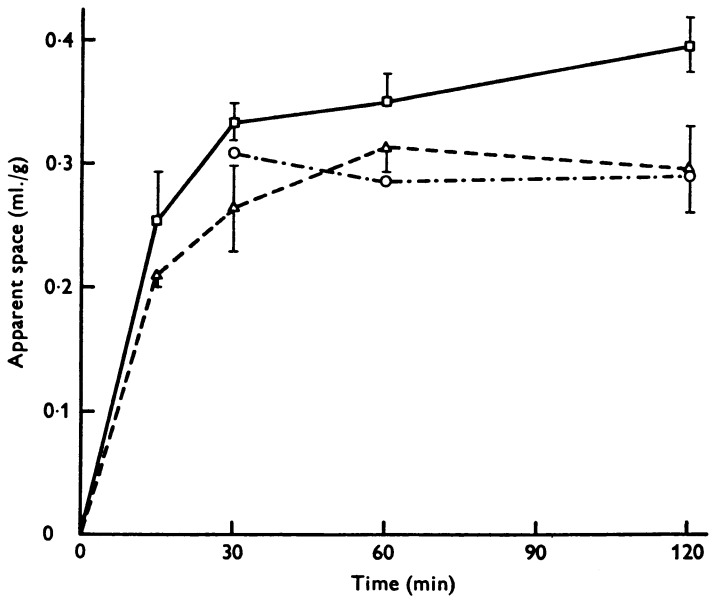
Text-fig. 1. Values for water content (A) and O_2 consumption (B) for rat parotid slices for various times throughout 2 hr incubation. Water values are means of six replications; O_2 consumption values represent four measurements each. The dispersions represent ± 1 S.E. of the mean.

osmotically and does not swell during the 2 hr period. The pooled mean for thirty-six measurements of water content was 73·7 ± 0·6 %. Similarly stable values have been obtained with rat submaxillary gland (Putney & Borzelleca, 1971), but not with the same gland from dog (Siegel, 1969).

O_2 consumption. The rate of O_2 consumption by the slices during the 2 hr incubation was quite constant (Text-fig. 1*B*); the pooled mean respiratory rate from 16 tissues was 20·3 ± 0·5 μl./g.min. This value is somewhat lower

than those obtained with other glands or other species (*ca.* 35 μl./g.min, Martin, 1967).

Extracellular space. Estimates of extracellular volume often vary when two or more supposedly impermeable substances are used (Goodford & Hermansen, 1961; Page, 1962; Barr & Malvin, 1965). Thus, in preliminary experiments, we measured the volumes of distribution of four ^{14}C-labelled carbohydrates after 30 min incubation. The space values obtained as the ratio of tissue (cpm/g) to incubation medium (cpm/ml.) concentration were



Text-fig. 2. Uptake of [^{14}C]sucrose (□) and [^{14}C]inulin (△) as a function of incubation time. Values for [^{14}C]sucrose, after correcting for the slow component seen in Text-fig. 3, are also plotted (○). The method of correction is explained in the text. Each point is the mean of four values, ± 1 S.E. of the mean.

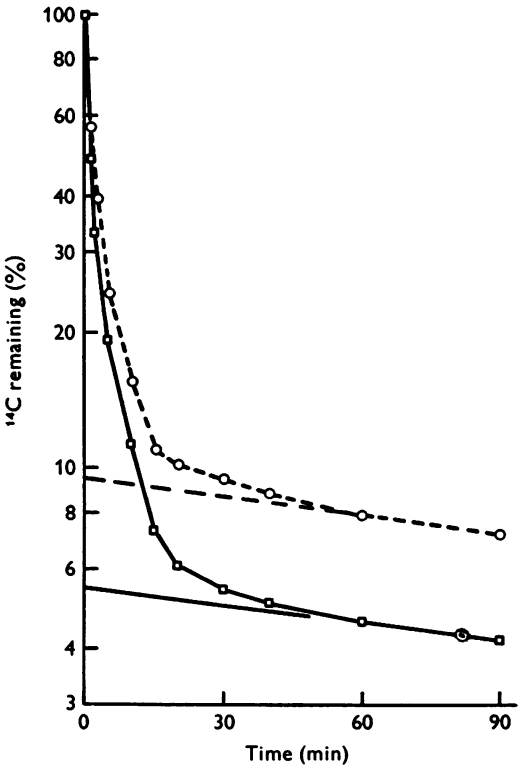
(ml./g, ± S.E., n = 4): inulin, 0·238 ± 0·030; sucrose, 0·328 ± 0·024; mannitol, 0·384 ± 0·29; mannose, 2·60 ± 0·52. The first three compounds, inulin, sucrose and mannitol, yielded values in inverse relation to their molecular size. The value for mannose, in exceeding 1·0, almost assuredly indicates cellular uptake of this substance. This possibility has been raised previously for the submaxillary gland (Putney & Borzelleca, 1971). Curiously, smooth muscle cells do not appear to accumulate mannose in this manner (Weiss 1966). In an attempt to delineate the mechanism(s) of this apparent size-dependent distribution, we followed the distributions of [^{14}C]inulin and

[^{14}C]sucrose (the two smallest values) as a function of incubation time. The data obtained (summarized in Text-fig. 2) show that the inulin space is stable between 60 and 120 min. The sucrose space, on the other hand, appears to drift upward in a manner similar to that seen by others in heart muscle (Tuttle, Witt & Farah, 1961; Baskin, Dutta & Marks, 1973). If the apparent slow increase in the sucrose space represents penetration into the cellular compartment, then the values extrapolated to zero time should equal that of inulin, the non-penetrating marker. Regression analysis of the sucrose values *vs.* time (30–120 min) was significant ($r = 0{\cdot}61$, $P < 0{\cdot}05$) and yielded slope and y-intercept values of 0·046 ml./g.hr and 0·310 ml./g respectively. The latter value agrees with the pooled 60–120 min inulin value of $0{\cdot}307 \pm 0{\cdot}019$ ml./g ($n = 8$). If the increasing sucrose values represent cellular penetration, then a cellular uptake of sucrose should be evident from efflux kinetics. This is indeed the case, as evidenced by Text-fig. 3. The time constants for efflux from 60 to 90 min were 287 min after 30 min uptake and 308 min after 60 min uptake. If the intercept values are converted to ml./g and divided by the uptake time, penetration rates of 0·056 ml./g. hr are obtained for the 30 min uptake and 0·053 ml./g. hr for the 60 min uptake. These values are slightly higher, but agree reasonably well with the slope obtained from Text-fig. 2. If the average of these two values are used to correct the sucrose values in Text-fig. 2, the corrected values appear stable with time and are not significantly different from the 60 and 120 min inulin values. In subsequent calculations, we have used the pooled inulin value of 0·307 ml./g. Dormer and Ashcroft (1974) report 0·25 ml./g for 30 min distribution of inulin which agrees with the 30 min value in Text-fig. 2, but does not seem to represent an equilibrium value. The technique of measuring the efflux of extracellular markers to determine their distribution is similar to that employed by Weiss (1966).

Cations. Text-fig. 4 summarizes the tissue contents of Na, K, Mg, and Ca in fresh tissue and slices incubated for various times up to 120 min. Fresh tissue was obtained as biopsy from the gland as soon as they were exposed in dissection. There appeared to be a slight increase in Na at the end of the incubation period and a slight decrease in potassium initially, though neither change was statistically significant. The stability of the values is in marked contrast to those obtained with submaxillary gland, where Na values rise and K values fall precipitously during incubation (Schneyer & Schneyer, 1962; Putney & Borzelleca, 1971). The pooled values from 15 to 90 min are summarized in Table 1. The Ca values are higher than for most mammalian tissues, which is probably due to the large amounts of Ca in the secretory granules (Wallach & Schramm, 1971). Table 1 also gives values for apparent intracellular concentration of Na

and K and their respective intracellular/extracellular concentration ratios. Higher values for K and lower values for Na have been reported *in vivo* by Schneyer & Schneyer (1960).

For Mg and Ca, Table 1 lists the cellular content per gram of cells rather than per millilitre intracellular water, because it is likely that only a small fraction of these metals are present in free solution intracellularly.



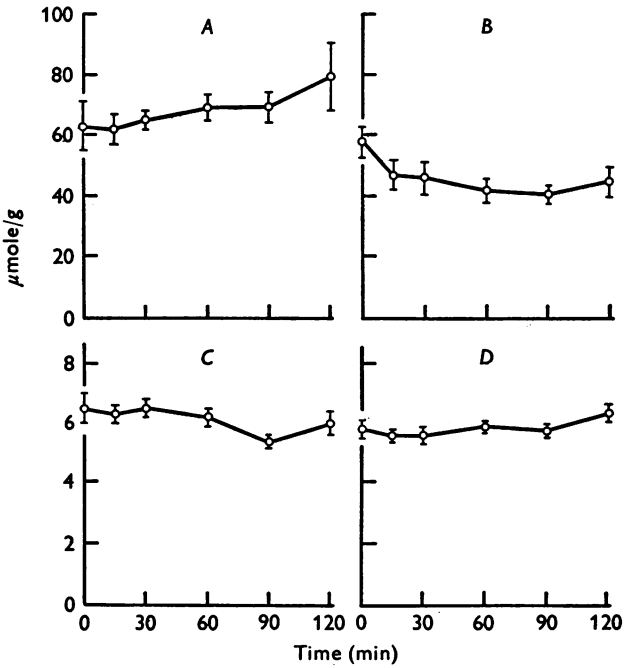
Text-fig. 3. Efflux of [^{14}C]sucrose from slices of rat parotid gland after exposure to [^{14}C]sucrose for 30 (□) or 60 (○) min. The ordinate values represent the percentage of total accumulated ^{14}C remaining in the tissue at each point in time. The linear extrapolations to zero were made through the 60 and 90 min values.

Secretory responses

Dose–response. Text-fig. 5 summarizes the effects of different concentrations of isoprenaline, carbachol and phenylephrine on α-amylase release. Since phenylephrine has weak β-agonist activity (Butcher *et al.* 1975), the phenylephrine solutions routinely contained 10^{-4} M D,1-propranolol. This concentration of propranolol was sufficient to block the partial β-agonist activity of phenylephrine as evidence by cyclic AMP data

(below) but was not sufficient to produce non-specific inhibitory effects since carbachol-induced secretion was not affected (below).

The data for carbachol, phenylephrine and isoprenaline well illustrate the superior efficacy of the β-adrenergic agent for induction of secretion. The rate of resting secretion was about 25 μ./g.min (0·1 %/min) for all

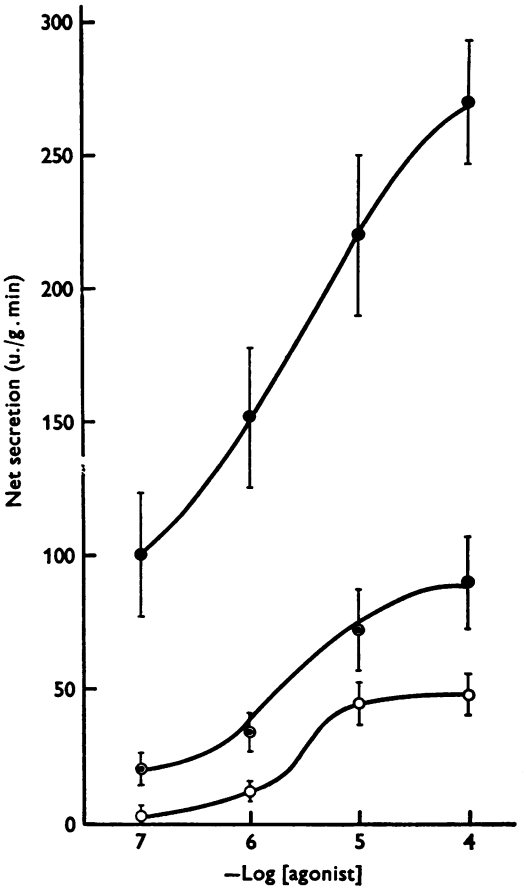


Text-fig. 4. Na (*A*), K (*B*), Ca (*C*) and Mg (*D*) contents of parotid gland slices at various times of incubation in KRT. The values at time zero were obtained from pieces taken directly from the anaesthetized animals. Each mean comprises observations from six animals. The dispersions represent ± 1 S.E. of the mean.

TABLE 1. Mean cation contents (15–90 min, n = 24) in rat parotid slices. Extracellular values are those for KRT. Intracellular values are per millilitre cell water assuming total cell water is 0·737 ml./g and extracellular space is 0·307 ml./g. Cellular values (for Mg and Ca) are similarly derived but expressed per g of cells rather than ml. cell water (i.e. solids are included). *I/E* values for Na and K are ratios of intracellular to extracellular concentration

	Na	K	Mg	Ca
Content (μmole/g)	66·1 ± 1·8	43·9 ± 1·5	6·10 ± 0·23	5·74 ± 0·07
Extracellular (μmole/ml.)	125·0	5·0	1·2	3·0
Intracellular (Na, K, μmole/ml.)	64·5	98·5	—	—
Cellular (Mg, Ca, μmole/g cells)	—	—	8·27	6·95
I/E	0·52	19·7	—	—

groups. Thus, though small in comparison to isoprenaline (270 μ./g.min., 1·05%/min), the maximum rates obtained with phenylephrine (48 μ./g.min, 0·19%/min) and carbachol (90 μ./g.min., 0·35%/min) represent 3- and 4·5-fold increases in secretion, respectively. Isoprenaline produced greater



Text-fig. 5. Net secretory responses (stimulated minus resting, μ/g.min) of parotid gland slices to several concentrations of isoprenaline (●), carbachol (⊙), and phenylephrine (○). In the phenylephrine experiments, 10^{-4} M propranolol was present. The lines were drawn freehand through points representing means of six determinations, ± 1 S.E. of the mean.

than a 10-fold increase in the rate of enzyme discharge. Expressed as percentage of total enzyme released per minute, the resting and stimulate rates for isoprenaline are comparable to those reported by Batzri & Selinger (1973) and by Butcher *et al.* (1975). The values obtained with carbachol are greater than those reported by Schramm (1968). Butcher

et al. (1975) have reported lower rates for phenylephrine in the presence of propranolol than our values obtained under similar circumstances. In these experiments the total recovery of enzyme (released plus remaining in the slice) averaged 30,000 μ/g and was not affected by any of the experimental treatments.

TABLE 2. Effect of blocking agents on secretion. Paired slices were used to measure amylase secretion by the agonists indicated in the presence and absence of the antagonists indicated. Secretion in the presence of agonist plus antagonist is expressed as % control (in the presence of agonist alone). Values are means ± S.E., (n) refers to the number of replications

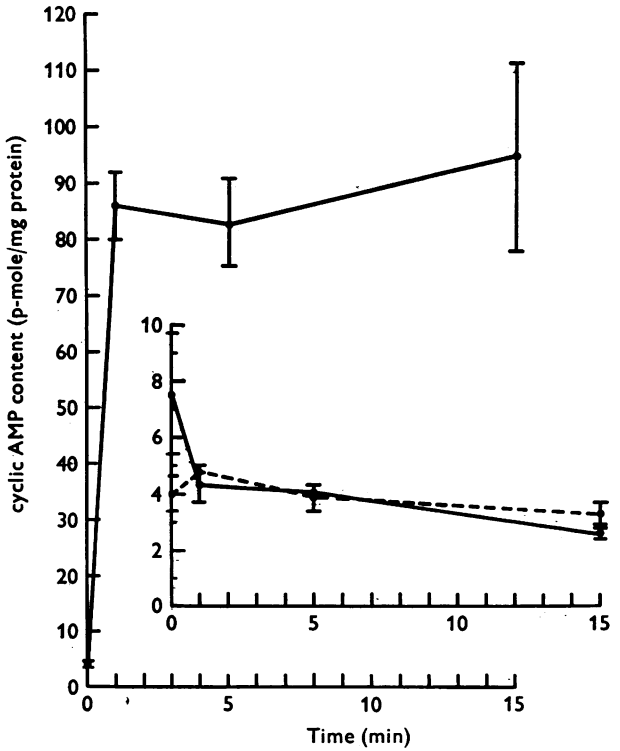
	Secretion (% control)			
Agonist (M)	Isoprenaline (10^{-5})	Carbachol (10^{-5})	Carbachol (10^{-5})	Phenylephrine (10^{-5})
Antagonist (M)	Propranolol (10^{-4})	Propranolol (10^{-4})	Atropine (10^{-4})	Phentolamine (10^{-4})
% Control ± 1 S.E. (n)	8·2 ± 4·9 (6)	92·2 ± 6·4 (7)	3·3 ± 2·7 (11)	5·6 ± 6·6 (6)

Table 2 summarizes the results of experiments designed to show the receptor specificity of the agonists. The failure of 10^{-4} M propranolol to affect carbachol induced secretion and the ability of phenylephrine to elicit secretion in the presence of 10^{-4} M propranolol (Text-fig. 5) suggest that these agents do not act through release of endogenous catecholamines. Responses to isoprenaline, carbachol and phenylephrine were completely blocked by propranolol, atropine and phentolamine, respectively.

Morphological evidence for exocytosis. The ultrastructure of acini from control tissue (Pl. 1) appears similar to that described by Scott & Pease (1959) and by Parks (1961). These cells are densely packed with granules to the extent that nuclei may be indented by the granules (Pl. 1, fig. *A*). The acinar lumina of unstimulated tissues are usually the size of one or two granules and are characteristically demarcated by junctional complexes (Pl. 1, fig. *B*). Lipid droplets and, occasionally, degenerated secretory granules similar to those suggested by Hand (1972) to result from starvation, were often found in the basal portion of acinar cells.

The complete secretion cycle of the parotid acinar cell (exocytosis of granular material and its resynthesis) induced either by injection of a β-adrenergic agonist (Amsterdam *et al.* 1969; Simson, 1969; Cope & Williams, 1973), or by sympathetic nerve stimulation (Garrett & Thulin, 1975) *in vivo* has been thoroughly documented. The initial phase of this cycle (the period of rapid discharge of preformed secretory material) can be induced *in vitro* by isoprenaline. After 5 min incubation with isoprenaline the lumina increase to the size of several granules, often maintaining the shapes of the

granules from which they were presumably derived (Pl. 2, fig. *A*, *B*). Several discharged granules with no apparent connexion to the original lumen can be seen in Pl. 2, fig. *A*. After 15 min (Pl. 2, fig. *C*) the change is more obvious. Flocculent material similar to that found in the lumen is evident in the enlarged area of discharged granules in Pl. 2, fig. *C*. We did not observe the formation of granular pseudopodia seen by others (Schramm, Selinger, Salomon, Eytan & Batzri, 1972) in response to β-adrenergic stimuli.



Text-fig. 6. Large graph: tissue content of cyclic AMP in parotid gland slices 1, 5, and 15 min after the addition of 10^{-5} M isoprenaline to the incubation medium. Inset: similar determinations made after introduction of 10^{-5} M carbachol (●——●) or 10^{-5} M phenylephrine (●– – –●) plus 10^{-4} M propranolol. In all three cases, the time zero values represent results of assays on slices taken just prior to the addition of agonist. Each point is the mean from three experiments, ± 1 S.E. of the mean.

Whether discharge of zymogen granules by cholinergic or α-adrenergic agonists occurs via exocytosis has not been documented. Pl. 3, fig. *A* shows a lumen after 5 min stimulation with carbachol which is similar in appearance to the exocytotic figures produced by isoprenaline in Pl. 2. As

with isoprenaline, after 15 min stimulation with carbachol (Pl. 3, fig. *B*) discharged granules occupy a progressively larger area of the cell. With phenylephrine, as with isoprenaline and carbachol, the connexions between discharged granules and the original lumina are not always obvious (Pl. 3, fig. *C*). It is possible to conclude from micrographs such as those in Pl. 3 that exocytosis of zymogen granules occurs upon stimulation with carbachol or phenylephrine, according to the morphological requirements given by Amsterdam *et al.* (1969) (progressive fusion of granules producing enlarged lumens).

In other experiments not shown here, 10^{-4} M propranolol, 10^{-4} M atropine, or 10^{-4} M phentolamine were included in the incubation media during and 10 min before a 5 min exposure to 10^{-5} M isoprenaline, 10^{-5} M carbachol or 10^{-5} M phenylephrine, respectively. Tissues were then fixed and prepared as before. As predicted from the secretion studies (Table 2), no structural evidence for exocytosis due to these procedures was observed.

The existence of a similar exocytotic process upon stimulation with α-adrenergic, β-adrenergic or muscarinic agents does not preclude the existence of unique structural changes caused by each of these. This possibility will be considered more extensively in a subsequent communication.

TABLE 3. Ca requirement for secretion. Paired slices were used to measure amylase secretion by each of the three agonists in the presence and absence of 10 mM-EGTA or 1·0 mM-$LaCl_3$ (carbachol and phenylephrine only). Secretion in the presence of agonist plus EGTA or La^{3+} is expressed as % control (in the presence of agonist alone). Values are means ± S.E., (n) refers to the number of replications

	Secretion (% control)		
Agonist (10^{-5} M)	Isoprenaline	Carbachol	Phenylephrine
+ 10 mM-EGTA	93·0 ± 5·7 (4)	16·0 ± 6·2 (4)	18·4 ± 9·0 (6)
+ 1 mM-$LaCl_3$	—	20·2 ± 9·2 (4)	− 11·5 ± 13·9 (6)

Effects on tissue cyclic AMP content. The results of cyclic AMP assays on tissues at various times of incubation in KRT containing isoprenaline, carbachol, or phenylephrine are summarized in Text-fig. 6. Isoprenaline elicited a 20-fold increase in tissue cyclic AMP content within one minute, carbachol and phenylephrine were without significant effect. Larger increases in response to 15 μM isoprenaline were obtained with female rats by Butcher *et al.* (1975).

Ca dependence. The addition of 10 mM-EGTA to the incubation media affected neither the resting secretion rates (not shown) nor the net secretion due to isoprenaline. Secretion due to carbachol or phenylephrine

was significantly diminished, however. These data are summarized in Table 3. Secretion due to carbachol or phenylephrine could also be blocked by the prior addition of 1 mM-La^{3+} to the KRT (Table 3).

DISCUSSION

Slices of rat parotid gland, incubated under the conditions described in Methods, appear to be physiologically stable *in vitro*. Constant values were obtained for respiration, content of water and cations, and extracellular space (Text-figs. 1, 2, 4) for up to 2 hr of incubation.

The data in Text-fig. 5 and Table 2 clearly demonstrate that the parotid cells contain α- and β-adrenergic and muscarinic type cholinergic receptors capable of inducing enzyme discharge. The ultrastructural evidence suggests that secretion induced through all three receptors (Pls. 2, 3) appears to involve classical exocytosis similar to that reported by others for the β-receptor (Amsterdam *et al.* 1969). These secretagogues caused no other consistent ultrastructural changes which could have been primarily responsible for the enzyme discharge measured here. Further, there were no obvious ultrastructural differences in the exocytotic process resulting from the three agonists. Phenylephrine and carbachol, however, also appeared to induce vacuole formation in the basal regions of the cells in a manner similar to that described by Schramm & Selinger (1975). Vacuole formation had been related to the abilities of cholinergic and α-adrenergic agents to induce K^+ release (Schramm & Selinger, 1975); neither vacuole formation nor K^+ release are considered characteristic of β-adrenergic mechanisms. Schramm & Selinger (1974, 1975) have used the ultrastructural marker of vacuole formation due to α-adrenergic and cholinergic agents together with observations of exocytosis due to β-adrenergic stimuli to show that all three receptors function in the same cell. Our observations of exocytotic figures due to all three receptors confirm their conclusions. Similar evidence for carbachol has been published by Maurs *et al.* (1974). None of the agents used in this study appeared to induce the formation of granular pseudopodia seen by others (Schramm *et al.* 1973; Selinger, Sharoni & Schramm, 1974*b*). This might be due to the use of osmotically balanced solutions for fixation in this study, or to differences in the condition of the animals. We used young starved animals (*ca.* 125 g) and observed glands more densely packed with granules than in the above study. The above investigators generally used younger animals (120–180 g) in earlier studies (Babad, Ben-Zvi, Bdolah & Schramm, 1967), but have used older rats in other investigations (260 g, Amsterdam, Schramm, Ohad, Salomon & Selinger, 1971). In the studies cited above showing pseudopodia formation, no information as to sex, strain or age

of animals is available. In earlier micrographs originating from the same laboratory, no such structures are evident (Amsterdam *et al.* 1969; Selinger *et al.* 1974*b*, fig. 2).

Despite the ability of the three agonists to ultimately induce exocytosis, all three receptor mechanisms obviously do not trigger the same sequence of steps preceding discharge. Activation of β-adrenergic receptors leads to an increase in the tissue levels in cyclic AMP (Text-fig. 6) in a manner similar to many other tissues (Robison, Butcher & Sutherland, 1971). Further, secretion rates equal to those with isoprenaline can be obtained with derivatives of cyclic AMP (Babad *et al.* 1967). No firm evidence has been obtained implicating Ca in an obligatory manner in β-adrenergic-induced secretion (Table 3, Batzri & Selinger, 1973; but see Selinger & Naim, 1970, discussed below).

Muscarinic and α-adrenergic receptors, on the other hand, do not appear to be functionally linked to adenylate cyclase (Text-fig. 6). Both appear, rather, to act through Ca-dependent mechanisms (Table 2). The nature of Ca action cannot, as yet, be determined with certainty. It is unlikely that Ca functions simply to preserve the functional status of the membrane and/or receptors, since Ca removal does not interfere with the electrophysiological responses of acinar cells to cholinergic or α-adrenergic agents (Petersen & Pedersen, 1974). Butcher (1975) has shown that the divalent cationophore A-23187 (see Reed & Lardy, 1972; Foreman, Mongar & Gomperts, 1973) can induce enzyme release only in the presence of extracellular Ca. Thus, activation of α-adrenergic or cholinergic receptors may lead to an enhanced permeability of the acinar cell membrane to Ca, with a subsequent increase in free intracellular Ca. However, Ca influx, within the time frame relevant to secretion, has not yet been shown to be augmented by cholinergic or α-adrenergic agents. Some negative findings have been mentioned (Selinger, Eimerl & Schramm, 1974*a*), although it is apparent that only total uptake of ^{45}Ca was measured, which would be unlikely to produce significant results.

The number of biological responses, including secretory responses, believed to be mediated by a Ca influx mechanism is large (Bianchi, 1968; Rubin, 1970). Likewise, there are many phenomena, secretory and otherwise, that can be ascribed to mechanisms mediated by cyclic AMP (Robison *et al.* 1971). Attempts have been made to implicate cyclic AMP in systems believed to act through Ca (protein release from exocrine pancreas, Ridderstap & Bonting, 1969; Bonting & de Pont, 1974; catecholamines from adrenal medulla, Peach, 1972; Poisner, 1973). Subsequent attempts to confirm these findings generally have yielded negative results (pancreas, Benz, Eckstein, Matthews & Williams, 1972; Williams, 1974; adrenal medulla, Jaanus & Rubin, (1974). Similarly, attempts have been made to

detect a role for Ca in β-adrenergic amylase secretion (Selinger & Naim, 1970; Dormer & Ashcroft, 1974). The former reference has often been cited as evidence for a role for Ca in the β-adrenergic response. Thus, Selinger & Naim (1970) found that prolonged Ca deprivation (120–180 min) produced a partial (30–60 %) reduction of amylase secretion in response to monobutyryl cyclic AMP or epinephrine. Such evidence seems insufficient to indict Ca in an obligatory manner since there is the possibility of non-specific effects of such a protocol (i.e. effects on tissue viability or ATP). That is not to say that the data of Selinger & Naim (1970) are not compatible with a mechanism utilizing less superficial Ca. It may be that activation of α- and β-adrenergic and muscarinic receptor mechanisms all ultimately lead to an increase in free intracellular Ca, with the cyclic AMP mediated mechanism achieving this by mobilizing an intracellular store. Definitive conclusions in this respect await more extensive investigation. To date, the only step in β-adrenergic induced salivary secretion indicated with reasonable certainty is an increase in cellular cyclic AMP. It should be mentioned that Dormer & Ashcroft (1974) have noted alterations in the subcellular distribution of ^{45}Ca due to epinephrine treatment, but in the absence of data with specific α- or β-agonists (or blockers) the significance of these data is not yet apparent.

Butcher (1975) has cited unpublished results of experiments wherein α-adrenergic and cholinergic agents elevated the level of quanosine-3′,5′-monophosphate (cyclic GMP) in parotid gland. However, the cyclic GMP concentration in the parotid gland is generally 1/800th of the cyclic AMP concentration (Durham, Baserga & Butcher, 1974) and is increased by β-adrenergic stimulants as well (Durham *et al.* 1974). Cyclic GMP appears to be involved in control of DNA synthesis (Durham *et al.* 1974); the nature of its role in enzyme secretion awaits further study.

The parotid gland may be almost unique among non-neuronal tissues in having α-adrenergic, β-adrenergic, and muscarinic cholinergic receptors in the same cell, particularly considering that all three receptor mechanisms lead to the same (rather than opposing) physiological result: exocytosis. Also, the occurrence of cyclic AMP and calcium dependent mechanisms in the same cell leading ultimately to the same response affords future investigators an excellent opportunity to study the interactions of calcium and cyclic nucleotides. The ultrastructural evidence challenges us to reduce these two mechanisms, at some level, to an identical molecular step.

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EXPLANATION OF PLATES

Electron micrographs of rat parotid gland. All scale bars represent 1 μm.

PLATE 1

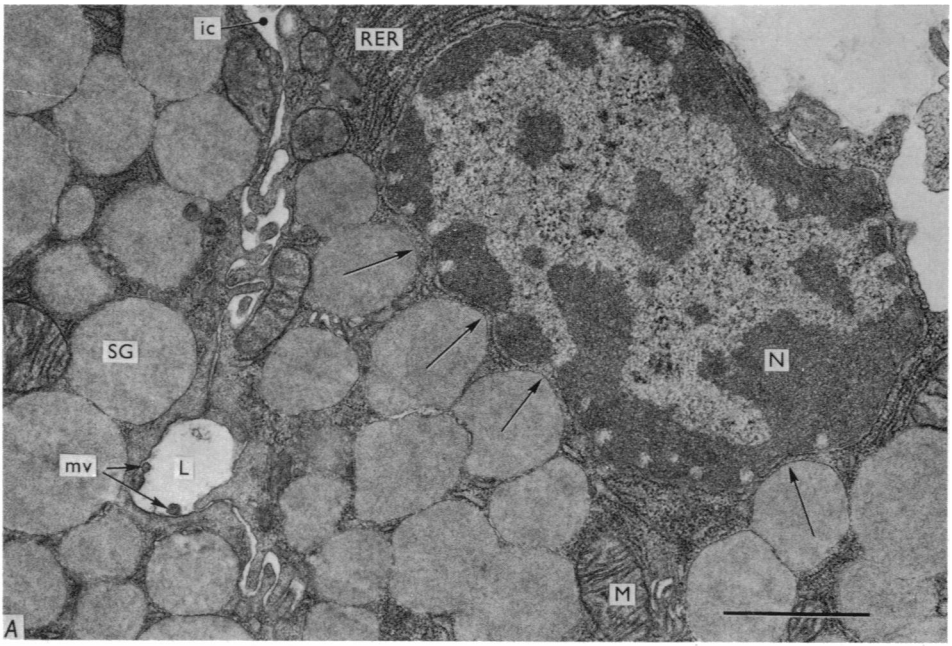
Lumina of acini from unstimulated tissue slices. *A*, Lumina of non-secreting acini are roughly the size of one granule or smaller. The nuclear membrane is often distorted (arrows) by closely packed secretory granules (SG). Although rough endoplasmic reticulum (RER) is usually found in parallel arrays in the basal portion of the cell, it can also be seen among granules and occasional mitochondria (M) throughout the cell. Intercellular clefts (ic) are narrow and convoluted. *B*, Lumina (L), recognizable by the location of junctional complexes (tight junctions, t, and desmosomal barriers, d), generally contain microvilli (mv).

PLATE 2

Parotid acinar cells from tissue slices incubated in 10^{-5} M isoprenaline. *A* and *B*, after 5 min incubation, the lumen (L) has increased in size and become irregular in shape from the fusion of secretory granules (L_1). Discharged granules (exocytotic figures, e), whose connexion with the lumen is presumably in another plane of section, can be seen in fig. *A*. The original lumen can be recognized by the location of desmosomes (d) and microvilli (mv). *C*, a longer incubation (15 min) results in greater areas of discharged granules (e). These areas in *C* contain a flocculent material of a similar density to that which is contained in the lumen, and is presumably of granular origin.

PLATE 3

Parotid acinar cells from tissue slices incubated in either 10^{-5} M carbachol or 10^{-5} M phenylephrine. *A*, 5 min incubation in carbachol. Fusion of secretory granules with lumen membrane in another plane of section has created several areas of discharged granules (e) grouped near the lumen (L). Granular material is present within these areas. *B*, 15 min incubation in carbachol. Successive fusion of secretory granules throughout this acinar cell has created many areas (e) reminiscent of the original granules. These areas are also filled with a slightly less electron-dense material similar to the substance observed in Pl. 2, *C*. *C*, 5 min incubation in phenylephrine. Exocytotic figures (e) as described in previous figures are also evident after stimulation with phenylephrine. This lumen (L) is located at the junction of four acinar cells. Serial sections through this area have shown the lumen to be continuous with L_1.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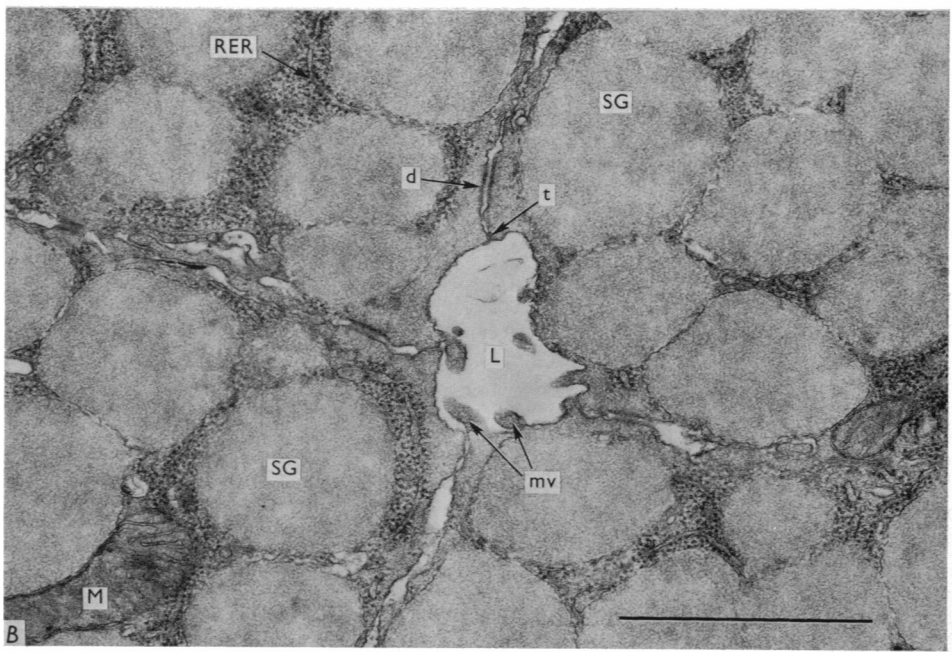
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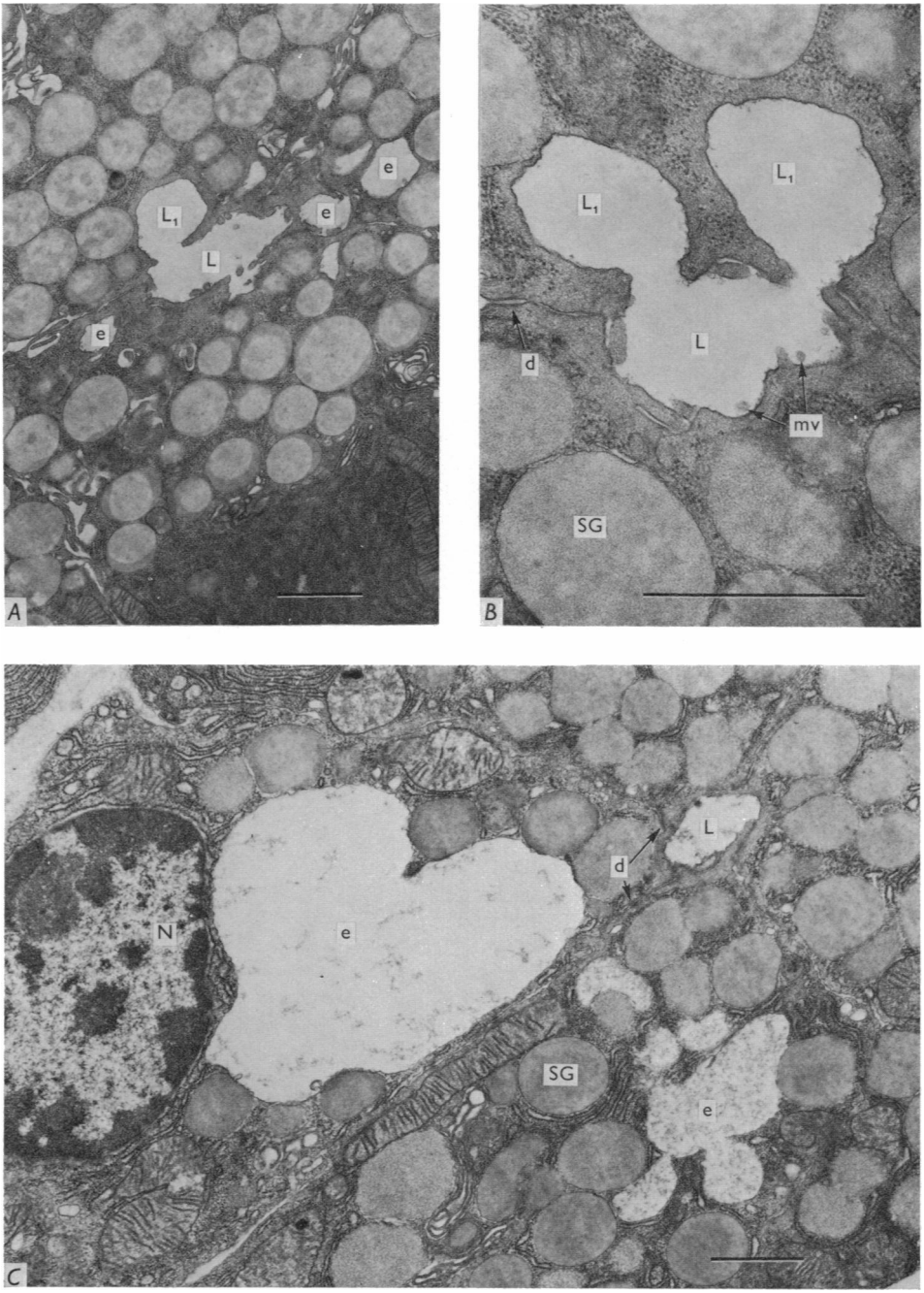
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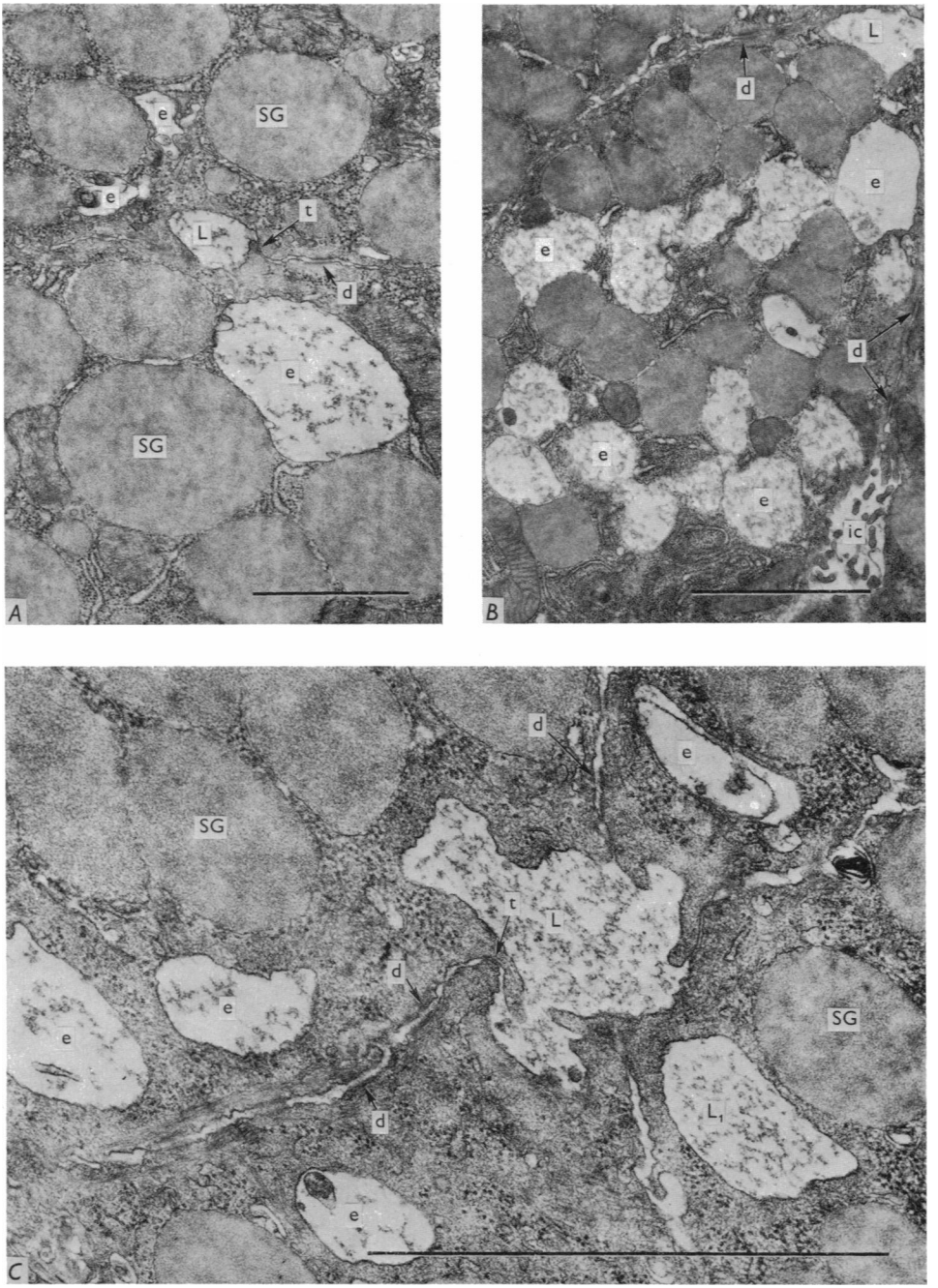
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