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Studies on Intracellular Transport of Secretory Proteins in the Rat Exocrine Pancreas

II. Inhibition by Antimicrotubular Agents*

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Summary. The possible role of microtubules and microfilaments in the secretory process of the rat exocrine pancreas was analysed *in vitro* using isolated pancreatic lobules. Colchicine and vinblastine as microtubule inhibitors, hexylene glycol as a microtubule stabilizer, and cytochalasin B as a disruptive agent for microfilaments were used in increasing concentrations to test their effects on protein synthesis, intracellular transport, zymogen discharge, and cellular respiration.

Colchicine only at 10^{-2} M concentrations inhibits protein synthesis, while vinblastine inhibits at 10^{-6} and 10^{-5} M by 20% and at 10^{-4} M by 55%. A similar inhibition is observed with 1.5% concentrations of hexylene glycol while cytochalasine B at 1,5 and 10 μg/ml is without effect on protein synthesis. Colchicine and vinblastine have their major effects on intracellular transport both in secretion studies and cell fractionation experiments. Colchicine in concentrations between 10^{-3} to 10^{-5} M inhibits discharge of newly synthesized proteins by 50%, while vinblastine shows a dose-response relationship of 40% inhibition at 10^{-6} M to 90% at 10^{-4} M. Discharge of amylase is uniformly reduced by 30% by both colchicine and vinblastine in the whole dose range. The pronounced effect of colchicine and vinblastine is evident in cell fractionation studies: both drugs inhibit the disappearance of protein radioactivity from microsomes and its appearance in zymogen granules; similarly the peak radioactivity in smooth microsomes (Golgi) appears delayed. No differential effect on the secretory process was observed with 1.5% concentrations of hexylene glycol or cytochalasin B at 1.5 and 10 μg/ml concentrations. A fines tructural analysis of microtubules and microfilaments in the exocrine pancreatic cell reveals their distribution in all parts of the cytoplasm and in relation to all cell organelles. Both systems (microtubules, microfilaments) seem to be connected, at least in certain areas of the cytoplasm and at the plasma membrane.

The reduction of transport efficiency by microtubule inhibitors results in a deposition of secretory material in the cisternal space of the rough endoplasmatic reticulum, which leads to the formation of paracrystals. Colchicine at 10^{-3} M concentrations leads to an enlargement of condensing vacuoles in the Golgi complex.

Key words: Exocrine pancreas — Intracellular transport — Colchicine — Vinblastine — Hexylene glycol — Cytochalasin B — Paracrystals.

Introduction

The current concept of stimulus—secretion—coupling in a variety of exocrine and endocrine glands involves the participation of cytoplasmic microtubules and/or microfilaments, which are believed to mediate the transport of secretory granules to their site of discharge (for review see Allison, 1973; Lacy and Malaisse, 1973; Olmsted and Borisy, 1973). The concept was largely formulated on the

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basis of *in vitro* experiments, in which antimicrotubular drugs (vinblastine, colchicine, deuterium oxide, hexylene glycol) interferring with the equilibrium between assembly and disassembly of tubulin into microtubular structures would lead to an inhibition of the secretory response.

Recent studies in the exocrine pancreas of the guinea pig have shown, that the secretory process involves a series of complicated steps, which occur in sequence and are associated with different compartments in the exocrine pancreatic cell (for review see Jamieson, 1972). Upon completion of biosynthesis secretory proteins are seggregated into the cisternal space of the rough endoplasmic reticulum (ER) and are transported to the Golgi complex involving small vesicles as intermediate "shuttle organelles". Condensing vacuoles pinching off from Golgi cisternae are transformed into zymogen granules, which are discharged upon stimulation of the gland. An analysis of the finestructural distribution of microtubules in relation to the different cell organelles revealed, that they initiate at the outer nuclear membrane, course between the profiles of the rough ER and the Golgi cisternae and are terminating at the inner leaflet of the plasma membrane (Kern, 1975). This distribution would suggest that if microtubules would be involved in intracellular transport phenomena they would not only mediate the translocation of secretory granules towards the plasma membrane but would also act on earlier phases of the secretory process. To study this in more detail isolated pancreatic lobules were incubated *in vitro* in the presence of various antimicrotubular/microfilamentous agents and their effect on the three major steps in the secretory cycle (protein synthesis, intracellular transport of newly synthesized proteins, discharge of zymogen granules) was compared with cellular respiration.

Materials and Methods

Male Wistar rats (S. Ivanovas, Kisslegg, Germany) 200 to 240 g in weight were used throughout the experiments. They had free access to drinking water and laboratory chow (Altromin R, Fa. Altromin, Laage/Lippe, Germany). They were killed by decapitation in light ether anesthesia, the pancreas was removed and placed in ice cold buffer solution. Pancreatic lobules were prepared according to Scheele and Palade (1975), and incubated in Krebs-Ringer solution containing 14 mM glucose, L-amino acids according to Eagle (1959) and 15 mM HEPES (N-2-hydroxyethyl-piperazine-2-ethane-sulfonic acid) instead of bicarbonate as buffer substance. pH was kept at 7.4 and in tracer experiments with L-leucine-4.5-^{3}H the concentration of unlabeled L-leucine was reduced to 10% of the regular concentration.

Incubation Procedures

a) Protein Synthesis. Sets of 5 pancreatic lobules were incubated for 1, 2 and 3 hrs in 10 ml Krebs-Ringer-Hepes buffer (KRH) containing 1 μCi/ml L-leucine-4.5-^{3}H and concentrations of colchicine and vinblastine from 10^{-2} to 10^{-6} M, cytochalasin B from 1 to 10 μg/ml or hexylene glycol in a concentration of 1.5%. Incubation vials were regularly gassed every 30 min. At the end of each incubation period the lobules were briefly washed with cold incubation medium and homogenized in 5 ml distilled water.

b) Intracellular Transport. This was analyzed by a radioassay for zymogen discharge (Jamieson and Palade, 1971) and by cell fractionation. In both procedures pancreatic lobules were pulse-labeled with 5 μCi/ml L-leucine-4.5-^{3}H for 4 min and the tracer amino acid was quickly removed by 3 washes on Büchner funnels with chase medium containing 2.0 mM L-leucine-^{1}H. For the radioassay 7 lobules were transferred to 25 ml flasks containing 10 ml of KRH, 0.3 mg/ml bovine serum albumine (BSA) and antimicrotubular/microfilamentous

agents in increasing concentrations. The lobules were stimulated to secrete by the addition of 5×10^{-6} M carbamylcholine. For cell fractionation studies sets of 40 to 50 lobules (around 400 mg wet weight) were pulse labeled for 5 min and further chase incubated in 10 ml KRH for an additional 10, 35 and 55 min. Sets of control lobules and those in the presence of increasing concentrations of antimicrotubular drugs were homogenized in 4.5 ml of 0.3 M sucrose. Fractions of zymogen granules, mitochondria and microsomes were prepared according to Jamieson and Palade (1967). The microsomes were further subfractionated for 3 hrs at $160{,}000 \times g$ on a discontineous gradient of sucrose between 1.0 and 2.0 M.

c) Discharge of Zymogen Granules. From the same set of pancreatic lobules used for the radioassay (b) 5 ml samples of the incubation medium (containing 5×10^{-6} M carbamylcholine) were removed after 30, 60, 120 and 180 min of incubation and replaced by new medium. From this 5 ml sample 4 ml were precipitated with tri-chloro-acidic acid (TCA, 10% final concentration) for determination of discharged labeled proteins. The remaining 1 ml was used for the determination of amylase. At the end of 3 hrs incubation the pancreatic lobules from secretion experiments were briefly washed and homogenized in 5 ml distilled water. 1 ml of the homogenate was precipitated in duplicate with TCA (for determination of protein radioactivity). Samples of the remaining volume served for the determination of amylase in the tissue.

Assay Procedures

a) Radioactivity Assays. Samples from incubation medium, homogenates and subcellular fractions were precipitated at 4° C with TCA overnight and washed twice with 5% TCA. The final pellet was dissolved in 1 ml 1 N NaOH. From this 0.1 ml were used for protein determination, the remaining was transferred to scintillation counting vials containing 10 ml Unisolve (Koch-Light-Laboratories, Colnbroock, England). After the addition of 3 ml distilled water Unisolve forms a gel. Counting was performed in a Nuclear Chicago liquid scintillation counter.

b) Chemical Assay. Proteins were determined by the methods of Lowry *et al.* (1951), using crystaline bovine serum albumine as standard. Amylase activity, defined according to Schramm and Dannon (1961) was measured by the procedure of Bernfeld (1955).

c) Cellular Respiration. This was determined by the production of $^{14}CO_2$ from palmitate-1-^{14}C as described by Jamieson and Palade (1968).

Electron Microscopy

Pancreatic lobules were fixed after isolation or *in vitro* incubation for various time periods by immersion in 2.5% glutaraldehyde-formaldehyde mixture buffered at pH 7.2 with 0.067 M Na-cacodylate with or without the addition of 3% tannic acid (Mizuhira and Futaesaku, 1974). Dehydration and embedding in Epon was performed according to standard procedures. Sections cut with a diamond knife on a LKB microtome were stained with 5% uranylacetate and lead citrate according to Reynolds (1963). They were examined in a Zeiss EM 9 S electron microscope.

Materials

All chemicals were reagent grade (Merck Chemicals, Darmstadt, Germany). L-amino acids, colchicine, hexylene glycol (2 methyl-2.4-pentandiol) and cytochalasin B were obtained from Serva Biochemica, Heidelberg. Vinblastine sulfate was kindly provided by S. Howe, E. Lily, Co., Bad Homburg, Germany. L-leucine-4.5-^{3}H (30–60 mCi/mole) and 1-^{14}C-palmitic acid (50 mCi/m mole) were purchased from Amersham Buchler, Braunschweig, Germany.

Results

1. Biochemical Findings

Under control conditions isolated lobules from the rat pancreas incorporate L-leucine-^{3}H at a linear rate for a period of 3 hrs *in vitro* incubation. The different agents used to inhibit the function of the microtubular/microfilamentous system

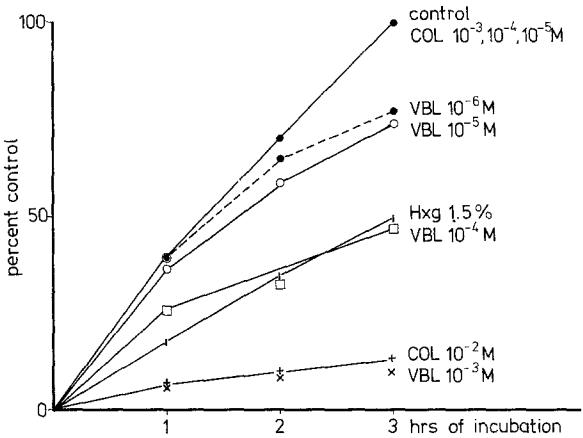

Fig. 1. Effect of increasing concentrations of colchicine (*COL*), vinblastine (*VBL*) and hexylene glycol (*Hxg*) on the incorporation of L-leucine-4.5-^{3}H into TCA-precipitable proteins. Values are expressed as percent of control (100% = 60,500 dpm/mg protein)

affect this rate of incorporation at various extent (Fig. 1). Colchicine only at 10^{-2} M concentrations blocks protein synthesis (by 90%) while 10^{-3} to 10^{-5} M concentrations are without any effect. On the other hand vinblastine shows a dose-dependant inhibition of protein synthesis, which amounts to 20% at 10^{-6} M and 10^{-5} M, 55% at 10^{-4} M and 90% at 10^{-3} M. Hexylene glycol, generally assumed to stabilize microtubular structure, inhibits protein synthesis by 55% at a concentration of 1.5% in the medium. Cytochalasin B used as a disruptive agent to the microfilaments has no effect on the rate of incorporation of radioactive leucine into protein. These findings on protein synthesis correlate largely with the effect of these agents on cellular respiration. This can be determined by the production of $^{14}CO_2$ from 1-^{14}C-palmitic acid. Fig. 2 shows the data obtained with the various drugs. Colchicine at 10^{-5} and 10^{-4} M concentration is ineffective, while 10^{-3} M reduces the production of $^{14}CO_2$ by 35% and 10^{-2} M by 65%. This is in contrast to the findings in protein synthesis, where 10^{-3} M colchicine was without effect. Using vinblastine as an antimicrotubular agent both its effect on protein synthesis and cellular respiration show a close dose-response relationship. The same applies to 1,5% concentrations of hexylene glycol. Cytochalasin B, which had no effect on L-Leucine incorporation increases the $^{14}CO_2$-production from ^{14}C-palmitate: 1% DMSO (Dimethylsulfoxide) alone which is used as solvent for cytochalasin B leads to an increase by 15%, 1 μg/ml cytochalasin B increases by 50%, 5 μg/ml by 28% and 10 μg/ml is back to control level.

A more pronounced and differential effect is observed, if the action of microtubule inhibitors is analysed on intracellular transport and discharge of secretory proteins. This is studied *in vitro* using isolated pancreatic lobules pulse labeled with tritiated leucine for 4 min and chase incubated for an additional 3 hrs in the presence of 5×10^{-6} M carbamylcholine as secretagogue. The discharge of labeled proteins into the medium can be taken as an indication of intracellular transport operations, while the release of amylase stands for the behaviour of the total

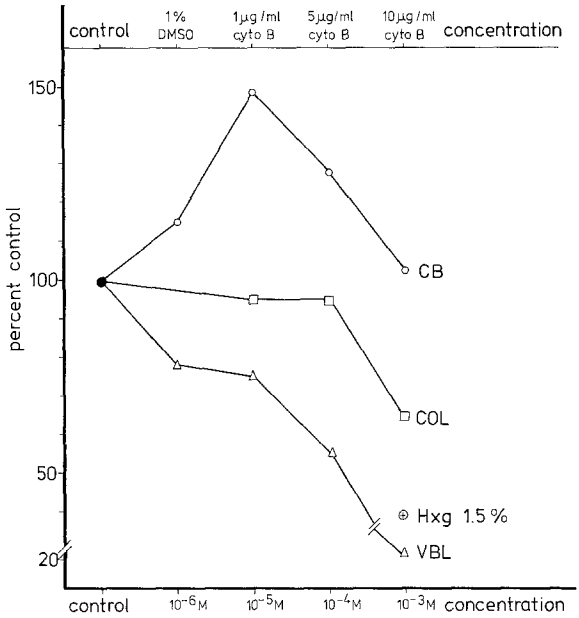

Fig. 2. $^{14}CO_2$-production from palmitate-1-^{14}C in the presence of microtubule and microfilament inhibitors. Pancreatic lobules are incubated for 30, 60 and 90 min in the presence of labeled palmitate, the $^{14}CO_2$ produced is absorbed by 20% KOH on filterpaper and counted. Values are expressed as percent of control

pool of zymogen granules. Only a small fraction of the granules is derived from new synthesis during the 4 min pulse incubation in tritiated leucine.

Under control conditions during the first 30 min of chase incubation about 1% of the total content in labeled proteins is discharged into the medium, while the release of amylase amounts to about 10% of total content (Figs. 3, 4). This difference is explained by the fact, that newly synthesized proteins have to undergo a series of transport steps before they are packed to zymogen granules and released into the acinar lumen. This takes a minimum of 30 min (Jamieson, 1972). The amylase appearing in the medium is derived from zymogen granules which are stored at the apical pole of exocrine cells and are ready to be released at instance. At the end of the 3 hrs incubation period both newly synthesized proteins and amylase have been released to the same extent (about 50% of their total content in the tissue).

The addition of antimicrotubular agents affects both pools of secretory proteins ("old" and newly synthesized) to a different extent. Discharge of amylase is uniformely reduced by about 30% from control levels by both colchicine and vinblastine, regardless of the dose (Figs. 3a, 4a). The effect of colchicine is not observed during the first hour of incubation, but becomes evident after 2 and 3 hrs. (Fig. 3a). Vinblastine at 10^{-6} M concentrations is without effect on amylase release while 10^{-5} and 10^{-4} M reduce it by 25 and 30% respectively.

However, the effect of both substances on discharge of newly synthesized proteins is much more pronounced. Colchicine in a range of 10^{-3} to 10^{-5} M con-

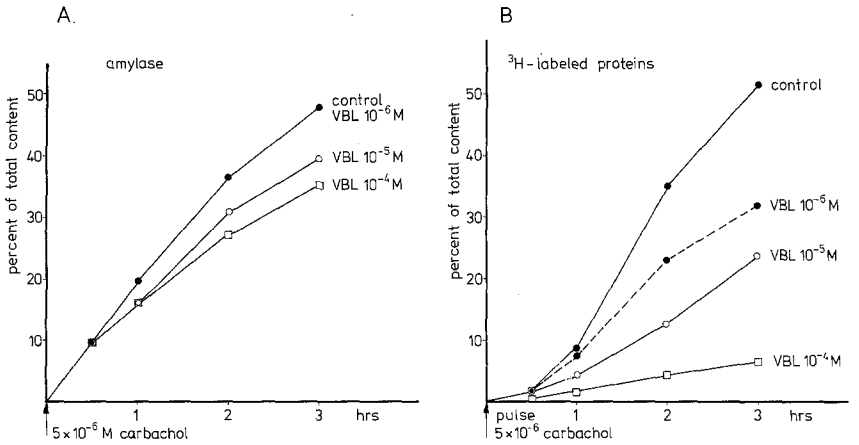

Fig. 3A and B. Effect of increasing concentrations of vinblastine (*VBL*) on discharge of amylase (A) and labeled proteins (B) stimulated by 5×10^{-6} M carbamylcholine in the medium. Incubation time is 3 hrs, data are expressed as percent of total content in the medium and the tissue

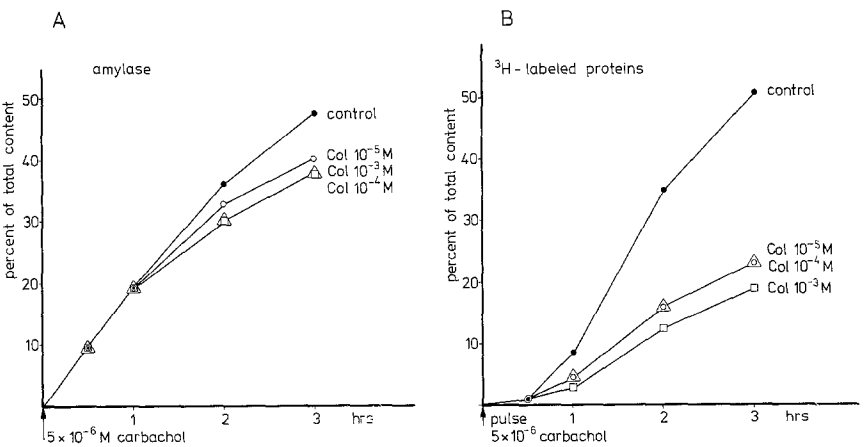

Fig. 4A and B. Carbamylcholine (5×10^{-6} M) stimulated discharge of amylase (A) and of labeled proteins (B) in the presence of increasing concentrations of colchicine (*COL*). Results are expressed as percent of the sum of amylase or protein radioactivity in medium and tissue

centration inhibits the release by 50%, while vinblastine shows a close dose-response relationship ranging from 40% inhibition at 10^{-6} M to 90% at 10^{-4} M (Figs. 3b, 4b).

This differential effect on the release of newly synthesized proteins compared to amylase release is not observed with hexylene glycol, a substance stabilizing microtubules and cytochalasin B, a mold metabolite which interferes with the function of microfilaments. Table 1 summarizes the effect of both drugs on stimulated discharge of amylase and labeled proteins from pancreatic lobules over a period of 3 hrs. It can be seen that hexylene glycol reduces the release of both

Table 1. Effect of cytochalasin B (CB) and hexylene glycol (Hxg) on discharge of amylase and labeled proteins stimulated by 5×10^{-6} M carbamylcholine ($n = 6$)

Conditions	Discharge of amylase (% of total content/3 hrs)	± SD	Discharge of labeled proteins (% of total content/3 hrs)	± SD
Control	47.4	1.9	51.0	2.8
DMSO (1%)	49.9	4.3	53.8	4.4
CB (10 μg/ml)	35.8	3.2	41.8	1.8
CB (5 μg/ml)	34.4	0.7	42.0	1.4
CB (1 μg/ml)	36.7	3.5	40.9	3.9
Hxg (1.5%)	40.8	0.8	37.7	5.3

secretory pools to the same extent (about 20%), while cytochalasin B in doses of 1, 5 and 10 μg/ml affects discharge of amylase slightly more (30%) then the release of labeled proteins (20% reduction from control levels). In the case of hexylene glycol the effect on secretion is combined with a 50% reduction of the rate of protein synthesis and of cellular respiration (Figs. 1, 2), while cytochalasin B has no effect on protein synthesis and increases dose-dependantly the rate of $^{14}CO_2$-production from ^{14}C-palmitate (Fig. 2).

From the results obtained with colchicine and vinblastine in secretion experiments it could be concluded, that both drugs have a predominant effect in inhibiting intracellular transport and interfer much less with the exocytosis process of zymogen granules. This possibility was further tested in cell fractionation studies: sets of pancreatic lobules were pulse labeled with 5 μCi/ml L-leucine-4,5-^{3}H and further chase incubated for an additional 10, 35 and 55 min in the presence of 10^{-4} M concentrations of vinblastine and 10^{-3} M colchicine. At the end of the pulse and at each time point of chase incubation the lobules were homogenized and fractionated by sucrose-gradient centrifugation to reveal zymogen granules, rough microsomes (representing mainly rough endoplasmatic reticulum) and smooth microsomes (mainly derived from elements of the Golgi complex). The specific radioactivity (dpm/mg protein) in each of these fractions is determined as a function of time in control lobules and after incubation with colchicine and vinblastine (Fig. 5). Under control conditions at the end of the pulse about 75% of the radioactivity is contained in the rough microsomal fraction (RM), smoth microsomes (SM) contain about 20% and zymogen granules less then 5%. Chase incubation for 10, 35 and 55 min results in a progressive decrease of specific radioactivity in the RM and concomitant increase in the zymogen granule fraction. The SM fraction shows a peak concentration at 10 min with slow decrease at 35 and 55 min. These results are in agreement with earlier studies of Jamieson and Palade (1967) in the guinea pig pancreas and reflect a directional transport from rough ER to zymogen granules with an intermediate step in the Golgi complex. *In vitro* incubation of lobules in the presence of 10^{-4} M vinblastine and 10^{-3} M colchicine change the kinetics of intracellular transport (Fig. 5). Both the decrease of specific radioactivity from RM and its increase in the zymogen granules is delayed, with colchicine the radioactivity in the RM fraction shows a plateau between 35 and

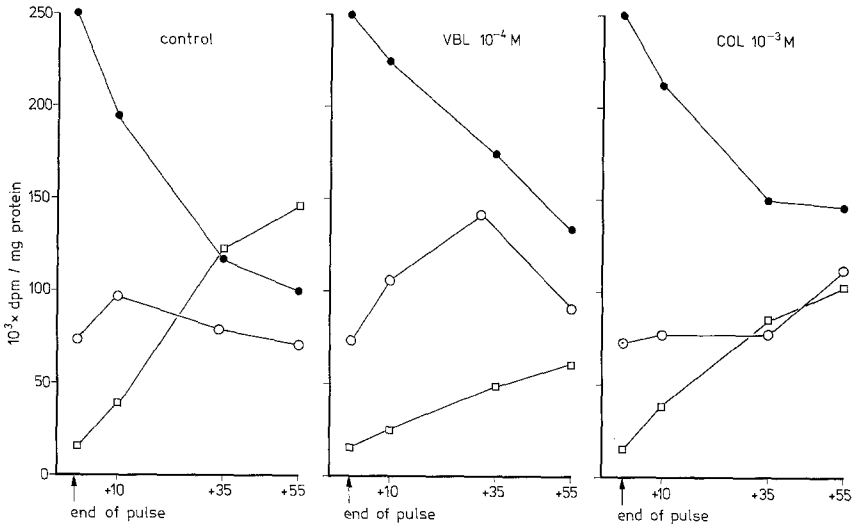

Fig. 5. Graphic presentation of cell fractionation studies. Sets of pancreatic lobules were pulse labeled for 5 min with leucine-^{3}H and chase incubated for an additional 10, 35 and 55 min under control conditions or in the presence of 10^{-4} M vinblastine (*VBL*) or 10^{-3} M colchicine (*COL*). The specific protein radioactivity is shown in the respective fraction of zymogen granules, rough microsome and smooth microsomes. ▫——▫ zymogen granules; •——• rough microsomes; ○——○ smooth microsomes

55 min of chase incubation. Both antimicrotubular agents shift the appearance of peak radioactivity in the SM fraction (corresponding to Golgi elements) from 10 min in controls to 35 min in the presence of 10^{-4} M vinblastine and to 55 min in the presence of 10^{-3} M colchicine.

2. Ultrastructural Findings

a) Finestructural Distribution of Microtubules and Microfilaments. Microtubules have only rarely been described in exocrine pancreatic cells (Kern and Ferner, 1971) while microfilaments generally are observed in the cytoplasm beneath the apical membrane and as core structure in the microvilli projecting into the acinar lumen. A detailed analysis of both structures in the exocrine cells of isolated rat lobules reveals their occurrance in all parts of the cytoplasmic space. As has been recently described in endocrine secretory cells (Warchol *et al.*, 1975; Kern, 1975) in the exocrine pancreatic cell of the rat microtubules originate in the outer nuclear membrane and extend over long distances through the cytoplasm (Fig. 6a). They course between elements of the rough endoplasmic reticulum and between or around the Golgi complex and are inserted in the lateral and apical plasma membrane. Since microtubules generally approach the plasma membrane under an angle of 5° to 15° their fixation in the membrane can best be studied in tangential sections through the plane of the membrane (Figs. 6b, 7a, b). It is then observed that microtubules terminate as such at the inner leaflet of the plasma membrane or that microfilaments are interposed between plasma membrane and the approaching microtubule. At the apical pole of the cell microtubules

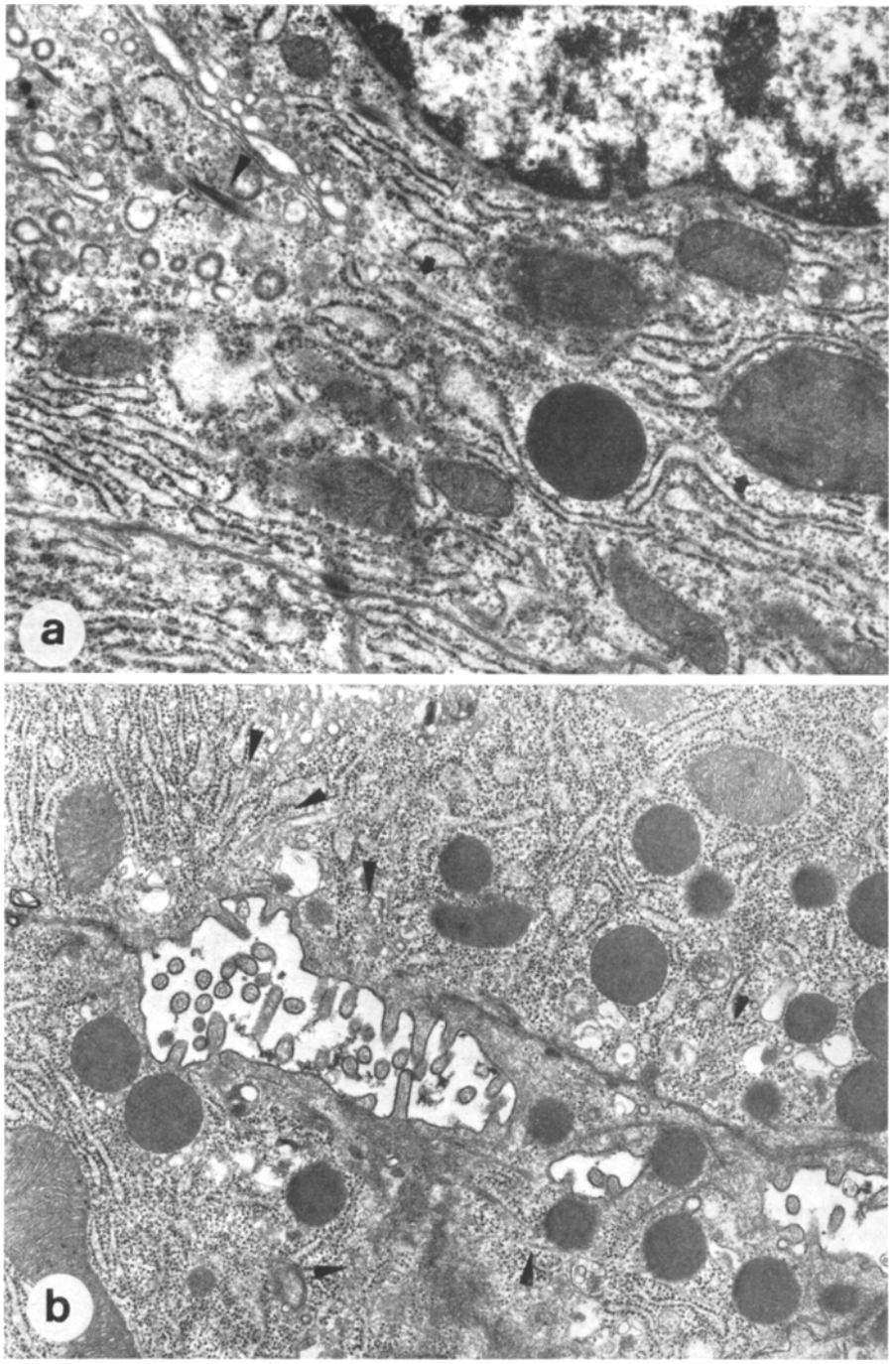

Fig. 6a and b. Finestructural distribution of microtubules and microfilaments in exocrine pancreatic cells from control lobules. (a) Microtubules (arrows) extending between profiles of rough ER to the Golgi complex, where they come in contact with a bundle of microfilaments (arrow head) Magnification, ×17,600, (b) feltwork of microfilaments beneath the apical plasma membrane. Several microtubules (arrow heads) in close procimity to this region. Magnification, ×19,200

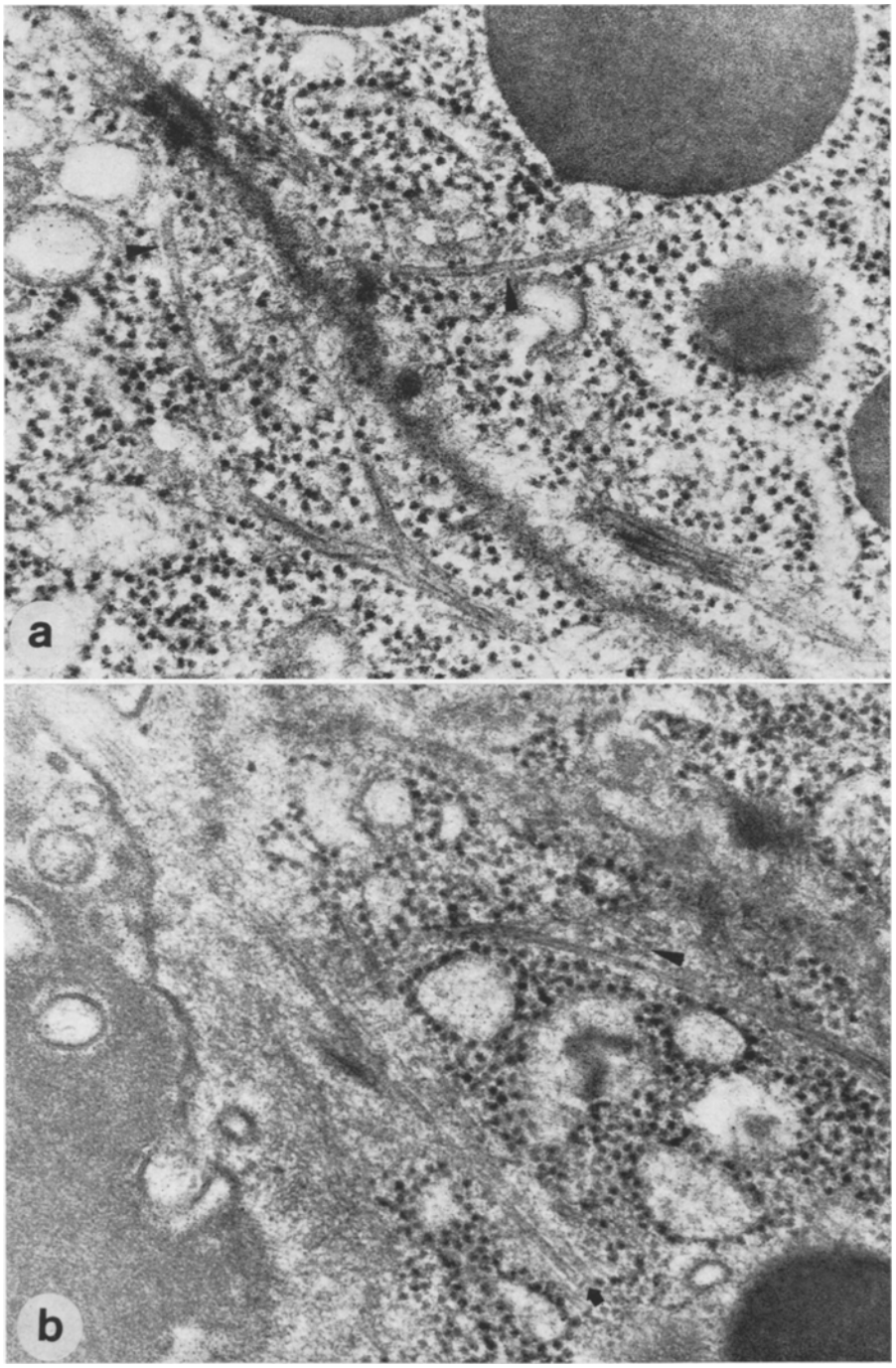

Fig. 7. Termination of microtubules at membranes (a) microtubules (arrow head) approaching the lateral plasma membrane (cut tangentially); (b) microtubules in proximity to the terminal web running in triplet (arrow) and doublet (arrow head). Magnification, ×66,000

are often arranged in parallel running in doublet or triplet together and terminating in the "terminal web" beneath the apical plasma membrane (Figs. 6b, 7b). This "terminal web" represents a feltwork of individual microfilaments (50 to 60 Å in diameter) which run in parallel to the apical surface of each exocrine cell (Fig. 6b). In the interior of each microvillus a bundle of the same filaments extends longitudinally from the tip of microvillus into the terminal web. At the sides of the cell the filaments of this apical network converge upon the junctional complex of adjoining cells (Fig. 6b). Bundles of similar microfilaments are, however, observed in all parts of the cytoplasmic matrix: they extend from the outer nuclear membrane (Fig. 6a) and are observed between the profiles of the rough endoplasmic reticulum and the Golgi complex (Fig. 8a). At the lateral plasma membrane desmosomes are often interconnected by a band of microfilaments of about 200 to 300 mμ in total thickness which runs in parallel to the plasma membrane and is symetrical in both neighbouring cells (Fig. 8b). Under favorable conditions of sectioning bundles of microfilaments connected to each other under an angle can be observed both in the cytoplasmic matrix and close to the plasma membrane. A regular lattice of fibers in two planes of sectioning can be revealed indicating the possibility of fusion of microfilament bundles from different directions in the cell (Fig. 8c, d). Occasionally microtubules are connected to the bundles of microfilaments.

b) Structural Changes Induced by Antimicrotubular Agents. The most pronounced changes in the finestructure of exocrine pancreatic cells are observed after *in vitro* incubation in the presence of 10^{-4} M vinblastine. Individual microtubules have largely disappeared from the cytoplasm, instead rectangular bodies of several μ in length and 0.2 to 0.5 μ in diameter are observed in the basal, paranuclear and especially abundant in the apical cytoplasm (Fig. 9a). At higher magnification sections parallel to the long axis of these bodies reveal an array of regular electron-dense lines spaced 280 Å from each other. In cross sections these bodies are composed of tubules with a diameter of 280 Å, which are packed together tightly and may be connected by lateral bridges (Fig. 9b). They closely resemble the tubulin crystals first described by Bensch and Malawista (1969) in fibroblasts. The finestructure of microfilaments along the apical and lateral plasma membrane is not altered by the vinblastine treatment. However, besides the tubulin-crystals dispersed in the cytoplasmic matrix the cisternal space of the rough ER contains numerous needle-like inclusions, which are randomly distributed in the cell (Fig. 10a). They do not occur in all pancreatic acini incubated *in vitro*, but are most common after vinblastine and less frequent with colchicine and hexylene glycol. At higher magnification the electron opaque material fills tightly the cisternal space and sometimes has a plate-like appearance (Fig. 10b). They resemble largely the paracrystals found in exocrine pancreatic cells after *in vitro* incubation with high concentrations of aromatic amino acids (Bieger and Kern, 1975). In these previous studies they have been isolated and identified as secretory proteins aggregated in the cisternal space of the rough ER. Incubation in 10^{-3} M concentrations of colchicine leads to a disappearance of microtubules. In most exocrine cells the regular arrangement of the Golgi complex has been changed: the stacks of Golgi cisternae are fragmented into smaller vesicles, which are filled with electron opaque material. Most Golgi complexes reveal one enlarged condensing vacuole,

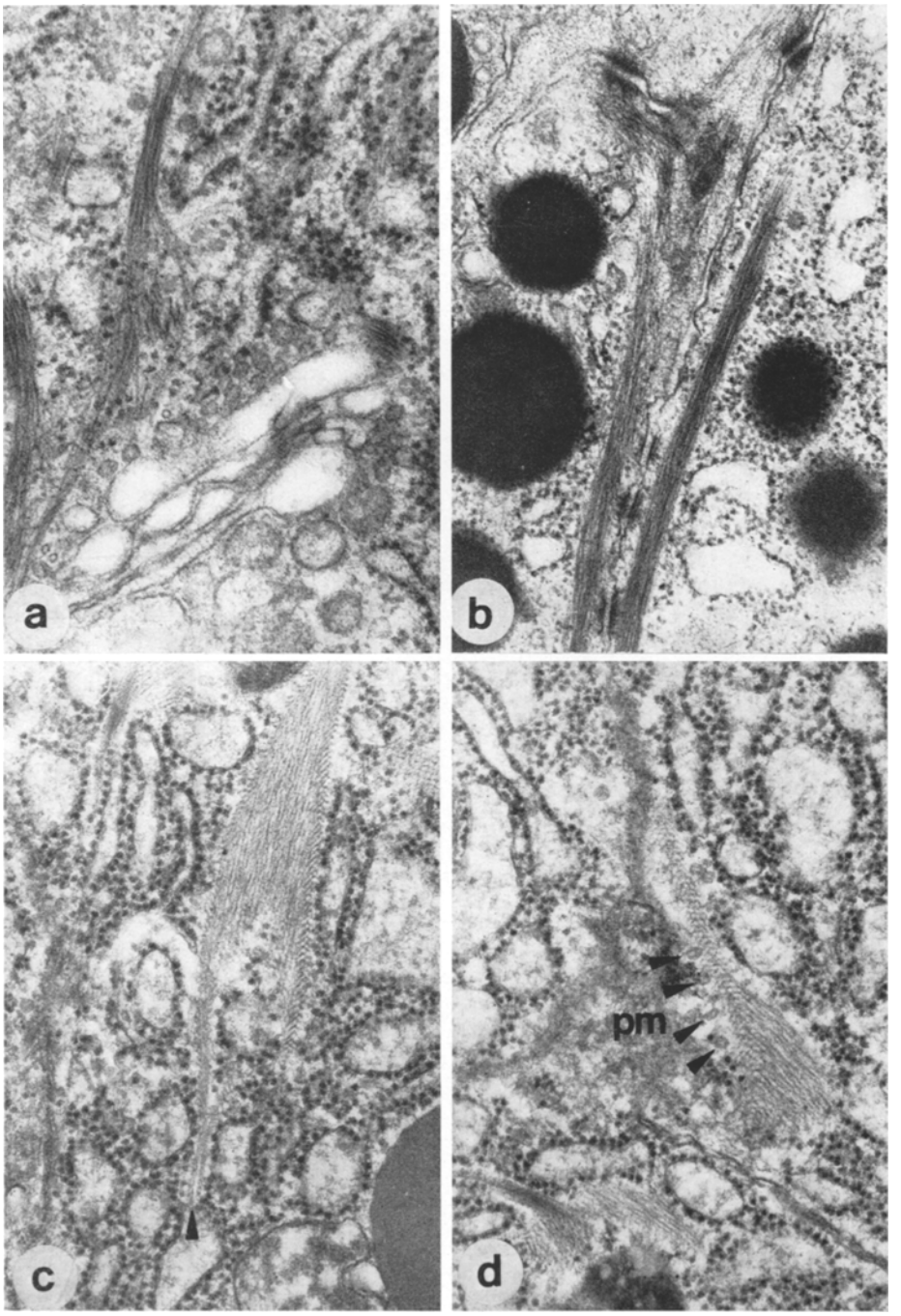

Fig. 8a—d. Bundles of microfilaments in different parts of the cytoplasm of normal exocrine cells. (a) between the rough ER and close to the Golgi complex; (b) parallel to the lateral plasma membrane in association with desmosomes; (c) and (d) lateral connection of two bundles of microfilaments one cut longitudinally, the other obliquely. This plane of sectioning creates the lattice-like appearance at the crossing points. In (c) two microtubules approach the microfilaments (arrow head), in (d) microtubules are seen in transverse section (arrow heads). *pm* plasma membrane cut tangentially. Magnification, ×55,000

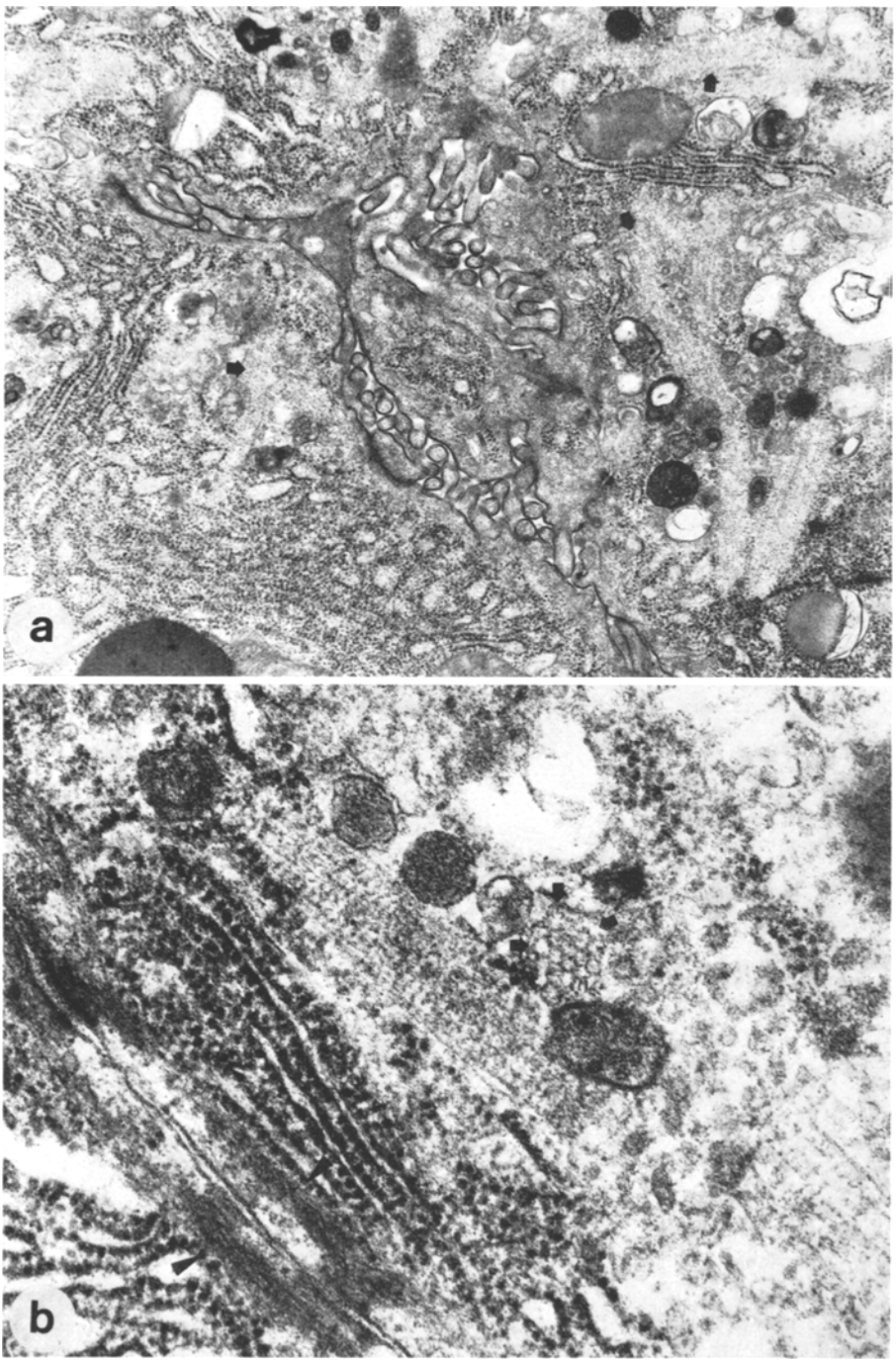

Fig. 9a and b. Tubulin crystals in exocrine pancreatic cells incubated for 3 hrs in the presence of 10^{-4} M vinblastine. (a) The crystals are most abundant in the apical cytoplasm of exocrine cells (arrows). Magnification, ×18,000. (b) Longitudinal and cross sections (arrows) of microtubules. They measure 280 Å in diameter, microfilaments are not changed (arrow heads). Magnification, ×68,750

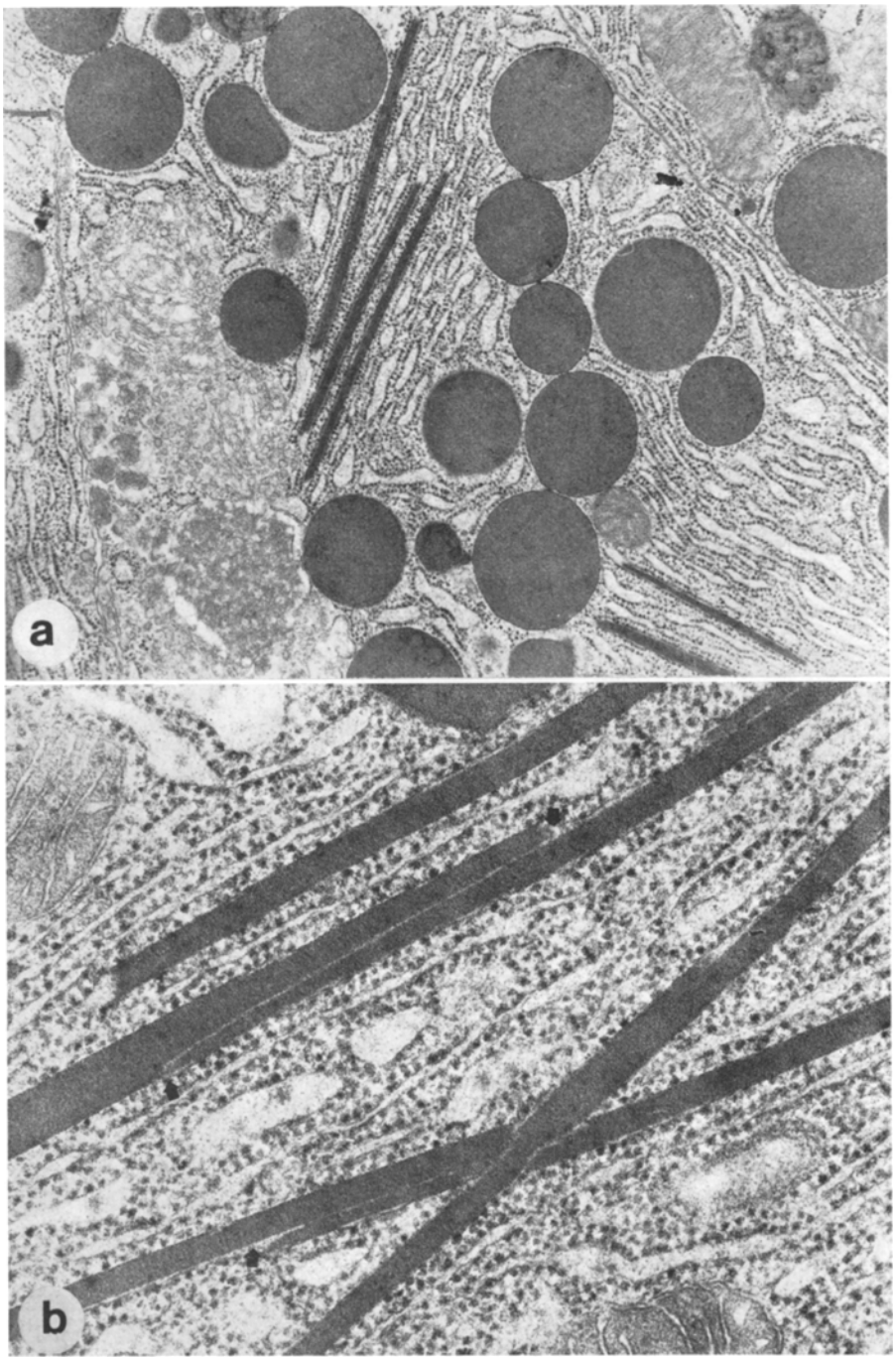

Fig. 10a and b. Needle-like aggregation of secretory proteins in the rough ER of exocrine cells, incubated for 3 hrs in 10^{-4} M vinblastine. (a) The paracrystals are 2–3 μ long and can reach close to Golgi complex. (b) They fill tightly the cisternal space of the rough ER, occasional brakes along the plane of the paracrystal are observed (arrows). Magnification, $\times 66{,}000$

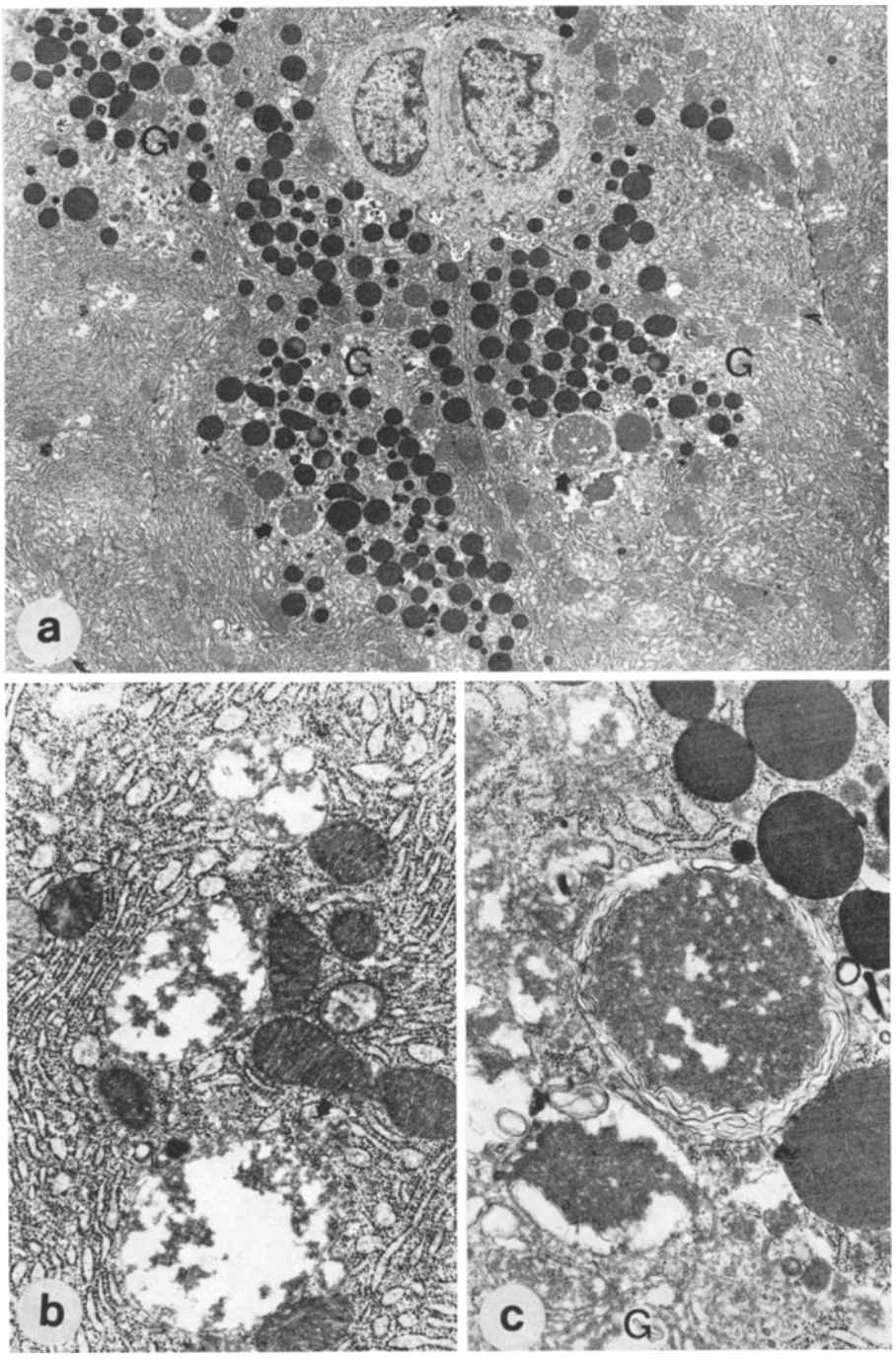

Fig. 11 a—c. Fine structural alteration after incubation in 10^{-3} M colchicine for 3 hrs. (a) At low magnification the fragmentation of the Golgi complex (*G*) and several enlarged condensing vacuoles (arrows) become obvious. Magnification, ×4,275. (b) One stage of enlarged condensing vacuole is derived from the dilatation of Golgi cisternae themselves, at the limiting membrane small vesicles (arrow) are observed fusing with the condensing vacuole. Magnification, ×16,000. (c) Enlarged condensing vacuole with signs of degeneration beneath the limiting membrane, *G* Golgi complex. Magnification, ×16,000

measuring up to 2 μ in diameter (Fig. 11). A fiber-like material in the interior just beneath the limiting membrane is a constant finding in these enlarged granules, while the other cell constituents are well preserved. As a result of an incubation in cytochalasin B the microvilli projecting in the acinar lumen largely disappear. At lower concentrations (1 μg/ml) the network of microfilaments beneath the apical plasma membrane is not significantly altered. At the highest concentration used (10 μg/ml) most acinar lumina appear enlarged with only sparse and very short microvilli present. In addition the terminal web of microfilaments has been disorganized, the apical cytoplasm contains focal masses of electron-translucent material with some short microfilaments contained in it. The disruption of the terminal web allows elements of the ER and free polysomes to reach up to the apical plasma membrane.

Discussion

Recent immunofluorescent studies with antibodies against actin (Lazarides and Weber, 1974; Gabbiani *et al.*, 1974) and tubulin (Weber *et al.*, 1975) have demonstrated two distinct intracellular networks of both structural proteins in a variety of eukaryotic cells. With antibodies against actin bundles of straight fibers of considerable diameter are observed crossing the cytoplasm in a few preferential directions. Phase contrast and electron microscopy reveal that the fluorescent fibers correspond to bundles of microfilaments, which also show an immunofluorescence with antibodies against myosin and tropomyosin. This indicates that the 3 structural proteins involved in contraction of muscle cells are also present in nonmuscle cells and are related to microfilaments. On the other hand, antibodies against tubulin demonstrate a delicate network of fine straight elements, which run from the outer nuclear membrane to the plasma membrane and cross the cytoplasmic matrix in all possible directions (Weber *et al.*, 1975). From the structural findings reported in this paper it could be concluded that the same distribution of microfilaments and microtubules applies to the exocrine pancreatic cell of the rat. Similar bundles of microfilaments can be traced everywhere in the cytoplasm, they are interconnected at focal points and also come in contact with microtubules (Figs. 6a, 8c). These span the whole cytoplasmic space from the nucleus to the plasma membrane in which they are inserted under low angle, again occasionally involving microfilaments (Fig. 7a, b). The widespread distribution of microtubules and microfilaments in a variety of cells has prompted abundant studies concerning their functional significance. Their possible role in the secretion process of exocrine and endocrine cells is only one topic in a large range of other biological activities (for review see Lacy and Malaisse, 1973; Wolff and Williams, 1973). Studies with antimicrotubular agents (colchicine, vinca alkaloids) in different secretory systems demonstrated a predominant inhibition of discharge of secretory products and no interference with earlier steps in the secretory process (Redman *et al.*, 1975). It is therefore generally assumed that microtubules participate in the translocation of membrane-bound particles, such as vesicles or secretory granules and not in the intracellular transport of unpacked secretory products, which occurs through the cisternal space of the rough ER to the Golgi complex. Only Kemper *et al.* (1973) reported an inhibition of transport of pro-parathyroid hormone from its site of synthesis (ER) to the site of conver-

sion into parathyroid hormone (Golgi) by microtubule inhibitors. Reports on the action of antimicrotubular agents on the secretory process of the exocrine pancreatic cell are scarse and contradictory. According to Benz *et al.* (1972) colchicine at 10^{-4} M concentrations had no effect on the discharge of amylase. The studies of Jamieson (1972) point to the fact, that both colchicine and vinblastine inhibit discharge by only 25 to 30% at 10^{-3} to 10^{-4} M concentrations, while at the same time they have a marked effect on protein synthesis. The last finding would indicate that both substances beside their disruptive effect on microtubular structure have a nonspecific cytotoxic action, which could affect the secretory process of the exocrine cell generally. Jamieson (1972) postulated that antimicrotubular agents should be able to inhibit discharge by 95% if a specific involvement of microtubules with this step of the secretory process would exist. Data presented in this paper at least partly fullfill this prerequisit—not for discharge of granules but for intracellular transport of newly synthesized proteins. Nevertheless nonspecific sideeffects of the different antimicrotubular agents cannot be ruled out. Considering colchicine, 10^{-3} to 10^{-5} M concentrations did not interfere with incorporation of amino acids into proteins, despite the fact that $^{14}CO_2$-production from ^{14}C-palmitate was reduced by 30% at 10^{-3} M. All 3 concentrations led to an inhibition of discharge of amylase by 20% and of newly synthesized proteins by 60%. Since the studies of Jamieson (1972) have demonstrated similar energy requirements for protein synthesis, intracellular transport and discharge of secretion granules the differential effect exerted by colchicine on the different steps in the secretory process cannot be explained by interference with energy production alone: there is no reduction of protein synthesis and a marked effect on intracellular transport and both processes depend on the supply of energy. Similar results are obtained with vinblastine: 10^{-4} M concentration lead to an inhibition of protein synthesis by 50%, which corresponds well with the reduction of cellular respiration. The discharge of newly synthesized proteins, however, is blocked by 90% over a period of 3 hrs. This action explains the deposition of secretory material in the cisternal space of the rough ER and the formation of paracrystals. A further substantiation for the conclusion that only part of the action of antimicrotubular agents is nonspecific comes from experiments with hexylene glycol: 1.5% concentrations reduce cellular respiration to a greater extent than 10^{-3} M colchicine or 10^{-4} M vinblastine and still its effect on discharge of amylase and newly synthesized proteins amounts to 25 to 30% from controls. Our results on the action of cytochalasin B on the exocrine pancreatic cell largely agree with Bauduin *et al.* (1975). We could, however, not observe an increase of unstimulated secretion by low doses (up to 1 μg/ml). The exact role of the intracellular network of microfilaments in the different phases of the secretory cycle is not clear, as is the participation of microtubules in this process.

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