EFFECT OF PANCREOZYMIN ON RAT PANCREATIC ENZYME BIOSYNTHESIS

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ABSTRACT

Pancreatic enzyme secretion in rats anesthesized by pentobarbital was stimulated by intravenous perfusion of the hormone pancreozymin, as indicated by a decreased amylase level in the pancreas and by specific, fine structural changes observed in an electron microscope. Rates of protein synthesis were determined by pulse labeling. Amylase, total protein, and valine were purified from pancreas and counted. Pancreozymin promotes an 8 to 10 times increase in the rate of biosynthesis of pancreatic enzymes, as compared to rats similarly anesthesized but without hormone. This stimulation effect is obtained very rapidly (2 hr) and is not inhibited by actinomycin D. Secretin alone has no effect, whereas pentobarbital is inhibitory.

INTRODUCTION

The secretion of pancreatic juice into the intestine is mediated in part by the polypeptidic hormones, pancreozymin and secretin. The specific physiological role of these two hormones is now well established. Pancreozymin is known to promote the secretion of hydrolytic enzymes from the pancreas, while secretin acts on the secretion of water and salts (Harper, 1967). Specific structural changes induced by pancreozymin have been observed in target acinar cells (Ichikawa, 1965; Hermodson, 1965). All structural and radioautographic studies (Caro and Palade, 1964) lead to the conclusion that pancreozymin might trigger the discharge of enzymes stored in the zymogen granules into the glandular lumen. Biochemical investigations of the process of synthesis and cellular transport of enzymes have been successfully achieved by using subcellular fractions identified by electron microscopy (Jamieson and Palade, 1966; Redman, 1967; Palade et al., 1962). However, it is still controversial whether increased enzyme secretion is paralleled by increased enzyme synthesis in the gland, or whether enzyme synthesis proceeds at a constant rate (steady state) while the rate of secretion varies. Possibly because of the different systems and techniques used to stimulate secretion or measure protein biosynthesis, opposite conclusions have been reached (Hokin, 1953; Siekevitz and Palade, 1958; Webster and Tyor, 1966; Kramer and Poort, 1968). Taking advantage of the methods set up in our laboratory to assay and purify pancreatic enzymes and to measure biosynthetic rates, we decided to investigate this question.

In order to get a maximal and continuous stimulation, the hormones were given to rats by perfusion; all rats were thus necessarily anesthesized. Stimulation was estimated from the enzyme level in the gland and from ultrastructural studies. The rate of enzyme synthesis was determined by measuring the incorporation of radioactive valine into purified enzymes and total protein after pulse labeling. Our results indicate a close relation between protein biosynthesis and secretion.

MATERIALS AND METHODS

Wistar male rats weighing between 200 and 250 g and fed ad lib. on a balanced diet were used.

Operation Procedure

All rats were injected intraperitoneally with pentobarbital (60 mg/kg), 30 min before the start of perfusion; a cannula (ϕ = 0.1 mm) was then inserted into the femoral vein at one end and connected to an automatic Braun distributor at the other end. Pancreozymin and secretin were perfused at the rate of 0.75 Ivy dog-units/kg per hr.[1] Control rats were perfused with the same volume of saline. In addition, all rats were perfused simultaneously with small doses of pentobarbital (1.5 mg/kg per hr) to maintain anaesthesia. 5 μCi of L-valine-^{14}C, dissolved in 0.4 ml of 1.25 mM HCl and 0.9% NaCl was injected through the femoral vein cannula, 10 min before death. The animals were killed by decapitation. The pancreas and liver were rapidly excised, weighed, rinsed with cold 0.9% NaCl, and analyzed either as individual or pooled specimens. Actinomycin D was dissolved in 1,2-propanediol at 2 mg/ml final concentration; when indicated, 2 mg/kg of body weight was given intraperitoneally, 15 min before injecting pentobarbital.

Purification Procedure

Fresh tissue (0.6–5.0 g) was minced and homogenized with 9 volumes of either water or 0.1 M phosphate buffer (pH 8.0) in a Potter-Elvehjem apparatus (clearance = 0.3 mm). The homogenate was sonicated at 10 sec intervals for 30 sec (8 amp audiofrequency current) to obtain a complete release of amylase, and filtered through gauze.[2] Amylase was purified by chromatography and glycogen precipitation (Marchis-Mouren et al., 1963). All preparative steps were carried out at 0°C. Protein and DNA were extracted from the sonicated pancratic homogenate by trichloroacetic acid precipitation (Schneider, 1957). Free intrapancreatic valine was extracted from the cold 5% trichloroacetic acid supernatant, purified on a Technicon amino acid analyzer, and counted (Palla et al., 1968).

Assays

The following, previously described assays we used: amylase was assayed by the colorimetric method, by means of dinitrosalicylic acid (Bernfield, 1955) with the use of soluble purified starch as substrate; amylase units were expressed as micromole of maltose liberated per minute; protein was determined by the method of Lowry et al. (1951) with horse albumin as a standard; DNA was analyzed by the diphenylamine method (Dische, 1930). The amount of amylase, or protein, of the homogenate was referred to DNA as a unit, (or as milligram) per microgram of DNA phosphorus. When indicated, these values were expressed as a percentage of the control values.

Counting Procedure

An aliquot of each radioactive sample was added to 10 ml of scintillation fluid (Bray, 1960) in a glass vial, and the radioactivity was measured in a Packard Tricarb spectrometer. Counts in amylase or in total protein were referred to micrograms of DNA-phosphorus.

Microscopy

Pancreas fragments (tail) were fixed for 2–4 hr at 0°C in 2% OsO_4 buffered at pH 7.4 with 0.1 M K

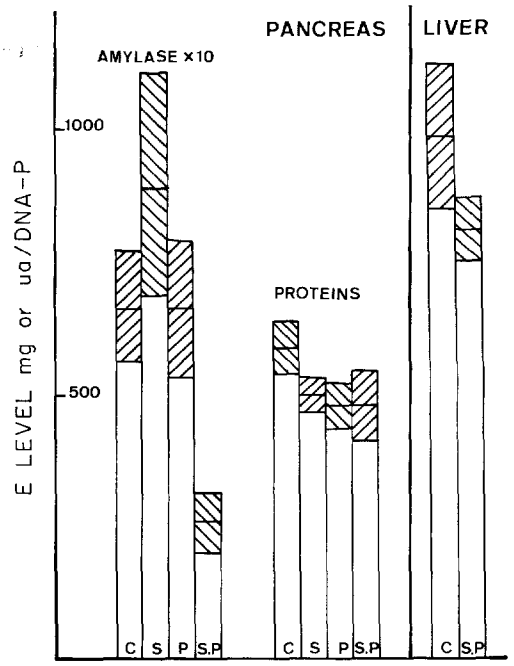


FIGURE 1 Effect of perfusion of pancreozymin (*P*) and/or secretin (*S*) on the level of amylase and total protein in pancreas and liver. All rats were perfused with the same dose of pentobarbital for 4 hr. In the control group (*C*) the hormones are omitted and replaced by saline. The level plotted in ordinate is expressed as milligrams of protein or units of amylase (*ua*) per microgram of DNA-P phosphorus. The level of amylase and protein was determined in four groups of six rats each. Confidence intervals (indicated by the hatching) are calculated by multiplying the standard error by the Fisher coefficient for a 95% probability.

[1] 75 Ivy dog-units = 300 Crick Harper and Raper Units.

[2] Without sonication, only 58% of total amylase is released; complete release (97%) was obtained by sonication.

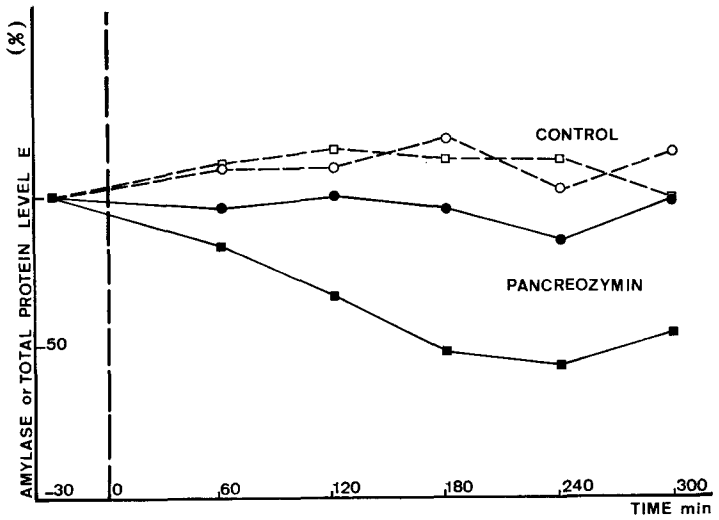


FIGURE 2 Effect of pancreozymin and secretin on the level of amylase and total protein in pancreas after various time of perfusion. A single injection of pentobarbital is given at 30 min before the start of perfusion (zero time). The conditions are the same as in Fig. 1. Control animals are perfused only with pentobarbital and saline (dotted line). Treated animals are perfused additionally with pancreozymin and secretin (solid line); total protein (circles); amylase (squares). Each value, a mean of four experiments of six rats each, is expressed as a per cent of the untreated animals.

Magnification markers for all figures indicate 1μ.

phosphate, dehydrated, and embedded in Epon. Thin sections were doubly stained with uranyl acetate and Pb citrate, and examined in a Philips EM 300.

Chemicals

L-Valine-^{14}C (95 mCi/mmole) was purchased from the Commissariat à l'Energie Atomique, Saclay; sodium pentobarbital from Lathevet Laboratory, Paris; pancreozymin and secretin from Boots Laboratory, Nottingham; and actinomycin D was a gift from Merck Sharp and Dohme, Rahway, N. J.

RESULTS

Effect of Pancreozymin on the Level of Amylase and Total Protein in the Pancreas

Stimulation of enzyme secretion by pancreozymin by depleting the zymogen granules should decrease the enzyme content in the gland. In this experiment (Fig. 1) rats were perfused with saline (*C*), with secretin (*S*) or with pancreozymin (*P*), or with both hormones (*S.P.*), for 4 hr; pancreas and liver were then excised and treated individually. Statistical studies were carried out on groups of six rats. The results reported in Fig. 1 indicate that, after perfusion for 4 hr, neither the amylase nor the total protein level is significantly modified when pancreozymin and secretin are used separately. However, when the hormones are perfused simultaneously, the amylase level is decreased by more than 50%, while only a slight effect is shown on total protein. No significant change is seen in liver protein. In the next experiment, pancreozymin and secretin were perfused simultaneously, the perfusion was started at zero time, and groups of six rats were killed when indicated (Fig. 2). The excised pancreases were pooled and treated together. Each point on the figure is a mean of four experiments on six rats. A progressive decrease of the amylase level (E) in the gland is observed ($dE/dt < 0$). A plateau which corresponds to 50% of the amylase level in the control is attained, 3 hr after the start of perfusion ($dE/dt = 0$). However, only a slight decrease (10–20%) of the total protein level is obtained.

Ultrastructural Studies

This work was carried out (Dr. Tasso) to check the secretory state of the acinar cells in

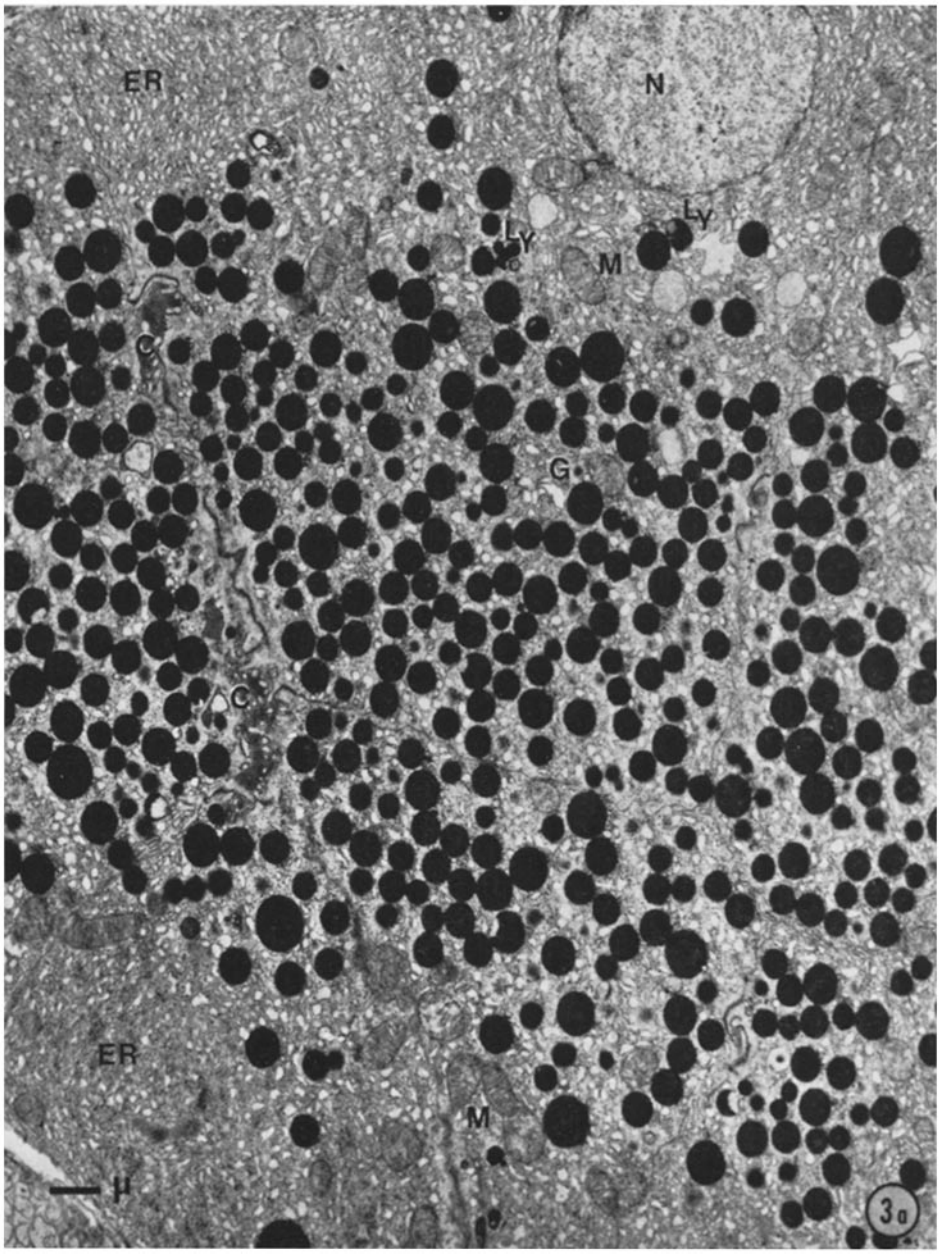
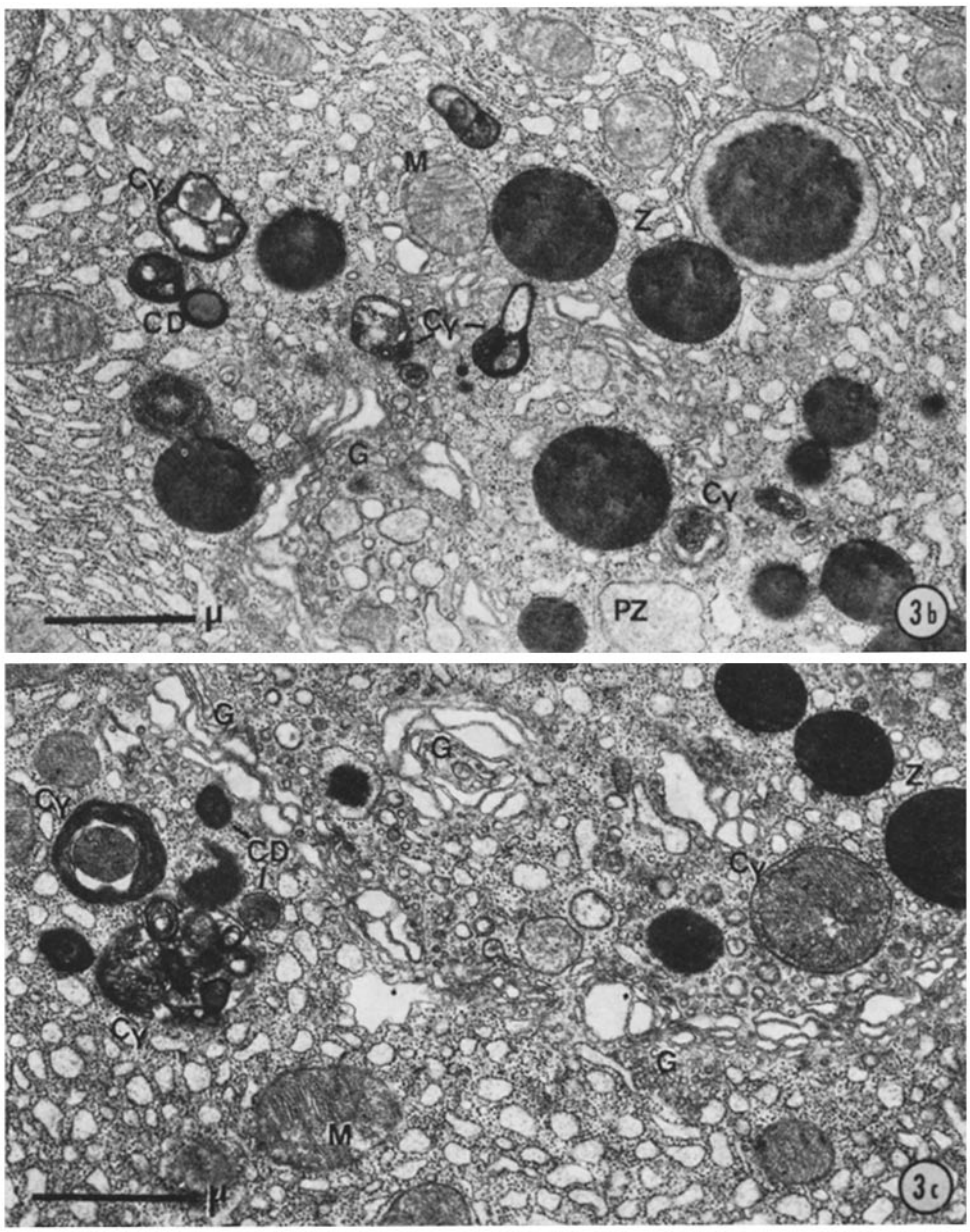


FIGURE 3 *a* Acinar cell fine structure from control rats perfused with saline and pentobarbital for 4 hr. Numerous zymogen granules are shown. Intercellular duct (*C*), lysosome (*Ly*), endoplasmic reticulum (*ER*), Golgi region (*G*), mitochondria (*M*), nucleus (*N*). × 6700.

secretin and pancreozymin, and secretin-perfused animals,[3] as compared to control rats perfused under the same conditions with the hormones omitted. All animals were perfused for 4 hr. As seen in Figs. 3 *a*, 3 *b*, and 3 *c*, acinar cells in control rats are heavily loaded with zymogen granules. Perfusion of secretin alone did not induce any

[3] Similar studies have been carried out on fasting dog by Dr. Frexinos (1968).



FIGURES 3 *b* and *c* Acinar cell fine structure from control rats perfused as in Fig. 3 *a*. Numerous cytolysomes (*Cy*) and dense bodies (*CD*) are found around the Golgi region (*G*). Zymogen granules (*Z*), prozymogen granules (*PZ*), mitochondria (*M*). Fig. 3 *b*, × 21,900; Fig. 3 *c*, × 20,200.

apparent morphological change in these cells. However, after 4 hr of pancreozymin plus secretin perfusion, characteristic changes of the morphological pattern are obtained, although, depending on the section observed, the number of zymogen granules is markedly reduced. In Fig. 4 *a* only a few granules accumulate in the apical region of the cell. In addition, the extension of the Golgi apparatus is remarkable (Fig. 4 *b*): the Golgi vacuoles and the cisternae become larger and more numerous, and the number of prozymogen granules is increased. One should also note

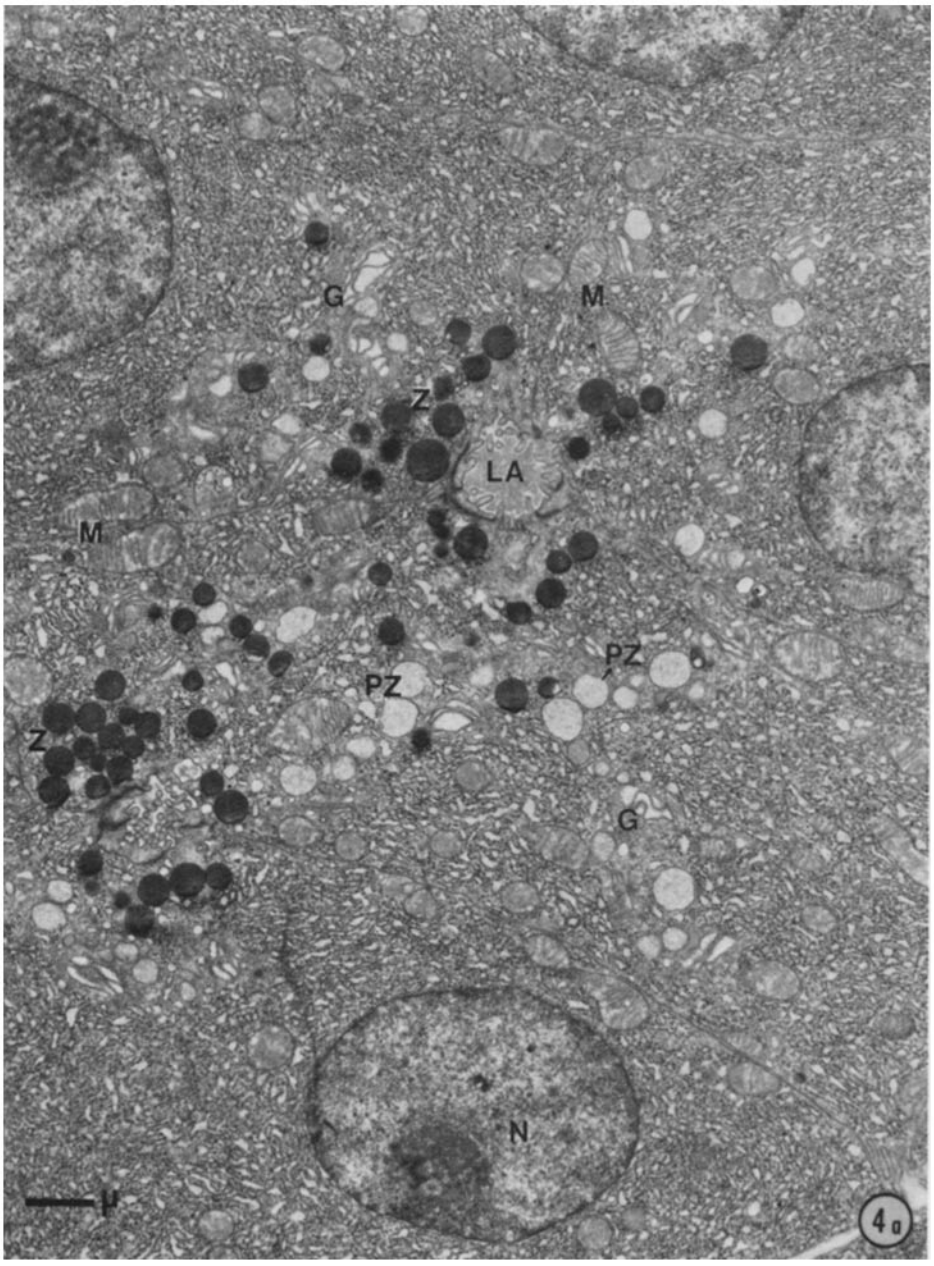


FIGURE 4 *a* Acinar cell fine structure from rats perfused with pancreozymin, secretin, and pentobarbital. A few zymogen granules (*Z*) are apparent around the lumen (*LA*) which is slightly expanded. Numerous prozymogen granules (*PZ*) are found near the Golgi region (*G*). Mitochondria (*M*), nucleus (*N*). × 9100.

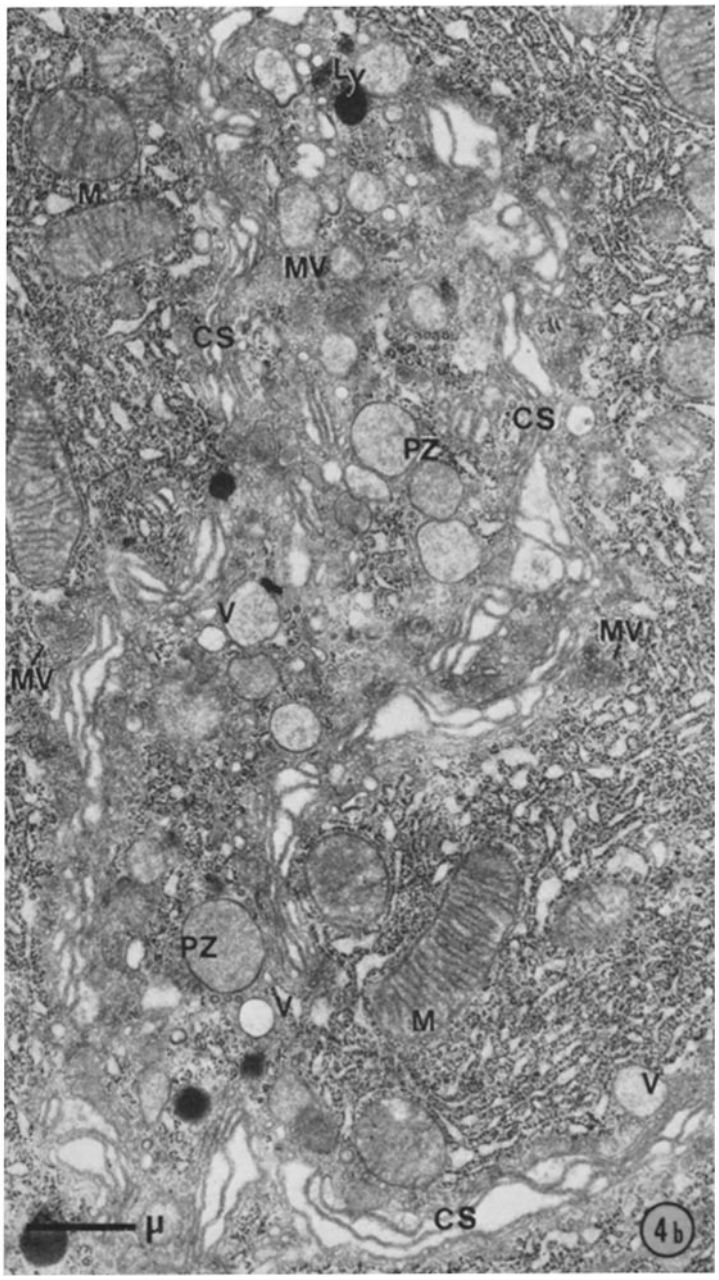


FIGURE 4 *b* Fine structure of an acinar cell from rats perfused with pancreozymin, secretin, and pentobarbital. The Golgi apparatus is widely expanded, with numerous microvesicles (*MV*), cisternae (*CS*), and condensing vacuoles (*V*), which mature as prozymogen granules (*PZ*). Lysosome (*LY*), mitochondria (*M*). × 14,400.

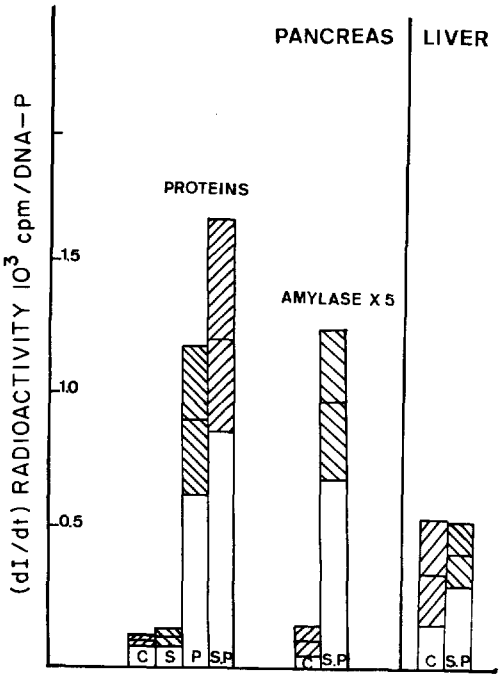


FIGURE 5 Effect of pancreozymin and secretin on the rate of L-valine-^{14}C-incorporation into amylase and total protein in pancreas and in liver. After 4 hr of perfusion (the conditions are the same as in Fig. 1), radioactive valine was injected through the cannula, and the rat was killed 10 min later. Symbols and statistical results are expressed as in Fig. 1. The amount of radioactivity incorporated in 10 min (ordinate) is expressed as 10^3 cpm/μg of DNA phosphorus (*DNA-P*).

that electron-opaque bodies, cytolysomes, and lysosomes were encountered in perfused control animals and hormone-treated animals at a significantly higher frequency than in unperfused normal and fasting animals, although these particles are unrelated to the secretion process.

Effect of Pancreozymin and Secretin on the Rate of Radioactive Valine Incorporation into Pancreatic Proteins

In the two next experiments, rats were perfused as previously described. Statistical studies were performed on rats perfused for 4 hr. Radioactive valine was injected 10 min before death; pancreas and liver were excised and analyzed individually. Amylase, total protein purified from pancreas, and total liver protein were assayed and counted. The results are given in Fig. 5. In each case, calculations were made from six specimens. No hormonal stimulation of valine incorporation (I) into liver protein is obtained. An 8- to 10-fold increase in radioactivity, due to pancreozymin alone or combined with secretin is found in both pancreatic protein and amylase, while perfusion of secretin alone is ineffective. The addition of secretin to pancreozymin does not promote a larger stimulation. Secretin and pancreozymin were thereafter routinely perfused together.

Kinetic studies were performed on groups of six rats perfused with or without hormones as indicated in Fig. 6; each point on the figure is a mean of four experiments. Rats were injected 10 min before death with radioactive valine. The pancreases were pooled, and total protein and amylase were purified. The rate of incorporation into control-rat proteins (lower curve) decreases as a function of time (similar curves, not shown, were found for amylase). Hormone addition induces a rapid rise in radioactivity (upper curve). After perfusion for 1–2 hr, both curves reach plateaus, which indicate that the rate of incorporation ($dI/dt = C_I$) of radioactive valine is 10 times higher in hormone-perfused rats than in control rats. The same stimulatory effect is observed when incorporation of label into pure amylase is measured. This stimulatory effect is not abolished by actinomycin D doses, high enough to inhibit 90–95% of RNA synthesis (unpublished results). Actinomycin was given 15 min before pentobarbital injection.

In order to know whether the observed increase in the incorporation rate of L-valine-^{14}C reflects an equal increase in the rate of protein synthesis, (dE/dt), free intrapancreatic valine was purified and counted. Since the specific radioactivity of the valine pool is known (Reboud et al., 1966) to fall after injection of the tracer, it was determined after various times of radioactive valine labeling. Rats perfused for 4 hr were injected with the tracer either 3, 6, or 10 min before being killed. L-valine was purified from pancreas homogenate and counted. Only slight differences in the specific radioactivity of the valine between hormone- and control rats were found (Table I).

DISCUSSION

Although a prolonged perfusion of pancreozymin probably does not mimick physiological conditions, the expected usually stimulatory effect was obtained as shown by our biochemical and ultrastructural studies.

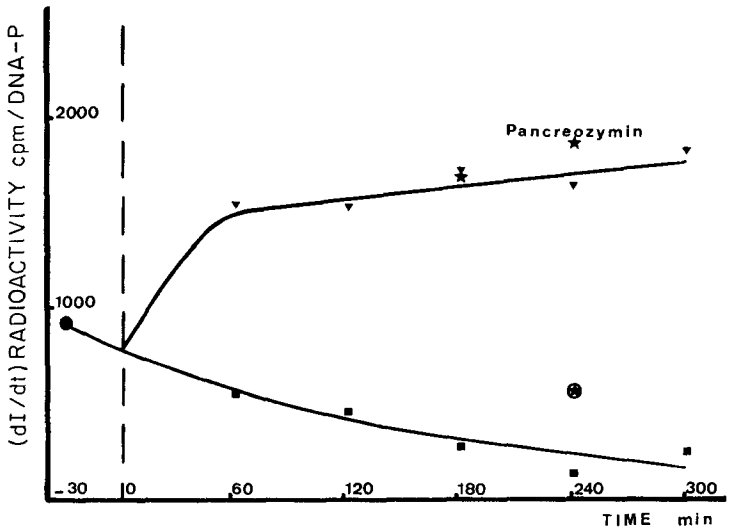


FIGURE 6 Effect of pancreozymin, secretin and actinomycin on the rate of valine incorporation into total protein after various times of perfusion. The conditions of perfusion are the same as in Fig. 2. When indicated actinomycin D was given intraperitoneally, 45 min before the start of perfusion. Each point is a mean of four experiments on six rats, each (except for the control group) treated with actinomycin D which represents one experiment. The radioactivity incorporated in 10 min plotted on the ordinate, is expressed as cpm/μg of DNA phosphorus. Control rats perfused with saline and pentobarbital (closed squares); rats perfused additionally with pancreozymin and secretin (closed triangles); rats before perfusion (closed circles); actinomycin- and hormone-treated rats (closed stars); and actinomycin given to control rats (encircled closed star).

TABLE I

Effect of Pancreozymin on the Specific Radioactivity of the Intrapancreatic Valine Pool

Valine incorporation time	Controls		Treated rats	
	Valine	Total protein	Valine	Total protein
min	*cpm/μmole*	*cpm/μg of DNA-P*	*cpm/μmole*	*cpm/mg of DNA-P*
3	110,650	61.5	95,800	663.0
6	66,700	111.0	41,200	1,115.5
10	51,400	136.0	44,000	1,079.0

The conditions are the same as in Fig. 2. Radioactive valine was injected either 3, 6, or 10 min before death. Free valine was purified from the pancreatic homogenate, analyzed on an amino acid analyzer, and counted. These results come from individuals. This explains why the incorporation value into total proteins, after 10 min, is smaller than the average value (see Fig. 6).

Since in both biochemical experiments, calculated confidence intervals (see legend for Fig. 1) are not overlapping, it was then valid to pool the pancreases and to calculate a mean value at each time of perfusion. One may ask why, in the two first experiments, simultaneous perfusion of secretin and pancreozymin was necessary in order to decrease the amylase level of the gland. It is likely that the effect of secretin is due to a good drainage of the enzymatic juice from the pancreatic ducts to the intestine, similar to the well-known "washing out" effect. Another point to discuss in this experiment is the observed rather slight decrease of the total protein level in the

pancreas as compared to that of amylase. It is known that the amount of exocrine pancreatic enzymes in the gland does not represent more than 40% of the total pancreatic protein. In our experiment, amylase depletion is about 50%, and the level of the nonexportable protein is of course unchanged; one may then calculate that the maximum effect on total protein is no higher than 20%. This explains the apparent discrepancy found in our results. A similar difference between total protein, amylase, and lipase levels had been previously observed when carbamylcholine was used for stimulation (Marchis-Mouren and Reggio, 1968).

A more direct study of the stimulation of secretion by pancreozymin is supported by the ultrastructural studies. However, two facts should be kept in mind when the micrographs are being interpreted: the rats are not fasting, and all animals are treated with pentobarbital. The zymogen granule content is usually low in nonfasting rats. The observed accumulation is thus possibly a side effect of the pentobarbital. Nevertheless, the pancreozymin effect on secretion is clearly demonstrated by the fine structural changes observed in the acinar cells as well as by the low level of amylase in the gland.

Pancreozymin is now well characterized, especially with respect to secretin, from a physiological point of view: it stimulates secretion of an enzyme-rich juice; from a cellular point of view, it induces fine-structural changes. Additional characterization is obtained from our experiments: pancreozymin promotes a specific stimulation of amino acid incorporation into pancreatic proteins. As noted, the same extent of stimulation of incorporation is obtained in total protein as in amylase. This result is not opposed to the differences observed in the levels of these substances reported here. It indicates, rather, that most of the valine radioactivity is incorporated into rapidly synthesized enzymes, while nonexportable protein incorporates negligible amounts of radioactivity because of its slow turnover.

However, the conditions under which this effect was obtained should be noted: that is, prolonged anaesthesia and perfusion may have influenced the results obtained. As seen in the kinetic experiment, the rate of valine incorporation into protein of control rats decreases because of the effect of prolonged pentobarbital treatment. This inhibitory effect is very rapid in pancreas as well as in other tissues (Marchis-Mouren and Reggio, 1968). Thus, in our experiment, pancreozymin seems to act at least in part by specifically reversing the pentobarbital effect on pancreas. Since pancreozymin is known to stimulate cellular respiration in pancreas (Davies et al., 1949), a possible explanation would be the reversal of the respiration depression presumably produced by the barbiturate. Nevertheless, it should be pointed out that the effect of pancreozymin is organ specific, since pentobarbital inhibition in liver is not reversed by the hormone. Actually, the stimulation of amino acid in incorporation as compared to normal rats is no more than two-fold.

The comparison of the specific radioactivity values for free pancreatic valine in control rats and treated animals has shown slight differences but they have only negligible bearing in the calculation of the rate of biosynthesis (Reboud et al., 1966). The results strongly suggest that pancreozymin increases the rate of synthesis of amylase and of total pancreatic protein by a factor of 10. A more rigorous measurement of the valine pool could have been attained by purification of valyl-tRNA. Unfortunately, the attempt to do this was unsuccessful. However, a possible error in the measurements reported due to valine from residual blood is very unlikely because its radioactivity decreases rapidly (Reboud et al., 1966).

In addition to the data from the biochemical studies, ultrastructural changes such as dilatation of the Golgi apparatus were observed, which support the concept of increased transport and biosynthesis of enzymes in acinar cells (Jamieson and Palade, 1966).

In view of the mode of action of pancreozymin, it should be noticed that the kinetic curve for the rate of incorporation of radioactivity in amylase (dI/dt) does not parallel the level of amylase (E) in the gland (Figs. 2, 6). If one assumes that the secretion rate is a first order process ($dE/dt = K_s E$) and the rate of synthesis a zero order process ($dE/dt = K_I$), the rate of amylase loss is:

$$\frac{dE}{dt} = K_I - K_s E.$$

From the kinetics of incorporation (Fig. 6) it can be seen that the value of C_I (the incorporation rate constant) is rapidly increased to a new value C'_I (the incorporation rate constant with hormone)

$$C'_I > C_I$$

and since this constant is proportional to the rate of synthesis constant (K_I) then:

$$K'_I > K_I .$$

The decrease of enzyme level shown in Fig. 2 ($dE/dt < 0$) can then only be accounted for by a rapid increase in the rate of secretion; thus, if K_s' is the secretion constant with hormone,

$$K'_s > K_s .$$

The enzyme level reaches a new steady state after 3 hr (Fig. 2); at this time a new equilibrium is reached between synthesis and secretion, and

$$\frac{dE}{dt} = 0, \text{ i.e., } K'_I = K'_s E',$$

where E' is the new level of enzyme.

From our data it may be concluded that both the secretion and the synthetic processes are affected by the hormone in no more than 1 hr, but it is not possible yet to decide which process is triggered first.

The regulatory mechanism in protein synthesis of this system is characterized by, in addition to its rapidity, a magnitude (10-fold) rarely observed in mammalian cells. Preliminary studies indicate that the hormonal effect is not abolished by actinomycin D. Pancreozymin, then, does not appear to act through modified transcription of DNA, since all *de novo* RNA synthesis is inhibited by the antibiotic (unpublished results). Moreover, this result is unexpected, from what we know of messenger RNA (mRNA) stability. The average mRNA stability in pancreas has been measured and its half-life is 4 hr (Marchis-Mouren and Cozzone, 1966; Cozzone and Marchis-Mouren, 1967). After a perfusion for 4 hr, at most half of the pancreozymin effect should be obtained; since this was not observed, a possible explanation is that, in pancreozymin perfused rats, mRNA is not rate limiting. Many working hypotheses concerning control at the translation level are apparent, and they will guide our future research.

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