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Effect of caerulein on protein synthesis and secretion in the guinea-pig pancreas

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Summary

1. Caerulein produced a striking increase in the rate of secretion of the guinea-pig pancreas. When tested *in vivo* with pancreatic slices its optimal concentration was found to be 2·5 ng/ml. *In vivo* the intravenous injection of 400 ng/kg and the subcutaneous injection of 4 μg/kg of the drug produced marked secretory responses.

2. Neither incubation of pancreatic slices *in vitro* for various lengths of time in the presence of caerulein nor the intravenous injection of the drug brought about any detectable changes in the rate of ^{14}C-L-leucine incorporation into protein of pancreatic slices incubated *in vitro*. By contrast, a two-fold increase in the incorporation of leucine was found in fasting animals injected subcutaneously with caerulein twice a day for 4 days. The same treatment in fed animals, or one single dose of caerulein injected subcutaneously 4 h before killing in guinea-pigs starved for 4 days did not affect the rate of incorporation. Moreover the long term treatment with the cholinergic segretagogue bethanechol in fasted guinea-pigs was also ineffective.

3. It is concluded (*a*) that in the guinea-pig pancreas, stimulation of enzyme secretion does not affect *per se* the rate of synthesis of digestive enzymes; and (*b*) that the increase of protein synthesis elicited by caerulein is critically dependent both on the nature of the segretagogue (that is, it is elicited by the polypeptide but not by a cholinergic drug) and on the experimental conditions (it is evident only in fasted animals after prolonged treatment).

Introduction

Numerous studies dealing with the effect of stimulation of enzyme secretion on the rate of protein synthesis in the pancreas have been reported (Daly & Mirsky, 1953; Allfrey, Daly & Mirsky, 1954; Ferreira Fernandez & Junqueira, 1955; Farber & Sidransky, 1956; Hokin, 1956; Busch, Allen & Anderson, 1959; Stöcker, 1962; Webster & Tyor, 1966; Peakall, 1967; Webster, 1968; Kramer & Poort, 1968; Poort & Geuze, 1969; Poort & Kramer, 1969). Though several conflicting results have been obtained in the past, recent studies seem to indicate that a substantial increase of the rate of protein synthesis is brought about by either feeding or pharmacological stimulation of secretion only in birds and frogs (Webster & Tyor, 1966; Peakall, 1967; Webster, 1968; Poort & Geuze, 1969); whereas in mammals protein synthesis proceeds at a constant rate, independently of secretion (Kramer & Poort, 1968; Poort & Kramer, 1969).

However, the evidence available is not unquestionable, both because the effects of different segretagogues have not been sufficiently investigated and because the results mentioned above concern acute experiments, where stimulation was obtained either by incubating pancreatic slices *in vitro* in the presence of segretagogue, by injecting a single dose of the drug intravenously or by feeding animals which had been starved for a short time. Very few prolonged experiments, implying repeated stimulation of pancreatic secretion, have been reported (Rothman & Wells, 1967).

We have reinvestigated the problem in the guinea-pig using caerulein, a segretagogue whose effect on pancreatic protein synthesis had never been investigated previously. This polypeptide, whose C-terminal heptapeptide closely resembles the C-terminal heptapeptide of the natural hormone pancreozymin, has recently been found to be much more active on pancreatic secretion than pancreozymin itself in several animal species (Bertaccini, de Caro, Endean, Erspamer & Impicciatore, 1969 ; Erspamer, 1970).

We have observed in acute experiments that doses of caerulein eliciting a massive secretion of digestive enzymes both *in vivo* and *in vitro* were unable to affect the rate of protein synthesis in the pancreas, but prolonged treatment in starved animals resulted in a conspicuous increase in protein synthesis. The latter effect could not be duplicated when a different type of segretagogue, namely bethanechol, was used instead of caerulein, thus suggesting that it was not simply due to the extrusion of secretory proteins but somehow specifically related to the segretagogue drug.

Methods

Male albino guinea-pigs, weighing 450–550 g (gift of Sigurtà Drug Corporation, Milan, Italy) were used. In most of the experiments they were fasted 20–24 h before being killed. In other experiments, animals had free access to food or were starved for a longer time (4 days).

Stimulation of secretion was obtained either by injection of segretagogues *in vivo* or by incubating pancreas tissue slices *in vitro* in the presence of the drugs. Incorporation of ^{14}C-L-leucine into protein was always studied on tissue slices incubated *in vitro*.

Stimulation in vivo. *In vivo* stimulation of secretion by caerulein (400 ng/kg) was obtained either by a single rapid intravenous injection in pentobarbitone anaesthetized animals or by the subcutaneous injection of 4 μg/kg. In this case conscious animals were given either single or repeated injection (two per day for 4 days) and were killed 4 h after the last dose. When bethanechol (carbamoyl-β-methyl-choline) was used as segretagogue it was given according to the latter schedule, in a dose of 600 μg/kilogramme.

Controls were treated analogously to experimental animals using saline and were killed according to the same schedule, in order to eliminate changes due to diurnal or day-to-day variations. Since, however, such variations could not be detected, values found in control animals are grouped together in the tables.

Stimulation in vitro. Stimulation *in vitro* was achieved by incubating pancreas tissue slices in the presence of caerulein (2·5 ng/ml) in Krebs Ringer bicarbonate

solution as indicated below. After incubation at 37° C for 10, 30, 90, and 180 min, slices were washed with a large volume of warm incubation medium and transferred to flasks containing radioactive medium for incorporation studies. Control slices, derived from the same pancreata as the experimental ones, were incubated according to the same schedule but without caerulein.

Slicing technique and incubation of tissue slices in vitro. Animals were stunned by a blow in the head and bled by section of the heart. Pancreata were quickly excised and immersed in ice-cold incubation medium (Krebs-Ringer bicarbonate solution, Krebs, 1950) equilibrated with 95% O_2 and 5% CO_2 to a final pH of 7·4, containing 14 mM glucose and supplemented with L-amino-acids at concentrations specified for Eagle tissue culture medium (Eagle, 1959), except that, for tracer experiments with ^{14}C-L-leucine, unlabelled leucine was omitted.

Tissue slices ~0·5 mm thick and weighing ~40–50 mg were made as described by Jamieson & Palade (1967a), using a Stadie and Riggs tissue slicing blade. Fifty ml flasks, containing 7 ml of the incubation medium, were loaded with eight to ten slices and gassed with 95% O_2 and 5% CO_2. After 10 min of equilibration at 0° C the incubation was carried out in a water bath at 37° C and agitated 80 times/min, for various lengths of time. At the end of the incubation the slices were washed carefully with ice-cold 0·3 M sucrose, placed in 2 ml of ice-cold 0·3 M sucrose and homogenized.

In tracer experiments uniformly labelled ^{14}C-L-leucine was added to give a concentration of 0·14 μCi/ml (0·4 mM). Unless otherwise indicated the time of incubation in the radioactive medium was 10 minutes.

Determination of pancreatic secretion in vivo. In guinea-pigs anaesthetized with pentobarbitone the pancreatic duct was cannulated with a soft, calibrated plastic tube. The volume of the secreted juice and, in some experiments, its protein content and amylase activity were determined at various times before and after the injection of either caerulein or bethanechol, as indicated in the text.

Determination of pancreatic secretion in vitro. This was made as described by Jamieson (1970). Tissue slices were incubated in the radioactive medium for 5 min, washed with warm, non-radioactive medium containing an excess of ^{12}C-L-leucine, and reincubated in the latter. After 80 min of incubation at 37° C the slices were transferred to a third flask containing non radioactive medium and various concentrations of caerulein, and incubated for 20 additional minutes. At the end of the incubation this medium was collected and centrifuged at 50,000 rev/min for 1 h in a L2 Spinco centrifuge (50 Ti rotor), in order to remove free organelles and cell debris ; the supernatant was decanted and trichloroacetic acid (TCA) was added to give a final concentration of 10%. The slices were homogenized in 0·3 M sucrose and protein precipitated with 10% TCA. The rate of secretion is expressed as the percentage of TCA-precipitable d.p.m. found in the medium with respect to the total TCA precipitable d.p.m. present in the system—that is, medium + slices.

Radioactivity and chemical assays. Aliquots of tissue homogenates and incubation media were precipitated at 4° C with TCA, 10% final concentration, for 1 h, and centrifuged. Aliquots (0·2 ml) of the cold TCA-soluble material were mixed with 10 ml of Bray's phosphor (Bray, 1960) and counted directly in a Pakard Tri-Carb liquid scintillation spectrometer. TCA precipitates were washed 3 times with cold 5% TCA, resuspended in 5% TCA at 90° C, for 15 min, extracted with 3 : 1

ethanol-ether, at 37° C, for min, dried, dissolved in hyamine hydroxide and mixed with a dioxane-toluene-base scintillation fluid.

All counted rates reported have been corrected for quenching (by means of an external standard) and for background.

Proteins were determined according to Lowry, Rosebrough, Farr & Randall (1951) on TCA precipitates dissolved in 1 N NaOH, using bovine serum albumin as standard.

DNA was measured according to Burton (1956), purified calf thymus DNA was used as standard. Amylase activity was estimated according to Bernfeld (1955). One amylase unit is defined as the amount catalysing the formation of 1 mg of maltose in 10 min incubation at 30° C.

Materials. All chemicals were reagent grade. L-amino-acids were purchased from Sigma Chemical Co., St. Louis. Uniformly labelled ^{14}C-L-leucine (specific activity 312 mCi/mmol) was obtained from the Radiochemical Centre, Amersham. Synthetic caerulein was kindly supplied by the Farmitalia Laboratories for Basic Research, Milan. Bethanechol (carbamoyl-β-methyl-choline) was from Merck, Sharp & Dohme, West Point. Hyamine hydroxide 10× was from Packard Instrument Co., Downers Grove. Solvents were from E. Merck, A.G., Darmstadt.

Results

Effect of caerulein on pancreatic secretion

Caerulein had a striking effect on pancreatic secretion both *in vitro* and *in vivo*. The *in vitro* experiments are based on the observation by Jamieson (1970) that most of the radioactive proteins synthesized in a short pulse labelling are transported during an 80 min chase incubation to the secretory granules near the apex of the cell. If segretagogues are now added to the system the slices promptly discharge radioactive secretory proteins which can be recovered in the incubation medium and counted. The radioassay is more sensitive than the determination of the activity of secretory enzymes and is uninfluenced by the previously secreted zymogens which are always stored in the duct system of the gland. In these experimental conditions caerulein proved to have a clear-cut effect, since the percentage of radioactive proteins recovered in the medium after 20 min incubation rose from ~5% in unstimulated control slices to ~30% in slices stimulated by 2·5 ng/kg of caerulein. With higher concentrations the effect was less evident (Fig. 1).

Results *in vivo* are analogous with those obtained previously in different animal species (Bertaccini *et al.*, 1969).

The rapid intravenous injection of caerulein in guinea-pigs anaesthetized with pentobarbitone and bearing cannulated pancreatic ducts produced a prompt and large increase of volume of secretion (Table 1). Since the juice was collected at short time intervals before and after stimulation the volumes were too small for biochemical analysis.

When stimulation was provided by a subcutaneous injection of caerulein (4 μg/kg) a large increase of the volume of the juice was observed, beginning ~15 min after the injection and lasting at least 2·5 hours. This juice was rich in protein and enzymes (Fig. 2A).

An increase in the pancreatic secretion analogous with that elicited by a subcutaneous injection of caerulein could be obtained by giving the animals bethanechol (600 μg/kg) by the same route (Fig. 2B).

Effect of stimulation of pancreatic enzyme secretion on incorporation of ^{14}C-L-leucine into protein

The effect of caerulein on ^{14}C-L-leucine incorporation into protein was investigated by means of three different types of experiment.

First of all pancreatic tissue slices were incubated *in vitro* for various time intervals either with caerulein at the concentration optimal for stimulating secretion or without caerulein and then transferred to the radioactive medium for a 10 min *in vitro*

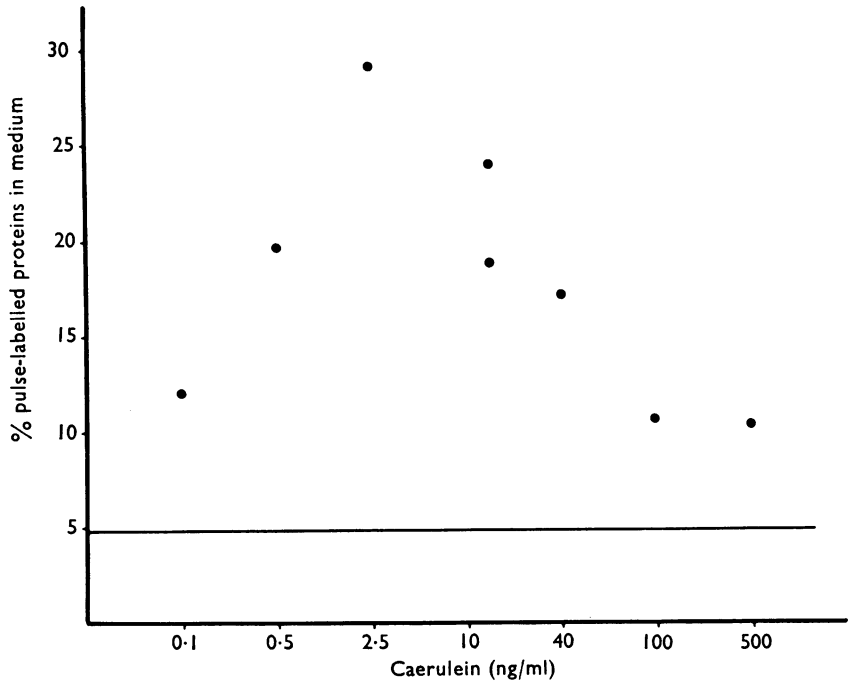

FIG. 1. Effect of caerulein on the rate of secretion of pancreas tissue slices incubated *in vitro*. Slices were first labelled for 5 min with ^{14}C-L-leucine, then chased for 80 min in a non-radioactive medium and finally reincubated for 20 min in the latter containing various concentrations of caerulein. The values shown indicate the amount of radioactive protein recovered in the medium expressed as percentage of radioactive proteins present in slices + medium. The secretion rate of the unstimulated controls is marked by the solid line.

ABLE 1. *Effect of a single intravenous injection of caerulein* (400 *ng/kg*) *on the total volume of pancreatic juice in guinea-pigs with acutely cannulated pancreatic duct, under pentobarbitone anaesthesia*

Animal number	Before stimulation μl/5 min	After stimulation μl/5 min
1	2·2	41·0
2	2·8	91·2

Values given are the averages of either three 5 min determinations made immediately before the injection or two 5 min determinations made immediately after the injection.

labelling. In these circumstances the incorporation of the labelled amino-acid was practically the same in stimulated and unstimulated slices (Table 2).

In a second series of experiments an acute stimulation of secretion was obtained *in vivo* by rapid intravenous injection of caerulein at a dose capable of producing a large increase of the *in vivo* secretion and a quasi-complete degranulation of the

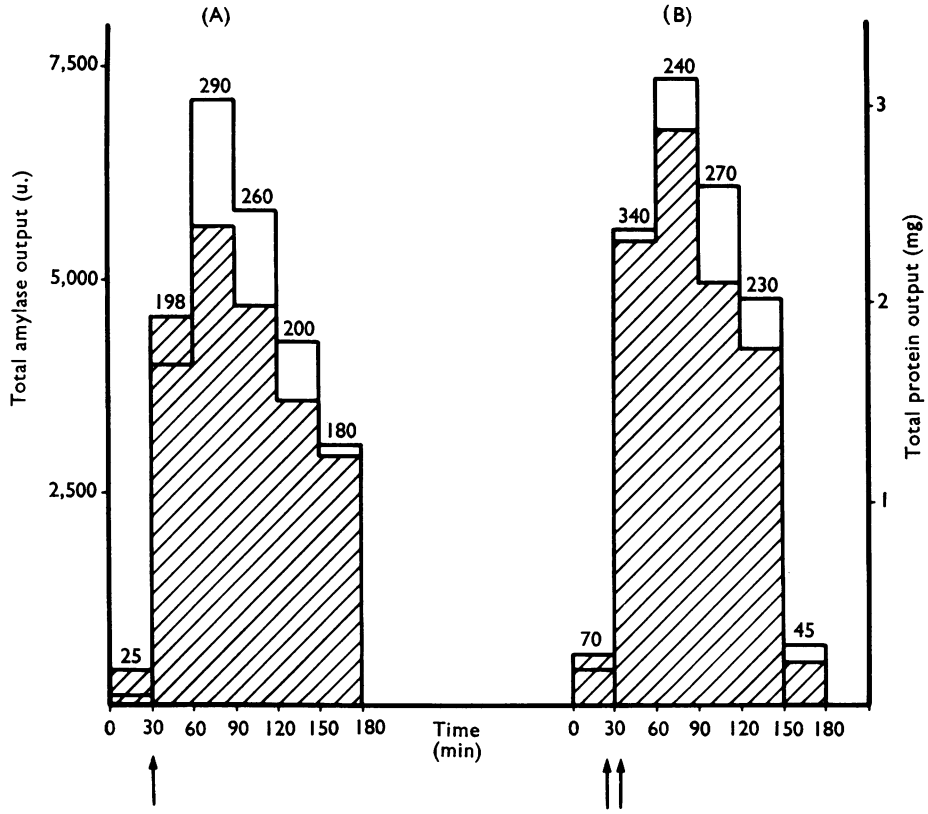

FIG. 2. *In vivo* effect of a single subcutaneous dose of either caerulein (4 μg/kg) (A) or bethanechol (600 μg/kg) (B) on the rate of pancreatic secretion in guinea-pig anaesthetized with pentobarbitone and bearing an acutely cannulated pancreatic duct. The injection of caerulein is marked by a single arrow, that of bethanechol with a double arrow. White columns indicate the total amylase output, hatched columns the total protein output; figures above columns indicate the total volume of the juice, in μl. Figures shown are averages of two experiments.

TABLE 2. In vitro *effect of caerulein* (2·5 *ng/ml*) *on* ^{14}*C-L-leucine incorporation into protein by pancreas tissue slices*

Preincubation time	Pretreatment	Activity/mg protein d.p.m.	Activity/mg DNA d.p.m.
10 min	Caerulein	604·0	28,940
	Saline	744·2	31,850
30 min	Caerulein	590·1	23,200
	Saline	525·6	24,300
90 min	Caerulein	711·3	20,581
	Saline	658·2	23,650
180 min	Caerulein	545·0	14,500
	Saline	494·1	16,500

Results are the average of two highly consistent experiments.
Slices were first preincubated for various time intervals in the presence of caerulein then labelled with ^{14}C-L-leucine for 10 minutes.

pancreas, as shown by electron microscopy (Meldolesi, unpublished observation). In pancreas slices incubated *in vitro* no significant difference of the rate of incorporation of ^{14}C-L-leucine into protein was observed, even if in some experiments the incorporation in stimulated slices derived from animals injected 90 and 180 min before they were sacrificed appeared slightly increased (Table 3).

Experiments on prolonged stimulation *in vivo* were made both in animals fasted throughout the whole experiment and in animals which had free access to food. Control animals fasted for 4 days exhibited a rate of ^{14}C-L-leucine incorporation into protein which was reduced with respect to fed controls to ~43% when calculated on a protein basis and to ~32% when calculated on a DNA basis. When caerulein was injected twice a day for 4 days at a dose known to be very active on secretion, no change of the *in vitro* protein labelling was observed in fed animals. In fasted animals, however, the same treatment elicited a 2-fold increase in the incorporation, calculated both on protein and DNA basis (Table 4).

In contrast, a single subcutaneous injection of caerulein, given 4 h before killing guinea-pigs fasted for 4 days did not produce any significant effects (Table 5).

TABLE 3. *Effect of a single intravenous injection of caerulein* (400 *ng/kg*) *on* ^{14}*C-L-leucine incorporation into protein by pancreas tissue slices*

In vivo treatment	Time before killing (min)	Number of experiments	Activity/mg protein d.p.m.	Activity/mg DNA d.p.m.
Saline	—	14	649·0 **319·2–1,199·0**	32,369·2 **17,620–58,580**
	10	2	532·5 **262·6–798·4**	30,220·0 **12,380–48,060**
	30	3	633·8 **385·0–857·2**	32,018 **23,076–37,400**
Caerulein	90	5	891·2 **606·1–1,458·0**	40,368 **25,500–60,230**
	180	3	856·0 **503·2–1,119·0**	43,273 **39,500–50,120**

Values given are averages; ranges are in italics

TABLE 4. *Effect of prolonged* in vivo *treatment with caerulein* (4 *μg/kg, subcutaneously, twice a day for* 4 *days*) *on* ^{14}*C-L-leucine incorporation into protein by pancreas tissue slices obtained from either fed or fasted guinea-pigs*

			Activity/mg protein			Activity/mg DNA		
Fasted or fed	No. of experiments	*In vivo* treatment	d.p.m.	% Increase over control	*P* value	d.p.m.	% Increase over control	*P* value
Fasted	6	Saline	523·0 ± 72·4			18,380 ± 1,646		
Fasted	5	Caerulein	1,091·8 ± 186·8	+108·6	$P<0.01$	34,480 ± 3,294	+87·5	$P<0.0005$
Fed	6	Saline	1,219·4 ± 124·2			57,086 5,860		
Fed	6	Caerulein	1,484·6 ± 289·0	+21·7	n.s.	68,584 11,542	+20·0	n.s.

Values given are averages ±S.E.
Animals were either fasted throughout the experiment or fed *ad libitum*. They were killed 4 h after the last injection.

In order to investigate whether the increase of protein labelling brought about by the prolonged treatment with caerulein was simply dependent on the extrusion of secretory proteins or related, somehow specifically, to this segretagogue, a different segretagogue, namely bethanechol, was used. Fasting animals were injected with the latter drug (at the dose known to elicit a large secretory response) according to the schedule used for the prolonged treatment with caerulein (two subcutaneous injections per day, for 4 days). No difference was detected in the rate of incorporation of ^{14}C-L-leucine in stimulated animals with respect to controls (Table 6).

Stimulation of pancreatic secretion with either caerulein (both *in vitro* and *in vivo*) never produced a change in the TCA soluble radioactivity of pancreas slices (not shown in tables). Since the TCA soluble radioactivity, under our experimental conditions, is primarily accounted for by intracellular ^{14}C-L-leucine not yet incorporated into protein, it seems likely that the specific radioactivity of the intracellular amino acid pool is not altered by the stimulation.

Discussion

Several conflicting results concerning the effect of stimulation of pancreatic secretion on the rate of protein synthesis have been published in the past (Daly & Mirsky, 1953 ; Allfrey *et al.*, 1954 ; Ferreira *et al.*, 1955 ; Farber & Sidransky, 1956 ; Hokin, 1956 ; Busch *et al.*, 1959 ; Stocker, 1962 ; Webster & Tyor, 1966 ; Peakall, 1967 ; Webster, 1968 ; Kramer & Poort, 1968 ; Poort & Geuze, 1969 ; Poort & Kramer, 1969). As pointed out by Poort & Kramer (Kramer & Poort, 1968 ; Poort & Kramer, 1969) it is possible to reconcile most of the discrepancies by taking into account the fact that in a protein-secreting gland the amount of protein present

TABLE 5. *Effect of a single subcutaneous injection of caerulein* (4 $\mu g/kg$) *on* ^{14} *C-L-leucine incorporation into protein by pancreas tissue slices obtained from fasted guinea-pigs*

No. of experiments	*In vivo* treatment	Activity/mg protein d.p.m.	% Increase over control	*P* value
3	Saline	436·6±79·1		
3	Caerulein	569·6±100·9	+37	n.s.

Values given are averages ±S.E.
Animals were fasted for 4 days; they were killed 4 h after the injection.

TABLE 6. *Effect of prolonged* in vivo *treatment with carbamoyl-β-methyl choline* (*bethanechol* 600 $\mu g/kg$, *subcutaneously, twice a day for* 4 *days*) *on* ^{14}*C-L-leucine incorporation into protein by pancreas tissue slices obtained from fasted guinea-pigs*

No. of experiments	*In vivo* treatment	Activity/mg protein d.p.m.	% Increase over control	*P* value	Activity/mg DNA d.p.m.	% Increase over control	*P* value
4	Saline	641·0± 102·0			26,266± 7,000		
4	Bethanechol	745·6± 78·0	+16·0	n.s.	26,694± 161	+2·0	n.s.

Values given are averages ±S.E.
Animals were fasted throughout the experiment; they were killed 4 h after the last injection.

depends on the secretory activity and is therefore variable. Since stimulation of secretion ultimately results in the extrusion of secretory proteins it is obvious that after stimulation the specific radioactivity of the total protein present in the tissue will be increased, even when protein synthesis *per cell* is unaffected.

Another possible reason for the above mentioned discrepancies lies in the fact than when tracer experiments are prolonged over the time needed for secretory proteins to be transported from the site of synthesis in the endoplasmic reticulum to zymogen granules radioactive proteins may be secreted at different rates from stimulated and non-stimulated cells, the result being an artifactual difference in the specific radioactivity of cellular proteins.

In order to cope with these problems we have calculated our results not only on a protein basis, but also on a DNA basis, as recommended by Kramer & Poort (1968). DNA-based results, being referred to a cellular constituent which is uninfluenced by the secretory activity of the cell, should be considered related to the proteosynthetic capacity of the single pancreatic cell.

Furthermore, the animal used in this work was the guinea-pig, whose pancreas cell physiology has been extensively studied by Palade and his associates (Caro & Palade, 1964; Jamieson & Palade, 1967a, b). In particular it is known that in guinea-pig pancreas slices incubated *in vitro* accumulation of radioactive secretory proteins into mature zymogen granules does not begin until 40 min after the beginning of incubation with the tracer (Jamieson & Palade, 1967b). Since in our experiments tracer incubations never lasted longer than 10 min, the possibility of radioactive proteins being secreted during our experiments can be discounted.

Our results on the lack of effect of an acute stimulation of secretion by caerulein on the rate of pancreatic protein synthesis in the guinea-pig are in agreement with those obtained by Poort & Kramer (Kramer & Poort, 1968; Poort & Kramer, 1969) in other mammalian species, using different secretory stimuli. Since it is known that most of the newly synthesized protein is accounted for by exportable (pro)enzymes (Jamieson & Palade, 1967b; Poort & Kramer, 1969), the constancy in the rate of the total protein synthesis that we observed under these conditions indicates that the synthesis of exportable protein does not undergo significant fluctuations. A different situation has been found in the pigeon, where stimulation *in vivo* (and not *in vitro*) brings about an increase of protein synthesis (Webster & Tyor, 1966; Peakall, 1967; Webster, 1968), and also in frogs, where feeding after prolonged starvation is equally effective (Poort & Geuze, 1969). Furthermore we have observed that in pancreatic slices from untreated fed guinea-pigs the incorporation of ^{14}C-L-leucine into protein is much greater than in those from 20–24 h fasted guinea-pigs; prolongation of fasting up to 4 days results in a further large decrease in the protein synthesizing capacity of the pancreas. This effect is particularly evident when results are calculated *per cell*—on DNA basis—since prolonged starvation brings about a marked increase of the DNA/protein ratio. In fasted but not in fed guinea-pigs repeated injection of caerulein results in a 2-fold increase of the ^{14}C-L-leucine incorporation into protein. Such an effect was not observed when pancreatic secretion was stimulated by a cholinergic drug.

These findings cannot yet be definitely interpreted. Caerulein is a versatile polypeptide whose spectrum of activity is very similar, or virtually identical, with that of pancreozymin (Bertaccini *et al.*, 1969; Erspamer, 1970). Given during starvation

when the rate of protein synthesis is greatly reduced and secretion of pancreozymin is also hampered, caerulein is capable of partially restoring the proteosynthetic activity of pancreatic cells, while in fed animals it is completely ineffective. Taken together these data seem to suggest that the decrease of protein synthesis during starvation may be partially dependent on the decreased secretion of pancreozymin and that caerulein restores protein synthesis by substituting for the endogenous hormone. If this were the case, pancreozymin would display at least two actions on the guinea-pig pancreas cell: one involving stimulation of secretion and the other concerned with the maintenance of the normal rate of protein synthesis. The latter effect would be fully evident only after a certain time from either restoration of pancreozymin secretion or prolonged treatment with pancreozymin-like drugs. This may explain why feeding starved rats and ferrets (Poort & Kramer, 1969) as well as acute caerulein stimulation of starved guinea-pigs did not produce a rapid increase of protein synthesis in the pancreas. In this respect it is interesting to recall that the possibility of pancreozymin playing a main role in the regulation of the synthesis of pancreatic digestive enzymes has been envisaged previously on the basis of indirect evidence (Rothman & Wells, 1967; Konijn, Guggenheim & Birk, 1969; Konijn, Birk & Guggenheim, 1970; Snook, 1969) and that gastrin, another hormone of the gut, having a chemical composition similar to pancreozymin, has been found to have a trophic action on the gastric and duodenal mucosae (Johnson, Aures & Yuen, 1969a; Johnson, Aures & Hakanson, 1969b).

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