Virchows Arch. A Path. Anat. and Histol. 367, 289—305 (1975)
© by Springer-Verlag 1975

Studies on Intracellular Transport in the Rat Exocrine Pancreas*

I. Inhibition by Aromatic Amino Acids *in vitro*

Wilfried Bieger and Horst F. Kern

Department of Anatomy, University of Heidelberg, Germany

Received May 9, 1975

Summary. In vitro incubation of rat pancreatic lobules in the presence of 10 mM concentrations of 2 natural (phenylalanine, tryptophane) and 2 modified aromatic amino acids (p-fluorophenylalanine, p-chlorophenylalanine) induces paracrystal formation in the cisternal space of the rough endoplasmic reticulum and in the acinar lumen. Aggregation of secretory material in transitional elements of the rough endoplasmic reticulum suggests tubular connection to the Golgi complex. Paracrystal formation is correlated with a disturbance of the three major phases in the secretory process of the exocrine cell. Incorporation of radioactive amino acids into proteins is inhibited by 10 mM concentrations of phenylalanine and tryptophane by 20 and 50% respectively and by p-chlorophenylalanine at 1 and 10 mM concentrations by 50 and 75%. The inhibition of protein synthesis is not due to a reduced intracellular concentration of radioactive precursor amino acids.

Intracellular transport of newly synthesized proteins as studied by a radioassay for zymogen discharge and by cell fractionation is similarly inhibited by phenylalanine, tryptophane and p-chlorophenylalanine at 10 mM concentrarions (20, 30, and 40% respectively).

Discharge of zymogens as measured by the secretion of amylase stimulated with 5×10^{-6} M carbamylcholine is reduced by 20% if 10 mM concentrations of phenylalanine, tryptophane or p-chlorophenylalanine are present in the medium. Paracrystals were isolated by differential centrifugation and their protein content compared with isolated zymogen granules. On sodium dodecylsulfate gel electrophoresis paracrystalline proteins show the same electrophoretic pattern as the content of zymogen granules.

Key words: Pancreas — Protein synthesis — Secretion — Amino acids — Paracrystals.

Introduction

Modified aromatic amino acids like p-chlorophenylalanine (p-Cl-Phe)[1] have been used as inhibitors for enzymes involved in serotonin metabolism (Koe and Weissman, 1966). It was postulated that p-Cl-Phe is incorporated into or close to the active site of tryptophane-5-hydroxylase and phenylalanine-4-hydroxylase which alter the activity of both enzymes (Gal and Millard, 1971). *In vivo* experi-

* A short communication on the same subject was presented at the 7th Symposium of the European Pancreatic Club in Dundee (July 4th–6th 1974).

** This research was supported by a grant from Deutsche Forschungsgemeinschaft (Ke 113/8). We gratefully acknowledge the technical assistance of Miss Waltraud Schmidt and Miss Helga Hollerbach. We thank Dr. J. D. Jamieson and Dr. A. Tartakoff for carefully reading the manuscript and their suggestions.

1 Abreviations: Phe, L-phenylalanine; Try, L-tryptophane; Tyr, L-tyrosine; p-Cl-Phe, D,L-para-chlorophenylalanine; p-F-Phe, D.L-para-fluorophenylalanine; p-Br-Phe, D,L-para-bromophenylalanine; RER, rough endoplasmic reticulum; Hepes, N-2-hydroxy ethyl-piperazine-2-ethane sulfonic acid; BSA, bovine serum albumin; SDS, sodium dodecylsulfate.

ments by Forssmann *et al.* (1970) using a single dose of p-Cl-Phe to study functional changes in serotonin-producing cells of the gut, led to the discovery of pronounced structural and biochemical changes in the exocrine pancreas (Bieger *et al.*, 1972; Forssmann and Bieger, 1973). The appearance of 3 to 5 μm long needle-like inclusions in the cisternal space of the rough endoplasmic reticulum (RER) is associated with a progressive decrease in protein secretion while volume secretion remains constant. These earlier studies assumed that p-Cl-Phe is incorporated into secretory proteins which then form insoluble aggregates in the cisternal space of the RER. Later experiments showed that p-Cl-Phe is quickly metabolized mainly to tyrosine[2]. The question remained whether aromatic amino acids in high intracellular concentrations could affect the normal transport operations from RER to Golgi complex in the exocrine cell. We turned to a pure *in vitro* system of isolated pancreatic lobules and tested whether natural and modified aromatic amino acids (Phe, Try, p-Cl-Phe, and p-F-Phe) are able to induce paracrystal formation in the RER and how this would affect the different sequential steps of the secretory process in the exocrine cell. Our experimental procedure followed mainly the classical studies of Jamieson and Palade (1967, 1968, 1971, 1972).

Methods

Animals. Male Wistar rats (S. Ivanovas, Kisslegg, Germany) 200 to 250 g in weight were used throughout the experiments. They were fed Altromin® laboratory chow (Fa. Altromin, Laage, Germany) and had free access to drinking water. They were killed by cardiac section under light ether anesthesia, the pancreas was removed and placed in ice cold incubation medium.

Preparation of Pancreatic Lobules. After removal of fat, mesentery and damaged tissue, pancreatic lobules were prepared according to Scheele and Palade (1975). Ice cold incubation medium was injected into the interstitium with a 20 ml syringe and N⁰ 20 needles. The resulting oedema makes visible lobules, which were excised in a size of 1 to 2 square millimeters using watchmakers forceps and fine scissors. While cutting, the tissue is held by interlobular connective tissue. Each rat pancreas (600 to 800 mg wet weight) gives about 80 to 100 lobules.

Incubation Procedure. Incubation was performed in Krebs-Ringer solution (Krebs, 1950) containing 14 mM glucose. The medium was gassed every 30 min with 95% O_2/5% CO_2. Bicarbonate was substituted by 15 mM Hepes to assure better constancy of pH. In all experiments the Krebs-Ringer-Hepes buffer was supplemented with L-amino acids according to Eagle (Eagle, 1959). For tracer experiments with L-leucine-^{3}H, L-phenylalanine-^{14}C or L-tyrosine-^{3}H the concentration of the respective unlabeled amino acids was reduced to 10% of the regular concentration. In secretion experiments the incubation medium contained 0.3 mg/ml BSA.

Pulse Labeling. For cell fractionation experiments and for a radioassay of zymogen discharge (Jamieson and Palade, 1971) 50 or 15 pancreatic lobules were loaded to a 25 ml flask containing 10 ml of incubation medium. L-leucine-4,5-^{3}H was added to a concentration af 10 μCi/ml, the flasks were gassed, kept on ice for 10 min, and then incubated at 37° C in o GFL-shaker bath (Gesellschaft für Labortechnik, Hannover, Germany) with agitation at 120 cycle/min. After 4 min the content of each flask was poured into Büchner funnels on glass filters and washed with warm chase medium containing 2.0 mM L-leucine-^{1}H. The lobules were transferred to 25 ml flasks containing 10 ml incubation medium, 5×10^{-6} M carbamylcholine and aromatic amino acids (Phe, Try, Tyr, or p-Cl-Phe) at 1 mM and 10 mM. After 30, 60, 120, and 180 min of incubation, 5 ml of the medium were removed from the flasks and replaced by new medium. From this 5 ml sample 4 ml were precipitated with TCA (10% final concentration). The remaining 1 ml of the medium was used for enzyme assays. At the end

2 Bieger, W.: unpublished results (1974).

of 3 hrs incubation the pancreatic lobules were briefly washed and homogenized in 5 ml cold distilled water, 1 ml of the homogenate was precipitated in duplicate by TCA and samples of the remaining volume served for enzyme determination.

Cell Fractionation. For analysis of intracellular transport operations and for isolation of paracrystalline inclusions in the RER we followed the fractionation scheme of Jamieson and Palade (1967). 40 to 50 lobules (=400 mg wet weight) were homogenized in 4.5 ml 0.3 M sucrose by three passes in a Brendle type homogenizer (Thomas, Philadelphia, N⁰A 14299) driven by a motor at 2800 rpm. Centrifugal forces were as in Jamieson and Palade (1967). For isolation of paracrystals from the RER we induced their massive formation *in vivo*. A single dose of 50 mg/100 g body weight p-Cl-Phe-methylester dissolved in saline was injected intraperitoneally 20 hrs prior to sacrifice. After homogenization cellular debris was removed. The zymogen granule fraction contained most of the recovered paracrystals. An enriched paracrystal fraction was obtained after incubation of the zymogen granule fraction for 30 min in 0.2 M $NaHCO_3$ at 0° C and pH 8.2 and consecutive centrifugation at $1000 \times g$ max for 10 min. This procedure leads to release of most of the zymogen granule content, while the paracrystals remain.

Assay Procedures

1. *Intracellular Amino Acid Pool.* According to Venrooij *et al.* (1972) the permeability of pancreatic plasma membrane for amino acids decreases with decreasing temperature and is negligible at 4° C. Using the pancreatic lobule preparation similar results were obtained. ^{14}C-inulin, added as marker for the extracellular space is totally removed by 5 washes with 5 ml isotonic saline at 0° C. During this procedure the intracellular concentration of free L-leucine-4,5-^{3}H decreases by less than 20%. At the end of each incubation period, pancreatic lobules were quickly cooled on ice, washed with saline, homogenated in 5 ml ice cold distilled water and precipitated with TCA. The TCA soluble radioactivity was taken as a measure of free intracellular radioactive precursor.

2. *Radioactivity Assays.* Samples from incubation medium, homogenates and subcellular fractions were precipitated at 4° C with TCA (final concentration 10%) overnight and washed two times with 5% TCA. The final pellet was dissolved in 1 ml 1 N NaOH and transferred to scintillation counting vials containing 10 ml Unisolve (Koch-Light Laboratories, Colnbrock, England), which forms a gel after the addition of 3 ml distilled water. Counting was performed in a Philips liquid scintillation counter. Counting rates have been corrected for quenching and background.

3. *Chemical Assays.* Proteins were determined by the method of Lowry *et al.* (1951), using crystalline bovine serum albumin as standard and DNA content in TCA precipitates according to Burton (1956) with calf thymus DNA (6×recryst.) as standard. Amylase activity, defined according to Schramm and Dannon (1961) was measured by the procedure of Bernfeld (1955).

4. $^{14}CO_2$-production from palmitate-1-^{14}C was measured as described by Jamieson and Palade (1968).

Electron Microscopy. Pancreatic lobules were fixed after isolation or incubation for various time periods by immersion in 2.5% glutaraldehyde-formaldehyde-mixture buffered at pH 7.2 with 0.1 M Na-phosphate. Particulate fractions were fixed with 2% glutaraldehyde, or 1% osmium tetroxide, both in 0.3 M sucrose. After dehydration in a graded series of alcohols and passage through propyleneoxide, tissue blocks were embedded in Epon 812 (Luft, 1961). Pellets of particulate fractions were oriented to allow sections through all layers of the pellet. Sections cut with a diamond knife on a LKB microtome were stained with 5% uranylacetate and lead citrate (Reynolds, 1963). They were examined in a Zeiss EM 9 S or a Philips EM 301 electron microscope.

Materials. All chemicals were reagent grade (Merck Chemicals, Darmstadt, Germany). L-amino acids, p-Cl-Phe and p-F-Phe were obtained from Serva Biochemica, Heidelberg; p-Br-Phe from Cyclo-Chemicals, Los Angeles. L-leucine-4.5-^{3}H (30–60 mCi/mole), L-tyrosine-3.5-^{3}H (30–60 mCi/mole) uniformly labeled L-phenylalanine-^{14}C (405 mCi/m mole) and 1-^{14}C-palmitic acid (50 mCi/m mole) were purchased from Amersham Buchler, Braunschweig, Germany.

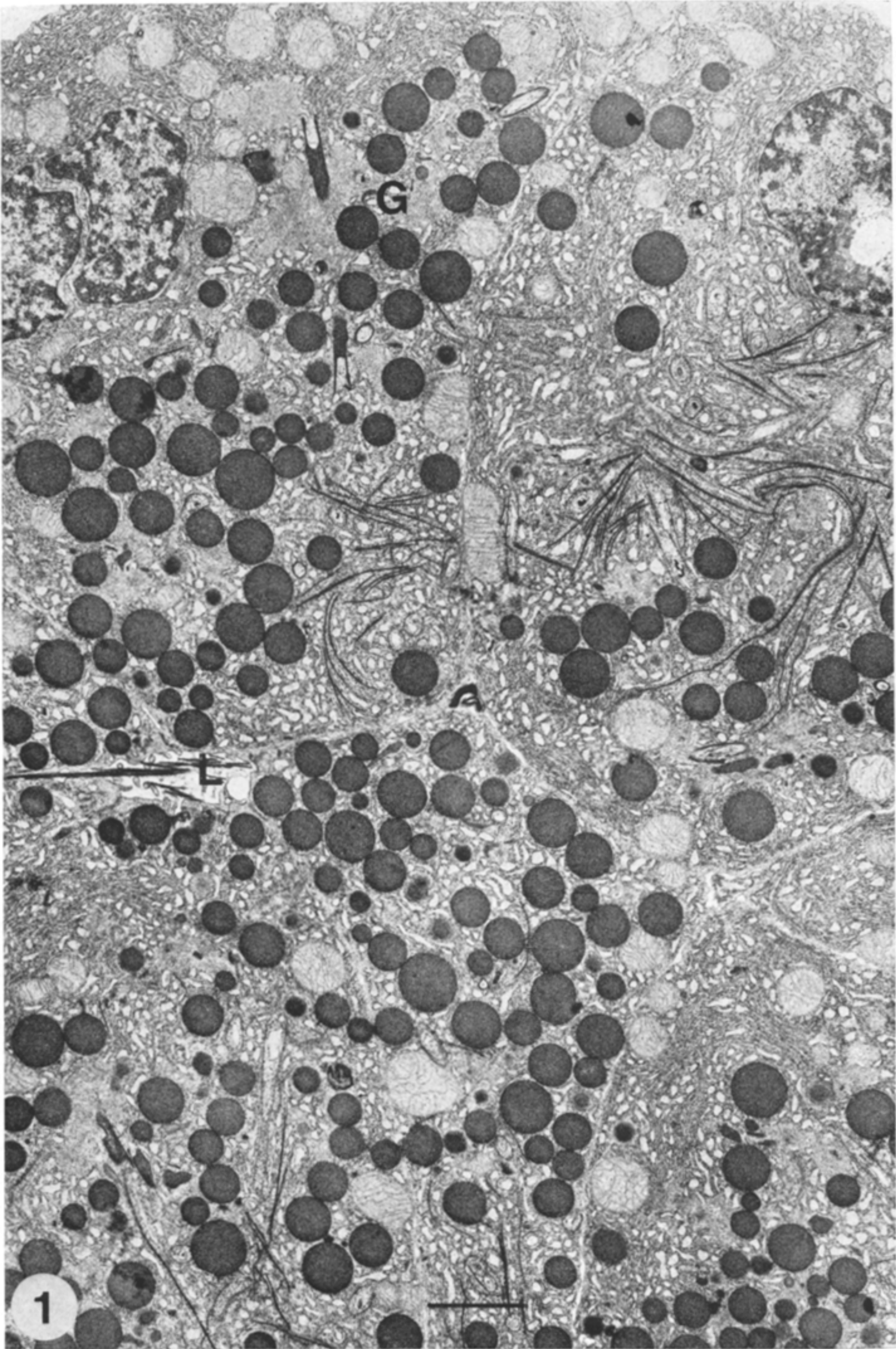

Fig. 1. Low magnification electron micrograph of several exocrine cells incubated *in vitro* for 3 hrs in 10 mM Phe. Paracrystals have formed in the RER of most cells and in the acinar lumen (L). Transitional elements filled with electron opaque material appear in Golgi region (*G*). $\times$ 6500. Scale marker = 2 μm

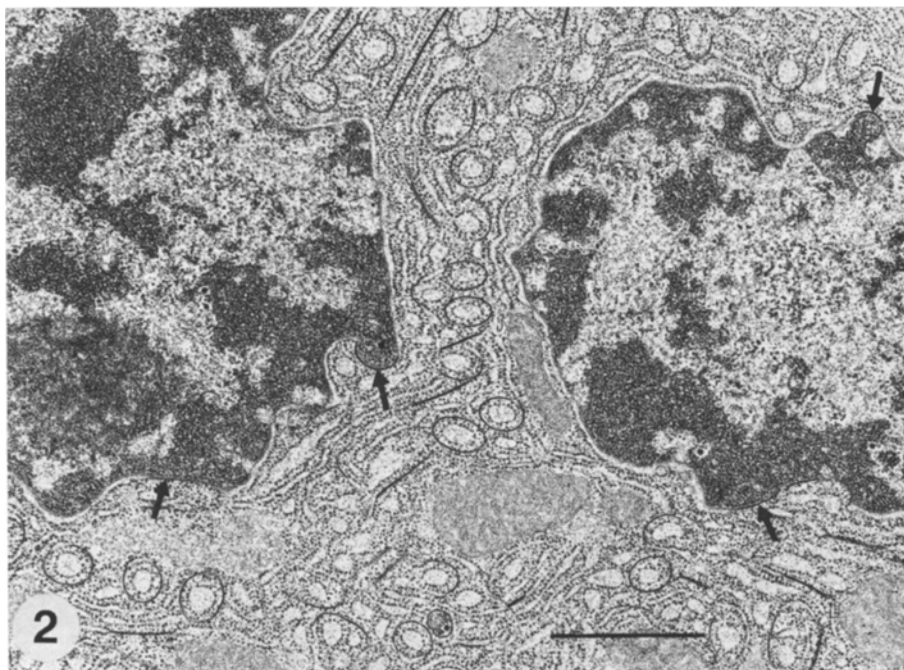

Fig. 2. Circular profiles of paracrystals in proximity to the cell nucleus. It can be seen, that in some segments of the perinuclear cisternae short paracrystals have formed (arrows). × 20000. Scale marker = 1 μm

Results

Morphology of Incubated Pancreatic Lobules. The fine structure of rat pancreatic lobules corresponds to the description of guinea pig pancreatic slices given by Jamieson and Palade (1967). *In vitro* incubation with three natural (Phe, Try, Tyr) and three halogenated aromatic amino acids (p-Cl-Phe, p-F-Phe, p-Br-Phe) induces the same structural changes as described after *in vivo* injections of p-Cl-Phe in rats (Forssmann and Bieger, 1973). The only exception was seen with tyrosine, since incubation at 10 mM leads to paracrystal formation only outside the exocrine cell in the acinar lumen. The following description therefore is given for one aromatic amino acid (Phe) and applies in general for all others tested, except tyrosine.

Already after 5 min of incubation small needle-like structures appear in the cisternal space of the RER and in the saccules of the Golgi complex. These needles are not observed inside condensing vacuoles or in mature zymogen granules. They lie free in the cisternal space and keep a distance from the limiting membrane. The size and incidence of these needle-like inclusions increases considerably if incubation is prolonged for 3 hrs. Most exocrine cells reveal a large number of 2 to 3 μm long structures of high electron opacity, which are mostly situated in the supranuclear region of the cell (Fig. 1). Depending on the plane of sectioning through the RER they appear as straight electron dense needles or as oval rings. They are also found in some areas of the perinuclear cisternae (Fig. 2). The in-

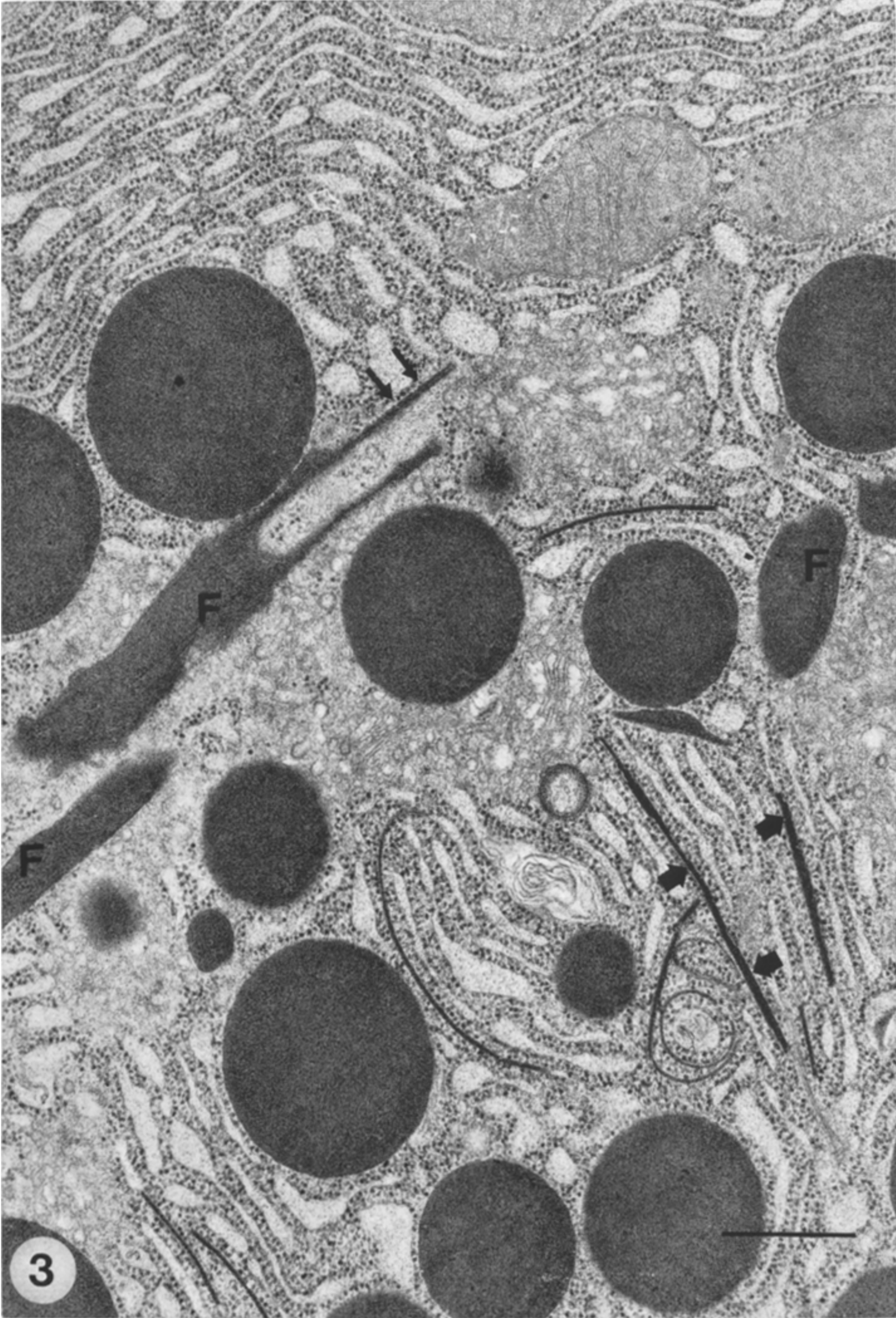

Fig. 3. Altered transitional elements of the RER filled with electron opaque material after *in vitro* incubation in 10 mM Phe for 3 hrs. Besides the normal Golgi cisternae and vesicles oblong shaped electron dense bodies are seen, sometimes with thin fibres included (*F*). They have tubular projections limited by a membrane, which is partly studded with ribosomes (small arrow). In the Golgi vicinity paracrystals are shown within the lumen of the RER. They fill tightly the cisternal cavity. (thick arrow). $\times$ 32000. Scale marker = 0.5 μm

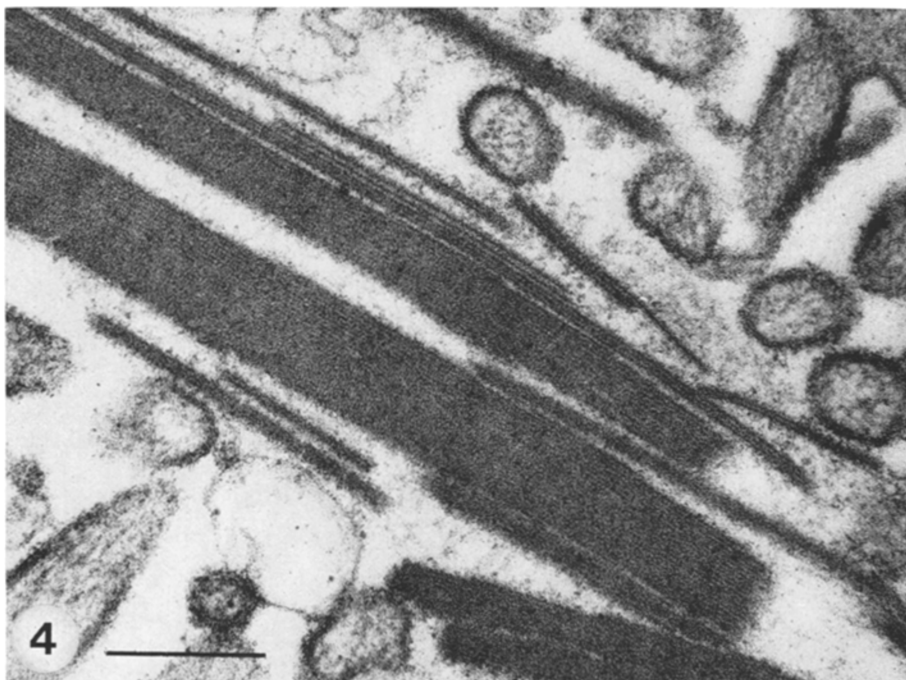

Fig. 4. The paracrystals formed in the acinar lumen are not bounded by a membrane and measure about 100 mμ in diameter and 1 to 2 μ in length. They are composed of parallel fibers (about 50 to 75 Å in diameter) with a center to center spacing of 80 to 100 Å. × 105000. Scale marker = 0.2 um

clusions fill totally the cisternal cavity of the RER, which seems to be compressed in its transverse diameter for the whole length of the needle-like inclusion. The bounding membrane of the RER is tightly apposed to the electron dense material in the cisternal space (Fig. 3). Higher magnifications reveals a substructure, which led to the term: paracrystals.

Another structural alteration in the exocrine cell which is observed after *in vitro* incubation of lobules in 10 mM concentrations of aromatic amino acids concerns the Golgi complex. In addition to the characteristic stacks of flattened saccules and small Golgi vesicles one observes one or more membrane-bound oblong structures with two or more slender processes (Fig. 1, 3). The content of the structure has the same electron opacity as mature zymogen granules. At higher magnification thin fibers are arranged within the structures (Fig. 3). Since the bounding membrane in some areas is studded with ribosomes and is smooth elsewhere, it is assumed that these structures correspond to transitional elements of the RER. No amino acids tested induced structural alterations in condensing vacuoles or mature zymogen granules. In the acinar lumen, however, paracrystalline inclusions are observed with all the amino acids including tyrosine (Fig. 1, 4).

Isolation of Paracrystals. To ensure a high yield of paracrystals in the RER 50 mg/100 g body weight p-Cl-Phe-methylester was injected *in vivo* 20 hrs prior to killing of the animals. After homogenization and removal of cellular debris a

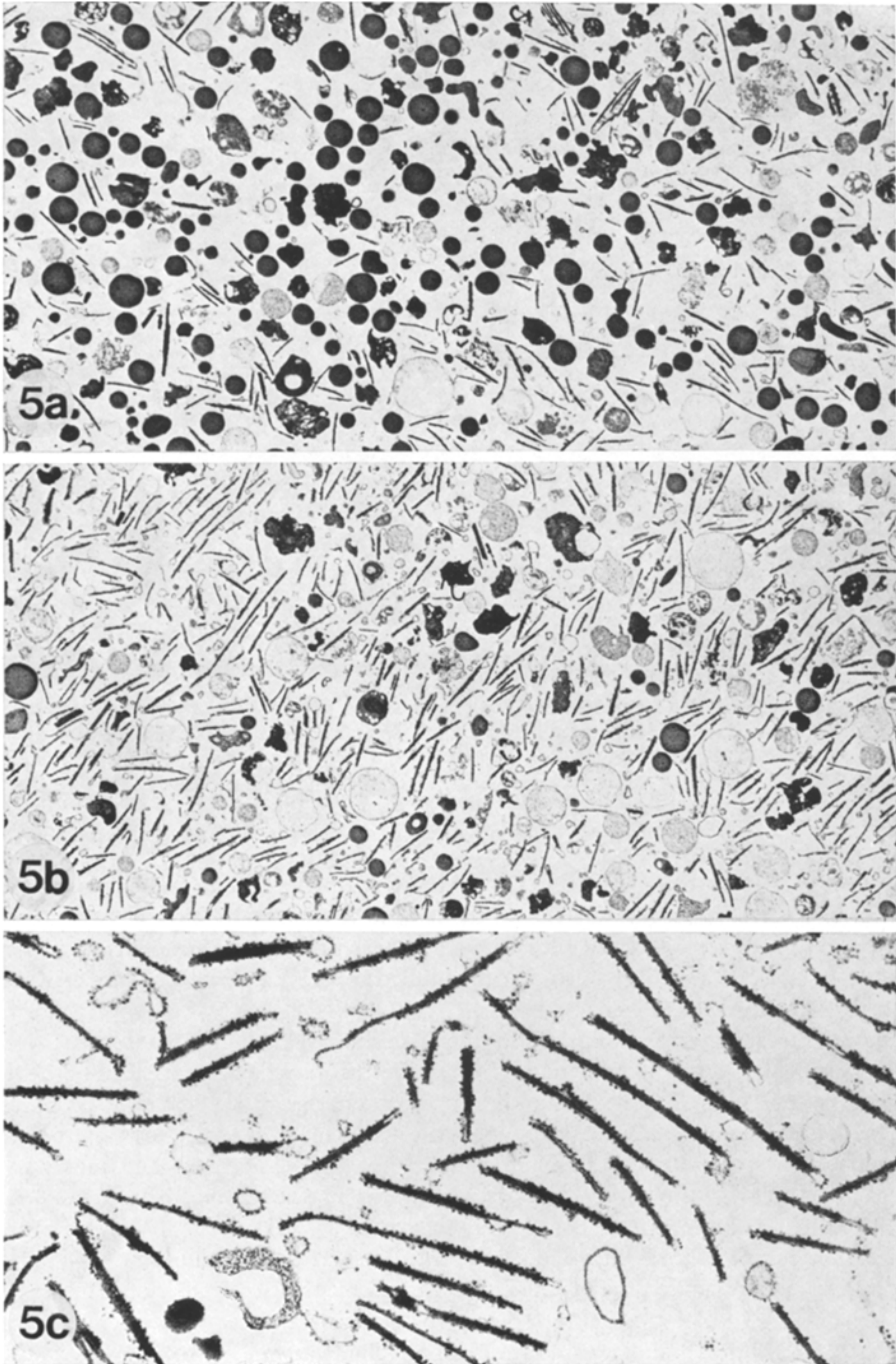

Fig. 5

zymogen granule fraction was prepared. Fig. 5 depicts two representative strata of the pellet from top to bottom.

Paracrystals with the adhering ER membrane sediment mainly together with mitochondria and phagosomes on top of the zymogen granule fraction, but are found in lower concentration throughout the pellet. All attempts to seperate zymogen granules and paracrystals on linear gradients between 1 and 2 M concentrations did not give satisfying results. Incubation of the resuspended zymogen granule fraction in 0.2 M $NaHCO_3$ at pH 8.2 for 30 min at 4° C leads to lysis of the zymogen granules while the majority of the paracrystals remains intact. After centrifugation at $1000 \times g$ for 30 min the resulting pellet contains mitochondria, fragments of membranes and mainly paracrystals (Fig. 5c). This fraction is resuspended in distilled water, sonicated for 30 sec and the protein precipitated by TCA (10% final concentration). After dissolution of the precipitate in Tris-phosphate buffer pH 6.7, proteins were seperated using the discontinuous SDS-gel system of Maizel (1971) with 13% acrylamide in the separating gel. Proteins from paracrystal fraction were compared with the proteins from isolated zymogen granules from control rats.

Both protein mixtures gave identical patterns of separation (Fig. 6), which corresponds largely to the results obtained in the analysis of guinea pig pancreatic juice or zymogen granule content (Tartakoff *et al.*, 1974). These findings support the hypothesis that paracrystals are formed by the aggregation of normal secretory proteins contained in the RER.

Effect of Aromatic Amino Acids on the Incorporation of L-*Leucine-^3H into Proteins.* To determine the effect of three natural and halogenated aromatic amino acids on the incorporation of a tracer amino acid into protein, sets of 5 lobules were incubated for 1, 2, and 3 hrs in media containing L-leucine-^3H and concentrations of phenylalanine, tryptophane and tyrosine from 1 to 10 mM and p-Cl-Phe and p-F-Phe from 0.1 to 10 mM. The amount of radioactivity incorporated into total protein was measured and compared with controls and also with sets of lobules incubated with tracer amounts of L-phenylalanine-^{14}C and concentrations of L-leucine from 1 to 10 mM. Tyrosine and leucine between 1 and 10 mM did not inhibit protein synthesis over a period of 3 hrs. All other aromatic amino acids at 10 mM concentration inhibited the incorporation of radioactive leucine (Table 1). The inhibition is most pronounced with halogenated derivates, especially with p-Cl-Phe. Despite the extent of the inhibition the tissue remained viable as indicated by the fact, that $^{14}CO_2$-production from palmitate-1-^{14}C was not changed compared with controls (Table 2). It also could be shown that paracrystal formation was independant of continuous protein synthesis, since 10 mM concentrations

Fig. 5. Micrograph of the bottom (Fig. 5a), and top (Fig. 5b) parts of a zymogen granule pellet. Fig. 5a consists of zymogen granules, mitochondria, lysosomes and some paracrystals. $\times$ 6800. Scale marker = 2 μm. The upper part of the pellet (Fig. 5b) is mainly composed of paracrystals contained in RER segments and some zymogen granules and mitochondria. $\times$ 6800. Scale marker = 2 μm. After incubation of the crude zymogen granule pellet in 0.2 M $NaHCO_3$ at pH 8.2 for 30 min and centrifugation for 30 min at $1000 \times g$ the resulting pellet contains a reasonably pure paracrystal fraction (Fig. 5c). Besides the paracrystals there are mitochondria and still very few zymogen granules. $\times$ 20000. Scale marker = 1 μm

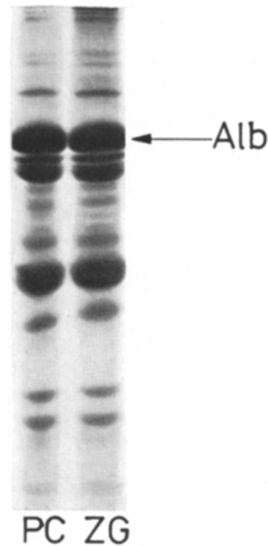

Fig. 6. SDS-gel analysis of zymogen granule content (*ZG*) and proteins of the paracrystal pellet (*PC*). Zymogen granules have been lysed by incubation for 30 min in 0.2 M $NaHCO_3$ at pH 8.2, centrifugated and the supernatant precipitated with TCA with BSA added as carrier. The arrow indicates the position of BSA (*ALB*). The paracrystals were prepared as described in the text. The two fractions were run in 3 mm slab gels. The direction of electrophoresis is from top to bottom. Although trace amounts of secretory enzymes are detected in the final pellet after lysis of zymogen granules, secretory protein recovery is significantly higher in the paracrystal pellet

of aromatic amino acids caused all structural alterations described earlier even in presence of 5×10^{-4} M cycloheximide. Moreover the inhibitory effect of cycloheximide was consistently increased by the addition of 1 and 10 mM p-Cl-Phe (Table 1). The inhibitory effect of aromatic amino acids on protein synthesis was not due to a decrease of radioactive precursor amino acids inside the cell as a result of competitive inhibition of transport across the membrane. Fig. 7 shows that there is an inverse relationship between the amount of inhibition of protein synthesis and the intracellular concentration of L-leucine-^3H, which is most significant for p-Cl-Phe and p-F-Phe.

Effect of Aromatic Amino Acids on Intracellular Transport and Discharge of Secretory Proteins. Recent studies by Jamieson and Palade (1967, 1968) have demonstrated that secretory proteins in the exocrine pancreatic cell undergo a series of intracellular transport operations within the membrane bound compartments of the cytoplasm. After synthesis on bound ribosomes, proteins are segregated in the cisternal space of the RER and transported to the Golgi complex to be packed into zymogen granules. We therefore tested how the formation of paracrystalline inclusions in the RER would affect the directional flow of secretory proteins through the cell. This was analysed using a radioassay for zymogen discharge (Jamieson and Palade, 1971) and cell fractionation techniques (Jamieson and Palade, 1967). In both cases pancreatic lobules are pulse labeled for 3 min and the movement of radioactive proteins is assayed either as a measure of their

Table 1. Effect of various amino acids on protein synthesis after 3 hrs incubation

Conditions		Radioactive precursor	Relative specific activity (%)	Specific activity (DPM/mg Protein)
Control		4.5-H^3-Leucine (1 μCi/ml)	100	496.500
PHE	1 mM		102.1 ±3.2	
	10 mM		83.5 ±8.7	
TRY	1 mM		104.7 ±6.2	
	10 mM		47.8 ±8.9	
TYR	1 mM		102.1 ±3.2	
	10 mM		104.3 ±4.7	
LEU	1 mM	Phenylalanine-1-C^{14}	104.8 ±2.4	321.000
	10 mM	(0.5 μCi/ml)	106.1 ±4.4	
p-Cl-PHE	0.1 mM	4.5-Leucine-H^3 (1 μCi/ml)	97.4 ±4.8	515.000
		Tyrosine-H^3 (1 μCi/ml)	101.4 ±9.1	527.000
	1 mM	4.5-Leucine-H^3	50.3 ±7.1	
		Tyrosine-H^3	61.5 ±8.5	
	10 mM	4.5-Leucine-H^3	28.4 ±4.7	
		Tyrosine-H^3	24.6 ±8.9	
Cycloheximide	5×10^{-4} M	4.5-Leucine-H^3	3.4 ±1.1	1750 (DPM/μg DNA)
+ p-Cl-PHE	1 mM		2.36±0.7	
	10 mM		1.4 ±0.8	

Table 2. Effect of aromatic amino acids on cellular respiration as production of $^{14}CO_2$ from palmitic acid-C^{14}

Conditions (2 h incubation)		Relative specific activity (%)	Specific activity (DPM/mg Protein)
Control		100	23.800
PHE	1 mM	102.5± 3.6	
	10 mM	101.3±4.2	
TRY	1 mM	104.6±2.8	
	10 mM	98.7±3.1	
p-Cl-PHE	0.1 mM	98.2±1.3	
	1 mM	93.5±6.2	
	10 mM	99.4±4.7	
p-F-PHE	1 mM	98.6±4.9	
	10 mM	99.6±2.8	
Cycloheximide	5×10^{-4} M	78.6±4.1	

discharge into the incubation medium as a function of time or by their translocation from RER (determined as specific activity in the microsomal fraction) into zymogen granules (measured as specific activity in the zymogen granule fraction).

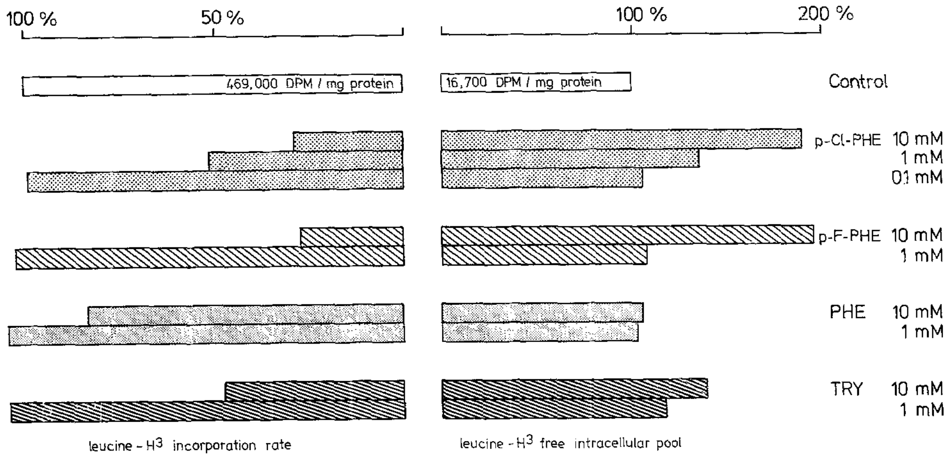

Fig. 7. Comparison of radioactive leucine incorporated into TCA-precipitable proteins (left side of the diagram) and contained free in the TCA-soluble fraction. The data are expressed as per cent of control lobules incubated for 3 hrs in the presence of 1 μCi/ml L-leucine-4.5-^{3}H. Addition of natural (Phe, Try) and modified aromatic amino acids (p-Cl-Phe, p-F-Phe) in increasing concentrations (0.1–10 mM) reduces the incorporation of the tracer amino acid into protein and increases simultaneously the TCA-soluble radioactivity

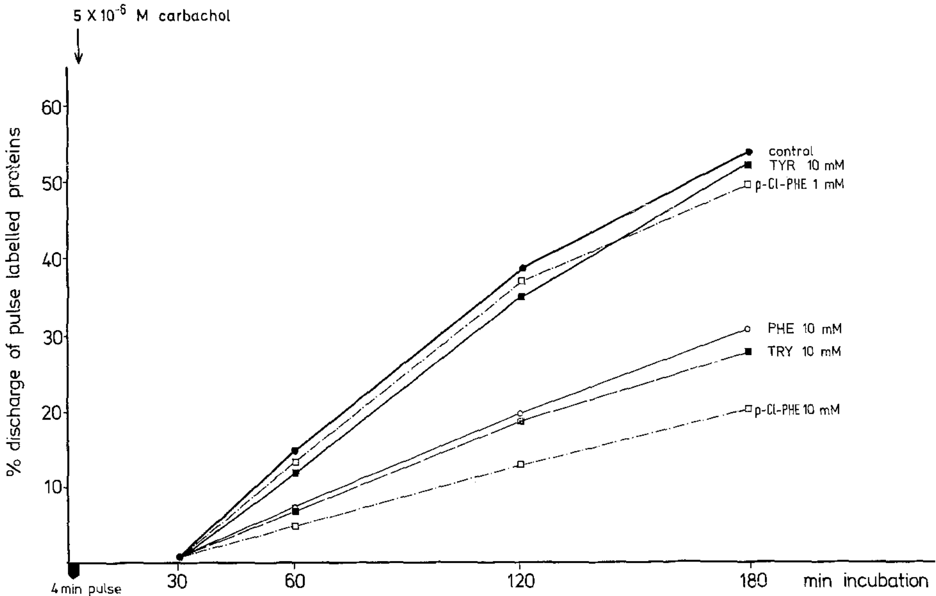

Fig. 8. Effect of various aromatic amino acids on induced discharge of labeled secretory proteins. Sets of 5 lobules were pulse labeled for 3 min with leucine-^{3}H and incubated for 180 min in chase medium with 5×10^{-6} M carbamylcholine and various aromatic amino acids added. The amount of pulse-labeled proteins appearing in the medium was determined after 30, 60, 120 and 180 min stimulation. The results are expressed as per cent of the sum of protein radioactivity in medium and lobules. The results are representative of 3 to 6 identical experiments

Table 3. Discharge of pulse labeled protein in the incubation medium

Conditions		Post pulse incubation time			
		30 min	60 min	120 min	180 min
Control		1.2 ± 0.6	15.1 ± 2.2	35.0 ± 2.6	54.1 ± 4.8
PCPA	1 mM	1.3 ± 0.6	14.2 ± 9.2	40.8 ± 1.9	51.9 ± 1.8
	10 mM	0.9 ± 0.4	3.7 ± 0.3	13.2 ± 1.4	20.1 ± 3.6
PHE	1 mM	1.5 ± 0.1	9.6 ± 2.1	39.5 ± 4.3	53.4 ± 1.8
	10 mM	1.1 ± 0.1	5.5 ± 0.6	20.9 ± 1.7	31.2 ± 1.2
TYR	1 mM	1.5 ± 0.1	10.1 ± 0.4	35.5 ± 3.1	52.8 ± 2.1
	10 mM	1.4 ± 0.2	9.8 ± 0.6	34.6 ± 2.3	53.2 ± 1.9
TRY	1 mM	1.1 ± 0.2	14.9 ± 2.1	38.1 ± 4.8	54.3 ± 1.9
	10 mM	0.9 ± 0.1	4.8 ± 1.4	19.1 ± 4.3	28.9 ± 3.4
LEU	1 mM	1.4 ± 0.3	9.1 ± 0.2	35.6 ± 2.7	52.4 ± 2.0
	10 mM	1.5 ± 0.1	9.6 ± 1.4	34.2 ± 3.6	53.1 ± 1.8

Fig. 8 summarizes the result of the radioassay experiments. Under control conditions 5×10^{-6} M concentrations of carbamylcholine in the medium will discharge about 50% of radioactive proteins synthesized during a 3 min pulse over a period of 3 hrs. This process reflecting normal intracellular transport operations is not affected by either 10 mM Leu or Tyr or by 1 mM p-Cl-Phe. However, 10 mM concentrations of Phe, Try and p-Cl-Phe, which morphologically induce paracrystal formation and Golgi alterations reduce the stimulated discharge of labeled proteins by about 50%. Table 3 gives the percentage of radioactive proteins released at each time point in detail. Since high concentrations of aromatic amino acids are only added at the end of the pulse, the first 30 min of chase incubation can not reveal a delay or change in rate of discharge. A change in rate becomes evident, however, by 60 min and is even more pronounced, if lobules are incubated in 10 mM aromatic amino acids before pulse. The inhibition of intracellular transport corresponds closely in time and dose dependency to the structural studies. This can be seen if cell fractionation is used to analyze the first 30 to 40 min of intracellular transport after a 3 min pulse. In correspondance to earlier studies with guinea pigs (Jamieson and Palade, 1967, 1968) it can be shown that after a 3 min pulse followed by a standard chase incubation of 37 min about 40% of the original radioactivity in the microsomal fraction (at the end of the pulse) has been transferred into zymogen granule fraction (Fig. 9). In the *in vitro* system used, this figure is referred to as a 100% transport efficiency. Addition of 10 mM concentrations of Phe, Try, and p-Cl-Phe decrease the transport efficiency obtained under control conditions by 20, 30, and 40% respectively (Fig. 9). The same *in vitro* concentrations of Tyr or Leu have no effect on intracellular transport.

Effect of Aromatic Amino Acids on Zymogen Discharge. As a representative of the different digestive enzymes present in mature zymogen granules, amylase activity has been determined in the medium after the addition of 5×10^{-6} M carbamylcholine. Under control conditions, 55% of tissue amylase is discharged

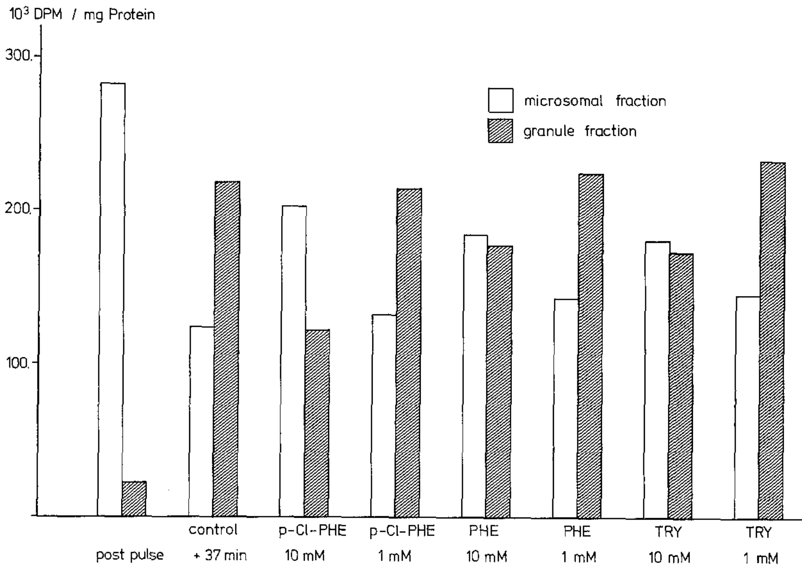

Fig. 9. Graphic presentation of cell fractionation studies. Sets of pancreatic lobules were pulse labeled for 3 min with leucine-^{3}H and the specific radioactivity determined in the zymogen granule and microsomal fraction (first set of columns, post pulse). Corresponding sets of lobules were chase incubated after the pulse for 37 min. It can be seen that at the end of pulse, the majority of labeled proteins is associated with the microsomal fraction. During chase incubation about 45% of the radioactivity in the microsomal fraction is transferred to the zymogen granule fraction under control conditions. Addition of 10 mM concentrations of Phe, Try and p-Cl-Phe inhibits this transfer of secretory proteins from microsomes to zymogen granules

into the medium over a 3 hrs period (Fig. 10). Tyr and Leu in 10 mM concentrations and p-Cl-Phe at 1 mM have no effect on this stimulated discharge, while 10 mM Phe, p-Cl-Phe, and Try reduce the discharge of amylase by 20%. The same values are obtained if chymotrypsinogen is measured. Resting secretion from lobules without secretagogue added is not affected by the various aromatic amino acids.

Discussion

Palade (1956) was the first to show that under certain physiologic conditions secretory proteins are precipitated in the cisternal space of the RER to form discrete dense masses referred to as intracisternal granules. Furthermore filamentous and fibrous structures in condensing vacuoles and in the content of acinar lumina have been described *in vitro* (Jamieson, 1972). Both observations suggest that secretory proteins in the exocrine pancreatic cell can form aggregates. In particular, the studies of Siekevitz and Palade (1958) show the enrichment of certain secretory proteins to be almost as high in intracisternal granule fraction as in zymogen granule fractions. The present study adds more information about complex formation among secretory proteins. Paracrystals are formed in the RER, the Golgi complex and also in the acinar lumen, where a high concentration of

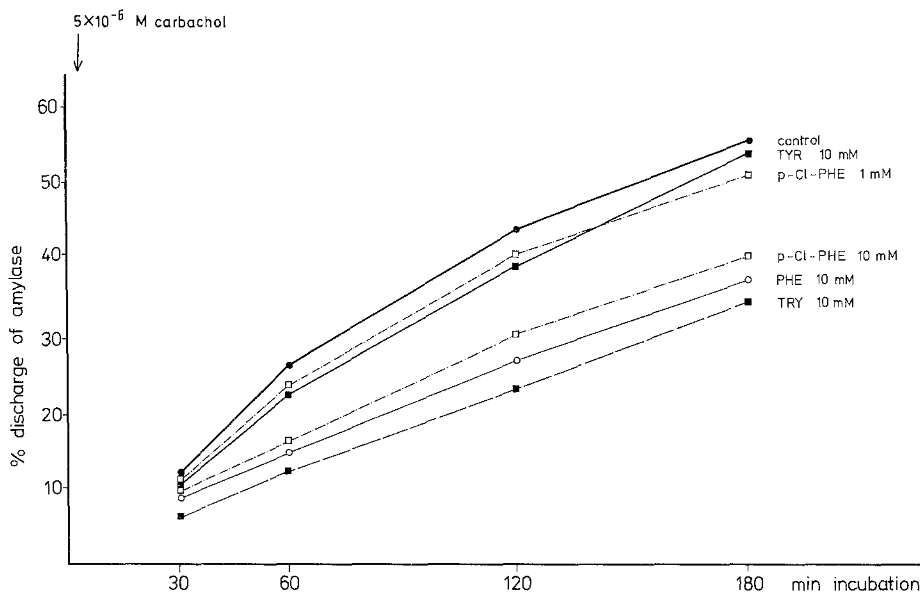

Fig. 10. Effect of various aromatic amino acids on induced discharge of amylase. The procedure corresponds to Fig. 7 and results are expressed as per cent of the sum of amylase in medium and tissue. They are representative of 3 to 6 identical experiments

secretory proteins is known to exist. Condensing vacuoles and mature zymogen granules never contain paracrystals. Earlier studies (Jamieson and Palade, 1971) have made evident, that secretory proteins are concentrated to an osmotically stable form during the maturation of condensing vacuoles to zymogen granules. Thus, the physicochemical state of the proteins inside the granules may preclude formation of paracrystals, which is possible during their passage through the cisternal space of the RER and after their discharge into the acinar lumen. The crystals are distributed at random in the supranuclear portions of the RER, but are contained only in a fraction of the cisternae. It is possible that this inhomogeneous distribution of paracrystals could reflect differences in concentration and/or composition of secretory proteins inside the different parts of the endoplasmic reticulum. It is also interesting to note that only certain parts of the perinuclear cisternae contain paracrystalline inclusions (Fig. 2). A second interesting structural feature concerns the Golgi complex. To date it is uncertain whether transport of proteins from the RER to the Golgi complex is mediated exclusively by transporting vesicles (Jamieson and Palade, 1967) or can also take place through tubular connections between both compartments as demonstrated in hepatic cells (Claude, 1970). The precipitation of secretory material in transitional elements of the RER produces pictures suggesting a limited number of direct tubular communications between RER and Golgi complex. The described morphological changes correspond to biochemical alterations of the secretory process. Though protein synthesis needs not to continue for paracrystals to form, protein synthesis is reduced by just those aromatic amino acids which at the same time induce paracrystal formation. The inhibition is not due to a decreased con-

centration of radioactive precursor amino acid. It can be speculated that the intricate spatial arrangement between ribosomal subunits and the ER-membrane could be disturbed by the paracrystals formed in the cisternal space, resulting in an interference with protein synthesis, but a number of other possibilities could be postulated. For the inhibition of intracellular transport and discharge of secretory proteins a similar structural explanation can be offered: formation of paracrystals renders the secretory proteins in the RER and in transitional elements less soluble and results in a disturbance of the orderly flow of proteins through the different compartments of the cell.

Similar paracrystals have been demonstrated in the mitochondrial mutant of Neurospora grassa (abnormal-1) and can be induced also in wild-type grown in ethidium or euflavine (Wood and Luck, 1971). These paracrystals are composed of a single polypeptide, which occurs normally in soluble form in the cytoplasm of Neurospora, but will crystallize if a mitochondrial defect is genetically present, or is induced by drugs.

In the case of paracrystals in the RER of exocrine pancreatic cells it can be excluded that they are precipitated aromatic amino acids themselves. This is indicated by the fact that 1.: tyrosine, despite its lower solubility reaches the same intracellular concentrations as e.g. phenylalanine, without inducing paracrystals *inside* the cell (only in the acinar lumina). 2.: paracrystals induced *in vitro* by high concentrations of aromatic amino acids remain, even if incubation is continued for an additional 2 hrs without aromatic amino acids present. 3.: preliminary binding studies of labeled aromatic amino acids with purified secretory proteins from the pancreas did not give any indication of a significant protein-amino acid affinity. Most important, 4.: proteins from isolated paracrystals showed identical electrophoretic mobility to the proteins within isolated zymogen granules. Earlier *in vivo* structural studies with p-Cl-Phe (Bieger *et al.*, 1972; Forssmann and Bieger, 1973; Forssmann *et al.*, 1970) have revealed that paracrystals are restricted to the exocrine pancreatic cell and are not observed in other secretory cells – parotid, islets of Langerhans or liver. Thus the proteins of other secretory cells either have substantially different physico-chemical properties or exogeneous amino acids are transported and metabolized differently in these tissues. It is therefore assumed that paracrystals arise from aggregation of normal secretory proteins, but the mechanism by which this occurs is not clear.

References

Bernfeld, P.: Amylases, α and β. Methods Enzymology **1**, 149–158 (1955)

Bieger, W., Wacker, Ph., Forssmann, W. G.: Zum Mechanismus der inhibitierten Pankreassekretion durch p-Chlorophenylalanin (PCPA). Verh. anat. Ges. (Jena) **66**, 87–98 (1972)

Burton, K.: A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of desoxyribonucleic acid. Biochem. J. **62**, 315–323 (1956)

Claude, A.: Growth and differentiation of cytoplasmic membranes in the course of lipoprotein granule synthesis in the hepatic cell. J. Cell Biol. **47**, 745–766 (1970)

Eagle, H.: Amino acid metabolism in mammalian cell cultures. Science **130**, 432–437 (1959)

Forssmann, W. G., Bieger, W.: Biosynthese falscher Proteine: Einbau von P-Cl-Phe in Enzyme des exokrinen Pankreas. Res. exp. Med. **160**, 1–20 (1973)

Forssmann, W. G., Wacker, Ph., Daldrup, J., Tesi, D., Rouiller, Ch.: Paracrystalline inclusions in the granular endoplasmic reticulum of exocrine pancreas after treatment with p-Cl-Phe.

7. Congr. int. Microsc. Electr., Grenoble. In Soc. Franc. Micr. Electr., Paris (F. Favard, eds.) **3**, 506–507 (1970)

Gal, E. M., Millard, S. A.: The mechanism of inhibition of hydroxylases in vivo by p-Cl-Phe: The effect of cycloheximide. Biochem. biophys. Acta (Amst.) **227**, 32–41 (1971)

Jamieson, J. D.: Transport and discharge of exportable proteins in pancreatic exocrine cells: *in vitro* studies. In: Current Topics in Membranes and Transport. (F. Bronner and A. Kleinzeller, eds.) New York: Academic Press, Inc. vol. 3, p. 273–338 (1972)

Jamieson, J. D., Palade, G. E.: Intracellular transport of secretory proteins in the pancreatic exocrine cell. I. Role of the peripheral elements of the Golgi complex. J. Cell Biol. **34**, 577–596 (1967)

Jamieson, J. D., Palade, G. E.: Intracellular transport of secretory proteins in the pancreatic exocrine cell. II. Transport of condensing vacuoles and zymogen granules. J. Cell Biol. **34**, 597–615 (1967)

Jamieson, J. D., Palade, G. E.: Intracellular transport of secretory proteins in the pancreatic exocrine cell III. Dissociation of intracellular transport from protein synthesis. J. Cell Biol. **39**, 580–588 (1968)

Jamieson, J. D., Palade, G. E.: Intrazellular transport of secretory proteins in the pancreatic exocrine cell. IV. Metabolic requirements. J. Cell Biol. **39**, 589–603 (1968)

Jamieson, J. D., Palade, G. E.: Condensing vacuole conversion and zymogen granule discharge in pancreatic exocrine cells: metabolism studies. J. Cell Biol. **48**, 503–522 (1971)

Koe, K., Weissman, A.: p-Cl-Phe: a specific depletor of brain serotonine. J. Pharmacol. exp. Ther. **154**, 499–516 (1966)

Krebs, H. A.,: Body size and tissue respiration. Biochem. biophys. Acta (Amst.) **4**, 249–256 (1950)

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., Randall, R. J.: Protein measurement with the folin phenol reagent. J. biol. Chem. **173**, 265–275 (1951)

Luft, J. H.: Improvement in the epoxy resin embedding methods. J. biophys. biochem. Cytol. **9**, 409–414 (1961)

Maizel, J. V.: Polyacrylamide gel electrophoresis of viral proteins. In: Methods in Virology (K. Maramorosh and H. Koprowski, eds.) vol. 5, p. 179–258. New York: Academic Press, Inc. (1971)

Palade, G. E.: Intracisternal granules in the exocrine cells of the pancreas. J. biophys. biochem. Cytol. **2**, 417–425 (1956)

Reynolds, E. S.: The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Biol. Cell **17**, 208–212 (1963)

Scheele, G. A., Palade, G. E.: Studies on the guinea pig pancreas. Parallel discharge of exocrine enzyme activities. J. Biol. Chem. **250**, 2660–2670 (1975)

Schramm, M., Danon, D.: The mechanism of enzyme secretion by the cell. I. Storage of amylase in the zymogen granules of the rat parotid gland. Biochem. biophys. Acta (Amst.) **50**, 102–112 (1961)

Siekevitz, P., Palade, G. E.: A cytochemical study on the pancreas of the guinea pig. II. Functional variations in the enzymatic activity of microsomes. J. biophys. biochem. Cytol. **4**, 309–318 (1958)

Tartakoff, A., Greene, L. J., Jamieson, J. D., Palade, G. E.: Parallelism in the processing of pancreatic proteins. In Advances in Cytopharmacology (B. Ceccarelli, F. Clementi and J. Meldolesi, eds.) vol. 2, p. 177–194. New York: Raven Press (1974)

Venrooij, W. J. van, Poort, C., Kramer, M. F., Jansen, M. T.: Relationship between extracellular amino acids and protein synthesis *in vitro* in the rat pancreas. Europ. J. Biochem. **30**, 427–433 (1972)

Wood, D. D., Luck, D. J. L.: A paracrystalline inclusion in Neurospora Crassa. Induction by ethidium, acridine, isolation, and characterization. J. Cell Biol. **51**, 249–264 (1971)

Wilfried Bieger, MD
Department of Anatomy
University of Heidelberg
D-6900 Heidelberg
Im Neuenheimer Feld 307
Federal Republic
of Germany