# MICROTUBULES AND PANCREATIC AMYLASE RELEASE BY MOUSE PANCREAS IN VITRO

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ABSTRACT

The effects of vinblastine and colchicine on pancreatic acinar cells were studied by use of in vitro mouse pancreatic fragments. Vinblastine inhibited the release of amylase stimulated by bethanechol, caerulein, or ionophore A23187. Inhibition required preincubation with vinblastine, and maximum inhibition was observed after 90 min. Inhibition was relatively irreversible and could not be overcome by a high concentration of stimulant. Inhibition could also be produced by colchicine although longer preincubation was required and inhibition was only partial. Uptake of [$^3$H]vinblastine and [$^3$H]colchicine by pancreatic fragments was measured and found not to be responsible for the slow onset of inhibition by these drugs. In incubated pancreas, microtubules were present primarily in the apical pole of the cell and in association with the Golgi region. Vinblastine, under time and dose conditions that inhibited the release of stimulated amylase, also reduced the number of microtubules. The only other consistent structural effects of vinblastine were the presence of vinblastine-induced crystals and an increased incidence of autophagy. The remainder of cell structure was not affected nor were overall tissue ATP and electrolyte contents or the stimulant-induced increase in $^{45}Ca^{++}$ efflux. It is concluded that the antisecretory effects of vinblastine and colchicine are consistent with a microtubular action, but that acinar cell microtubules are more resistant to the drugs than many other cell types.

Microtubules have been implicated in the release of a number of hormones and proteins including insulin (15–17, 20), thyroid hormones (10, 40, 43, 44), catecholamines (28, 29), pituitary hormones (11, 14), parathyroid hormone (8, 30), collagen (9), salivary gland glycoprotein and amylase (6, 33), low density lipoproteins (24, 37), and albumin and other plasma proteins (18, 31). Most of this work is based on the use of antimitotic drugs known to exert an effect on microtubules, especially colchicine and the vinca alkaloids. These agents interact with the primary microtubular protein, tubulin (45), and cause the disappearance of formed microtubules.

Microtubules have been observed in all types of cells examined since the advent of aldehyde fixation, and a protein presumed on the basis of its colchicine-binding activity to be tubulin has also been found in a number of secretory tissues (5, 22, 36, 44). It is not clear, however, whether all of the antisecretory actions of antimitotic drugs are mediated by an effect on microtubules since, in many cases, high concentrations of the drugs have been used which may produce other effects (45). Furthermore, in some cases minimal or no inhibition has been observed, although generally no morphological studies were carried out to determine that microtubules had been affected. It is thus not

yet clear whether microtubules are important as part of a general mechanism for protein packaging and release. Possibly, differences observed in studies of different secretory processes may reflect an inherent difference in microtubules or in tissue penetration of the drugs.

While the pancreatic acinar cell has been a prototype for studies of protein synthesis and packaging (12, 26), only recently have studies on the control of digestive enzyme release been carried out. Cholinergic agonists and the hormone cholecystokinin-pancreozymin, which stimulate massive discharge of enzymes, depolarize the cell and release intracellular $Ca^{++}$ (7, 21, 27). A rise in intracellular free $Ca^{++}$ is believed to increase release of zymogen granule contents by exocytosis.

In the present work, the effect on pancreatic amylase release of agents acting on microtubules, particularly vinblastine, has been studied. We have investigated: (*a*) to what extent vinblastine and cholchicine inhibit protein secretion from pancreatic exocrine cells; (*b*) where in the sequence of stimulus-secretion coupling this inhibition takes place; (*c*) whether this inhibition can indeed be correlated with the disappearance of microtubules from these cells; and (*d*) what structural consequences of the loss of microtubules might explain the inhibition of protein secretion. The slow onset and limited effectiveness of these drugs on pancreatic tissue which we observed further motivated us to determine whether their tritiated analogs were actually taken up by the tissue.

## MATERIALS AND METHODS

All studies were carried out with pancreases obtained from male White Swiss mice which had been fasted overnight before use. The pancreas was removed and divided into four or five 20–25-mg fragments which were incubated in 3 ml Krebs-Henseleit bicarbonate (KHB) medium equilibrated with 95% $O_2$-5% $CO_2$ (41). Incubation was carried out in 25-ml Erlenmeyer flasks maintained at 37°C and shaken at 90 cycles/min. A preincubation was carried out to allow washout of amylase from damaged cells and for penetration and action of the compounds studied. Incubation for 30 min in the presence or absence of secretagogues was used to measure stimulation of amylase release. $^{45}Ca^{++}$ efflux was measured with a perfusion apparatus as previously described (41).

### *Analytical Procedures*

Amylase activity released into the medium by pancreatic fragments was assayed by the method of Rinderknecht et al. (32) using the dye-coupled substrate, amylose azure. ATP was measured in perchloric acid extracts of pancreatic fragments fluorometrically by the method of Lowry et al. (19). Pancreatic total water content, inulin space, and $Na^+$ and $K^+$ content were determined as previously reported (39). Uptake of [$^3$H]colchicine and vinblastine was measured as previously described in studies on thyroid glands (40, 43). Thin layer chromatography on silica gel plates was used to establish purity of the radiolabeled compounds and to check for tissue metabolism (40, 43). After incubation of pancreatic fragments for the specified time with medium containing the radioactive compound, the tissue was rinsed, weighed, and dissolved in 0.5 ml Protosol (New England Nuclear, Boston, Mass.). 10 ml of scintillation fluid consisting of 2 vol toluene to 1 vol Triton X-100 containing 4 g/liter butyl PBD was added and, after the decay of chemiluminescence, radioactivity was measured. Quenching was corrected by use of an internal standard.

### *Electron Microscopy*

Pancreas was fixed for 90 min at 37°C in 1.5% glutaraldehyde-1% paraformaldehyde in 0.08 M sodium cacodylate buffer, pH 7.4. Tissue was postfixed in 2% $OsO_4$ in 0.08 M cacodylate at 2°C for 2 h and then dehydrated in ethanol, passed through propylene oxide, and embedded in British Araldite. Thin sections were doubly stained with uranyl acetate and lead citrate and examined in a JEM 100B electron microscope at 60 kV. For estimation of microtubular content sections were photographed at a magnification of 12,000 and photographically enlarged to a magnification of 30,000. For each experimental condition at least 10 prints were examined from each of five pancreases. In all cases control as well as drug-treated pancreatic fragments from the same mouse were processed and examined.

### *Materials*

Colchicine was obtained from Sigma Chemical Co., St. Louis, Mo., [$^3$H]colchicine from New England Nuclear, Boston, Mass., and caerulein from Calbiochem, San Diego, Calif. Vinblastine and A23187 were gifts from Dr. Robert Hammil of Eli Lilly and Company, Indianapolis, Ind. [$^3$H]Vinblastine was a gift from Dr. J. Wolff of the National Institutes of Health and had been prepared by a modification (5) of the method of Owellen and Donigan (25).

Vinblastine and colchicine were dissolved directly in KHB. Stock solutions of bethanechol and caerulein in water were prepared and an aliquot was added to provide the specified concentration. The calcium ionophore A23187 was dissolved in ethanol with an equivalent amount of ethanol (0.5%) added to control flasks.

## RESULTS

The effect of vinblastine on basal and stimulated amylase release from in vitro mouse pancreas is

shown in Fig. 1. Vinblastine at concentrations of $10^{-6}$–$10^{-4}$ M had little effect on basal amylase release but reduced release stimulated by bethanechol and caerulein in a concentration-dependent manner. Since release evoked by caerulein was inhibited to the same extent as that evoked by bethanechol, vinblastine must be inhibiting more than just the cholinergic receptor. A standard 90-min period of preincubation was chosen after experiments shown in Fig. 2 had been carried out with varying lengths of preincubation. When vinblastine was present only during stimulation of release there was no inhibition, while preincubation with the drug for 30 min before stimulation led to a maximum of about 50% inhibition, and preincubation for 90 min led to a maximum of 85–90% inhibition. After 90 min preincubation with vinblastine, it was not necessary to have the drug present during the incubation period, as shown in Table I. In other words, there was no rapid reversibility of vinblastine inhibition once established.

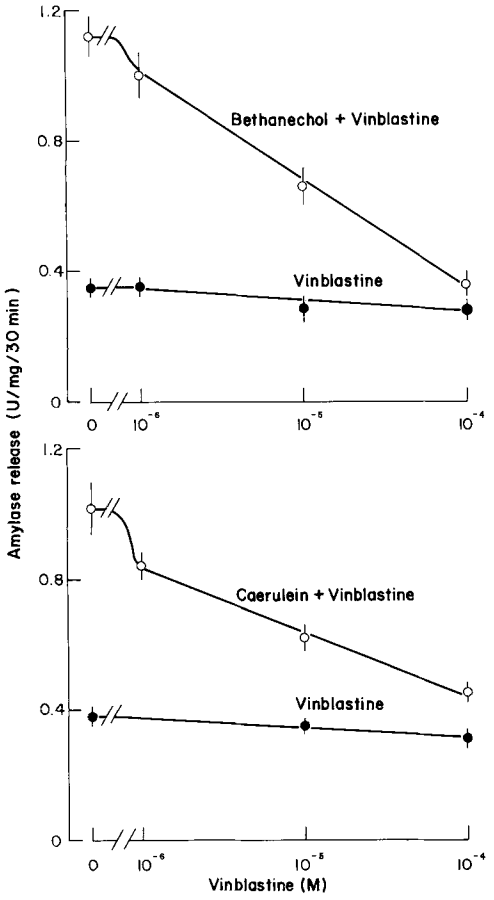



FIGURE 1 Effect of vinblastine on basal, bethanechol-stimulated, and caerulein-stimulated amylase release by mouse pancreas in vitro. Pancreatic fragments were preincubated for 90 min in the specified concentration of vinblastine and then incubated for 30 min in the same medium with bethanechol ($3 \cdot 10^{-5}$ M) or caerulein (10 ng/ml) added as indicated. All values are the mean ± SE for five to eight pancreases.

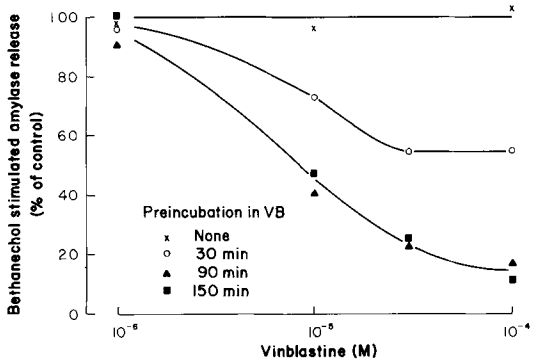



FIGURE 2 Effect of preincubation time on the vinblastine inhibition of bethanechol-stimulated pancreatic amylase release by mouse pancreas in vitro. Each curve represents pooled data from experiments such as shown in Fig. 1 and is constructed by subtracting basal from the bethanechol-stimulated amylase release for each concentration of vinblastine and expressing it as the percent of control stimulation. Thus, 100% is the difference between bethanechol-stimulated and basal amylase release in the absence of vinblastine.

TABLE I

*Absence of Rapid Reversibility of Vinblastine Inhibition of In Vitro Pancreatic Amylase Release*

| Addition to medium | Amylase release (U/mg/30 min) | |
|---|---|---|
| | Unstimulated | Bethanechol ($3 \cdot 10^{-5}$ M) |
| None | 0.28 ± 0.03 | 1.31 ± 0.11 |
| Vinblastine | | |
| Preincubation and incubation | 0.26 ± 0.03 | 0.41 ± 0.03 |
| Vinblastine | | |
| Preincubation only | 0.21 ± 0.02 | 0.41 ± 0.03 |

Pancreatic fragments were sequentially incubated for 90-, 60-, and 30-min periods. Vinblastine ($3 \cdot 10^{-5}$ M) was present during the initial 90 min (preincubation only) or during the entire period (preincubation and incubation) as specified. Unstimulated or bethanechol-stimulated amylase release was measured during the terminal 30-min incubation. All values are the mean ± SE of 9–12 pancreases.

When the effects of varying the concentration of bethanechol in the presence of a submaximal concentration of vinblastine were studied, there was no indication that a high concentration of stimulant could overcome the vinblastine inhibition (Fig. 3). Both the relative irreversibility and "noncompetitive" nature are similar to results of a recent study of vinblastine inhibition of in vitro thyroid secretion (40).

Recently, it has become possible to bypass the early steps of stimulus-secretion coupling by use of the calcium ionophore A23187 to artificially introduce $Ca^{++}$ into the pancreatic cell and stimulate amylase release (35, 42). Vinblastine was found to inhibit amylase release initiated by A23187 as shown in Table II. That vinblastine is inhibiting a later, rather than an earlier step in stimulus-secretion coupling is confirmed by the finding that the increased efflux of $^{45}Ca^{++}$ from prelabeled pancreatic fragments is not affected by a high concentration of vinblastine. An example of the bethanechol-stimulated efflux of $^{45}Ca^{++}$ and the lack of effect of vinblastine is shown in Fig. 4. In seven experiments with vinblastine at a concentration of $3 \cdot 10^{-5}$ M, which inhibits amylase release by about 75% (Fig. 2), the maximum increase in $^{45}Ca^{++}$ efflux was 215% ± 31% as compared to 180% ± 21% for the increase measured when vinblastine was absent.

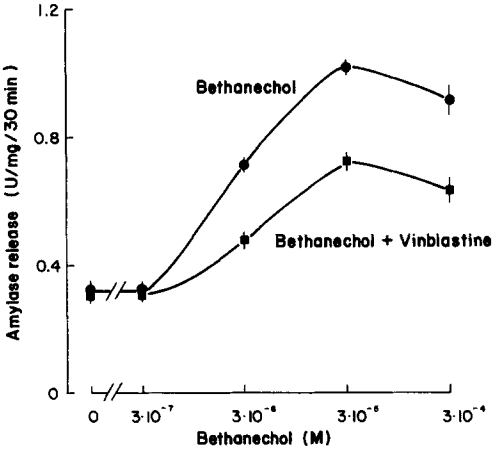



FIGURE 3 Effect of vinblastine on amylase release from mouse pancreas induced by varying concentrations of bethanechol. Amylase release was measured during a 30-min incubation period after 90 min preincubation, both carried out in the presence (■—■) or absence (●—●) of vinblastine ($10^{-5}$ M). All values are the mean ± SE for five to seven pancreases.

TABLE II

*Inhibition of Bethanechol- and A23187-stimulated In Vitro Pancreatic Amylase Release by Vinblastine*

| | Amylase release (U/mg/30 min) | | |
|---|---|---|---|
| Vinblastine | Unstimulated | Bethanechol ($3 \cdot 10^{-5}$ M) | A23187 ($1 \cdot 10^{-5}$ M) |
| 0 | 0.25 ± 0.02 | 0.87 ± 0.09 | 0.49 ± 0.03 |
| $10^{-4}$ M | 0.26 ± 0.04 | 0.34 ± 0.03 | 0.28 ± 0.03 |

Pancreatic fragments were preincubated for 90 min in medium with or without vinblastine and then incubated for 30 min in new medium with the addition of bethanechol or A23187 as specified. All medium contained the normal concentration of $Ca^{++}$ (2.56 mM). All values are the mean ± SE of five to seven values.

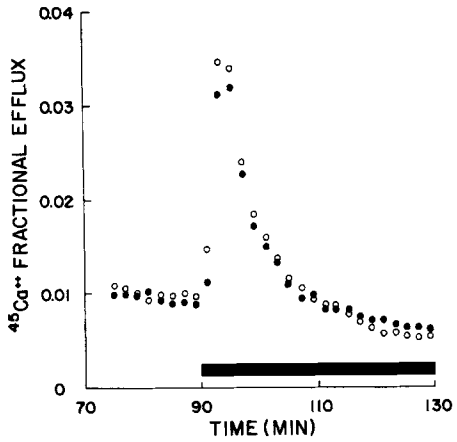



FIGURE 4 Lack of effect of vinblastine on the bethanechol-stimulated increase in $^{45}Ca^{++}$ efflux from mouse pancreas in vitro. Fractional efflux of $^{45}Ca^{++}$ is plotted as a function of washout time after 1 h of loading with $^{45}Ca^{++}$. Bethanechol ($3 \cdot 10^{-5}$ M) was added at 90 min (black bar) to both halves of a mouse pancreas superfused separately with KHB (●) or KHB with $3 \cdot 10^{-5}$ M vinblastine (○).

Inhibition of bethanechol-stimulated amylase release could also be produced by colchicine which interacts with the microtubule at a different site from vinblastine. As shown in Fig. 5, it was necessary to use high concentrations of colchicine and a long preincubation time to inhibit amylase release to any significant extent.

Uptake of vinblastine and colchicine into pancreas does not appear to be a rate-limiting factor for inhibition of secretion. As shown in Fig. 6, both vinblastine and colchicine rapidly accumulate in pancreas with tissue:medium ratios as high as 40 for vinblastine and 7 for colchicine. There was

no metabolic alteration of either label as the extracted compound chromatographed similarly to the original material. About 10 times as much vinblastine is taken up as colchicine, and the vinblastine uptake curve shows more than one kinetic component. Both the rapid component of vinblastine uptake and all of colchicine uptake are largely complete at a time when microtubules are still present, as will be shown later. While the relation of tissue uptake to microtubular binding of the drugs is not clear, slow entry of the drugs into the cell appears unlikely as an explanation of the high concentrations and long preincubation time necessary to inhibit amylase release.

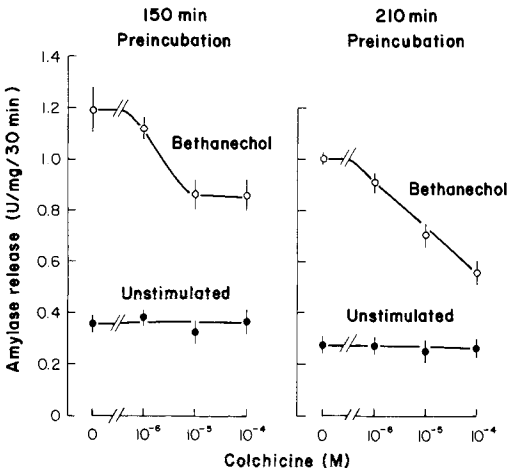



FIGURE 5 Effect of colchicine on basal and bethanechol-stimulated amylase release by mouse pancreas in vitro. Pancreatic fragments were preincubated for 150 or 210 min in the specified concentration of colchicine and then incubated in the same medium with or without bethanechol ($3 \cdot 10^{-5}$ M). All values are the mean ± SE for four to nine pancreases.

Electron microscope studies were carried out to assess the effects of the compounds used on pancreatic fine structure. In control incubated pancreas, microtubules were found primarily in the apical portion of the cell and in the Golgi region as shown in Fig. 7. There was no obvious structural relationship between microtubules and the zymogen granules, the apical membranes, or other organelles. In pancreatic fragments incubated with $3 \cdot 10^{-5}$ M vinblastine for 90 min, such as shown in Fig. 8, microtubules were no longer discernible, although most other aspects of the cell structure and organization appeared normal. When tissue was examined after only 30 min in medium containing a similar concentration of vinblastine, a few microtubules were invariably present. After 90 or 150 min incubation with vinblastine at concentrations of $3 \cdot 10^{-5}$–$10^{-4}$ M, vinblastine-in-

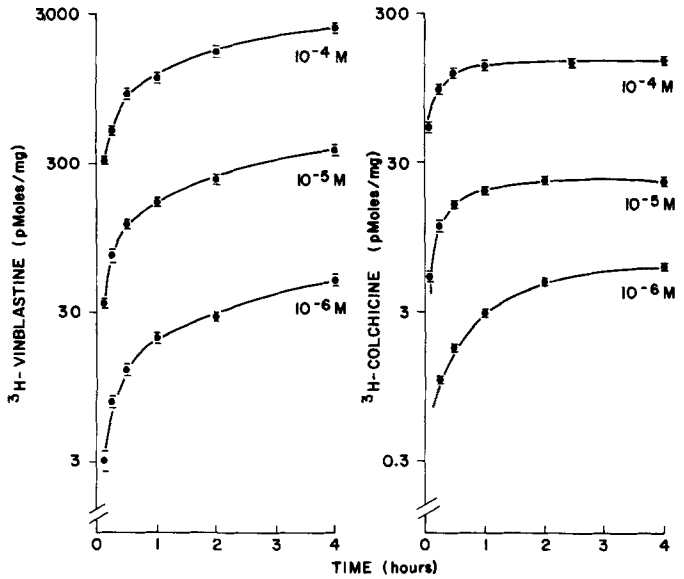
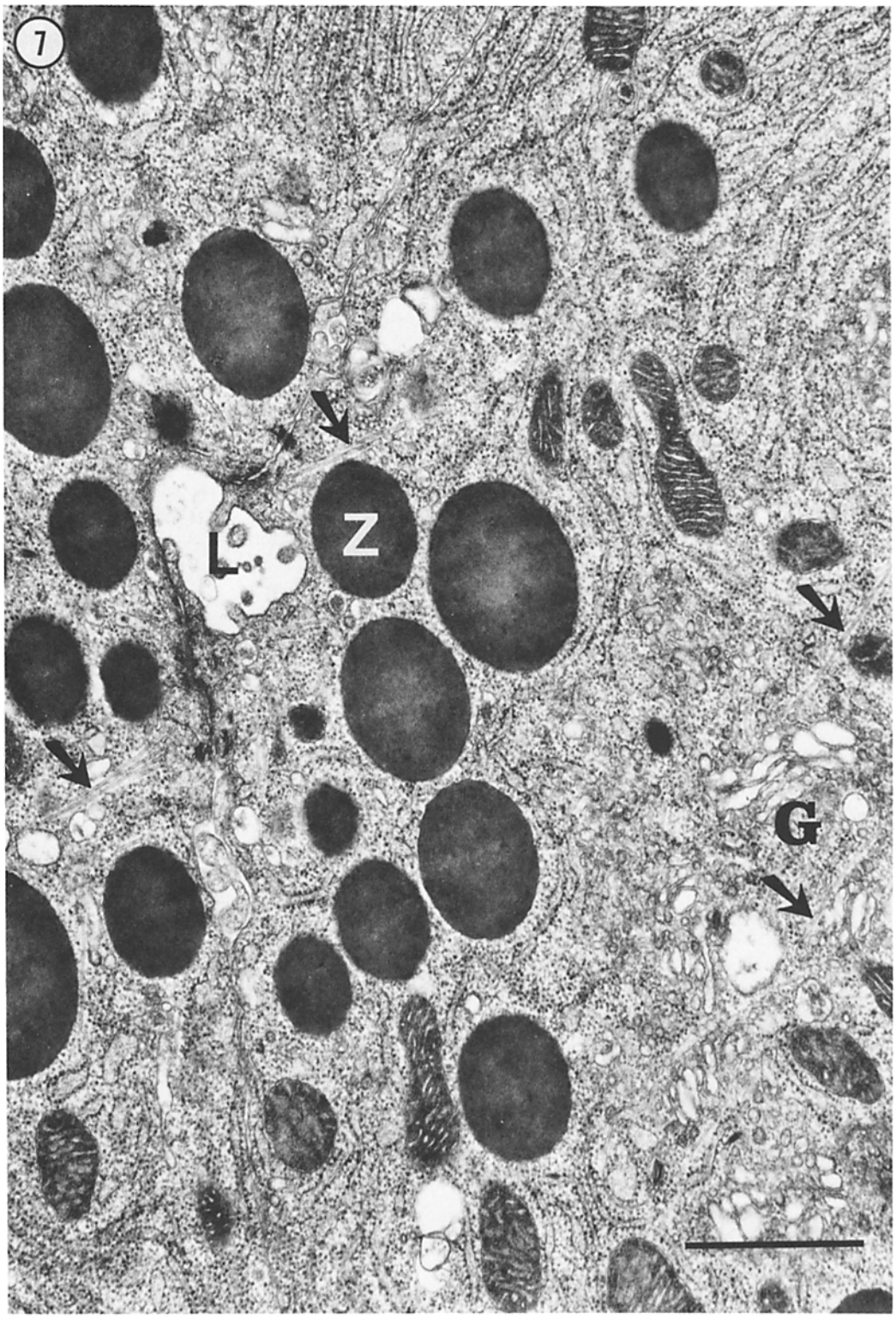



FIGURE 6 Uptake of [$^3$H]vinblastine (left panel) and [$^3$H]colchicine (right panel) by mouse pancreatic fragments in vitro. Drug concentration in the tissue is plotted as a function of time for the medium concentrations shown. All points are the mean ± SE for three to five pancreases.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duced crystals were seen as well as an increased number of autophagic vacuoles. Colchicine ($3 \cdot 10^{-5}$ M) was also found to reduce or abolish microtubules but only after 150 or 210 min incubation; no effect was seen after 90 min incubation. As shown in Figs. 9 and 10, prolonged incubation with vinblastine (210 min, $3 \cdot 10^{-5}$ M) had no effect on the general organization of the internal membrane compartments of the cells even though microtubules must have been absent for some time.

While the antisecretory effects of vinblastine and colchicine are consistent with a microtubular site of action, other possibilities should be considered, especially because of the high concentrations of the agents used. Pancreatic secretion is known to be dependent on metabolic energy. Agents such as DNP and cyanide will inhibit stimulated amylase release, presumably by reduction of cellular ATP (13). Table III shows the effects of high concentrations of vinblastine and colchicine on mouse pancreatic ATP content. While DNP and cyanide each reduced ATP levels by over 90%, neither vinblastine nor colchicine had any effect. To analyze further the functional intactness of pancreatic cells, water, sodium, and potassium content were measured. As shown in Table IV, these indices of cell membrane integrity were not affected by vinblastine or colchicine.

## DISCUSSION

The present work clearly shows that antimitotic agents known to affect microtubules can inhibit the stimulation of pancreatic amylase release if applied for a long enough period of time. Earlier negative results (4, 12) used shorter preincubation times which in our studies also produced little inhibition. These results again point out the need for preincubation in in vitro studies with drugs affecting microtubules (43, 44), and suggest the necessity of morphological confirmation that the drugs have in fact affected microtubules before one concludes that microtubules are not necessary for secretion. That the drugs are affecting protein release and not synthesis is shown by the normal content of zymogen granules (Figs. 8, 10) and amylase content (J. A. Williams, unpublished data). Jamieson (12) also found that concentrations of colchicine and vinblastine lower than $10^{-4}$ M did not inhibit incorporation of [$^3$H]leucine into guinea pig pancreas.

The striking inhibition of stimulated amylase release contrasts with the slight degree of inhibition of basal release. While this suggests that the two types of release occur by separate mechanisms, it is not clear how much of basal release is due to leakage from damaged cells. Inhibition of stimulated but not basal amylase release is not unique to antimitotic agents. Similar inhibition is produced by metabolic inhibitors (13), removal of $Na^+$ from the medium (39), tetracaine (42), and cytochalasin B (2; J. A. Williams, unpublished data).

Since vinblastine was more effective than colchicine in inhibiting stimulated amylase release, we examined its ultrastructural effects in more detail. Microtubules were normally present in the apical pole of the pancreatic cell and in the Golgi region. No obvious structural relation between microtubules and granules or luminal membrane was seen, nor was there obvious change in number or location of microtubules in pancreas stimulated with bethanechol or A23187 (unpublished data). Vinblastine essentially abolished microtubules, while other structures in the apical pole of the cell remained normal (Fig. 8). At short incubation times (30 min for vinblastine, 90 min for colchicine) when inhibition was negligible or incomplete, microtubules were present. In addition to

TABLE III

*Effect of Microtubule-Active Drugs and Metabolic Inhibitors on ATP Content of Mouse Pancreas in vitro*

| Addition to medium | ATP content |
|---|---|
| | *nmol/mg* |
| None | 2.59 ± 0.20 (9) |
| Vinblastine ($10^{-4}$ M) | 2.33 ± 0.14 (6) |
| Colchicine ($10^{-4}$ M) | 2.86 ± 0.13 (6) |
| Cyanide ($10^{-3}$ M) | 0.19 ± 0.07 (6) |
| Dinitrophenol ($2 \cdot 10^{-4}$ M) | 0.17 ± 0.06 (6) |

Pancreatic fragments were incubated in KHB with the specified addition for 90 min. All values are the mean ± SE of the number of values shown in parentheses.

FIGURE 7 Electron micrograph of mouse pancreas incubated for 90 min in KHB. Microtubules indicated by arrows are found around the acinar lumen ($L$) and in the Golgi area ($G$). $Z$, zymogen granules. × 28,000. Bar = 1 μm.

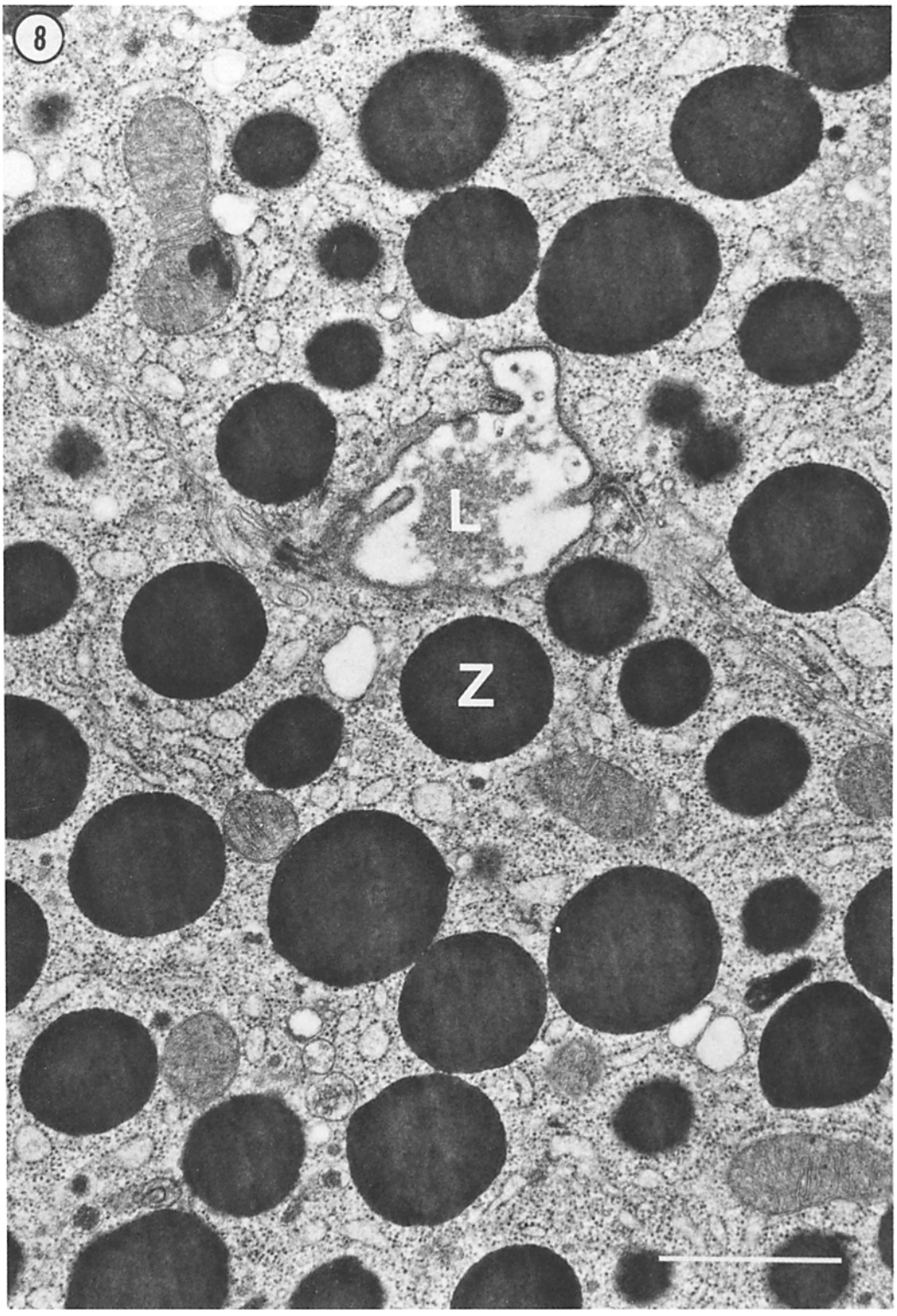
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TABLE IV

*Effects of Vinblastine and Colchicine on Pancreatic Water and Electrolytes*

| Additions to medium | Total $H_2O$ | Inulin space | $Na^+$ | $K^+$ |
|---|---|---|---|---|
| | % | % | *nmol/mg* | *nmol/mg* |
| None | 81.21 ± 0.42 | 27.0 ± 0.9 | 81.5 ± 2.8 | 95.1 ± 1.6 |
| Vinblastine ($10^{-4}$ M) | 80.39 ± 0.81 | 26.8 ± 1.1 | 81.8 ± 4.4 | 98.6 ± 3.2 |
| Colchicine ($10^{-4}$ M) | 81.57 ± 0.41 | 26.0 ± 1.0 | 76.8 ± 4.0 | 104.5* ± 2.6 |

Mouse pancreatic fragments were incubated for 3 h in KHB containing 0.3 μCi/ml [$^3$H]inulin and the additions specified before determination of water and electrolyte contents. All values are the mean ± SE for five determinations.

* $P < 0.05$ as compared to control.

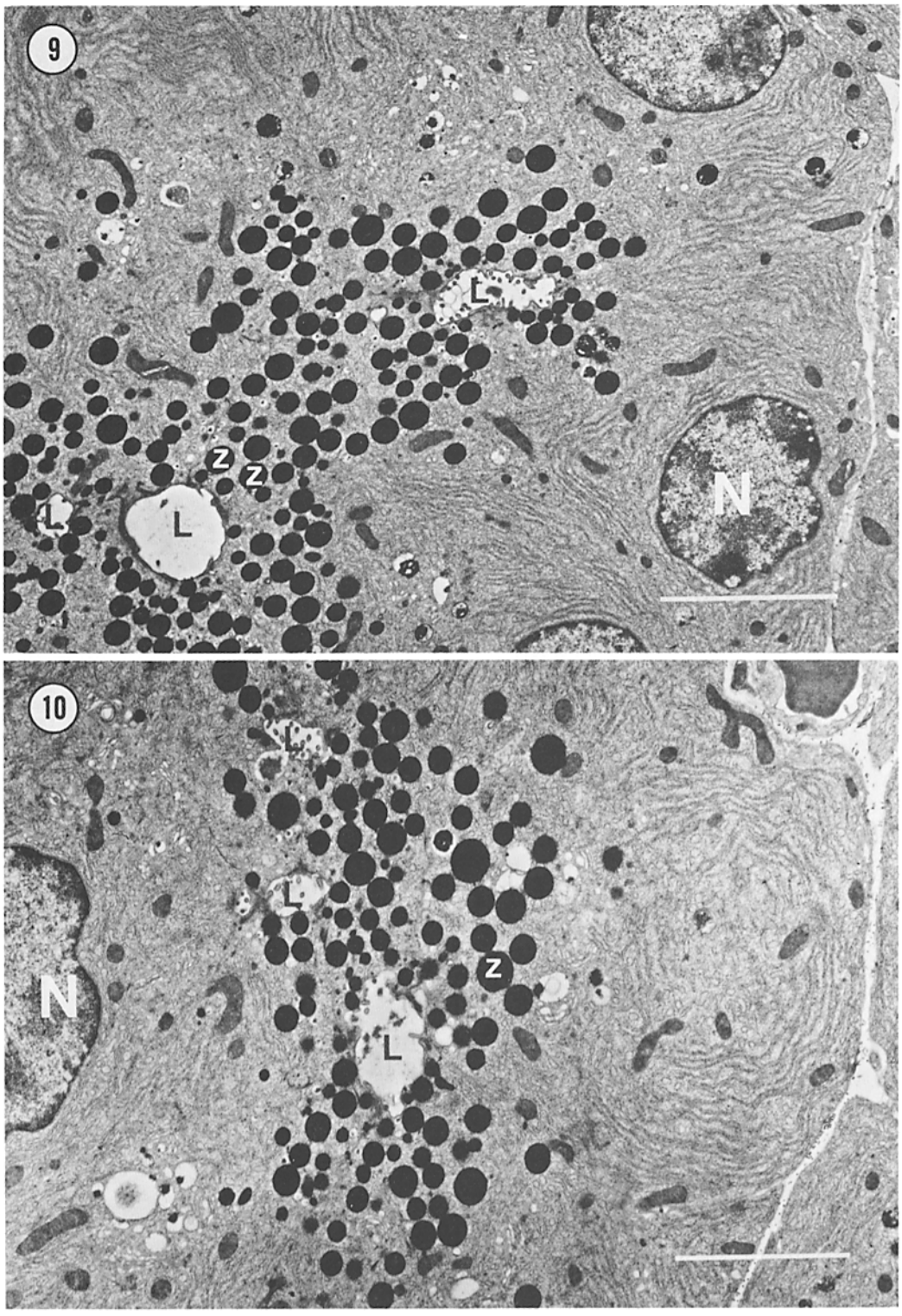
causing the disappearance of normal microtubules, vinblastine at concentrations of $3 \cdot 10^{-5}$ M or higher induced characteristic crystalloid structures (3) and frequent autophagic vacuoles (1). The overall cell polarity was not, however, affected by vinblastine even after prolonged incubation so that formed microtubules should have been absent for several hours (compare Figs. 9 and 10). Thus, other factors besides microtubules must contribute to overall maintenance of cell shape in pancreatic acini.

Morphological studies are thus consistent with a microtubular site of action for the antimitotic drugs and suggest that the delayed inhibition reflects a relative resistance of the microtubules in pancreatic cells to the drug. Vinblastine appears to act faster than colchicine as in the case of inhibition of thyroid secretion (40). In both the pancreas (Fig. 6) and the thyroid (40, 44), tissue uptake of the drugs is rapid. The relation between tissue uptake, binding to tubulin, and disaggregation of tubules, as well as the possibility of alternative binding sites, remains to be established. A possible explanation of the delayed action is that the drugs prevent only polymerization of microtubules, and the rate of disappearance of formed microtubules depends on the rate of continual turnover.

While the current data showing parallel inhibition of stimulated amylase release and disappearance of acinar cell microtubules are consistent with a requirement for microtubules in amylase release, other possibilities have been considered. Pancreatic secretion is acutely dependent on metabolic energy and is inhibited by blockers such as cyanide and dinitrophenol (13). Vinblastine does not appear to act in this mode, since ATP levels as well as tissue $Na^+$ and $K^+$ levels are unaffected. Membrane stabilizers such as local anesthetics, tranquilizers, and detergents will inhibit amylase release (42; J. A. Williams, unpublished data) and vinblastine can, at high concentrations, act as such an agent (34). Inhibition of pancreatic secretion, however, occurs at lower concentrations. Furthermore, membrane stabilizers block the bethanechol-stimulated increase in $^{45}Ca^{++}$ efflux while vinblastine does not (Fig. 5). While alternative mechanisms of action cannot be eliminated, at least vinblastine and colchicine do not appear under present conditions to be inhibiting secretion by acting as general cell poisons. Larger amounts of vinblastine in vivo, however, have been shown to cause toxic necrosis of the pancreas after 24 h (23).

If antimitotic agents are inhibiting amylase release by abolishing formed microtubules, then the role of microtubules in the release process must be considered. Knowledge of how microtubules participate in secretion is a problem faced in studies of a number of secretory cells and one for which there is at present no clear solution. Various workers have suggested that microtubules may guide granules to the membrane (16), interact with microfilaments (20), or organize the distribution of cell membrane components (38). Further work is in progress to distinguish among these possible mechanisms for microtubular participation in the secretory process.

FIGURE 8 Electron micrograph of apical area of mouse pancreas incubated 90 min in KHB with $3 \cdot 10^{-5}$ M vinblastine. Microtubules are absent but other cell constituents are normal. Labels as in Fig. 7. × 28,000. Bar = 1 μm.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FIGURES 9 and 10 Electron micrograph of acinar structure of mouse pancreas incubated for 210 min in KHB (Fig. 9) or KHB containing $3 \cdot 10^{-5}$ M vinblastine (Fig. 10). *L*, lumen; *Z*, zymogen granules; *N*, nucleus. Note similar structural polarization in tissue treated with vinblastine. × 5,400. Bar = 5 μm.

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