

SECRETION OF OLD VERSUS NEW EXPORTABLE PROTEIN IN RAT PAROTID SLICES

Control by Neurotransmitters

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ABSTRACT

The possibility that old and new secretory granules do not mix and that older exportable protein can be secreted preferentially was tested on parotid gland *in vitro*. Slices from fasted animals were pulse labeled for 3 min with L-[³H]leucine. Subcellular fractionation showed that after a 90-min chase period, the formation of new labeled secretory granules was mostly completed. The ratio of label in secretory granules to label in microsomes increased 250-fold during the period 5–90 min postpulse. After the 90-min chase, a submaximal rate of secretion was initiated by adding a low concentration of isoproterenol to the slices. Preferential secretion of old unlabeled exportable protein was evident from the finding that the percent of total amylase secreted was 3.5-fold greater than the percent of labeled protein secreted. Preferential secretion of old unlabeled exportable amylase was undiminished even when the chase period before addition of isoproterenol was extended to 240 min. Such long chase incubations were still meaningful due to the fact that the spontaneous rate of amylase release and radioactive protein release from the slices was negligibly low. A high isoproterenol concentration added to the slices after a 90-min chase produced the following results. An initial phase of preferential secretion of old unlabeled protein was soon replaced by secretion of a random mixture of new and old exportable protein. Electron micrographs indicated that high rates of secretion involved sequential fusion of secretory granules so that the lumen extended deep into the cell where the new labeled granules were presumably located. At low rates of secretion, the lumen showed no such deep extensions. Experiments were also conducted on slices from glands which had been largely depleted of old granules by prior injection of isoproterenol into the animals. Secretion of labeled protein from such slices stopped with the export of 80% of the labeled protein. This finding indicates that about 20% of the radioactive protein is cellular nonexportable protein and that the slices are capable of exporting the entire amount of secretory protein which was synthesized *in vitro*.

In addition to the β -adrenergic receptor which mediates protein secretion, the parotid acinar cell also possesses an α -adrenergic and a cholinergic receptor both of which cause K⁺ release, vacuole formation, and water secretion. Activation of

either of the latter two receptors in conjunction with the β -adrenergic receptor increased randomization of the protein secreted.

It is concluded that in the rat parotid acinar cell there is little spontaneous mixing between old granules near the luminal cell membrane and new granules coming up behind from the Golgi complex. The neurotransmitters which induce secretion produce the observed randomization.

The pathway of intracellular transport of exportable protein in exocrine glands was established several years ago (7, 12-14, 24). It was shown that protein newly synthesized on the ribosomes moves into the cisternae of the endoplasmic reticulum and from there to the Golgi complex, to emerge finally as a membrane-bounded package for export, the secretory granule. The newly produced granule joins the population of previously formed granules which congregate near the apex of the cell facing the lumen.

The granules might be in random motion so that new and old granules mix or, alternatively, the new granules line up behind the older granules, thus assuming a position farthest away from the lumen into which secretion will take place. In rabbit parotid gland, the newly formed granules remained basally located even several hours after labeling (7). In contrast, in guinea pig pancreas, the newly labeled granules readily became evenly distributed throughout the granule population (13). It is not known whether the hormones which cause secretion bring about any mixing of old and new secretory granules. Previous work on guinea pig pancreatic slices indicated that the discharge of old and new secretory protein can occur in random fashion (14). However, as discussed later, the experimental conditions may determine whether secretion will be random or whether old exportable protein will be discharged preferentially. In the present study, these questions are approached by pulse labeling the exportable protein in rat parotid slices and further incubating the slices without adding inducers of secretion until the new exportable protein has arrived in the secretory granule population. Only then, various neurohormones are added to cause secretion of the granule content. The relative rate of amylase secretion is compared with the relative rate of radioactive protein secretion. The experiments indicate that there is little spontaneous mixing of old and new granules, that submaximal activation of the β -adrenergic receptor causes preferential secretion from old granules and that the additional activation of the α -adrenergic receptor or the cholinergic receptor increases the mixing of old and new secretory material.

MATERIALS AND METHODS

Treatment of Rats

Albino male rats weighing 180-250 g were kept as previously described (27). Unless otherwise noted, animals fasted overnight were used. For some experiments, animals were injected with reserpine in order to deplete sympathetic nerve endings of catecholamines (20) and cause massive accumulation of secretory protein (26). Fed rats received 0.25 mg reserpine intraperitoneally 40 and 20 h before the experiment. Glands partially depleted of secretory protein were also prepared. For this purpose, fed animals received 1 mg of isoproterenol intraperitoneally 2 h before removal of glands. The catecholamine was dissolved just before the injection in 0.5 ml of 0.15 M NaCl containing 0.1 mM ascorbic acid to prevent oxidation.

Incubation of Parotid Slices

All stages of tissue preparation and experiments were carried out in nutrient medium F-12 (11, 7) at 37°C with continuous shaking under a mixture of 95% O₂, 5% CO₂. Rats were killed by cervical dislocation and bled by cutting through the heart. Parotid slices were prepared as previously described (27) with the following modifications. Preincubation of 5-10 min was carried out with constant gassing through an F-12 medium which was without both L-leucine and Ca⁺⁺. This was a leucine-less medium available. The omission of Ca⁺⁺ is of no significance. The slices at one gland equivalent/milliliter medium were pulse labeled for 3 min with 0.1 mM L-[³H]leucine (20-40 μ Ci/ml). The pulse was terminated by washing the slices with F-12 medium containing 1 mM leucine and 0.3 mM Ca⁺⁺. The slices were divided into equal portions, each equivalent to two glands and were further incubated under the O₂/CO₂ mixture in stoppered 50-ml Erlenmeyer flasks containing 5 ml of the latter medium. At the end of the incubation which served as a chase period, the procedure of gassing was changed to permit rapid sampling of the medium during secretion. Rubber stoppers with inlet and outlet gassing tubes were placed on the flasks, and the gas mixture was passed over the medium continuously. Shaking was stopped only briefly for removal of medium aliquots. A zero-time aliquot was taken immediately before addition of an inducer of secretion. Catecholamines were added to the medium in a solution containing 2-mercaptoethanol and ascorbic acid so that the final concentration of the latter two reagents in the incubation medium was 1 mM and 0.1 mM, respectively. These reagents prevent oxidation of the hormones. Secretion was monitored as

previously described (27). A single aliquot served for the determination of both amylase and radioactive protein. Secretion of both is expressed as percent of total released from the tissue into the medium. Results are given in the figures after subtraction of the value for the medium at zero time. Each experiment was repeated at least three times.

Preferential Ratio

The percent of total amylase in the slices which is secreted into the medium serves as a measure of secretion of all exportable proteins (24). The percent of total pulse-labeled protein which is secreted into the medium serves as a measure of secretion of new exportable protein. This latter term is justified as a close approximation since 80%, at least, of the labeled protein is exportable protein (24, and the present work). The preferential ratio is defined as percent of total amylase secreted per minute divided by percent of total radioactive protein secreted per minute. The preferential ratio gives a measure of the extent at which old unlabeled exportable protein is secreted in preference to the new labeled protein. Thus, a high number signifies a high degree of order whereby old secretory protein is released while the newly formed labeled secretory protein is still retained in the cell. In contrast, random secretion of old and new protein is expressed by a ratio of one. In some experiments, the rate of secretion of amylase and of labeled protein changed during the incubation period. Therefore, preferential ratios were calculated separately for each time interval during which the rates were essentially constant. For key experiments which were repeated many times, the preferential ratio is given as the mean \pm standard error of the mean.

Isolation of Subcellular Fractions

Subcellular fractionation after pulse labeling was performed as described previously (15). At the end of the chase period and before homogenization, the parotid slices were washed twice by sedimentation at 1,500 g for 1 min in cold homogenization medium. The 250-g supernate of the homogenate, which was loaded on the gradient, and the subfractions, which were collected from it, were assayed for amylase content. The amount of protein and radioactivity in protein were measured after overnight dialysis of the fractions against distilled water. Further precipitation by trichloroacetic acid and extractions by solvents did not remove significant amounts of counts.

Analytical Procedures

Amylase was determined by the Bernfeld method (5) and protein according to the procedure of Lowry et al. (17). Radioactive protein was measured as follows: samples received 2–3 mg of bovine serum albumin and trichloroacetic acid to give a concentration of 10% wt/vol in a vol of 3 ml. The precipitate after centrifugation was dissolved in 0.1 ml of 1 N NaOH and reprecipitated

by 3 ml of 10% trichloroacetic acid. The final precipitate was dissolved in 0.2 ml of Soluene 350 (Packard Instrument Co., Inc., Downers Grove, Ill.) and counted in a toluene base scintillation mixture. Counting efficiency was about 30%.

Electron Microscopy

Procedures were essentially as described by Amsterdam et al. (1). Just before fixation, the slices were transferred from the F-12 incubation medium into freshly gassed Krebs-Ringer bicarbonate medium containing 4% vol/vol glutaraldehyde. It should be emphasized that parotid gland can be well preserved for electron microscopy provided the tissue is thoroughly oxygenated up to the instant of addition of the glutaraldehyde fixative.

Materials

Nutrient mixture F-12 was obtained as a powder from Grand Island Biological Co., Grand Island, N. Y., and liquid nutrient mixture F-12, without L-leucine and Ca^{++} , from Bio-Lab Ltd. Laboratories, Jerusalem, Israel. L-[3H]leucine was a product of Nuclear Research Centre, Negev, Beer-Sheba, Israel. Reserpine was purchased from Teva Middle East Pharmaceutical and Chemical Works, Jerusalem, Israel, and Soluene 350. All other chemicals were of highest purity available.

RESULTS

Time Required for Transport of New Protein to the Secretory Granules and for Its Subsequent Secretion

It was intended to initiate secretion only after the new pulse-labeled secretory protein had already reached the secretory granule population. Thus, transport to the granules would occur without lumen enlargement (1), water flow (4), or any other effects which are caused by the inducers of secretion. For this purpose, it was necessary to establish the time interval required for the newly synthesized protein to reach the secretory granule population. Pertinent findings are presented in Table I and Fig. 1. Table I shows that the distribution of amylase and protein among the subcellular fractions does not change much from 5 to 90 min postpulse. Therefore, the decline in labeling of the microsomes and the striking rise in labeling of the secretory granules during the above time period must be due to the net transport of labeled protein from one cellular structure to the other. Changes in the distribution of labeled protein are more reliable criteria for transport than the often used parameter of specific radioactivity (cpm/mg protein). This is because the latter, being a ratio, is

TABLE I
Distribution of Radioactive Protein and Amylase in Subcellular Fractions of Parotid Slices after Pulse Labeling

Fraction	Chase period:	Radioactive protein in fraction		Protein in fraction		Amylase activity in fraction		Specific radioactivity	
		5 min	90 min	5 min	90 min	5 min	90 min	5 min	90 min
		%		%		%		cpm/mg protein	
Microsomes		60	9	18	15	0.6	0.7	16	6
Secretory granules		1	31	25	23	50	42	0.2	13
Mitochondria		15	9	10	10	4	4	7	8
Postmicrosomal supernate		29	37	45	42	47	53	3	8
Recovery (sum of fractions)		105	86	98	90	102	100		

Slices were prepared from parotid glands of 12 fasted rats. Propranolol ($1 \mu\text{M}$) was present in all incubation media to prevent secretion caused by endogenous catecholamines. After the pulse, the slices were washed and divided into four equal portions: one portion was fractionated immediately (5 min from the onset of the pulse), the other portions were further incubated in stoppered 250-ml Erlenmeyer flasks containing 20 ml of chase medium and were fractionated at 20, 90, and 120 min postpulse (see also Fig. 1). The results are expressed as percent of the total amount which was loaded on each gradient tube. The average load contained 45 mg of protein, 0.36×10^6 cpm in protein, and 13,000 U of amylase.

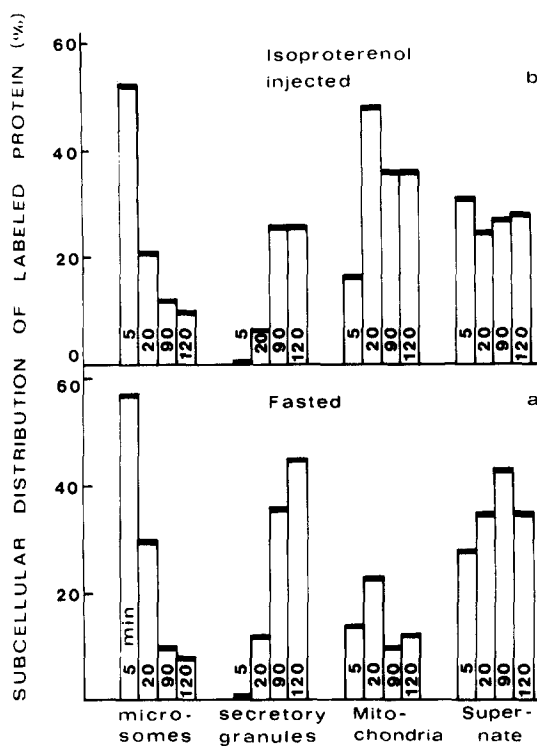


FIGURE 1 Changes in the distribution of radioactive protein in subcellular fractions from parotid slices as a function of time after pulse labeling. (a) Glands from fasted animals were used. Experimental details are given in Table I. The results are shown as percent of the sum of all fractions which is taken as 100. The numbers in the bars show the time in minutes from the onset of the pulse to the beginning of homogenization. (b) As in (a), but glands were from isoproterenol-injected rats which were thus partially depleted of secretory protein. In order to

more sensitive to errors, and gives no information on the relative amount of material transported (see Table I). Fig. 1a gives the results of transport in slices from fasted animals which contain a huge amount of stored secretory granules. It is shown that the percent of total radioactivity which is in the secretory granule fraction rises from 0.9% to 36% in the period of 5–90 min after the pulse of labeled leucine. During this time period, the ratio of percent label in secretory granules to percent label in microsomes increased 250-fold. It can be seen that extension of the chase period to 120 min caused only a small further increase in the percent of label in the secretory granule fraction. Relatively, large amounts of amylase and labeled protein are found in the postmicrosomal supernate. The amounts do not change much from 5 to 120 min after labeling. As observed and discussed in earlier work, secretory proteins in the supernate seem to derive from secretory granules and microsomes damaged during homogenization (4).

Transport was also tested on glands which had been emptied of most of the secretory protein by injection of isoproterenol into the animal 2 h before the experiment (Fig. 1b). The experiment with these slices differed from the experiments with slices from fasted animals, in two respects. The incorporation of radioactivity per gland was three to five times higher in the slices which had

have a sufficient amount of secretory granules for fractionation, two glands from fasted rats were added to the labeled slices before homogenization. The average load per gradient tube was: 67 mg protein, 1.7×10^6 cpm, and 11,000 U of amylase.

been partially depleted of exportable protein. A very considerable amount of radioactivity sedimented in a light fraction together with the mitochondria even at 120 min postpulse. This latter fraction is under further study to establish its relation to a presumed intermediate fraction in transport previously described (24). It might also be similar to the small storage granules formed in pancreas slices after stimulation (14). The transport in the partially depleted slices (Fig. 1*b*), as in slices from fasted animals (Fig. 1*a*), seemed to be nearly complete at 90 min postpulse.

The fractionation experiment showed that a considerable amount of newly formed exportable protein reaches the granule fraction 20 min postpulse. The incorporated label in this fraction is probably sequestered in the early maturation stages, the condensing vacuoles (12, 1), which may or may not function in secretion. It was therefore of interest to determine at what time after pulse labeling the newly formed protein can be secreted. It is shown that when isoproterenol is added 12 min after the beginning of the pulse, there is no significant release of labeled protein for an additional 30 min (Fig. 2). During this time, 20% of total amylase was secreted. Thus, the new protein is not available for secretion for a period which is apparently longer than the period required for transport of a considerable percentage of the new protein to the granule fraction. After about 50 min postpulse, the labeled protein is secreted at a constant rate which is comparable to the rate of amylase secretion. According to the fractionation studies in Fig. 1 and the secretion experiment in Fig. 2, it is evident that most of the newly formed labeled protein is available for secretion 90 min postpulse. This chase period was therefore adopted for subsequent studies.

Release of Amylase and Labeled Protein before the Addition of an Inducer of Secretion

26 experimental systems were analyzed for the amount of amylase and labeled protein released during the 90-min chase period. The mean calculated rate of basal amylase release into the medium was $0.040 \pm 0.003\%$ of total in the slices per minute. The mean calculated rate of basal release of labeled protein was $0.026 \pm 0.002\%$ of total in the slices per minute. Thus, when an inducer of secretion was added after 90 min of chase, in order to test preferential secretion of old

unlabeled amylase, the slices still possessed more than 96% of their original amylase content and more than 97% of their newly labeled protein.

It should also be noted that because basal amylase release was insignificant, very low concentrations of neurohormone could be tested as inducers of secretion. Thus, $0.05 \mu\text{M}$ isoproterenol produced a rate of amylase secretion (Fig. 3*a*) which was 10 times higher than the rate of basal enzyme release discussed above.

Secretion of Amylase and Labeled Protein Induced by Isoproterenol

The major pathway of protein secretion in the parotid gland operates via the β -adrenergic receptor (26). This receptor can be activated selectively by the synthetic catecholamine isoproterenol which does not activate the α -adrenergic receptor. When a saturating concentration of isoproterenol was added to the labeled slices after the 90-min chase period, secretion began in an ordered fash-

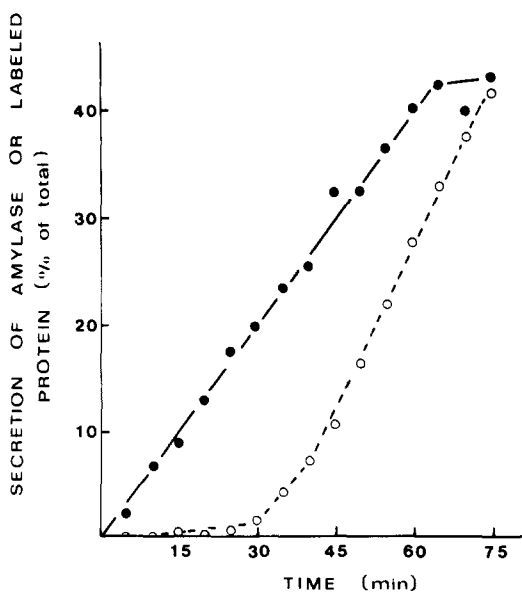


FIGURE 2 Secretion of amylase and labeled protein after pulse labeling. After a 3-min pulse, slices were washed and incubated in the chase medium for an additional 5 min. Secretion was induced by $1 \mu\text{M}$ isoproterenol 12 min after the beginning of the pulse. Total amounts in the system were 10,000 U of amylase and 0.42×10^6 cpm in protein. At zero time of isoproterenol addition, 0.3% of total amylase and 1.0% of total labeled protein were found in the medium. Percent amylase secretion ($\bullet - \bullet$); and percent labeled protein secretion ($\circ - - \circ$).

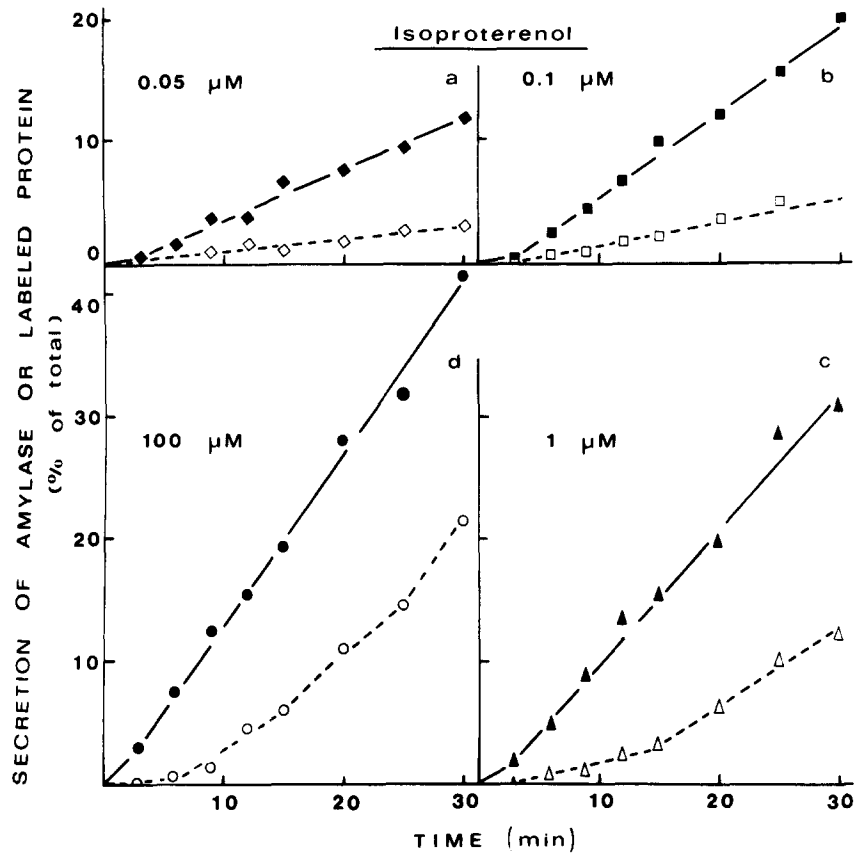


FIGURE 3 Effect of different concentrations of isoproterenol on the secretion of amylase and labeled protein. Rats received a single injection of reserpine 24 h before the experiment. Different amounts of isoproterenol were added to the incubation medium 90 min postpulse. The average total amounts per system were 13,000 U of amylase and 0.2×10^6 cpm in protein. Percent amylase secretion (—); and percent labeled protein secretion (---).

ion so that old unlabeled secretory granules discharged preferentially. However, as secretion continued, randomization set in gradually (Fig. 3d). For the first 6 min, the preferential ratio was 15; from 6 to 15 min it was only 2, and after 25 min it was 1. The possibility was therefore tested that randomization is a function of the intensity of the secretory process. It is shown in Fig. 3a-d that preferential secretion of old unlabeled amylase can indeed be better maintained when the rate of secretion is reduced by lowering the concentration of isoproterenol. Thus, at $0.1 \mu\text{M}$ isoproterenol the preferential ratio was 3.4 throughout the period of the experiment of 30 min. By the end of that period, 20% of total amylase had been secreted. In another experiment with slower secretion, a preferential ratio of 3 was maintained even for 90 min during which 25% of total amylase was

secreted. In contrast, the system discussed above which contains $100 \mu\text{M}$ isoproterenol (Fig. 3d) showed extensive randomization already after 9 min when only 12% of total amylase had been secreted.

In summing up a considerable number of experiments, it was found that when the rate of secretion of amylase exceeds $0.5\text{--}0.7\%/min$, preferential secretion of unlabeled amylase is very brief, and the system soon switches to the secretion of a random mixture of labeled and unlabeled exportable protein. It should be noted that the preferential ratio for a given set of conditions can vary considerably as shown in Table II. The mean preferential ratio for the data in Table II was 3.7 ± 0.6 . Quite different figures were obtained for seven experiments with high isoproterenol ($1\text{--}100 \mu\text{M}$) where the mean preferential ratio beyond

the first 15 min was only 1.3 ± 0.1 . The higher preferential ratio obtained at low isoproterenol concentration still does not exclude the possibility that slow mixing of the granules occurs in the cell during the chase period. To test this possibility, a chase period of 90 min was compared with a chase period of 240 min. It is shown in Fig. 4 that the preferential ratio is not decreased by extending the chase period to several hours beyond the time period required for functional maturation of the new granules.

Electron Microscopy

Random secretion might be the result of penetration of the lumen towards the cell center where the new granules are presumably located. To examine this possibility, electron micrographs of slices showing rapid and slow secretion were compared. Fig. 5a shows that under conditions of rapid secretion for 15 min the lumen has grown deep into the cell by sequential fusion (1, 4) of secretory granules. 34% of total amylase was secreted during the 15-min incubation. Fig. 5b shows acini under conditions of slow secretion for 60 min, during which a comparable amount of amylase, 25%, was secreted. Under these conditions, no extensive lumen penetration into the cell is seen. This latter system also failed to show deep penetrations when examined earlier, 15 min after addition of isoproterenol. The appearance of the acini under slow secretion conditions is comparable to that of resting acini (Fig. 5c). A considerable number of micrographs were examined to determine the percent of cells exhibiting penetration by the lumen. Under conditions of slow secretion,

only 9% of the cells demonstrated penetration as compared to 70% under conditions of fast secretion.

Effect of $N^6, O^{2'}$ Dibutyryl Adenosine 3',5'-Monophosphate (Dibutyryl cAMP)

It was of interest to test dibutyryl cAMP because adenosine 3',5'-monophosphate (cAMP) is the second messenger in the β -adrenergic pathway which causes enzyme secretion in the parotid gland. It was shown that the butyryl derivative causes, at first, slow secretion which increases with time to a high rate (2). Fig. 6 shows that during the first 20 min of slow secretion induced by dibutyryl cAMP, the preferential ratio is relatively high. Subsequently, when the rate of amylase secretion increases, random secretion sets in. The pattern is quite similar to that produced by high isoproterenol (Fig. 3d). Lower concentrations of dibutyryl cAMP produced a much extended lag period with less clear results.

Effect of the Amount of Exportable Protein in the Gland on the Preferential Secretion of Old Unlabeled Protein

The findings so far presented indicate that the preferential secretion of older unlabeled amylase might well be a function of the amount of such material present in the cell at the time of labeling. Therefore, an experiment was set up with glands containing different amounts of exportable protein. Glands from fasted animals and reserpine-injected animals both of which contain large

TABLE II
Variability of the Preferential Secretion of Old Unlabeled Exportable Protein

Exp no.	Chase period	Period of secretion	Rate of amylase secretion (a)	Rate of labeled protein secretion (b)	Preferential ratio (a/b)
	<i>min</i>	<i>min</i>	<i>% of total per min</i>	<i>% of total per min</i>	
1 <i>a</i>	90	30	0.50	0.07	7.0
1 <i>b</i>	90	30	0.40	0.09	4.5
2 <i>a</i>	90	60	0.40	0.18	2.2
2 <i>b</i>	90	60	0.32	0.14	2.2
2 <i>c</i>	240	60	0.35	0.10	3.5
2 <i>d</i>	240	60	0.28	0.07	4.0
3	90	90	0.30	0.12	2.5

The results are from various experiments with glands from fasted rats. In all systems, secretion was induced by 0.08 μ M isoproterenol. *a* and *b* as well as *c* and *d* are duplicates within a given experiment. During the period of secretion shown in the table, the preferential ratio was nearly constant.

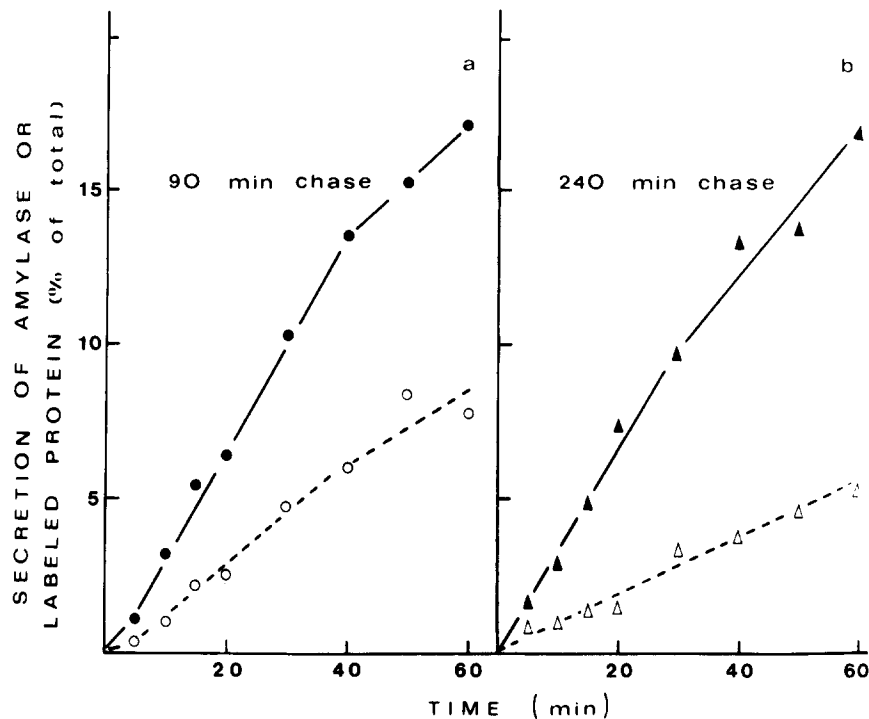


FIGURE 4 Secretion of amylase and labeled protein 4 h after pulse labeling. Secretion was induced 90 or 240 min after the pulse by addition of $0.08 \mu\text{M}$ isoproterenol. The percent secretion at each time-point is the average value from duplicate systems. The average total amounts per system were: 11,000 U of amylase and 0.6×10^6 cpm in protein. The zero time values in the medium for (a) (90-min chase) were 3% of total amylase and 1.5% of total labeled protein. For (b) (240-min chase) the zero time values were 12% of total amylase and 3% of total labeled protein. Percent amylase secretion (—); percent labeled protein secretion (---).

amounts of secretory protein were compared with glands from freely fed animals and from rats which had been injected with isoproterenol to cause extensive depletion of secretory protein. The findings are presented in Fig. 7. The highest preferential ratio is observed with slices from fasted animals. Slices from reserpinized animals showed only a transient high preferential ratio. Part of the reason may be the high rate of amylase secretion (0.7%/min) which was noted already to cause randomization. Slices from fed animals showed a somewhat lower preferential ratio than those from fasted animals. Interestingly, the slices which had been partially depleted of exportable protein *in vivo*, by isoproterenol injection, showed a preferential ratio of less than one. This means that there was some preference for the secretion of newly formed labeled protein over the residue of old protein. Presumably, some of the older exportable protein remaining after injection of the animal

with isoproterenol may be in acini which do not respond to the low concentration of isoproterenol added to the slices. A similar experiment was performed but with adding a high concentration of isoproterenol to the slices in order to determine what portion of the newly formed protein is available for secretion (Fig. 8). It is demonstrated that secretion of labeled protein almost stops when about 80% of it has been secreted. In another experiment, 85% of the labeled protein was secreted. Since some of the labeled protein is probably cellular, nonexportable, it is apparent that the slices discharged the entire amount of new secretory protein. It should be noted that these slices from isoproterenol-injected animals incorporated much more L- ^3H leucine into protein but contained much less amylase than slices from fasted animals. Thus, the term percent of total secreted has for this system a different connotation than in the case of slices from fasted animals.

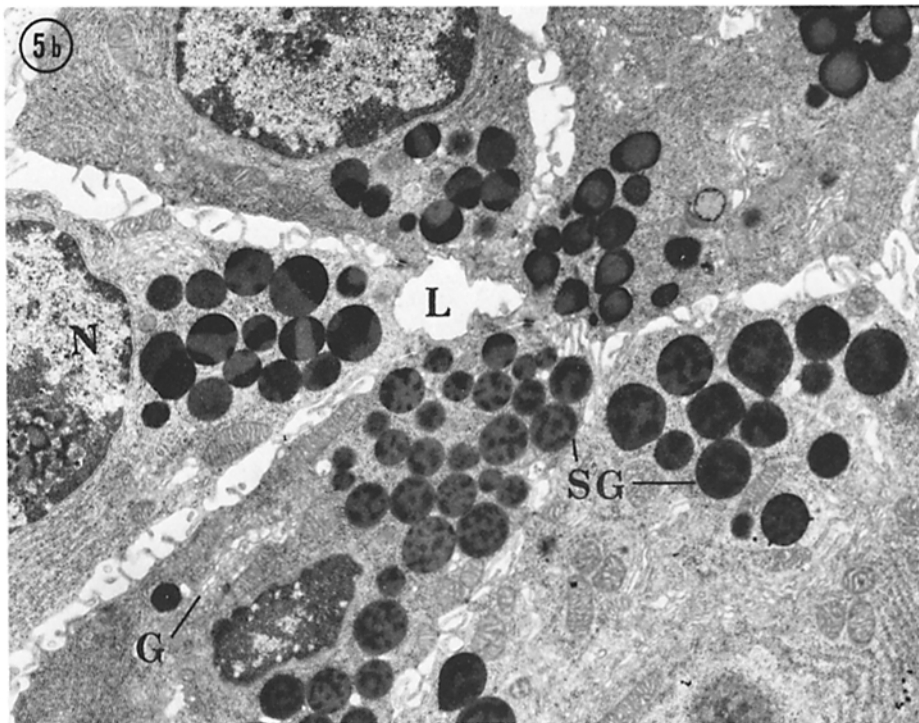
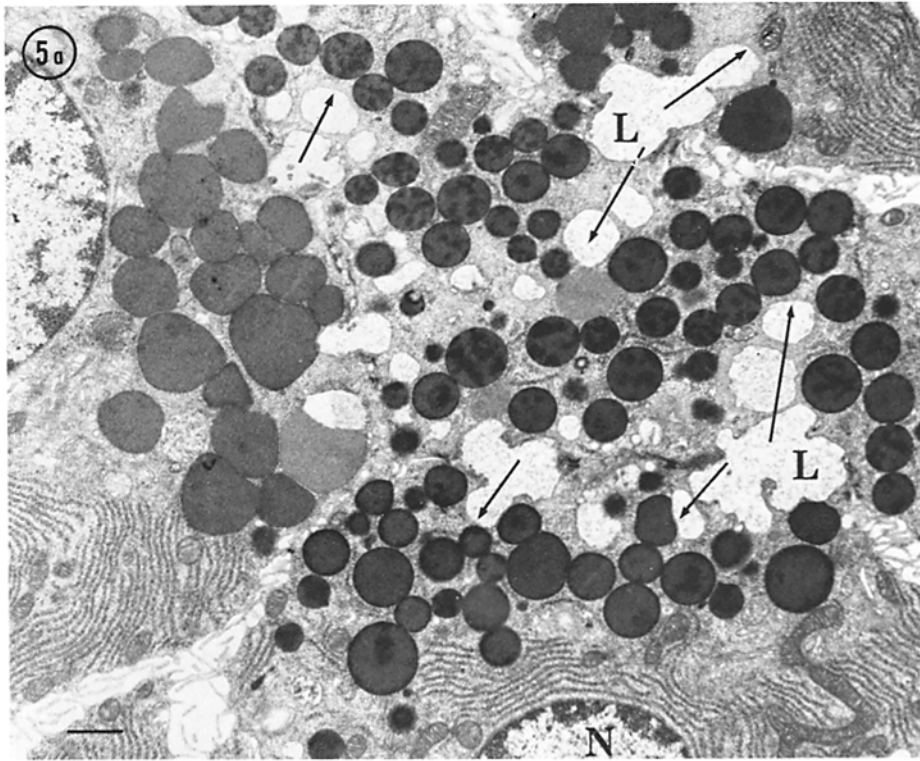
Effect of α -Adrenergic and Muscarinic Cholinergic Agents

Norepinephrine through the α -adrenergic receptor and carbamylcholine through the muscarinic receptor cause K^+ release, vacuole formation, and water secretion in the acinar parotid cell (3, 4, 18, 19, 25). It was therefore of interest to study the effect of these receptor-response pathways on the secretion of new secretory protein, which is mediated by the β -adrenergic receptor. For this purpose, secretion was induced by norepinephrine in the presence and absence of the α -adrenergic blocking agent, phentolamine. It is shown in Fig. 9*b* that norepinephrine plus phentolamine produces preferential secretion of unlabeled protein. This system is essentially the same as the low isoproterenol system (Fig. 3*a*). However, in the absence of phentolamine, when the α -adrenergic response is also activated, secretion is almost random (Fig. 9*a*). It can be seen that the α -adrenergic action causes a twofold increase in the rate of secretion of labeled protein but has little effect on the secretion of amylase. In seven such experiments, the mean preferential ratio was 1.3 ± 0.1 for norepinephrine and 2.8 ± 0.4 for norepinephrine plus phentolamine. Thus, the α -adrenergic response clearly contributes to the randomization of the secretory material. It should be noted that the medium for these experiments contained 1.8 mM Ca^{++} in order to maximally activate the α -adrenergic response (28). Experiments at 1.8 mM Ca^{++} did not affect the preferential ratio when isoproterenol served as the inducer of secretion (cf. Fig. 10*b*). Similar experiments were carried out with carbamylcholine which is capable also of causing slow amylase secretion (6, 21, 23). Fig. 10 shows the action of carbamylcholine in the presence and absence of isoproterenol. It is evident that carbamylcholine by itself causes preferential secretion of unlabeled amylase. In spite of the fact that the rate of amylase secretion is rather low, it is higher than the control without addition of an inducer of secretion (cf. Fig. 10*a* and *c*). In an experiment not shown, 1 μ M carbamylcholine produced a preferential ratio of 5 which was maintained during a 90 min incubation while 10% of total amylase was secreted. When carbamylcholine was added to isoproterenol, it caused only a partial randomization of secretion (cf. Fig. 10*d* to 10*a* and *b*). In five experiments with carbamylcholine alone the mean preferential ratio was 3.4 ± 0.8 , and in seven experiments with carbamylcholine plus isoproterenol it was 1.7 ± 0.3 .

DISCUSSION

A decade ago studies on parotid gland slices established some basic facts about the synthesis of labeled exportable protein, its intracellular transport and its subsequent secretion through the action of epinephrine (24). It was calculated that most of the label is incorporated into exportable protein and not into cellular protein. The study also showed quite clearly that newly synthesized amylase is not immediately available for secretion, and it was therefore concluded that the amylase must first be incorporated into secretory granules before it can be exported from the cell. In vivo experiments showed a similar lag period before exportable protein of the parotid gland became available for secretion (29). Such a lag period after labeling was also demonstrated for pancreas (14). However, as shown in the present work, once the new labeled protein has reached the granules the bulk of it is readily available for secretion. Slices from partially depleted glands secreted within 60 min 80% of the labeled protein that the slices had synthesized. Thus, there is now direct proof that at least 80% of the label incorporated into protein of such slices is indeed exportable protein. These findings, like the other results of the present work, are highly significant owing to the fact that release of exportable protein by the slices was almost absolutely dependent on addition of an inducer of secretion even after incubation of the slices for several hours.

While all the new labeled protein transported to the granules can apparently be secreted, it is not distributed randomly in the granule population. The present findings clearly show that under certain conditions unlabeled exportable protein is secreted preferentially. Before discussing the conclusions that can be drawn from this finding, the possibility must be considered that the unlabeled amylase secreted preferentially is enzyme that had already been extracellular, in the acinar lumen and ducts at the time of labeling. The following arguments seem to rule out this possibility. Labeling of the thin slices (1 mm³) was followed by washing and a 90–240-min incubation before the addition of neurotransmitter to induce secretion. It is quite unlikely that considerable amounts of the extracellular amylase could have been retained in the lumen of thin slices throughout such a long period of shaking. Preferential secretion of unlabeled amylase was maintained even when 25% of total amylase was secreted. This represents a very large absolute amount of exportable protein in



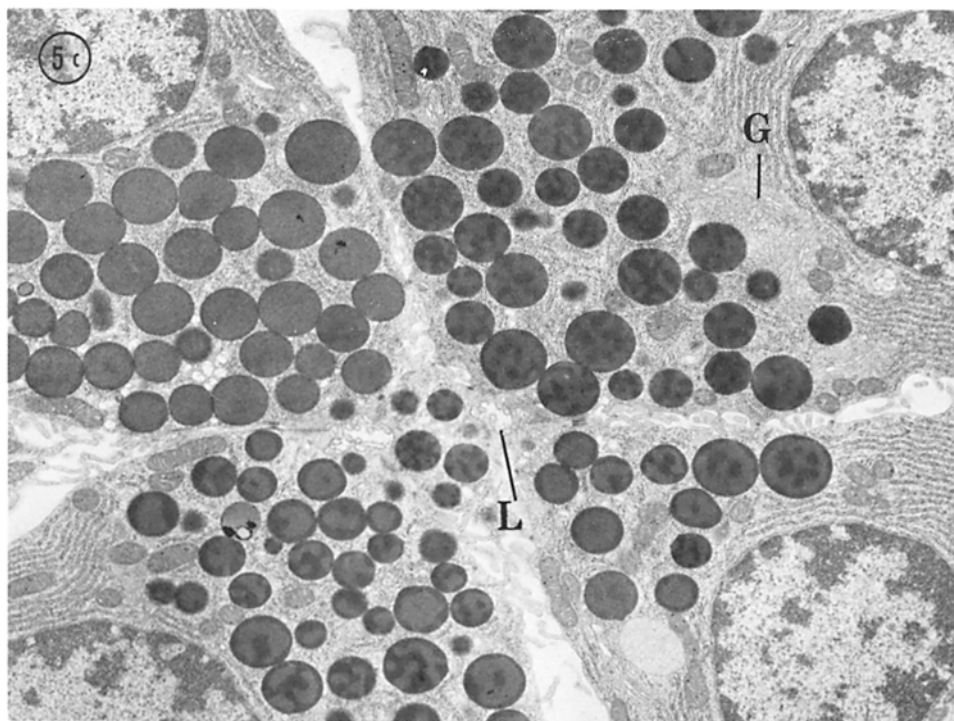


FIGURE 5 Electron micrographs of parotid slices fixed during high and low rates of secretion. Parotid slices from fed rats were incubated in F-12 medium. After 5 min of preincubation, isoproterenol was added, and secretion was followed as described under Materials and Methods. At the end of incubation time, about one-fifth of the slices were removed for fixation while the remaining slices were used for the determination of total amylase. (a) Fast secretion induced by 10 μ M isoproterenol for 15 min. Notice the penetration (arrows) of lumen (L) into the cell. Nucleus, N. (b) Slow secretion induced by 0.1 μ M isoproterenol for 60 min. In contrast to rapid secretion shown in a, slow secretion maintains a cellular organization of lumen and secretory granules similar to that of the unstimulated control, c. Golgi complex, G, secretory granules, SG. (c) 60-min incubation without addition of isoproterenol. Magnification in a, b, and c is $\times 8,000$. The bar in a is 1 μ M.

glands from fasted animals which were used for these experiments. Furthermore, activation by norepinephrine of the α -adrenergic receptor which causes water secretion (4), in conjunction with the β -adrenergic receptor, did not increase the rate of secretion of unlabeled amylase.

Carbamylcholine which also induces water secretion (9) produced a very slow and constant rate of amylase release which would not be expected if large amounts were located in the lumen. These considerations justify the conclusion that the preferential secretion of unlabeled amylase truly represents priority of older secretory granules in the fusion with the cell membrane at the lumen. The most likely and plausible explanation for this observation is that the older granules are more proximal to the lumen than the new granules just leav-

ing the Golgi area. Thus, when secretion was initiated through the selective action of isoproterenol the older granules closest to the lumen were initially more likely to be involved in the fusion process. When the isoproterenol concentration was high, producing high rates of amylase secretion, the system soon switched to random secretion of new and old amylase. This phenomenon can be readily interpreted with the aid of the electron micrographs. We have shown *in vivo* (1) and *in vitro* (4) that at high rates of secretion the lumen rapidly "grows" towards the center of the cell by sequential fusion of the secretory granules with the cell membrane. Thus, the new labeled granules at the center of the cell soon became just as available for fusion with the lumen as older granules which had been bypassed and remained

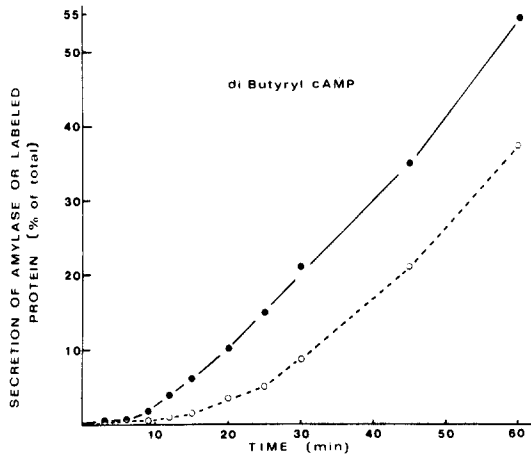
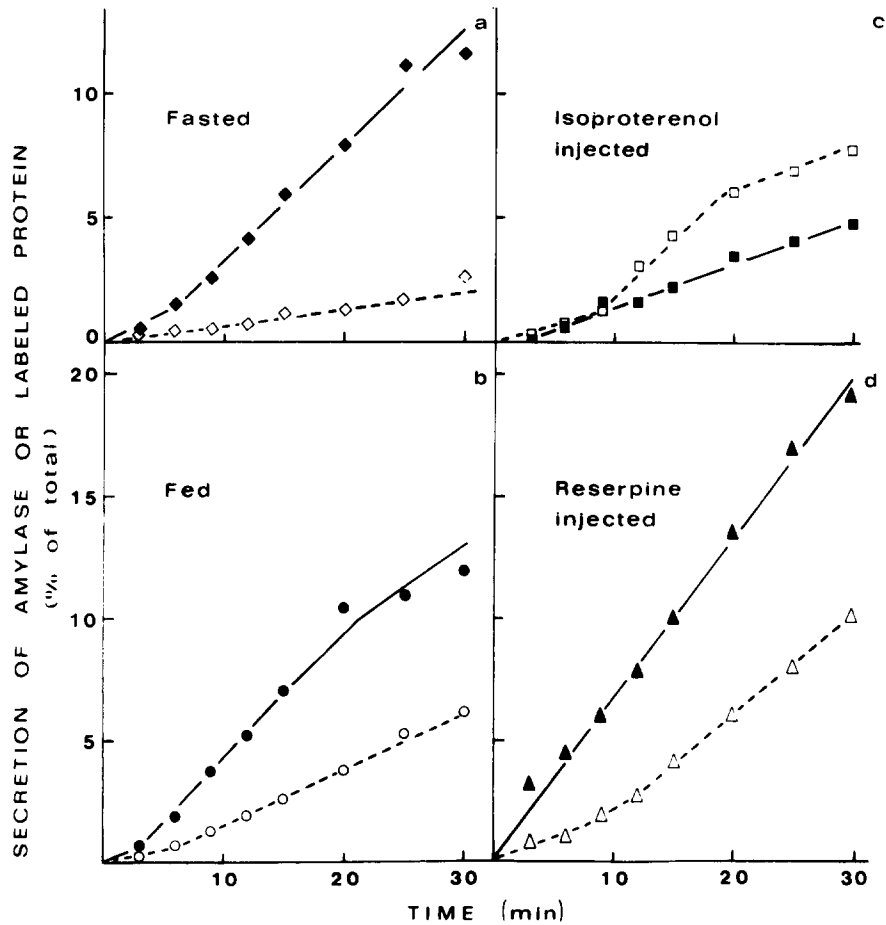


FIGURE 6 Secretion of amylase and labeled protein induced by dibutyl cAMP. Secretion was induced 90 min postpulse by 1 mM dibutyl cAMP. The system contained 13,000 U of amylase and 0.55×10^6 cpm in protein. Percent amylase secretion (—); percent labeled protein secretion (- -).

near the original apex of the cell. However, at low isoproterenol concentration, the rate of granule fusion with the cell membrane was slow, so that the empty granule membrane after fusion was presumably resorbed into the cell (1). Under these conditions, sequential fusion would rarely occur, the lumen would increase in size only slowly, without deep interdigitations, and thus the new secretory granules at the center of the cell would not be so readily available for secretion. It should be noted that resorption of the lumen membrane into the cell after fusion apparently proceeds at a rate sufficiently high to explain the present findings (1). While the electron micrographs cannot prove this hypothesis, they are in line with it.

The experiments show that 90 min after labeling it is possible to obtain either preferential secretion or random secretion, according to the isoproterenol concentration. It is thus evident that at 90 min the new granules are perfectly capable of participating in the secretory process. Preferential



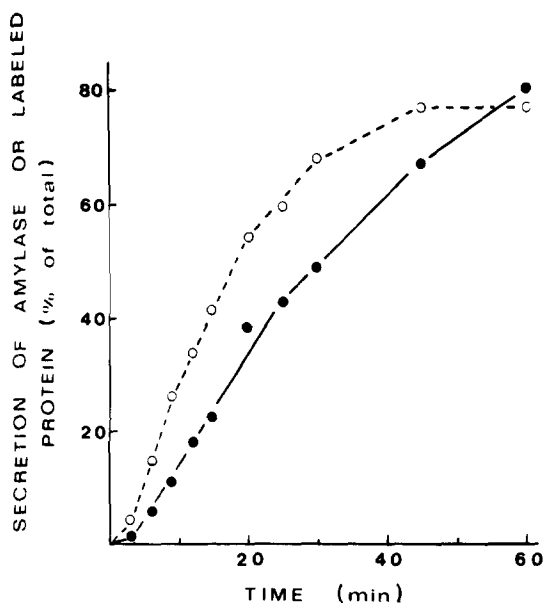


FIGURE 8 Secretion of amylase and labeled protein by glands from isoproterenol-injected rats. Slices equivalent to four glands instead of the usual two were taken for this experiment. All incubations were carried out in F-12 medium containing 1 μ M propranolol. The slices were pulse labeled with 20 μ Ci/ml L-[3 H]leucine. At 90 min postpulse, 20 μ M isoproterenol was added to overcome the blocking of the β -adrenergic receptor by propranolol and to achieve a maximal rate of secretion. The system contained 5,000 U of amylase and 3×10^6 cpm in protein. Percent amylase secretion (—); percent labeled protein secretion (---).

secretion of old granules can therefore not be ascribed to the immaturity of new granules. The fact that preferential secretion remained undiminished even 240 min after labeling also establishes this conclusion.

Another means to cause random secretion of new and old granules was the activation of the α -adrenergic receptor in addition to the β -adrenergic receptor. This can occur physiologically, since norepinephrine released from nerve endings activates both receptors. It is possible that the K^+ release, water secretion, and vacuole formation caused by the α -adrenergic response slow down the rate of resorption of granule membranes, thus permitting sequential fusion of granules even at relatively slow rates of secretion. Sequential fusion would cause random secretion of new and old granules as explained above. It is also possible that water secretion through the acinar cells caused by the action of the α -adrenergic receptor produces true mixing of granules. However, carbamylcholine which has been shown to cause the same effects as the α -adrenergic agents (18, 19, 25) and which also produces some amylase secretion (6, 21, 23) caused preferential secretion of old unlabeled protein. Thus, it seems less likely that water secretion *per se* causes mixing of granules in the cell. In combination with isoproterenol, carbamylcholine caused partial randomization of the protein secreted. The combination of the latter two agents produced essentially the same effect as norepinephrine acting on the α - and β -receptors simultaneously.

It must be emphasized that the term "preferential secretion of old granules" has been used throughout the present study as a loose, rather relative term. This is dictated by the variable factors which affect the extent of preferential secretion: inactivation of suboptimal concentrations of the labile catecholamines (oxidation, methylation, and uptake into vestige nerve endings), the distance of the new granules from the lumen in a cell filled to a variable extent with old granules, and

FIGURE 7 Secretion of amylase and labeled protein by glands containing different amounts of exportable protein. The animals were treated as described under Materials and Methods. Slices from differently treated rats were labeled separately with 40 μ Ci/ml L-[3 H]leucine. (a) Fasted rats: secretion was induced by 0.08 μ M isoproterenol 90 min postpulse. Each time-point in the graph represents the average of duplicate systems. Total amounts: 8,800 U of amylase, 0.93×10^6 cpm in protein. (b) Fed rats. Total amounts: 8,000 U of amylase, 3×10^6 cpm in protein. (c) Isoproterenol-injected rats. Because of the low content of exportable protein in these glands, slices equivalent to three glands were taken for each system. All incubations were carried out in F-12 medium containing 1 μ M propranolol to block the secretion caused by the residual isoproterenol which had been injected into the animal. In order to overcome the blocking of the β -adrenergic receptor at the end of the chase period, and to induce secretion at a moderate rate, isoproterenol was added to a concentration of 7 μ M instead of the usual 0.08 μ M. The system contained 4,900 U of amylase and 3.5×10^6 cpm in protein. (d) Reserpine-injected rats. The system contained 10,700 U of amylase and 0.95×10^6 cpm in protein. Percent amylase secretion (—); percent labeled protein secretion (---).

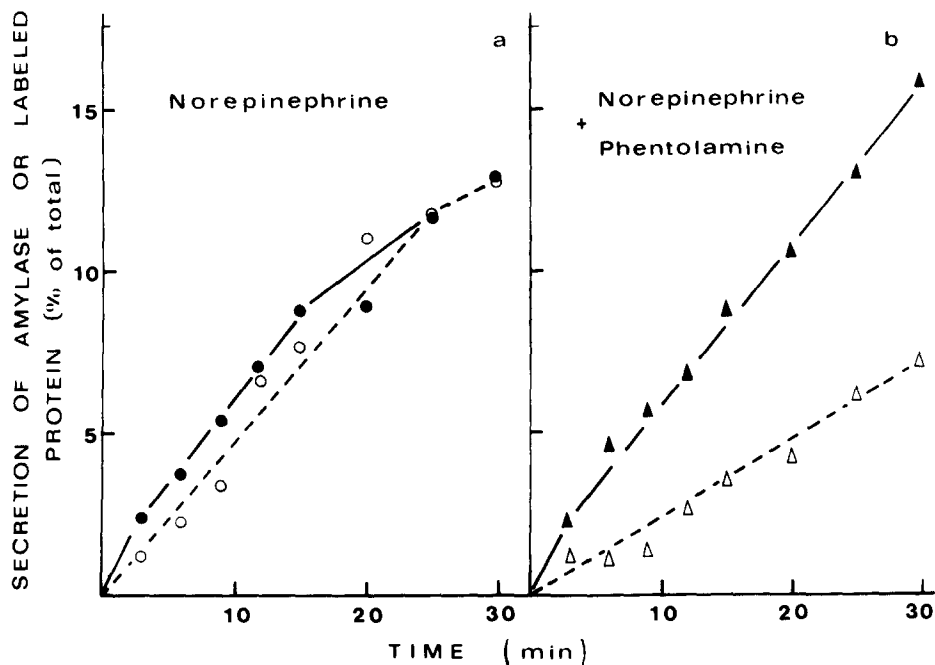


FIGURE 9 The effect of the α -adrenergic response on the preferential secretion of old unlabeled protein. The Ca^{++} concentration during the chase and secretion periods was 1.8 mM. Secretion was induced 90 min postpulse. (a) Norepinephrine, 1 μM ; (b) Norepinephrine, 1 μM plus phentolamine, 10 μM ; Phentolamine was added 5 min before addition of norepinephrine. Systems contained 9,300 U of amylase and 0.35×10^6 cpm in protein. Percent amylase secretion (—); and percent labeled protein secretion (---).

the factors which determine sequential fusion versus resorption of the lumen membrane back into the cell. With all these variables in mind, the results herein reported and discussed were qualitatively reproducible, i.e. low isoproterenol always produced some degree of preferential secretion of old secretory protein.

The present experiments show that there is little mixing of old and new granules in the absence of the neurotransmitters which cause secretion. Preferential secretion of old unlabeled protein was evident even 4 h after labeling. Thus, it should be concluded that the granules are not free to move about in the cell and that there is no rapid exchange of stored protein between the granules. It is of interest to note that the secretory granules in mast cells were also observed to be stationary (8). In rabbit parotid gland, new and old granules also seem not to mix in the resting cell (7). However, there is no information on their distribution during secretion. Findings which indicate random secretion of old and new protein were reported for guinea pig pancreas (14, 22). However, these data were obtained under conditions where secretion was rather active spontaneously or by addition of

hormone, before the arrival of the new labeled protein in the secretory granules. Thus, the lumen might have been considerably enlarged, and older granules were probably secreted to quite an extent before the labeled protein reached the granules. The question whether the pancreas can demonstrate preferential secretion of old unlabeled protein therefore remains open. Secretion of old versus new exportable material is being actively studied also with regard to catecholamine release from nerve endings (16). However, it should be pointed out that these small molecules of neurohormone cannot serve as models for studying secretion of protein. It is well known, for instance, that the catecholamines can be taken up by the granules from a soluble pool in the cell and even from the extracellular postganglionic space (10). With regard to the proteins of the chromaffin granules, it has been shown that these are secreted 4 h after labeling of the adrenal medulla (30). The data do not permit a decision whether preferential secretion takes place. Thus, it remains to be seen whether secretory systems other than the parotid gland show preferential secretion of old secretory protein.

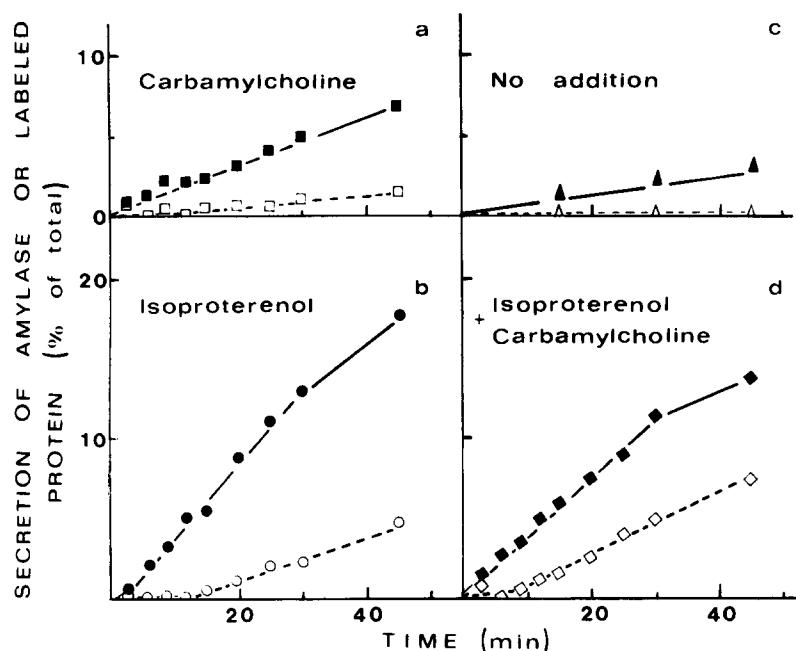


FIGURE 10 The effect of the cholinergic response on the preferential secretion of old unlabeled protein. The Ca^{++} concentration during the chase and secretion periods was 1.8 mM. Secretion was induced 90 min postpulse. (a) Carbamylcholine, 100 μM ; (b) isoproterenol, 0.1 μM ; (c) control of secretion without additions; (d) isoproterenol, 0.1 μM plus carbamylcholine, 100 μM . Each system contained about 13,000 U of amylase and 0.15×10^6 cpm in protein. Percent amylase secretion (—); percent labeled protein secretion (---).

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