# UNSTIMULATED SECRETION OF PROTEIN
# FROM RAT EXOCRINE PANCREAS CELLS

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ABSTRACT

Our earlier work demonstrated that the rate of protein synthesis in the exocrine cells of
the rat pancreas is constant in different physiological states, including prolonged fasting.
In this study we have followed the fate of the protein in the pancreatic cells of the fasting
animal in vivo as well as in vitro. The data were obtained by quantitative radioautography
and by biochemical determinations. In nonanesthetized, fasting rats, without cannulated
pancreatic duct, some 80% of the proteins synthesized at a given time leaves the cell within
12 hr by way of secretion, intracellular breakdown not being important. Two mechanisms
of fasting secretion exist. The first, starting at a slow rate after 20 min, is inferred to result
from fortuitous contacts of young secretory granules with the apical cell membrane. The
rate of secretion is the same in vivo as in vitro, at least during the first 4 hr after pulse
labeling. Within 7 hr about 20% of the total amount of newly synthesized protein has left
the cell. The second mechanism consists of an orderly movement of the mass of secretory
granules towards the apical cell membrane as caused by the continuous assembly of new
granules. The granules that come into contact with the cell membrane are discharged.
It takes about 7–12 hr for secretory protein transported in this way to reach the cell mem-
brane. The addition of new secretory granules to those present is essential for the second
mechanism, for the blockade of protein synthesis by cycloheximide decreases the rate of
this phase of secretion without interfering with the secretory process proper. Atropin does
not inhibit the fasting secretion in vitro, nor does extensive washing of the tissue slices,
excluding possible secretagogues as important factors in fasting secretion.

## INTRODUCTION

In previous work (Kramer and Poort, 1968;
Poort and Kramer, 1969) we confirmed the con-
clusion reached earlier by Hokin (1956) that the
rate of protein synthesis in the acinar cells of the
mammalian pancreas is constant and independent
of the degree of filling with secretory granules and
of the action of secretory stimuli.

This finding implies that per unit time as much
protein must be eliminated from fully granulated
cells as is synthesized during that time. This
elimination can take place via secretion or by

intracellular breakdown. Protein elimination may
result in a continuous renewal of the store of
secretory granules, or it can affect only newly
formed secretory protein, the store being sta-
tionary.

Secretion during fasting was observed in phys-
iological experiments on several species. Grossman
(1958) and Alphin and Lin (1959) reported it for
the rat, the quantity of protein secreted by one
cell per hour being estimated at $8.3 \times 10^{-6}$ µg by
Junqueira et al. (1957). Fasting secretion in

rabbits was observed by Baxter (1931) and in dogs by Scott et al. (1940). In all these experiments secretion observed after cannulation of the pancreatic duct was taken to be spontaneous. However, it is conceivable that in these experiments the cannulation itself acts as a stimulus for the secretion, e.g., by relieving a physiological intraductal pressure.

We have followed the loss of pulse labeled protein from the pancreatic cells of rats fasted for 44 hr. We compared this loss in vivo with the loss of similarly labeled protein in vitro. The experiments in vitro were performed to exclude as completely as possible the action of stimulatory effects present in the intact organism.

In the present investigation experiments in vivo were done on intact fasted rats, in which the loss of pulse labeled secretory protein from the pancreas was ascertained over a time interval of 10 hr. In a few additional experiments, we checked whether a contingent diurnal variability in fasting secretion might influence the results and whether the rate of protein loss found depends on the state of congestion of the gland cells with secretory granules. In order to exclude the effect of persisting secretory impulses in the fasting animal, we included experiments in vitro.

## MATERIALS AND METHODS

### Animals

Albino Wistar rats were obtained from Centraal Proefdieren Bedrijf TNO, Zeist, The Netherlands. They were used in groups. The animals in one group were all killed on the same day, the first three or four 1 hr after injection of radioactive amino acid (see below), the other animals at varying time intervals between 2 and 10 hr after the injection, three or four at a time. Each group consisted of only male or female animals in a narrow weight range (e.g., a group of males, weighing 191–227 g; a group of females, weighing 149–164 g). At the onset of the experiments all rats had fasted for 44 hr, with free access to water. Before this they were fed ad libitum with granular food, the main components being digestible protein (20.1% of the dry matter) and carbohydrate (62.1%). The animals were killed by decapitation.

### Administration of Chemicals In Vivo

L-leucine-4,5-$^3$H (The Radiochemical Centre, Amersham, England; SA 1 Ci per mmole) in saline (1 mCi per ml) was injected into the tail vein at a dose of 100 $\mu$Ci per 100 g of body weight. The time of injection of radioactive leucine was, unless otherwise mentioned, around 8:00.

Cycloheximide (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 10% (v/v) ethanol in physiological saline to a final concentration of 267 mg per 100 ml. It was injected intraperitoneally at a dose of 0.4 mg per 100 g of body weight.

Pilocarpine-nitrate (Brocacef, Utrecht, The Netherlands) was dissolved in physiological saline to a final concentration of 100 mg per ml. It was injected into the tail vein at a dose of 20 mg per 100 g of body weight.

### Microscopy

A portion of the pancreas bordering the spleen was excised and fixed for 24 hr in 4% (w/v) formaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C. The tissue was then rinsed for 24 hr in the same buffer with 6.8% (w/v) sucrose, at room temperature, before dehydration and embedding in paraffin wax. Unstained 5 $\mu$ sections were studied with phase contrast microscopy to estimate the quantity of secretory granules present in the acini.

### Radioautography

Sections of 5 $\mu$ were covered with Kodak AR 10 stripping film, dried, and stored in a refrigerator for 5 days. The film was developed with Kodak D 19 b at 18°C and fixed with a Na-thiosulfate (10% w/v) and K-metabisulfite (1% w/v) solution in distilled water. The preparations were stained with methyl green–pyronin (Kurnick, 1955) for 6 min and differentiated with 70% ethanol.

The radioautographic results were quantitated by counting the silver grains over six centrally sectioned acini per animal and by dividing their number by the number of nuclei (about 65) visible in these acini. The resulting quotient (the number of grains per "mean cellular area") is used as an index of the radioactivity present in protein *per cell* (Kramer and Poort, 1968).

In the course of the experiments the radioautographic procedure was changed in that the time of development increased from 4 to 5 min. As a result, the latter (II) series of experiments had a higher yield of silver grains than the first (I) series.

### Experiments In Vitro

Pancreas was taken out of the rat within 3 min after decapitation, nonpancreatic tissue being removed. The gland was cut into fragments of about 10 × 1 × 1 mm, and 300 mg of these fragments were incubated in 3 ml Krebs-Henseleit-bicarbonate buffer (Krebs, 1950), containing 2.5 mg glucose

and 1.3 $\mu$Ci L-leucine-$^{14}$C (The Radiochemical Centre; specific radioactivity 312 mCi per mmole) per ml. The other 18 amino acids were present in a concentration of $10^{-4}$ M. Incorporation time was 3 min at 37°C.

After this, the incubation medium was replaced by 7 ml of the same medium containing unlabeled L-leucine (1 mg per ml) instead of leucine-$^{14}$C, and after 15 min the unlabeled medium was renewed. Thereafter the postincubation was continued for several hours. All washings and incubations were performed at 37°C under an atmosphere of 95% $O_2$ and 5% $CO_2$. At various times 0.5 ml was taken from the medium. Portions of 0.1 ml were counted in a Packard liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.), after addition of 0.3 ml formic acid and 10 ml of Bray's counting solution. To other portions of 0.1 ml an equal volume of 20% trichloroacetic acid (TCA)[1], containing 1 mg of unlabeled L-leucine per ml, was added and, after centrifuging, the radioactivity of 0.1 ml of the supernatant was determined in the same way. From these two data, the radioactivity of the TCA-insoluble material can be calculated by subtraction.

The amount of protein present in the medium was measured by the biuret reaction, with albumin as a standard.

In some experiments cycloheximide was added to the medium, 1 hr after pulse labeling, to a final concentration of $10^{-3}$ M. In some other experiments atropine sulfate (Brocacef) was added to the postincubation medium (1.5 $\times$ $10^{-4}$ M).

The viability of the tissue fragments over the whole 4 hr period of incubation was checked on four parameters: the rate of incorporation of radioactive leucine, the ability to respond to a secretory stimulus, the constancy of the amount of soluble radioactivity present in the medium, and finally the ultrastructure of the pancreatic cells.

The rate of incorporation of radioactive leucine was measured after 10 min, 2 and 4 hr preincubation. Fragments of one pancreas were divided into six equal portions and each portion was incubated in 2 ml medium without radioactive leucine, but containing unlabeled leucine in a concentration of 0.18 mM L-leucine. After 10 min, 2 and 4 hr, respectively, the incubation medium was replaced by fresh medium containing, in addition, L-leucine-$^{14}$C in a concentration of 1.3 $\mu$Ci per ml. After 10 min the incorporation was stopped by the addition of an excess of cold medium containing 1 mg leucine per ml. The tissue fragments were washed three times with this cold medium and then homogenized in the same medium. In portions of the homogenate, DNA was determined (Burton, 1956). Other portions were

[1] Abbreviations:TCA, trichloroacetic acid.

used for counting the radioactivity, precipitable with 10% TCA containing an excess of unlabeled L-leucine. The precipitate was washed with 5% TCA with L-leucine and finally dissolved in formic acid. The radioactivity was determined after addition of 10 ml of counting solution (Bray, 1960). The number of counts per minute per microgram DNA was used as an index of the radioactivity incorporated into protein per cell.

The radioactivity incorporated into protein per cell after 2 and 4 hr of preincubation amounted to 67 and 73%, respectively, of the value obtained after a preincubation time of only 10 min (means of two experiments with two glands each; the radioactivity amounted to about 300 cpm per $\mu$g DNA, when preincubation time was 10 min). From these results one may conclude that the ability to synthesize protein does not decrease during the continued incubation of the pancreatic tissue, after a not too drastic fall in the first period.

The ability to respond to a secretory stimulus was checked by two experiments. Fragments of one pancreas were divided into two equal portions, pulse labeled in two vials for 10 min as described before, and postincubated for 3 hr. Then pilocarpine nitrate was added to the medium in the second vial, in a final concentration of $5.10^{-4}$ M. The amount of TCA-insoluble radioactivity present in the medium was measured in portions of 0.5 ml taken from the medium after 3 (before addition of pilocarpine) and 4 hr of postincubation. It is expressed per milligram dry TCA powder from the slices. The results of these experiments are mentioned in Table I. They

TABLE I

Response of Slices of Pancreas from Fasting Rats during Prolonged Incubation after Labeling with Leucine-$^{14}$C for 10 min

| | Amount of radioactivity in protein secreted into the medium during | | |
| | 3 hr of post-incubation* | 4 hr of postincubation* | |
| | | Pilocarpine in the medium during the 4th hr of postincubation | |
| | | − | + |
|---|---|---|---|
| Exp. 1 | 100‡ | 110 | |
| | 100 | | 201 |
| Exp. 2 | 100 | 107 | |
| | 100 | | 500 |

* Postincubation followed a 10 min period of labeling with subsequent washing.

‡ The radioactivity of the TCA-insoluble material in the medium after 3 hr of postincubation amounts to about 200 cpm per mg dry TCA powder from the slices.
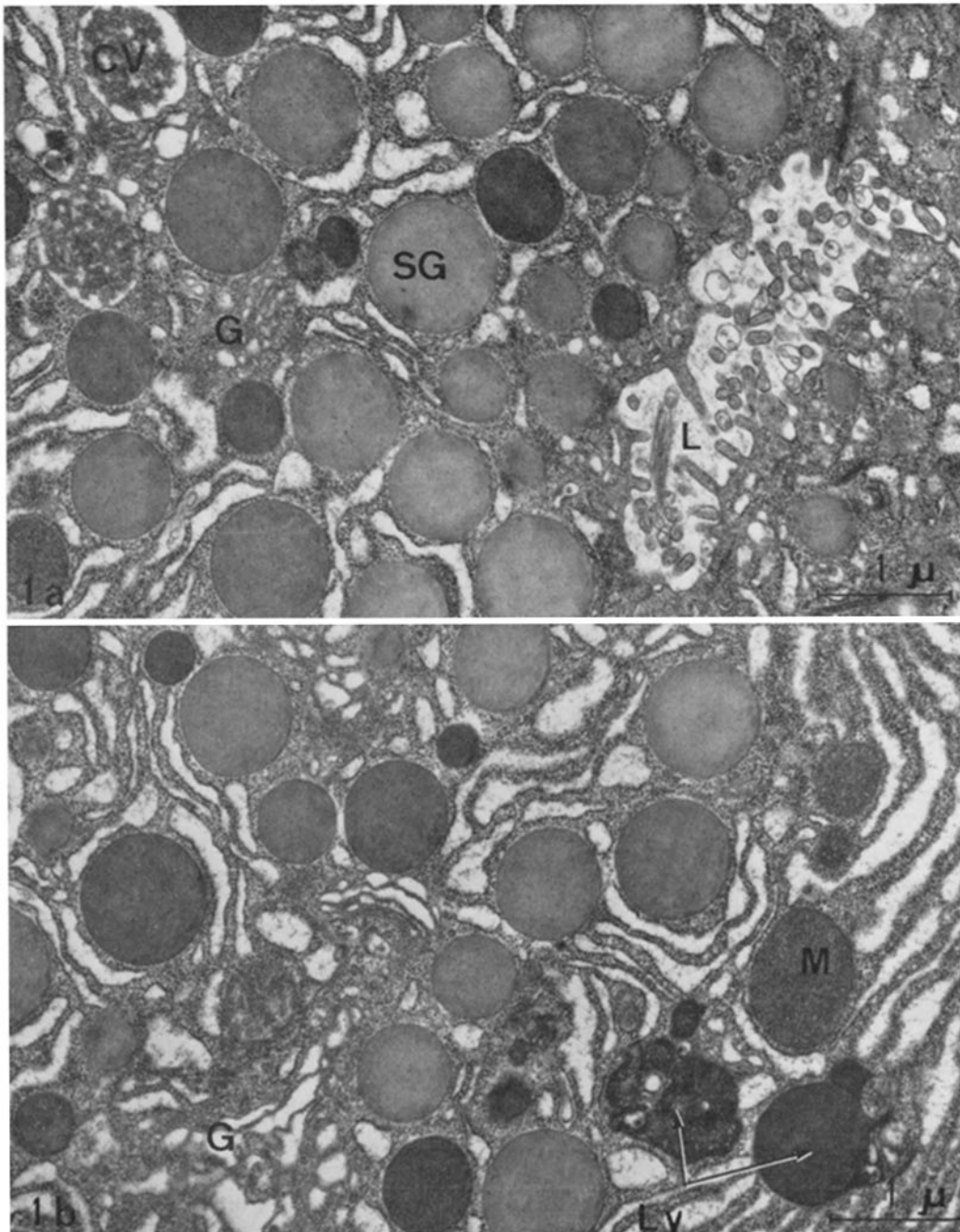
FIGURE 1  Ultrastructure of acinar cells from slices of pancreatic tissue, incubated for 6 hr. Representative examples of the good preservation of structure. The vesicles in the lumen (L) in Fig. 1 a indicate some cell damage. Lysosome-like bodies (Ly), shown in Fig. 1 b, are increased in number as compared with sections made from nonincubated tissue. (CV, condensing vacuoles; G, Golgi apparatus; M, mitochondria; SG, secretory granules). The slices were fixed with a mixture of 2% glutaraldehyde and 1% $OsO_4$ in 0.1 M phosphate buffer at pH 7.4, in-block stained with 1% aqueous solution of uranyl acetate, and embedded in Epon. Thin sections were poststained with lead citrate. × 20,000.

show the persistence of the ability of the cells to respond to secretory stimuli over a preincubation period of at least 4 hr.

Thirdly, the decrease rather than an increase of the amount of TCA-soluble radioactivity in the medium after pulse labeling and washing can be seen as additional evidence for a good viability of the tissue fragments during the 4 hr period of incubation. The TCA-soluble radioactivity present in the medium after 2, 3, and 4 hr of postincubation amounted to 89, 81, and 83%, respectively, of the value obtained after a postincubation period of only 1 hr (means of two experiments with two animals each; the TCA-soluble radioactivity in the medium amounted to about 800 cpm per mg dry TCA powder of the slices, when measured after 1 hr of postincubation after a labeling period of 10 min and subsequent washing).

Finally, most cells of the slices showed a quite normal ultrastructure, even after 6 hr of incubation (Fig. 1). The somewhat distended appearance of the cisternae of the rough endoplasmatic reticulum (RER) is also present in the micrographs of incubated slices of the pancreas of the guinea pig as presented by Jamieson and Palade (1967 a).

# RESULTS

## Loss of Radioactive Protein from the Cells In Vivo

Radioactivity incorporated after an intravenous injection of radioactive leucine is slowly lost from the acinar cells of rats previously fasted for 44 hr. The loss of radioactivity per cell as measured radioautographically follows a two-phase pattern (Fig. 2). During the first 7 hr some 20% of the radioactive protein is lost from the acinar cells, 70% during the next 3 hr period. The radioactivity still present 24 hr after injection of leucine-$^3$H (13%) is not incorporated into exportable protein, for stimulation of the secretion by pilocarpine at that time did not diminish the amount of radioactivity per cell (Kramer and Poort, 1968).

Most groups (animals killed at the same day) showed this low rate of loss of radioactive protein during the first period after pulse labeling. There were three groups, however, in which 40–60% of the radioactivity had left the cells within 4 hr after labeling, instead of the normal 10–22%.
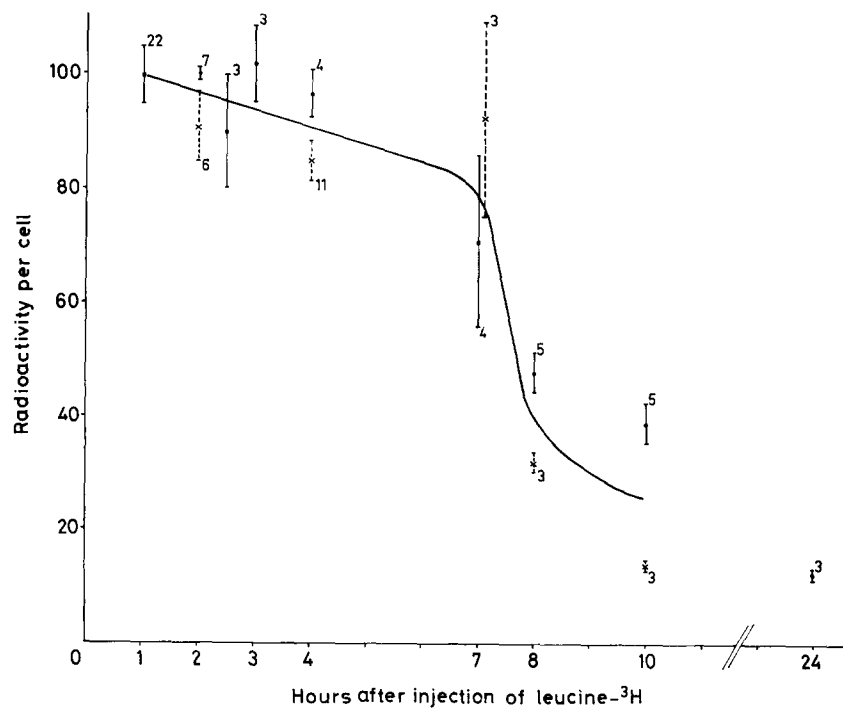


FIGURE 2   Loss of radioactive protein per cell in the rat pancreas during continued fasting after injection of leucine-$^3$H. Means ±SEM; number of animals mentioned for each point. ●, series I (100% = 9.0 silver grains per mean cellular area; ✕, series II (100% = 15.1 silver grains per mean cellular area).

TABLE II

*Presence of Radioactive Protein in Pancreatic Ducts of Fasting Rats after Injection of Leucine-³H*

| Time after injection of leucine-³H | Number of silver grains per 100 $\mu m^2$ of ductal lumen (means ±SEM) | Number of animals |
|---|---|---|
| *min* | | |
| 10 | 1.0* ± 0.2 | 4 |
| 20 | 4.9 ± 1.1 | 4 |
| 30 | 5.3 ± 2.2 | 6 |
| 60 | 6.8 ± 1.3 | 6 |
| 120 | 25.4 ± 4.7 | 6 |

* Background amounts to 0.4 ± 0.2.

The most probable explanation is that in these cases some secretory stimulation was still effective even after 44 hr of fasting. The data found for the animals of these three groups are *not* presented in the figures and tables.

Although no loss of radioactive protein could be measured radioautographically during the first 2 hr after labeling, secretion takes place as early as 20 min after injection, as evidenced by the labeling of the duct contents (Table II).

The amount of radioactivity incorporated into protein and discharged from the cells of the fasting rat pancreas did not vary with time of administration of the label (Table III; a.m. or p.m.).

TABLE III

*Incorporation of Leucine-³H into Protein and Loss of Radioactive Protein per Cell in the Rat Pancreas during Continued Fasting, in the Morning and in the Afternoon*

| Series | hr after injection of leucine-³H | Number of silver grains per mean cellular area (means ±SEM)* | |
|---|---|---|---|
| | | a.m. ‡ | p.m. § |
| I | 1 | 8.9 ± 0.5 (3) | 8.8 ± 0.6 (6) |
| | 2 | 8.9 ± 0.2 (4) | 9.1 ± 0.2 (3) |
| II | 1 | 15.9 ± 1.3 (4) | 15.2 ± 0.8 (4) |
| | 4 | 12.4 ± 0.7 (4) | 11.9 ± 0.6 (3) |
| | decrease | −22% | −22% |

* Number of animals in parentheses.
‡ Leucine-³H injected at 8:30.
§ Leucine-³H injected at 13:30.

TABLE IV

*Differences in Rate of Loss of Radioactive Protein per Cell in Pancreas of Fasting Rats with Different Degrees of Filling with Secretory Granules during Continued Fasting after Injection of Leucine-³H*

| hr after injection of leucine-³H | Number of silver grains per mean cellular area (percentage of the value found 1 hr after injection of leucine-³H; means ±SEM*) | | |
|---|---|---|---|
| | Degree of filling with secretory granules‡ | | |
| | + | ++ | +++ |
| 2.5 | 60 ± 5 (5) | 90 ± 10 (3) | |
| 3 | 67 ± 16 (3) | 102 ± 7 (3) | 130; 114 (2) |
| 4 | 52 (1) | 88 ± 4 (15) | 84 ± 9 (5) |

* Number of animals in parentheses.
‡ A series of control determinations showed a good agreement between the subjectively estimated degree of filling with secretory granules and the amount of amylase activity per microgram DNA in the same gland. A unit of amylase activity is that liberating 1 $\mu$mole of maltose per min at 37°C (Bernfeld, 1951).
The results were: +: 25–31 units/$\mu$g DNA (4 rats); ++: 47, 57–79 units/$\mu$g DNA (9 rats); +++: 64, 81–99 units/$\mu$g DNA (4 rats).

The results mentioned so far were obtained from glands selected for having acini showing a normal (+ +) degree of filling with secretory granules. "Normal" filling was found in 70% of the rats after fasting for 44 hr. In animals in which less (+) granules were present, the accelerated loss of radioactive protein appeared to start earlier, as demonstrated by the data in Table IV.

## Loss of Radioactive Protein from the Cells In Vitro

When slices of pancreas are incubated for 3 min in a medium containing radioactive leucine, this amino acid is incorporated into protein. If incubation is continued in the presence of cold leucine, radioactive protein appears in the incubation medium. During the first 20 min of incubation no radioactive protein is found in the medium (Table V). From 40 min onwards the amount of radioactivity in protein in the medium increases with time, up to 3–4 hr, after which time enzymatic proteolysis starts. The results of two typical experiments are shown in Fig. 3. The radioactivity in protein, present in the medium after 4 hr of incubation, amounts to 13.8% (SEM = 1.7; five experiments) of the total radioactivity incorporated into the pancreas slices. Apparently, the radioactive protein lost from the cells of the slices during a 4 hr period of incubation forms only a small part of the radioactivity incorporated into protein during the first 3 min of incubation. This is in good agreement with the small loss of protein radioactivity found in vivo during the first 4 hr after injection of radioactive leucine into fasting animals (Fig. 2).

The addition of atropin to the medium did not

decrease the rate of secretion of labeled protein from the cells in vitro, as is shown in Table VI.[2]

## Influence of Cycloheximide on the Loss of Radioactive Proteins from the Cells In Vivo and In Vitro

Cycloheximide was injected intraperitoneally, 1 hr after the administration of leucine-$^3$H. This interval was chosen to allow about 70% of the radioactive protein synthesized after the injection of leucine-$^3$H to reach the condensing vacuoles and secretory granules (Jamieson and Palade, 1967 b). Cycloheximide in the dose used (0.4 mg per 100 g body weight) inhibited the incorporation of leucine-$^3$H into protein of the acinar cells by about 80% up to 8 hr after its administration (Table VII). The animals did not die, but they showed a profuse diarrhea.

The rapid loss of radioactivity in protein, normally occurring in the acinar cells of fasting rats between 7 and 10 hr after incorporation, was evidently inhibited by cycloheximide (Fig. 4). However, the intravenous injection of pilocarpine, 1.5 hr before the death of the animal, results in secretion of a large part of the radioactive protein still present in the acinar cells of rats treated with cycloheximide (Table VIII).

In addition to these experiments in vivo, slices of pancreas of fasting rats, labeled with leucine-$^{14}$C during 3 min, were postincubated for 4 hr, and during the last 3 hr cycloheximide was added to the medium at a dose of $10^{-3}$ M. At this dose cycloheximide prevented protein synthesis nearly completely (98% inhibition of incorporation of L-leucine-$^{14}$C in three experiments), but no inhibition of secretion was observed during the last 3 hr of incubation[3].

### TABLE V

*Protein Radioactivity Released into the Medium by Slices of Pancreas from Fasting Rats during Incubation after Labeling with Leucine-$^{14}$C for 3 min*

| Time of incubation | cpm in TCA-insoluble fraction of medium per mg dry TCA powder from the slices | | | |
| --- | --- | --- | --- | --- |
| | Exp. 1 | Exp. 2 | Exp. 3 | Exp. 4 |
| *min* | | | | |
| 3 + 17 | 0 | 0 | 0 | 0 |
| 3 + 37 | 0 | 6 | 9 | 9 |
| 3 + 57 | 4 | 31 | 42 | 18 |

[2] We did not use atropin in vivo, since we showed that atropin (0.1 mg per g body weight, intravenously) decreased the rate of incorporation of leucine-$^3$H by 20%, 1 hr after its administration. A lower rate of fasting secretion might be attributed, therefore, to a lower rate of protein synthesis (see next paragraph) as well as to an inhibition of the acetylcholine activity.

[3] In fact the rate of in vitro secretion during 4 hr of incubation was increased by 50% in the presence of $10^{-3}$ M cycloheximide. The same effect has been described for puromycin during 2 hr of incubation by Bauduin et al. (1967).
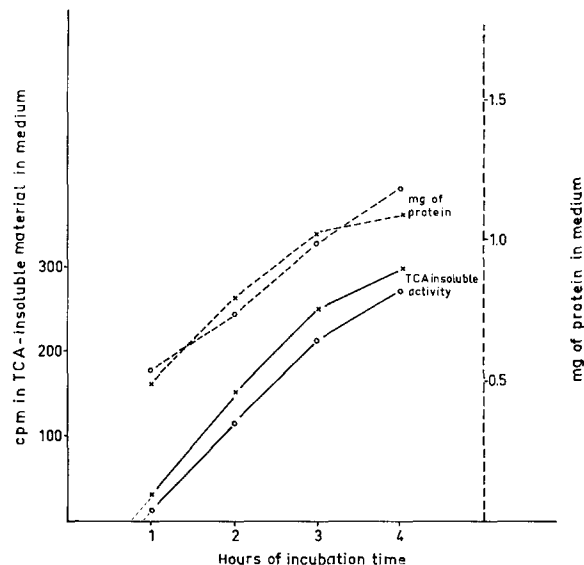
FIGURE 3 Protein (----) and protein radioactivity (———) released into the medium by slices of pancreas from fasting rats during incubation after labeling with leucine-$^{14}$C for 3 min. Data are expressed as cpm per mg dry TCA powder of the tissue (X and O represent one animal each).

## TABLE VI

Protein Radioactivity Released into the Medium by Slices of Pancreas from Fasting Rats during Incubation in the Presence of Atropin Sulfate, after Labeling with Leucine-$^{14}$C for 3 min

| Time of post-incu-bation | cpm in TCA-insoluble fraction of medium per mg dry TCA powder from the slices* | | | | | |
|---|---|---|---|---|---|---|
| | Exp. 1 | | Exp. 2 | | Exp. 3 | |
| | Control | Atropin | Control | Atropin | Control | Atropin |
| hr | | | | | | |
| 2 | 85 | 95 | 106 | 92 | 75 | 60 |
| 3 | 156 | 123 | 116 | 140 | 145 | 140 |

* In each experiment the slices from one pancreas are divided into equal parts, one for control, the other getting atropin during postincubation.

## DISCUSSION

The results of our experiments demonstrate the continuous loss of protein from the cells of the exocrine rat pancreas after a long period of fasting (Fig. 2). Such a continuous removal of protein was expected in view of the continuous and constant protein synthesis demonstrated earlier in these cells (Kramer and Poort, 1968; Poort and Kramer, 1969).

The method used—assessing the loss of protein from the gland rather than measuring the secre-

## TABLE VII

Incorporation of Leucine-$^3H$ into Protein per Cell during 10 Min after Its Injection in the Rat Pancreas, after Administration of Cycloheximide

| hr after injection of cycloheximide | Number of silver grains per mean cellular area (mean ±SEM)* | |
|---|---|---|
| | | % |
| 0 (controls) | 15.9 ± 1.5 (7) | 100 |
| 1 | 3.1 ± 0.3 (6) | 19 |
| 8 | 3.2 ± 0.1 (3) | 20 |

* Number of animals in parentheses.

tion produced—obviates the need for cannulating the duct and the concomitant risk of introducing unwanted stimuli, and allows for the actual measurement of the rate of elimination of proteins synthesized by the cells. The method cannot, however, in itself distinguish between protein elimination through secretion and protein catabolism.

Yet, in view of the scarcity of morphological indications of intracellular protein breakdown, we feel that most of the protein lost leaves the cell as secretion. The finding of labeled proteins in the duct system in accordance with the presumed onset of secretion (Table II) supports this hypothesis. Accordingly, we propose that the bulk of protein
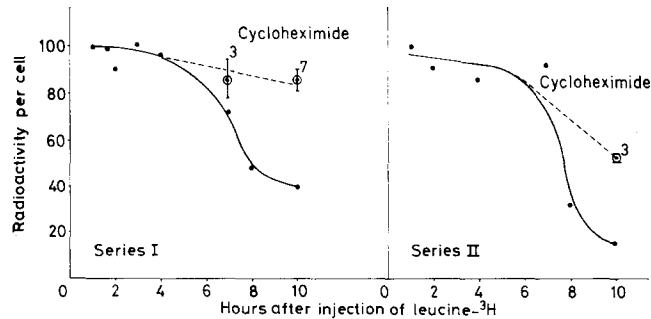
FIGURE 4 Loss of radioactive protein per cell in the rat pancreas during continued fasting after injection of leucine-$^3$H (——; same data as in Fig. 1), and the effect of cycloheximide injected 1 hr after leucine-$^3$H (----). Means ±SEM; number of animals mentioned for each point.

TABLE VIII

Influence of Cycloheximide and Pilocarpine on the Radioactivity in Protein Retained in the Cells of Fasting Rats, 10 hr after Injection of Leucine-$^3$H

| Intravenous injection | | Number of silver grains per mean cellular area (means ±SEM)* | |
|---|---|---|---|
| Materials | hr before death | | |
| | | | % |
| Leucine-$^3$H | 1 | 15.1 ± 0.6 (14) | 100 |
| Leucine-$^3$H | 10 | 2.1 ± 0.1 (3) | 14 |
| Leucine-$^3$H<br>Cycloheximide | 10<br>9 | 7.8 ± 0.2 (3) | 52 |
| Leucine-$^3$H<br>Cycloheximide<br>Pilocarpine | 10<br>9<br>1.5 | 5.8 ± 0.8 (3) | 38 |

* Number of animals in parentheses.

lost by the cells during the 10 hr after synthesis leaves the cells by way of secretion.

The secretion of radioactive protein follows a two-phase pattern (Fig. 2). The relatively slow rate of secretion during the first hours after labeling in vivo starts at about 20 min after synthesis (Table II). Since the first radioactive protein molecules enter the condensing vacuoles at that time (Jamieson and Palade, 1967 b), it is tempting to see the beginning of this phase as the result of a precocious contact of some of the condensing vacuoles with the cell membrane, though such a fusion has never been observed. Fusion between smooth vesicles (transporting protein from RER cisternae to Golgi areas) and the cell membrane cannot be excluded as a secondary cause of early secretion of labeled protein.

The uniformly slow rate of secretion in this

phase found in most but not all of the experiments in vivo seems to represent a minimum rate resulting from the absence of external stimuli acting on the pancreas cells in fasting animals. This is confirmed by the outcome of the experiments in vitro in which secretagogues, notably pancreozymin, had been largely diluted by repeated washings; yet, the cells secreted at a similar rate as in the intact fasting rat ($\sim$14% in 4 hr after pulse labeling). A contingent release of acetylcholine in the experiments in vitro could be excluded by the finding that the addition of atropin to the medium did not further reduce the release of protein (Table VI), which confirms experiments on pancreatic slices from guinea pigs by Jamieson and Palade (1971).

It should be noted that the experiments in vitro, which covered the first phase only, confirm the secretory nature of protein elimination in this

phase, in that there is no increase of TCA-soluble label in the incubation medium. But, for the highly improbable case of selective reutilization, such an increase would have to be expected if protein breakdown constitutes a major pathway of protein elimination in nonstimulated cells.

In vitro the first radioactive protein is found outside the cells between 20 and 40 min after pulse labeling (Table V and Fig. 3). Jamieson and Palade (1967 b, 1971), working with pancreatic slices from guinea pigs, observed radioactive protein in the medium even within 10 min. When they subtracted the radioactivity released by the *tissue* at 0°C (being interpreted as leakage or cell damage), they, too, found the secretion of radioactive protein to start between 20 and 40 min. The avoidance of cold washings in our system explains the absence of leakage of radioactive protein in our experiments.

About 7 hr after the synthesis of labeled protein the second phase in its history sets in, during which the greater part leaves the cell at a much higher rate (Fig. 2). Morphological signs of intracellular breakdown of secretory granules being absent and the proteins in the ducts being still radioactive at that time, one may assume that in this second phase, too, the labeled proteins are secreted rather than degraded. The late start of the second, main phase of secretion is interpreted as follows. The radioactive secretory granules assembled after the relatively short period of precursor uptake are deposited in a more or less definite layer in the cell region apical to the Golgi apparatus. This layer is gradually moved towards the cell apex by the continuous assembly of new unlabeled granules and finally reaches the apical cell membrane where the granules start to be secreted. At about 12 hr after its synthesis, all radioactive secretory protein has left the cell.

The effect of cycloheximide supports this interpretation. By blocking the protein synthesis, it brings the production of secretory granules to a standstill. Consequently, the radioactive granules formed before cycloheximide was administered are no longer pushed towards the cell membrane and the second phase of secretion is far less prominent (Fig. 4).

A possible objection to this explanation could be that by the action of cycloheximide the synthesis of certain enzymes, needed e.g. for the process of membrane fusion, is interrupted too. If those enzymes would have a high rate of turnover, their concentration could rapidly fall below

a critical value. The effect of pilocarpine, however, on cells in which cycloheximide has inhibited protein synthesis to a fair degree and for a long time (9 hr), shows that in that case secretion of stored secretory granules still can be stimulated (Table VIII). Thus, if fasting secretion depends on the same enzymatic mechanism as stimulated secretion, there is no interference of cycloheximide with the secretory process proper. Apparently, there is a sufficient store of enzymes needed for the secretory mechanism.

Dependency of the second phase of secretion on the formation of new secretory granules being an explanation for the rapid loss of secretory protein from 7 hr after its synthesis onwards, this rapid loss could conceivably be a mere consequence of a diurnal rhythm in either synthetic activity or secretory stimulation. From the results of Table III, however, we conclude that a diurnal rhythm in rate of protein synthesis or in rate of secretion does not exist in the pancreatic cells of the fasting rat during the time of the experiments.

The discussion till now has been based on the assumption that all secretory protein is gathered into secretory granules. Indeed, Jamieson and Palade (1967 a, b) have demonstrated that 70% of all labeled amino acid incorporated in the pancreas cells is found in those granules. Since about 25% of the label is incorporated into "constitutive" protein (see discussion in Poort and Kramer, 1969), few if any secretory protein molecules pass through the cell freely.

24 hr after the injection of leucine-$^3$H, 13% of the radioactive protein is still present in the cell (measured radioautographically and biochemically). Since this radioactivity is not removed from the cells by pilocarpine (Kramer and Poort, 1968), it is present not in secretory but in constitutive protein. Assuming that about 25% of the label is built into constitutive protein, the loss of this nonexportable protein can be deduced to have a half-life of at least 24 hr. The actual secretory protein radioactivity is found by subtracting, in Fig. 2, the constitutive protein radioactivity present in the cell from the over-all radioactivity value.

Our concept of secretion implies that the secretion of the granule contents depends essentially on the chance that the granules come into contact with the luminal cell membrane. Stimulated secretion might result from an enhanced intracellular movement and/or an increased motility of the cell membrane, both factors increasing the number of contacts of granules with the cell membrane.

Additionally, stimulation may imply an increase in the ability of the granule and cell membranes to fuse with each other. The latter capacity apparently persists to a degree in unstimulated cells, for in these cells, too, secretion takes place once a balance is struck between the number of granules produced and the number of granules coming into sufficiently close contact with the luminal cell membrane to be discharged.

The onset of the second phase at about 7 hr after pulse labeling—and, indeed, the biphasic character of secretion—is found only in fasting animals whose pancreas cells contained a fairly large amount of granules (designated by us as + + or + + +). In a number of unstimulated, fasting rats less granules (+) were present per cell. In those animals more radioactive protein was lost during the first few hours after pulse labeling (Table IV). As we have been unable to find differences in rate of protein synthesis in fasting animals with clearly different numbers of granules in their pancreatic cells (Poort and Kramer, 1969), the persistence of secretory stimuli, in spite of the fasting condition, offers the most likely explanation of the lower number of granules present and of the higher rate of early secretion of radioactive protein in these animals. The same may apply to the few animals with a relatively high rate of protein discharge during the first 4 hr, in spite of a normal (+ +) number of secretory granules, if one takes into account the rather broad variety in intracellular amount of secretory protein covered by the + + estimation (Table IV, footnote ‡).

In conclusion, we submit that the course of the unstimulated secretion of newly synthesized protein by the rat pancreas as observed in favorable circumstances is mainly determined by a gradual and orderly movement of secretory granules through the apical cytoplasm towards the luminal cell membrane. The displacement occurs mainly because the continuously and constantly synthesized protein is being accumulated into granules in the Golgi complex.

In the literature, data are found which support our finding of a low rate of secretion of radioactive protein in rats during the first hours after pulse labeling. In monkeys, 4 hr after intravenous injection of methionine-$^{75}$Se, the pancreas still contains 90% of the amount of radioactivity present 1 hr after the injection (Zuidema et al., 1963). In dogs, only 7% of the amount of radio-

activity present in the gland 2 hr after intravenous injection of methionine-$^{75}$Se is secreted during the two subsequent hours (Goidsenhoven et al., 1967).

In man, the unstimulated rate of secretion of radioactive protein reaches its maximal value 8 hr after the injection of methionine-$^{35}$S (Kukral et al., 1965). This interval is in good agreement with the observed acceleration in loss of radioactive protein 7 hr after labeling of rats.

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