SECRETORY PROTEIN SYNTHESIS IN THE STIMULATED RAT PAROTID GLAND

Temporal Dissociation of the Maximal Response from Secretion

JOHN H. LILLIE and SEONG S. HAN

From the Dental Research Institute and Department of Anatomy, University of Michigan Medical School, Ann Arbor, Michigan 48104

ABSTRACT

Administration of the β-adrenergic drug, isoproterenol (IPR), affects the release of 98% of stored amylase from rat parotid gland acinar cells. A period of 6 h elapses from the onset of secretion to the maximum [^{14}C]phenylalanine (Phe) incorporation into total protein and amylase. 10 h after IPR administration the rate of [^{14}C]Phe incorporation into total protein was no longer elevated above that of control. Incorporation into amylase, however, remained elevated above the control by 2.3 times. This latent period may reflect: (*a*) reduced amounts of available ATP which occurs as a result of the process of secretion as well as (*b*) the time required for reorganization of cellular organelles and membranes after secretion. The latent period after IPR-induced secretion appears similar to the latent period which has recently been reported to occur after physiologic release of amylase from the parotid gland during the diurnal feeding cycle of the rat. These observations support the existence of a positive feedback system operant in the parotid acinar cell linking the release of secretory proteins with their synthesis. The period of greatest protein synthesis is, however, temporally dissociated from the secretory process.

INTRODUCTION

Efforts to understand the interrelationship between the synthesis and release of secretory proteins by the acinar cells of digestive glands have been made by various investigators using differing experimental models and yielding varying results. Considering only the exocrine models of the pancreas and parotid glands of rodents, some investigators have found no change in the rate of protein synthesis after stimulation of glandular secretion (1, 2, 3, 4) while others (5, 6, 7) have reported increases in the rate of synthesis of different classes of proteins after gland stimulation. Similar differences of opinion exist concerning changes in amino acid transport across acinar cell membranes after gland stimulation. Gromet-Elhanan and Winnick (8) have reported a decrease in amino acid transport in the parotid after stimulation, and Grand (9) reported little or no flux in the amino acid pool of a similarly stimulated parotid system. However, Barka (10) and Ekfors and Barka (11) have reported a transient increase in the amino acid transport rate in the submandibular gland.

After consideration of various exocrine secretory models, the rat parotid gland was chosen for our study because it: (*a*) is a totally serous salivary gland having sufficient size to provide an adequate quantity of tissue for biochemical analyses, (*b*) has

been shown to release within 1–2 h nearly all stored secretory products in response to isoproterenol (IPR),[1] and (*c*) is a model in which the dynamics of the secretory process have been well characterized (12, 13, 14). The present report was undertaken to determine if the extremes of secretion granule storage (maximal—characteristic of the glands of fasted animals/minimal—approximated by IPR-stimulated glands) had any effect on the rate of amino acid incorporation and, if so, to establish the temporal relationship between the release of secretory proteins and their subsequent synthesis. Throughout the study comparisons between gland tissues from experimental (IPR stimulated) and control (sham stimulated) animals were made based either on their content of DNA or nonsecretory proteins in order to avoid the errors caused by hydration in the use of wet tissue weight or by the choice of a total protein index in a secretory model in which 50% of all the protein of the gland is exportable[2] (15).

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 250 ± 15 g were used. The animals were fed pelleted Purina Rat Chow ad libitum and housed under controlled light conditions (6:00 a.m. to 8:00 p.m.) for 4 days before beginning experiments in an attempt to eliminate irregular secretory cycles. After acclimatization food was withheld for 15 h before beginning the experiment. Experiments were initiated between 8:00 and 9:00 a.m. (time 0), when animals were injected intraperitoneally (i.p.) with either physiologic saline or freshly prepared IPR in saline (0.8 mg/100 g body weight [b.w.] in 0.5 ml/250 g b.w.). Animals were treated by one of two methods depending upon the nature of the particular experiment: (*a*) killed by a blow to the head before biochemical studies or (*b*) anesthetized with chloral hydrate (i.p. injection of 7% chloral hydrate, 0.5 ml/100 g b.w.) in preparation for structural observations.

Incubation Procedures

Assessments of [^{14}C]phenylalanine(Phe) incorporation into total protein (perchloric acid [PCA]-insoluble protein),[3] into water-soluble proteins of the postmicrosomal supernatant and determination of the content of [^{14}C]Phe in the acid-soluble pool were made on parotid gland slices employing an in vitro labeling procedure modified from Jamieson and Palade (16).

After killing the animals the parotid glands were quickly removed and placed in Eagle's minimum essential medium (MEM) at 37°C and gassed with 95% O_2 and 5% CO_2. Pieces approximately 1–1.5 mm on a side were prepared from the periphery of the gland. This minimized the inclusion of ductal elements which run primarily through the central region of the gland. The slices were placed in an appropriately identified diffusion chamber which was in turn suspended in a 25 ml Erlenmeyer flask containing 10 ml of nonradioactive MEM and incubated in a shaker bath (70 cycles/min agitation) while being gassed continuously with the O_2/CO_2 mixture. The diffusion chambers used in these experiments were made by gluing cheesecloth over one end of a Plexiglas cylinder 12 mm in diameter, 2 mm long with a 1 mm thick wall. Tissue slices were put in one such cylinder and a second was then sutured over the first to complete the chamber.

Tissue slices from other animals were placed in similar chambers and likewise held in the nonlabeled medium (MEM; 0.2 mM [^{12}C]Phe) until all tissues for a given experiment had been collected (~50 min). After draining and blotting the chambers of excess nonlabeled medium, they were simultaneously placed in MEM minus [^{12}C]Phe to which [^{14}C]Phe (315 mCi/mM) was added. The level of [^{14}C]Phe incorporation into acid-insoluble protein and the [^{14}C]Phe content of the free amino acid pool were determined after a 30 min incubation in the labeling medium containing 0.23 μCi/ml of [^{14}C]Phe (0.7 μM Phe). For gel electrophoretic studies, [^{14}C]Phe content of the medium was increased to 2.92 μCi/ml

[1] *Abbreviations used in this paper:* b.w., body weight; i.p., intraperitoneal; IPR, isoproterenol; MEM, Eagle's minimum essential medium without glutamine containing Earle's salts; PCA, perchloric acid; Phe, phenylalanine; PMS, postmicrosomal supernatant; PTA, phosphotungstic acid; RER, rough endoplasmic reticulum; R_f, relative electrophoretic mobility; SDS, sodium dodecyl sulfate.

[2] All animals were ad libitum fed until 15 h before beginning an experiment. At that time food was removed; however, free access to water was maintained throughout the experiment.

[3] Castle, Jamieson, and Palade have shown that phosphotungstic acid was found to be a necessary additive to precipitate certain "large molecules" which were not precipitated by PCA or trichloroacetic acid alone (48). Using samples obtained from a water homogenate of tissue slices incubated in [^{14}C]-Phe, we found there were no differences in the acid-precipitable counts after precipitation and washing with 0.5 N PCA or 0.5 N PCA and 0.5% PTA. The physical nature and apparent quantity of the precipitate was, however, markedly different.

(8.9 μM Phe) and the incubation period extended to 1 h.

Analysis of [^{14}C]Phe Incorporation into total Protein and Estimation of Amino Acid Pool Size

After incubation in the [^{14}C]Phe containing medium, the capsules were removed, blotted, and rinsed in two changes (30 s each change) of nonlabeled medium and then placed in 0.3 N PCA at 4°C. Tissue slices were then weighed in order to provide an amount of tissue in which the content of DNA and incorporated radioactivity would satisfy the sensitivity of the DNA assay and the statistical requirements for scintillation counting (approximately 16 m of tissue) and were subsequently homogenized in cold 0.3 N PCA and centrifuged. The supernatant was used to estimate the [^{14}C]Phe available in the PCA-soluble pool while fractionation of the pellet according to the method of Hinrichs et al. (17) was used to extract the proteins and DNA. DNA concentration was determined spectrophotometrically at 260 nm, using calf thymus DNA as the standard. The PCA supernatant was counted in a toluene-Triton X-100 scintillation mixture containing 2,5-diphenyloxazole and 1,4-bis-(5 phenyloxazole)-benzene. The acid precipitate was solubilized in Soluene (Packard Instrument Co., Inc., Downers Grove, Ill.) and counted in a toluene scintillation mixture using the same fluors. Radioactivity was determined in a Packard Tri-Carb 3320 liquid scintillation spectrophotometer and adjustments for quenching were made using an external standard. Counts were expressed in counts per microgram DNA for the reasons discussed in the Introduction. Data derived from incorporation studies of [^{14}C]Phe into total protein were statistically analyzed using a program of the University of Michigan IBM 360/67 computer.

Incorporation of [^{14}C]Phe into Amylase

While the great majority of counts in the PCA-precipitable protein has been reported to be incorporated into secretory proteins (7, 18, 6), an electrophoretic examination of the water-soluble, postmicrosomal supernatant was performed to determine the exact percentage of [^{14}C]Phe incorporated into amylase and/or other secretory proteins. Tissue slices from parotid glands of two animals were labeled as mentioned previously, pooled, and homogenized in distilled water. After centrifugation at 10,000 *g* in a refrigerated centrifuge the supernatant was used to prepare a postmicrosomal fraction by further centrifugtion at 105,000 *g* for 1 h in a refrigerated Spinco Model L preparative ultracentrifuge. The protein concentration of the resultant supernatant was determined (19) and diluted in a sodium phosphate buffer (pH 7.0) containing 0.1% sodium dodecyl sulfate (SDS) and 0.1% β-mercaptoethanol to a concentration of 200–300 μg/ml. Further preparation of the samples for electrophoresis on SDS-polyacrylamide gels was done as described by Ruddon and Rainey (20).

Proteins dissolved in aqueous solutions containing high concentrations of SDS have electrophoretic mobilities on polyacrylamide gels which are a function of their molecular weights. Therefore, electrophoresis of the prepared samples was done without previous removal of unincorporated [^{14}C]Phe (21). Samples were first run at 2.5 mA per gel for 20 min and then at 7 mA per tube for 4½ h. Experimental and control gels were run, stained, and destained simultaneously to allow densitometric comparisons between gels. Quantitative densitometric tracings of Coomassie brilliant blue-stained gels were made using a Gilford 2400 spectrophotometer equipped with a linear transport scanner set at an absorbance of 3.00 and a wavelength of 550 nm. Relative electrophoretic mobilities of protein bands were calculated from these tracings based on the bromphenol blue front. The radioactivity of the various protein bands was measured in the toluene-Triton X-100 scintillation mixture after serially slicing the gels in 1 mm transverse sections and solubilizing the slices in 0.5 ml of 30% H_2O_2 at 37°C for 36–48 h.

Localization of the electrophoretic band associated with amylase was accomplished by: (*a*) detecting which bands were deleted from the control pattern when a water-soluble protein preparation was made of tissues from animals which had discharged their exportable protein in response to IPR 1 h before death, (*b*) observing a gross reduction in the staining intensity of one of these bands after precipitation of amylase as an enzyme-substrate (glycogen) complex (22) from other proteins soluble in a water-ethanol solution, and (*c*) comparing the suspected amylase band with the electrophoretic migration of twice crystallized, hog pancreatic amylase. Four major bands whose protein content remained constant after IPR treatment of the animal were chosen as representative of nonexportable water-soluble proteins. The constant nature of these bands was determined by preliminary experiments in which the amount of protein loaded on to the experimental and control gels was related through the amount of DNA contained in a given homogenate sample. That is, it was found that there was approximately a 35% relative increase in the content of DNA per unit of wet tissue weight 1 h after IPR administration reflecting the release of stored secretory product. The proportion of experimental gland tissue in the original homogenate was therefore reduced to 65% of the control tissue before the preparation of the postmicrosomal super-

natant (PMS). The protein content of the control preparation was then determined and adjusted by dilution to give 200 μg/ml (20 μg per gel) and the experimental preparation diluted by an equal volume. In this way, even though a given unit of each preparation varied in the concentration of soluble proteins, each was derived from an approximately equal cell population. Similarly, adjustments of the control and experimental PMS preparations to equalize the content of DNA were made at 2, 6, and 10 h. Electrophoresis and subsequent staining of these preparations showed that certain bands in the pattern were decreased in staining intensity (concentration) after IPR administration while others remained constant. It was from these latter bands that four, easily recognized, major bands were chosen as representative of the nonexportable proteins. Concentrations of these proteins were determined spectrophotometrically (absorbance at 550 nm) and the average absorbance of these four bands used as a basis for normalizing the counts per minute of ^{14}C in the bands of exportable protein in the control and experimental animals. By using the average absorbance for these four proteins, any minor variability encountered between the relative absorbances of the bands was minimized. Differences between the average absorbance of control and experimental nonexportable proteins (bands 1, 2, 3, and 5) in any of the three time periods studied were $<1.3\%$, while extreme differences were observed between the secretory proteins. The absorbance of the amylase band in experimental preparations, for example, at 2, 6, and 10 h was 13.4%, 21.6%, and 39.6% of the nonstimulated gland value.

Determination of Amylase in the Tissues

The content of parotid gland amylase extractable from the tissue was also monitored over the experimental period studied (10 h) so that the rate of [^{14}C]Phe into secretory proteins could be related to the restitution of gland amylase.

Tissue amylase was extracted by homogenization of the tissue in distilled water. After centrifugation the supernatant was assayed for its amylase activity. α-Amylase activity was determined by hydrolysis of a Cibachron Blue F3GA-amylose substrate. The substrate was prepared as a slurry in a phosphate buffer (pH 7.0) containing 0.02 M sodium chloride. For each alpha bond hydrolyzed, a molecule of dye is released into solution (23). The substrate and an appropriate dilution of the sample are mixed and incubated for 15 min at 37°C in a shaker bath. The reaction is then quenched by the addition of cold phosphate buffer at pH 4.3, the mixture centrifuged, and the absorbance of the supernatant measured at 625 nm in a Bausch and Lomb Spectronic 20 using a red filter and red sensitive phototube. Parallel aliquots were precipitated with 0.5 N PCA and the DNA extracted from the precipitate by acid hydrolysis at 70°C. DNA content of the hydrolysate was determined using the diphenylamine reaction (24) and comparing it to known concentrations of calf thymus DNA. Expression of amylase activity extracted from tissues was made as dye units per milligram DNA.

Electron Microscopy

For electron microscopy anesthetized animals were perfused via left ventricular injection with prewarmed (37°C) 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 (25). Approximately 70 ml were perfused over a 10 min period. After removal and dicing the gland tissue, fixation in the perfusate was continued for 1 h. The tissues were then rinsed in 0.1 M phosphate buffer containing 4.5% sucrose before postfixation in buffered 1% osmium tetroxide. After fixation, tissues were dehydrated through graded alcohols and propylene oxide, then embedded in Araldite: Epon (26). To assess the quality of fixation and the variation in morphology between individual lobules, sections 1 μm in thickness were stained with 1% toluidine blue (pH 9.3) and surveyed before thin sectioning. Thin sections were stained with uranyl acetate and lead citrate.

RESULTS

Evaluation of the In Vitro Incubation Procedure

Parotid slices incubated at 37°C for 1 h in MEM containing carrier-free [^{14}C]Phe (0.7 μM; the lowest concentration of Phe used in these studies) showed linear incorporation of the precursor into PCA-insoluble proteins (Fig. 1). Light- and electron microscopy of tissues incubated for 1½ h at 37°C in the above-labeled medium generally showed good acinar cell preservation. The formation of large cytoplasmic vacuoles similar to those described by Batzri et al. (27) were demonstrable in some focal areas. Dilation of individual rough endoplasmic reticulum (RER) cisternae, nuclear pycnosis, and vesiculation of the Golgi apparatus were observed only in those cells disrupted during slice preparation. The secretory granules contained within intact cells were well preserved.

Incorporation of [^{14}C]Phe into Total Protein

6 h after in vivo administration of IPR, slices of parotid gland acini incubated for 30 min in

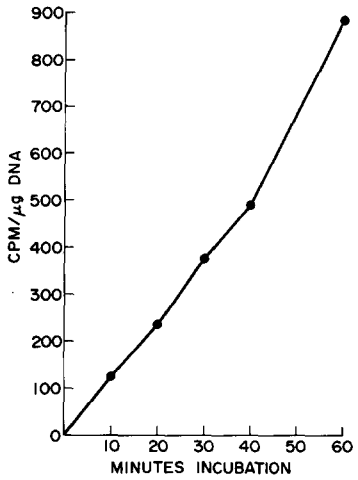

FIGURE 1 Incorporation of ^{14}C into PCA-insoluble proteins of parotid gland slices incubated in MEM containing [^{14}C]Phe (0.7 μM, 315 mCi/mM). Slices were prepared from parotid glands of ad libitum-fed rats killed at 9:00 a.m.

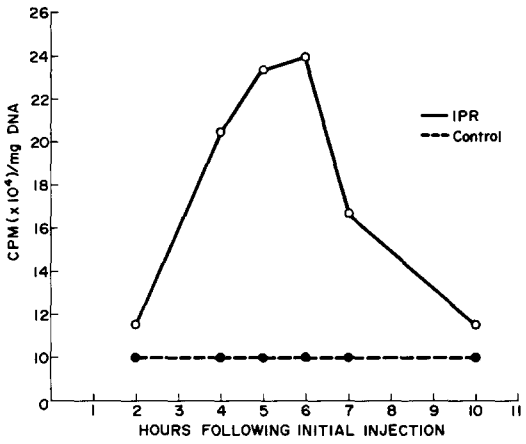

FIGURE 2 In vitro incorporation of [^{14}C]Phe into PCA-insoluble proteins of the rat parotid gland at various times after the administration of IPR. Incubation of gland slices was carried out for 30 min using the medium and concentration of the labeled precursor described in Fig. 1.

medium containing [^{14}C]Phe demonstrated a 2.5-fold increase of radioactivity in PCA-precipitable protein when compared to control slices ($P < 0.005$; five animals in each group) (Fig. 2). This 2.5-fold increase represented the maximum [^{14}C]Phe incorporation for the time periods studied—2, 4, 5, 6, 7, and 10 h after IPR injection. A total of five experimental and five saline-injected controls were used for each time period. In fact, the rate of [^{14}C]Phe incorporation did not begin to significantly ($P < 0.005$) increase above control levels until 4 h after IPR administration, while tissue samples 10 h after treatment no longer showed increased incorporation rates ($P > 0.5$).

Identification of Protein Bands of the SDS-Polyacrylamide Gels

Coincident to the discharge of stored secretory materials induced by IPR, SDS-polyacrylamide gels of water-soluble proteins in the PMS showed reduction in the protein content of bands 4, 6, 7, and 8 (numbers arbitrarily assigned for ease of reference) when compared with control tissue gel profiles (Fig. 3 *a*). Bands 1, 2, 3, and 5 were not reduced after stimulated secretion and were assumed to represent nonexportable proteins. Their relative electrophoretic mobility (R_f) values were found to be 0.19, 0.22, 0.26, and 0.48, respectively. As discussed, the densitometric tracings of these bands (1, 2, 3, and 5) provided the basis for relating the ^{14}C incorporation data from control and experimental PMS preparations. The amylase band was identified by a two-step precipitation sequence using ethanol followed by glycogen. The addition of ethanol to the water-soluble proteins of control tissues resulted in the partial precipitation of an amylase-glycogen complex as was shown by a concomitant reduction of amylase activity in the resulting supernatant. Electrophoretic separation of this preparation (Fig. 3 *b*) shows near-total loss of band 4 with an unmasking of a lower molecular weight protein ($R_f - 0.33$) which formerly appeared only as a shoulder of band 4 ($R_f - 0.37$) in the control pattern. Band 4 was then considered to represent amylase. As an additional check on the identity between amylase and band 4, twice-crystallized hog pancreatic amylase was found to migrate in a fashion identical with the band 4 of control preparations (Fig. 3 *b*)

Distribution of [^{14}C]Phe within Water-Soluble Proteins of the PMS

The position of nonexportable and exportable proteins, especially that of amylase, having been established on the gels, the amount of [^{14}C]Phe incorporated into the various bands was determined in control and IPR-treated animals. Fig. 4 shows the densitometric tracings of a control gel (A; ~25 μg of protein per gel) and an experimental (IPR injected) gel (B) derived from labeled

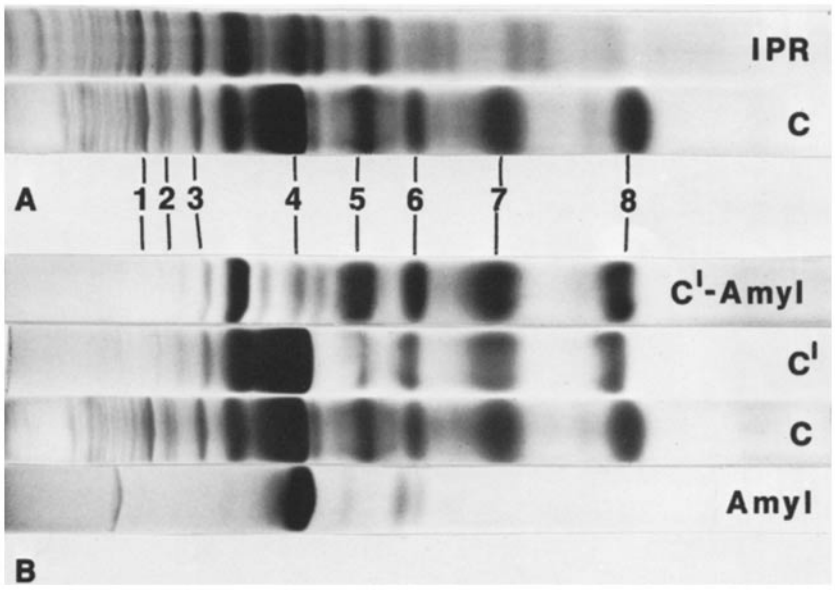

FIGURE 3 SDS-polyacrylamide gels demonstrating: (A) The composition of the postmicrosomal water-soluble proteins of the rat parotid gland 1 h after IPR-induced secretion and sham stimulation. (B) Localization of the electrophoretic band associated with amylase through: (1) absorption of the enzyme from other soluble proteins after exposure to glycogen (C^1-amylase); (2) comparison of the proposed amylase band with the electrophoretic migration of twice-crystallized hog pancreatic amylase. C^1 represents the electrophoretic pattern of an ethanolic solution of control proteins of the PMS. Gels for this figure were chosen for their photographic quality rather than their quantitative densitometric values. *C* = control.

parotid slices obtained at the 6 h time period. Superimposed on each tracing is the corresponding radioactivity profile. Counts derived from control and experimental gels are related to each other through staining intensities of nonexportable proteins (bands 1, 2, 3, and 5), thus allowing semiquantitative comparisons. While [^{14}C]Phe incorporation into nonexportable proteins (bands 1, 2, 3, and 5) showed little variation in the IPR-treated specimen when compared to the control pattern, exportable proteins (bands 4, 6, 7, and 8) were found to have significantly increased cpm in the amylase region with the [^{14}C]Phe incorporation into the major secretory protein, amylase (band 4), exceeding that of the control by 5.3-fold. In addition to these studies at the 6 h time period, results from similar preparations at 2 and 10 h are tabulated in Table 1. From these data a latency in the maximal stimulation of secretory protein synthesis can be recognized. Results of the 10 h time period indicate that [^{14}C]Phe incorporation into amylase was still some 3.3 times that of the control. While these observations on the synthesis of amylase would appear to contradict the findings regarding [^{14}C]Phe incorporation into total protein, the following features of the two studies should be considered: (*a*) The amylase study used *water-soluble* proteins while the incorporation into total protein utilized *PCA-insoluble* proteins. The latter include a very large number of proteins, some of which turn over very slowly; thus the total [^{14}C]Phe in PCA-insoluble proteins cannot be strictly compared to amylase alone. (*b*) Longer incubation times (60 min) were used for the gel (amylase) studies than the studies of [^{14}C]Phe into total protein (30 min). The longer labeling time would serve to accentuate differences between control and experimental tissues. There is an indication that differences may in fact exist in the total protein studies. The elevated mean of the experimental group at 10 h while not statistically significant with a 30 min incubation *might* be significant were the incubation thus extended to 1 h. The important correlation of these two studies was that by 10 h the peak of synthetic activity had passed. Table I also summarizes the effect of IPR on the PCA-soluble [^{14}C]Phe pool. While some fluctuations in the pool can be demonstrated, they are not marked and are probably insignificant.

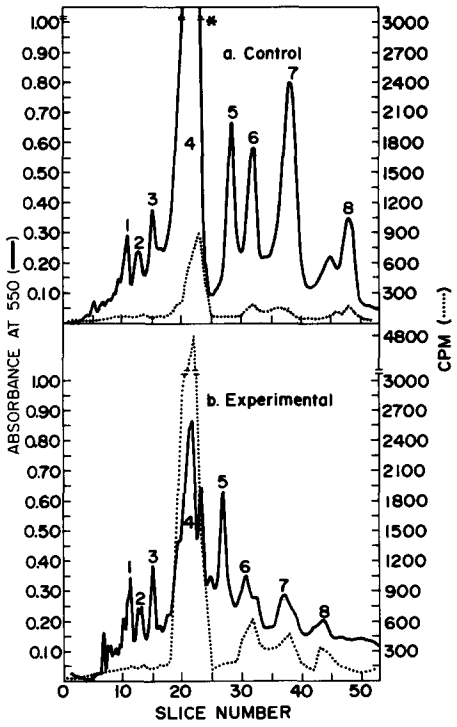

FIGURE 4 Densitometric profiles of SDS-polyacrylamide gels prepared from water-soluble proteins of the postmicrosomal fraction. The incorporation of [^{14}C]Phe into the various proteins present in the gels (~25–30 μg per gel) is also depicted. The counts per minute data from A and B are quantitatively related through their concentration of nonsecretory proteins (peaks 1, 2, 3, and 5). The Materials and Methods section describes the procedures used for normalizing the amounts of protein loaded onto the gels.

* Equals one quarter total height of α-amylase peak; based on estimates from serial dilutions of sample run on parallel gels.

TABLE I

The Effect of IPR Administration on Protein Synthesis in Rat Parotid Gland Slices

Time after IPR*	Radioactivity of amylase band‡ (cpm, % of control)	Radioactivity of pool (cpm, % of control)
2	145 ± 6	92.2
6	533 ± 11	100.7
10	330 ± 8	95.7

Incorporation of ^{14}C into the amylase band after SDS gel electrophoresis of the postmicrosomal water-soluble proteins. The percentages shown for the amylase study are absolute values of the cpm found in the amylase band not cpm/absorbance 550. The table also demonstrates a relatively stable, free Phe pool at the three time period studied.

* 0.8 mg/100 g b.w.

‡ Samples within each time period were compared through their content of nonsecretory proteins (average for three gels at each time period).

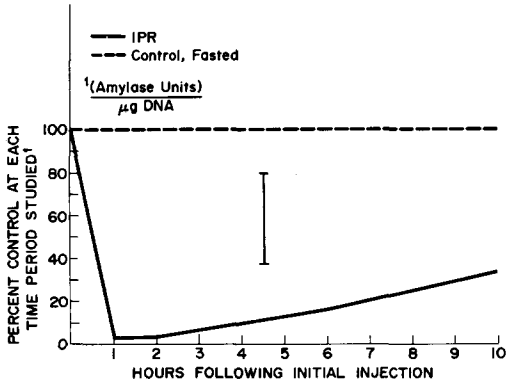

FIGURE 5 Restitution of parotid gland amylase after IPR-induced secretion. The bracket appearing in the center of the graph denotes the diurnal range of tissue amylase found in ad libitum-fed animals (MacCallum and Lillie, 1973).[4]

Levels of Parotid Gland Amylase

Amylase levels after IPR-induced secretion and during resynthesis are shown in Fig. 5. Administration of IPR led to the release of approximately 98% of tissue amylase within 1 h when compared with this enzyme's level in the fasted control gland. While accumulation of resynthesized amylase had only reached 38% of the fasted control content by 10 h, this value falls within the range of diurnal fluctuations for the rat parotid gland.[4] The amount of amylase activity observed at the time points sampled in our study also are in agreement with earlier reports (12, 13) regarding restitution of amylase activity after IPR-induced release.

Ultrastructural Observations

The fine structure of acinar cells obtained from fasted animals was essentially the same as that reported by previous workers (28, 29, 30, 31). Although variations were observed in the number of secretion granules present within acinar cells of fasted animals, the number of granules within the majority of cells studied was quite large (Fig. 6). The most frequently observed pattern was that of

[4] MacCallum, D., and J. Lillie. 1973. In preparation

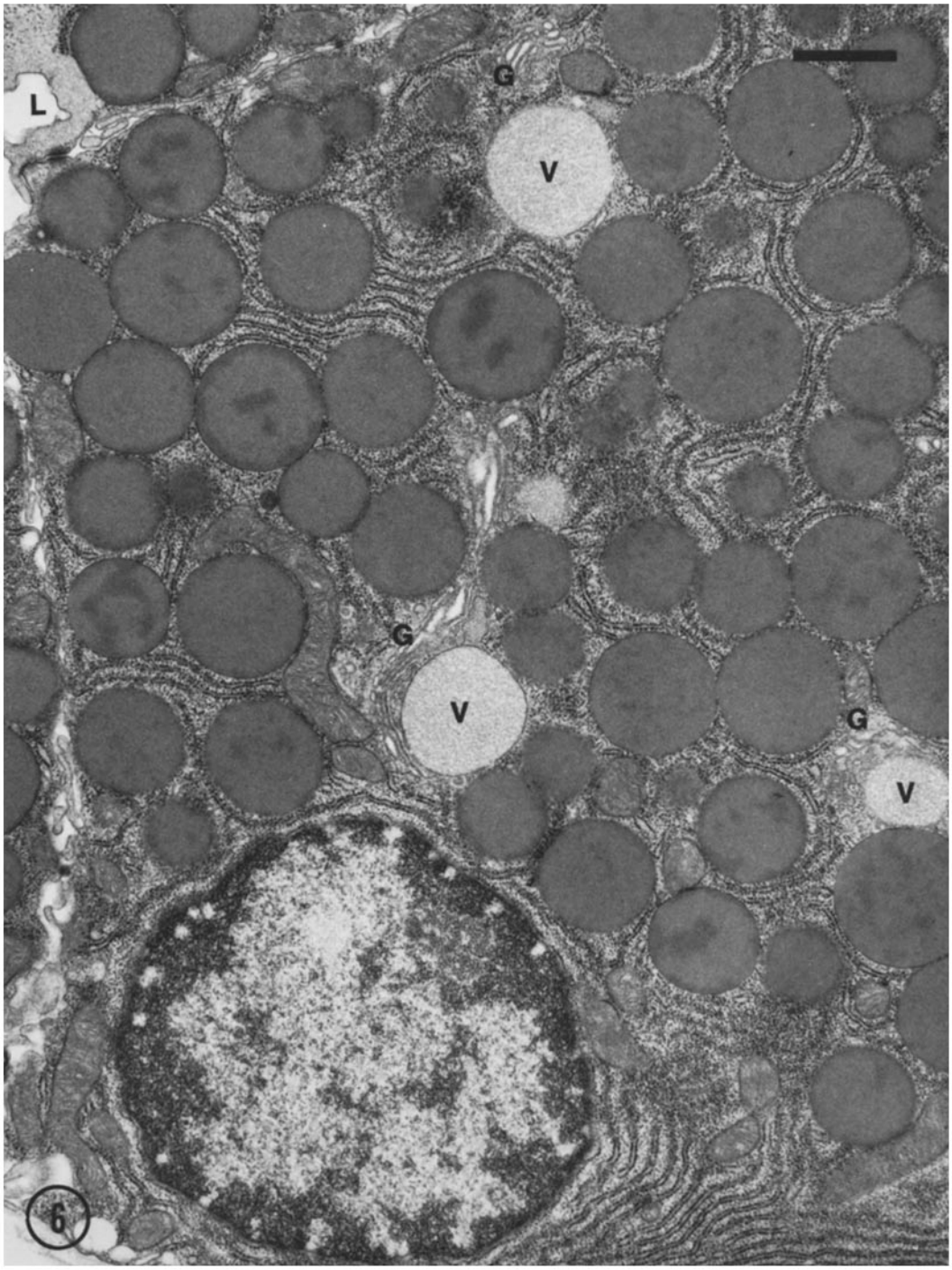

FIGURE 6 Parotid acinar cell taken from an animal fasted for 16 h. Mature secretion granules fill the cytoplasm. Golgi cisternae (*G*) and associated electron-lucent condensing vacuoles (*V*) are present in three different regions of the cytoplasm (× 15,000; bar on all micrographs = 1 μm, *L* = lumen).

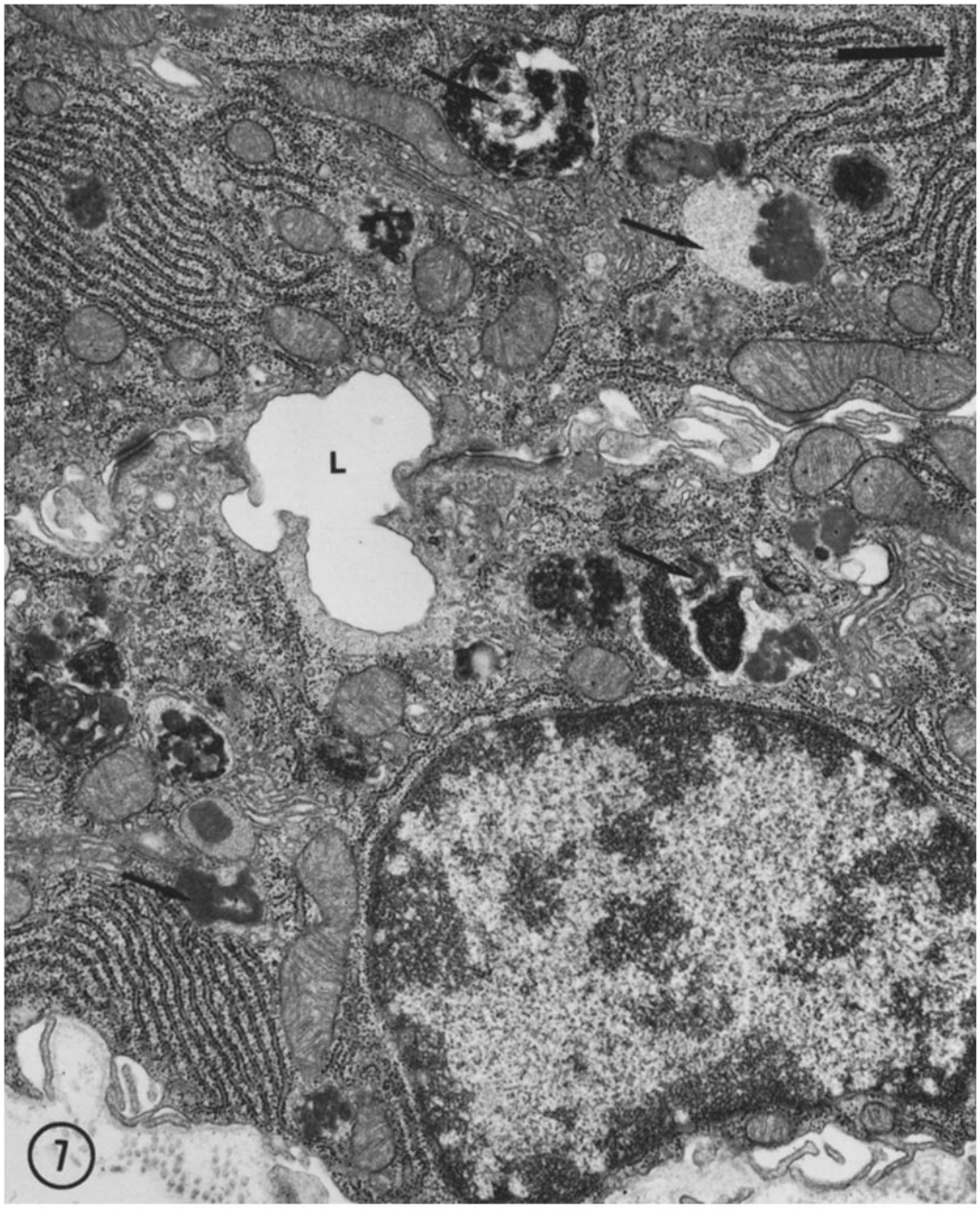

FIGURE 7 Parotid acinar cells 2 h after IPR administration. Secretion granules are absent and the lumen (*L*) is slightly enlarged and has an irregular border. Numerous RER arrays and autophagic vacuoles of varying composition (arrows) are typical of this time period. × 15,000.

large numbers of granules occupying the apical three-quarters of the cytoplasm with a lesser number interspersed between the RER cisternae. The number of secretory granules in the fasted animals was always greater than that of animals killed 10 h after IPR administration or ad libitum-fed animals killed between 5:00 and 6:00 p.m. (unpublished observation). 2 h after IPR injection, acinar cell

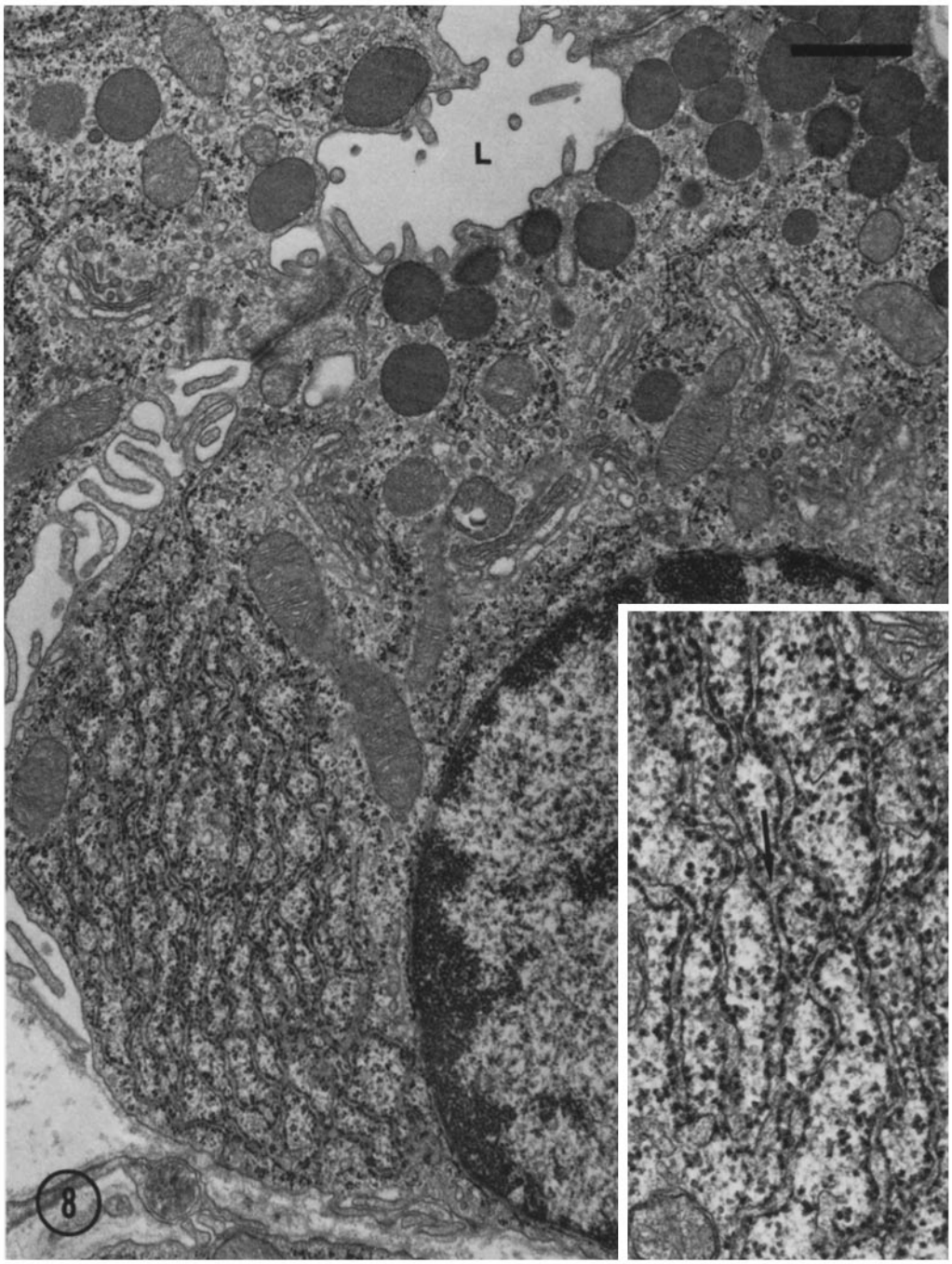

FIGURE 8 Parotid acinar cells 6 h after IPR administration. Newly formed secretion granules are located in the cell apices. These granules are consistently smaller in size (0.6 ± 1 μm) than those found in fasted animals (1.2 ± 0.1 μm). The Golgi apparatus is usually as depicted here, a contiguous series of vesicles and lamellae which occupy a considerable portion of the supranuclear cytoplasm. Acinar cells at this time period frequently possessed a distinctive arrangement of the RER. Adjacent cisternae repeatedly apposed and separated from one another in an irregular fashion. Occasionally, points of union (arrow) between the cisternae could be observed. The number of polyribosomes applied to the cisternal membranes also appeared reduced. The significance of this particular form of RER is unclear. × 17,000, *inset*, × 39,000.

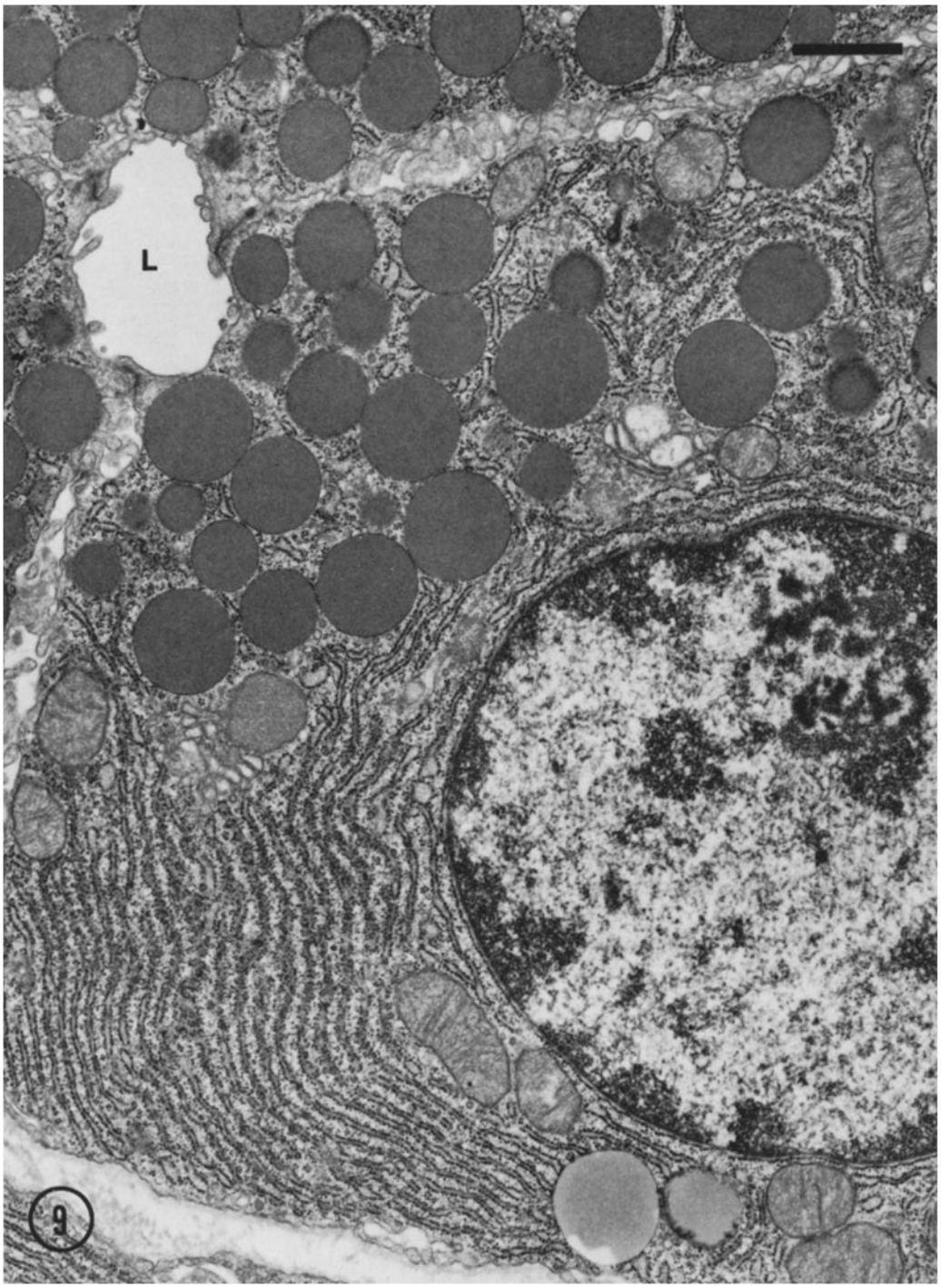

FIGURE 9 Parotid gland acinar cell 10 h after IPR administration. An increase in both the number and size of the secretion granules is evident when compared with the 6 h post-IPR period. The Golgi apparatus is dispersed into smaller units and the RER has returned to a more typical conformation. × 16,000.

structure was markedly altered. The cells were smaller and the intercellular spacing notably increased. Acinar lumina presented a gently scalloped contour and were moderately larger in size than those found in fasted animals (Fig. 7). Occasionally one or two secretion granules were found in the apex of the cells; however, most cells did not contain any secretion granules. In addition to mitochondria, RER, and the Golgi complex, a wide variety of inclusions were observed in these cells. Some of these were of an autophagic nature, being membrane-bound clumps of RER or altered mitochondria surrounded by whorls of membranes. Other inclusions were characterized by masses of electron-opaque, flocculent material. These latter inclusions were bounded by membranes. Alternatively, masses of this same flocculent material appeared to lie free in the ground cytoplasm with no discernible bounding membrane (Fig. 7). 6 h after IPR administration, the acinar cells had expansive Golgi complexes with moderate numbers of Golgi-associated, immature secretory granules (Fig. 8). Small numbers of electron-opaque secretion granules occupied the apical portions of the cell. In many of the cells (approximately 60%) studied at this time period, the RER presented an unusual appearance. One cisterna appeared to repeatedly converge and diverge from an adjoining one much in the fashion of several very irregular figure eights piled on top of one another. The number of polyribosomes applied to the cisternal membranes appeared reduced, resulting in short segments of smooth membrane alternating with segments bearing polyribosomes. 10 h after IPR injection (Fig. 9), the acinar cells demonstrated increased numbers of apically located secretion granules. These zymogen granules were also considerably larger (1.2 ± 0.1 μm) than those observed at the 6 h interval (0.6 ± 0.1 μm) though some granules similar to those seen at the earlier period were occasionally observed in the most apical portions of the cytoplasm. The Golgi complex and associated condensing vacuoles and immature secretion granules were still prominent. The membranes of the RER appeared covered by attached polyribosomes, and the cisternae were arranged in the usual extensive, parallel arrays.

DISCUSSION

It has been established that drugs which induce secretion may not simultaneously stimulate protein synthesis (3). Secretion can also occur in the absence of protein synthesis (6, 32). These observations do not, however, preclude a relationship between secretion (release) and synthesis. A number of studies (4, 7, 33) including this one have demonstrated increased rates of protein synthesis after stimulated secretion when the rate of synthesis was measured some time after the period of secretion. There does, however, appear to be a temporal dissociation of secretion from synthesis at least in the parotid gland.

A 6 h interval between the onset of secretion and the peak of protein-synthetic activity, as observed in our system, has also been depicted in studies of the mastication-dependent diurnal cycle of rat parotid secretion (7). And more recently Johnson and Sreebny have reported the effect of IPR as a substitute for mastication in the induction of secretion (34). While both of their studies support the concept of a reciprocal relationship between secretion and synthesis in the rat parotid gland, attention is not drawn to certain temporal aspects of their work. Both studies clearly demonstrate that 4–6 h after the onset of induced secretion (either by mastication or IPR) there is a maximum stimulation of protein synthesis. Despite the difference between our reference index (DNA) and theirs (total gland weights), and difference in labeling techniques (in vivo vs. in vitro), the results from the two groups of investigators are quite comparable. The maximal synthetic response in both instances was appreciably separated from the onset of secretion. While the Johnson and Sreebny (34) study showed this response to occur 4 h after IPR administration, the 6 h period was not sampled. Mongeau et al., using totally in vitro methodology, have also found early dissociation of protein synthesis and amylase secretion after hormonal stimulation of the pancreas.[5] Utilizing DNA as an index of tissue cellularity, their results show that protein synthesis is initially depressed. However, 30–45 min after in vitro stimulation protein synthesis is significantly increased. This rapidity of pancreatic response is in agreement with this tissue's general activity levels as indicated by the rates of pancreatic secretory protein synthesis and transport (49, 50).

One of the ways in which the latency observable under these conditions could be accounted for may be related to the gland's energetics. Release of

[5] Mongeau, R., Y. Couture, J. Dunnigan, and J. Morisset. 1973. Submitted for publication.

secretory materials from the parotid gland is energy dependent (35), presumably reducing ATP stores within the cell. Concomitant with catecholamine-stimulated secretion there is a rise of cyclic AMP (36, 37), thus adding an additional, though smaller, drain on ATP. Paralleling the onset of secretion is a rise in intracellular Ca which has been shown to be essential for secretion (38, 39, 40). High concentrations of ionic Ca also, however, inhibit oxidative phosphorylation, the major source for the genesis of high energy phosphates within the parotid gland (41, 42, 43). In this regard, it is interesting to note that, while parotid gland mitochondria contain 10 times the amount of Ca found in liver mitochondria, they are approximately 15 times more sensitive to the inhibitory effects of Ca++ on respiration (41). The continued presence of the adenyl cyclase stimulant in the medium (in vitro) or gland (in vivo) may lead to continued cyclic AMP generation until subsequent buildup of intracellular ionic Ca finally inhibits adenyl cyclase activity (39). Both cyclic AMP generation and high levels of intracellular ionic Ca for the duration of the secretory process (about 2 h in the IPR-treated rat parotid gland [44]) would continue to reduce the level of ATP within the cell. The decrease of existing pools of ATP during secretion and the marked reduction in the cells' capacity to generate new ATP would appear to delay the onset of energy-dependent protein synthesis (45). At the moment, this line of reasoning to explain the latency of protein synthesis is at best inferrential. It would appear, however, that investigation along the lines stated above would be warranted.

Morphologic aspects which may further explain the length of the period between release and increased synthesis may reflect the necessity for reorganization of cellular organelles after the active process of secretion. Though the extreme purge of secretory materials affected by IPR treatment exceeds the limit of the normal secretory range, the IPR system does have some ultrastructural similarity to reported findings in the normal gland. Reorganization of the RER and Golgi complex and an apparent increase in autophagy, shown by this study and by others (13 and 14) dealing with the IPR-stimulated parotid, are similar to those reported (46) in the gland of the ad libitum-fed rat. It would appear reasonable to suspect that such intracellular reorganization would also require some energy and be a necessary antecedent to secretory protein synthesis. Description of these morphologic changes after both secretion induced by feeding (46) as well as IPR (13 and 14) and observations of the latent period between secretion and synthesis of proteins in the normal diurnal cycling gland (7), suggest that our observations of the IPR-treated parotid are not merely a reflection of cell damage, a proposition recently advocated by Simson (47).

The long labeling periods used in this work, which are longer than those employed in earlier studies on the guinea pig pancreas appear justified. It has been shown that the secretory cycle of the parotid acinar cell in the ad libitum-fed rabbit (48) is basically the same as that of the guinea pig pancreas (49, 50), except that past the condensing vacuole stage, intracellular transport is much slower. For example, autoradiographic grain counts demonstrated that the mature granules were labeled to a greater degree than any other intracellular compartment 6 h after a 4 min pulse of [^{3}H]leucine. This prolonged storage phase allows relatively long labeling periods without concern for the loss of incorporated material through secretory expulsion (exocytosis).

It may be a matter of semantics, but it would appear that any stimulation which induces release of a stored exocrine product does not in itself stimulate the synthesis of secretory protein, but rather the reduced level of intracellular secretory protein calls for a compensatory burst of synthetic activity. The parotid gland model suggests that the synthesis of its exportable proteins, especially that of amylase, is controlled through a positive feedback mechanism which is latently expressed in response to the low levels of stored secretory material.

We gratefully acknowledge the help and encouragement of Dr. Donald K. MacCallum in the preparation of this manuscript.

This work was supported by National Institutes of Health Fellowship DE 43007 and grants DE 02731 and DE 03348.

Received for publication 14 March 1973, and in revised form 30 August 1973.

REFERENCES

1. HOKIN, L. E., and M. R. HOKIN. 1953. *J. Biol. Chem.* **203**:967.
2. POORT, C., and M. F. KRAMER. 1969. *Gasteroenterology*. **57**:689.
3. JAMIESON, J. D., and G. E. PALADE. 1971. *J. Cell Biol.* **50**:135.

4. FARBER, E., and H. SIDRANSKY. 1956. *J. Biol. Chem.* **222**:237.
5. RAPP, G. W. 1961. *J. Dent. Res.* **40**:1225.
6. GRAND, R., and P. R. GROSS. 1969. *J. Biol. Chem.* **244**:5608.
7. SREEBNY, L. M., D. A. JOHNSON, and M. R. ROBINOVITCH. 1971. *J. Biol. Chem.* **246**:3879.
8. GROMET-ELHANAN, Z, and T. WINNICK. 1963. *Biochim. Biophys. Acta.* **69**:85.
9. GRAND, R. J. 1969. *Biochim. Biophys. Acta.* **195**: 252.
10. BARKA, T. 1971. *Exp. Cell. Res.* **64**:371.
11. EKFORS, T., and T. BARKA. 1971. *Exp. Cell Res.* **66**:11.
12. BYRT, P. 1966. *Nature (Lond.).* **212**:1212.
13. AMSTERDAM, A., I. OHAD, and M. SCHRAMM. 1969. *J. Cell Biol.* **41**:753.
14. SIMSON, J. V. 1969. *Z. Zellforsch. Mikrosk. Anat.* **101**:175.
15. SCHRAMM, M. 1968. *Biochim. Biophys. Acta.* **165**: 546.
16. JAMIESON, J. D., and G. E. PALADE. 1967. *J. Cell Biol.* **34**:577.
17. HINRICHS, J., R. PETERSEN, and R. BASERGA. 1964. *Arch. Pathol.* **78**:245.
18. SCHRAMM, M., and A. BDOLAH. 1964. *Arch. Biochem. Biophys.* **104**:67.
19. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. *J. Biol. Chem.* **193**:265.
20. RUDDON, R. W., and C. H. RAINEY. 1971. *FEBS Lett.* **14**:170.
21. REYNOLDS, J. A., and C. TANFORD. 1970. *J. Biol. Chem.* **245**:5161.
22. LOYTER, A., and N. SCHRAMM. 1962. *Biochim. Biophys. Acta.* **65**:200.
23. KLEIN, B., J. A. FOREMAN, and R. L. SEARCH. 1970. *Clin. Chem.* **16**:32.
24. BURTON, K. 1968. *Methods Enzymol.* **12**:163.
25. KARNOVSKY, M. J. 1965. *J. Cell Biol.* **27**:137A. (Abstr.).
26. PALEVITZ, B. A., and E. H. NECOMBE. 1970. *J. Cell Biol.* **45**:383.
27. BATZRI, S., Z. SELINGER, and M. SCHRAMM. 1971. *Science (Wash. D. C.).* **174**:1029.
28. SCOTT, B. L., and D. C. PEASE. 1959. *Am. J. Anat.* **104**:115.
29. RUTBERG, U. 1961. *Acta Odontol. Scand.* **19**:7.
30. PARKS, H. F. 1961. *Am. J. Anat.* **108**:303.
31. HAN, S. S. 1967. *Am. J. Anat.* **120**:163.
32. JAMIESON, J. D., and G. E. PALADE. 1971. *J. Cell Biol.* **48**:503.
33. WEBSTER, P. D., and M. P. TYOR. 1966. *Am. J. Physiol.* **211**:157.
34. JOHNSON, D. A., and L. M. SREEBNY. 1973. *Lab. Invest.* **28**:263.
35. BDOLAH, A., and M. SCHRAMM. 1962. *Biochem. Biophys. Res. Commun.* **8**:266.
36. BDOLAH, A., and M. SCHRAMM. 1965. *Biochem. Biophys. Res. Commun.* **18**:452.
37. SCHRAMM, M., and E. NAIM. 1970. *J. Biol. Chem.* **245**:3225.
38. SELINGER, A., and E. Naim. 1970. *Biochim. Biophys. Acta.* **203**:335.
39. RASMUSSEN, H. 1970. *Science (Wash. D. C.).* **170**: 404.
40. POISNER, A., and J. HONG. 1973. The Proceedings of Symposium on Cytopharmacology of Secretion. Raven Press, New York. In press.
41. FEINSTEIN, H., and M. SCHRAMM. 1970. *Eur. J. Biochem.* **13**:158.
42. BAUDIN, H., M. COLIN, and A. DUMONT. 1969. *Biochim. Biophys. Acta.* **174**:722.
43. BABAD, H., R. BEN-ZVI, A. BDOLAH, and M. SCHRAMM. 1967. *Eur. J. Biochem.* **2**:96.
44. BYRT, P. 1966. *Nature (Lond.).* **212**:1212.
45. MAHLER, H. R., and E. H. CORDES. 1966. Biological Chemistry. Harper and Row Publishers, New York. 802.
46. HAND, A. 1972. *Am. J. Anat.* **135**:71.
47. SIMSON, J. A. V. 1972. *Anat. Rec.* **173**:437.
48. CASTLE, J. D., J. D. JAMIESON, and G. E. PALADE. 1972. *J. Cell Biol.* **53**:290.
49. JAMIESON, J. D., and G. E. PALADE. 1967. *J. Cell Biol.* **34**:577.
50. JAMIESON, J. D., and G. E. PALADE. 1967. *J. Cell Biol.* **34**:597.