INHIBITORY EFFECT OF COLCHICINE ON AMYLASE SECRETION BY RAT PAROTID GLANDS

Possible Localization in the Golgi Area

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ABSTRACT

Colchicine inhibited amylase secretion by isolated rat parotid glands only 6 h after administration of the drug in vivo. This delayed effect was not the result of the inability of the drug to reach its reaction site. When parotid glands were emptied of their secretory granules by isoproterenol treatment, the subsequent replenishment of cells with granules was inhibited by colchicine. Colchicine concomitantly produced alterations of the Golgi complexes, the cisternae of which were reduced in size and surrounded by clusters of microvesicles. Incubation of parotid glands with colchicine for prolonged durations failed to alter stored amylase secretion as stimulated by isoproterenol, but it inhibited the release of *de novo* synthesized enzyme. Colchicine-binding activity of high speed supernatant parotid gland homogenates, likely to represent cytosolic microtubular protein, was similar to that of brain. Another colchicine-binding activity, firmly bound to the particulate fraction of homogenates, was found, of which a part may represent membrane-located microtubular protein. An assembly-disassembly cycle of microtubules appears to exist in the parotid gland, as in the liver. However, only 14% of tubulin was found to be polymerized as microtubules in parotid glands as opposed to 40% in the liver. The present data suggest that colchicine primarily inhibits the transfer of secretory material towards or away from the Golgi complexes but not the hormone-stimulated secretion of stored amylase.

Despite the numerous experiments and speculations concerning the role of microtubules in secretory processes, it is still uncertain whether or not these organelles are directly involved in hormone-regulated secretory mechanisms. In several exocrine and endocrine glands, only a weak and/or delayed inhibitory effect of high colchicine concentrations has been demonstrated on the secretory process that follows hormonal stimulation (6, 17, 41). In contrast, in other organs such as the liver, the release of very low density lipoproteins or other proteins is inhibited rapidly by low colchicine concentrations (18, 19, 26, 27, 32, 38). Recent reports have suggested that the inhibitory action of colchicine may be mediated at the level of the Golgi complex since exposure of osteoblasts (10), chondrocytes (24) and pancreatic islets (25) to colchicine or other mitotic-spindle inhibitors led to an accumulation of Golgi-associated vacuoles and vesicles, together with a dispersion of the dictyosomes throughout the cytoplasm. Similarly, colchicine was shown to decrease protein secretion

by the liver (19, 32), a decrease that was accompanied by an intracellular accumulation of the nonsecreted proteins (19), particularly within the Golgi complexes (32).

The present study represents an attempt to differentiate between two possibilities of colchicine action outlined above, i.e., a distal one on hormone-stimulated secretion of stored secretory granules and a more proximal one presumably located in the Golgi region. The successful accomplishment of such a study depends on the availability of an organ with high capacity for storage of its exportable material which should be released promptly after stimulation, and in which the replenishment of the storage pool by newly synthesized material should not be limiting for a maximal secretory response. To this end, parotid glands were selected as they are a model in which mature secretory granules represent up to 37% of the volume of the resting cell, and in which complete degranulation can be achieved within 60 min by stimulation with a β-adrenergic agent (8). α-Amylase, the major component of the exportable proteins, is easily measurable. Furthermore, parotid glands have the advantage of being relatively large, easily accessible, and suitable for in vitro studies. We, therefore, investigated the effect of colchicine on the release of stored amylase or on the release of newly synthesized proteins in rat parotid glands. In order to characterize and possibly localize the site of action of colchicine in these glands, we studied the uptake and binding characteristics of tritiated colchicine, as well as the interference of colchicine with the microtubular system of parotid glands.

MATERIALS AND METHODS

Animals

Male albino rats of the Wistar strain, bred in these laboratories and weighing 250–350 g were used throughout these studies. They were fed *ad libitum* with UAR laboratory chow (Villemoisson, Epinay/Orge, France).

Tissue Incubations

Rats lightly anesthetized with ether were killed by section of the heart. Parotid glands were removed, sliced (about 1 mm^3) with scissors whilst in incubation medium gassed with O_2/CO_2 (95:5 vol/vol), and incubated following the method of Castle et al. (7). Owing to the presence of fibrous tissue, no attempt was made to dissect individual lobules. Following a preincubation (37°C, 10 min), floating adipose tissue was aspirated. Parotid gland slices were collected on a Büchner funnel and distributed (portions of about 400 mg wet weight) into plastic vials containing 4 ml of medium. Vials contained a plastic grid, fitting the inner diameter, which was immersed halfway in the incubation medium, allowing the pieces to lie in it while being gassed from below. Incubations were carried out at 37°C. At various time intervals, tissue slices were separated from the medium on a Büchner funnel and homogenized (all-glass homogenizers) in 1.5 ml of sodium phosphate buffer (30 mM, pH 6.9) containing 4 mM NaCl. Homogenates were used for measurements of DNA, ATP, potassium, amylase activity, isolation of amylase, and measurement of labeled proteins from tritiated leucine (1 mM, 20 μCi per vial), while incubation media were used for isolation or determination, respectively, of amylase or labeled proteins.

The incubation medium consisted of Ham's solution (Nutrient mixture F 12) (13) containing β-hydroxybutyrate (5 mM). When the incubation period exceeded one hour, penicillin and streptomycin were added to the medium (7).

In Vivo-In Vitro Experiments

In experiments to investigate the effect of long-term treatment with colchicine on normal or granule-depleted parotid glands, the experimental design shown in Fig. 1 was adopted. Isotonic saline (0.33 ml containing 0.25% ascorbic acid per 100 g body weight) or D,L-isoproterenol (0.33 ml of a freshly prepared 1% solution, containing isotonic saline and 0.25% ascorbic acid, per 100 g body weight) was injected intraperitoneally (2). One hour later, colchicine (0.1 ml of a 50 mM solution, in isotonic solution, per 100 g body weight) was administered into a femoral vein. This amount of colchicine administered in vivo resulted in a tissue colchicine level (measured via the use of tritiated colchicine) of 3×10^{-5} M 30 min, and 1×10^{-5} M 12 h after the injection. Animals were sacrificed at various intervals, parotid glands were removed, sliced, and incubated, and tissue amylase content plus amylase released into the medium were assayed.

Biochemical Determination

DNA was measured according to Hubbard et al. (14), potassium was determined by flame photometry, and ATP was assayed by the luciferin-luciferase technique (1). Amylase activity was measured by the procedure described by Danielsson (9). The enzyme was purified from homogenates and media according to Schramm and Loiter (34). Labeled proteins were isolated by precipitation with 10% trichloracetic acid (TCA). The sediment was washed twice with 5% TCA, heated for 15 minutes at 90°C, washed with ethanol, ethanol-ether (1:1), and ether, and finally dissolved in 1 N NaOH and counted in Insta-Gel in a liquid scintillation counter. Proteins were measured according to Lowry et al. (20).

Colchicine-Binding Assay and Studies on Microtubules

Colchicine-binding activity was measured by the assay of [^{3}H]colchicine binding previously described for liver high-speed supernate (28). For measuring the uptake of [^{3}H]colchicine by the tissue, parotid gland slices after incubation were briefly washed on a Büchner funnel with a solution of unlabeled colchicine (10^{-4} M), weighed, homogenized in a detergent-containing scintillation mixture (Insta-Gel) and counted. Microtubules from rat brain were isolated as described by Shelanski et al. (36). The proportion of total tubulin assembled as microtubules in parotid glands was measured by the use of a technique previously described for the liver (28). The method is based on the lability of microtubules in the cold and their stabilization by glycerol. Colchicine-binding activities of high-speed supernates of "stabilized" and "unstabilized" parotid gland homogenates give the proportion of free and total tubulin, respectively. The difference between these two measurements is an estimate of that portion of tubulin which is assembled as microtubules. For obtaining these measurements, parotid slices (0.4 g) were homogenized in MES-MEG buffer (i.e. 10^{-1} M morpholino-ethane sulfonic acid (MES), pH 6.5, 5×10^{-4} M $MgCl_2$, 10^{-3} M EGTA, 10^{-3} M GTP), with or without glycerol (4 M) as a microtubule-stabilizing agent.

Light and Electron Microscope Studies

Parotid slices were immersed in glutaraldehyde (4%) in 0.1 M phosphate buffer (pH 7.4) and finely chopped with a razor blade. After overnight fixation, the tissue was washed (1 h) in several changes of phosphate buffer, post-fixed (2 h) in phosphate-buffered osmium tetroxide (2%), dehydrated in increasing ethanol concentrations, and embedded in Epon 812 (21). Thin sections were contrasted with uranyl acetate and lead citrate and examined in a Zeiss EM 9 or Philips EM 300 microscope. Sections approximately 1 μm thick were also cut for light microscopy and stained with toluidine or methylene blue (30).

Chemicals

[^{3}H]Colchicine and L-[4,5-^{3}H]leucine were purchased from the Radiochemical Centre (Amersham, Buckinghamshire, England), Ham's solution from Grand Island Biological Company (Grand Island, N. Y.), and Insta-Gel from Packard Instrument Internatl. S.A. (Downers Grove, Illinois). Vinblastine was kindly donated by Eli Lilly and Co. (Indianapolis, Indiana). The other reagents were of analytical grade and obtained from E. Merck A.G. (Darmstadt, West Germany) or Fluka A.G. (Buchs, Switzerland).

Statistics

Statistical significances were calculated by Wilcoxon's test as modified by Mann and Whitney (22). The values for p are indicated in the tables and figures as follows: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

RESULTS

Effect of Pretreatment with Colchicine In Vivo on Amylase Secretion by Subsequently Isolated Parotid Glands

The finding that colchicine does not markedly modify amylase secretion during a relatively short incubation time (6, 39) was confirmed and extended in these studies. Neither 10^{-4} M colchicine nor 10^{-7}–10^{-4} M vinblastine had any effect on basal or isoproterenol-induced amylase secretion after a 1-h incubation period (data not shown). However, when rats were treated in vivo with colchicine for 6 h at least, following the experimental design summarized in Fig. 1, subsequently isolated and isoproterenol-stimulated glands released less amylase into the medium than did the controls (i.e. saline-treated animals) (Fig. 2).

In similar experiments, the parotid glands were first emptied of their stored granules by in vivo treatment with isoproterenol 1 h before in vivo colchicine or saline administration (experimental design of Fig. 1). As shown in Fig. 3, 1 h after isoproterenol injection of the animals, the amylase content of their parotid glands was reduced to very low values. However, within 12 h the amylase content of the glands of these isoproterenol-treated animals had been partly restored (compare absolute values of the isoproterenol-pretreated groups [Fig. 3] to that of the untreated group [Fig. 2]). Up to 6 h after colchicine administration, the secreted portion of amylase was similar to that remaining in the tissue, and no effect of colchicine could be detected. 11 h after colchicine administration, the portion of secreted amylase was found to be markedly smaller than that of controls, while intracellular amylase concentrations were similar for both groups (Fig. 3). When, in the above mentioned experiments, released and intracellular portions of amylase were added (an index of total amylase content), an increase of this index during the experimental period of 11 h was evident (Fig. 2). As can be seen from Figs. 2 and 3, the total amylase content was reduced in the respective groups of rats treated with colchicine for 11 h. This decrease was mainly at the expense of the secreted portion of the enzyme. These experiments were completed by light and electron

microscope examination, as illustrated in Figs. 4–6. It was observed that control parotid glands (i.e., tissues obtained from animals that were not treated with colchicine) contained a dense population of secretory granules (Fig. 4*a*) and a well-developed Golgi complex (Fig. 5*a* and 6*a*). 11 h after isoproterenol treatment, cells were not as well granulated as the controls, although an appreciable buildup of granules was seen, particularly around the apical pole of the cells (Fig. 4*c*). There was a slight increase in the number of lipid droplets in such cells compared to controls. After treatment of the animals with colchicine alone, the population of granules in subsequently isolated parotid glands was unaffected (Fig. 4*b*) but the Golgi complex showed marked alterations. Cisternae were reduced in size and surrounded by clusters of microvesicles. Few condensing vacuoles were observed in the altered Golgi regions. In addition, there was an accumulation of autophagic vacuoles, as previously described in colchicine-treated liver (18, 37) as well as in vinblastine-treated exocrine pancreas (16) and liver (3). Lipid droplets were more conspicuous in parotid cells obtained from animals treated with colchicine than in those obtained from isoproterenol-treated rats. Parotid cells obtained from colchicine plus isoproterenol-treated rats showed alterations similar to those described in glands obtained from animals treated with colchicine alone, i.e., the Golgi region was similarly altered (Fig. 6*b*), and there was, in addition, a marked reduction in the number of secretory granules (Fig. 4*d*) which were small and irregularly shaped (Fig. 5*b*, Fig.

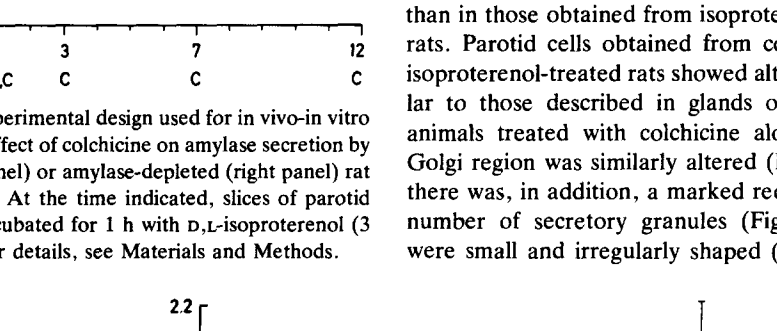

FIGURE 1 Experimental design used for in vivo-in vitro studies of the effect of colchicine on amylase secretion by normal (left panel) or amylase-depleted (right panel) rat parotid glands. At the time indicated, slices of parotid glands were incubated for 1 h with D,L-isoproterenol (3×10^{-5} M). For details, see Materials and Methods.

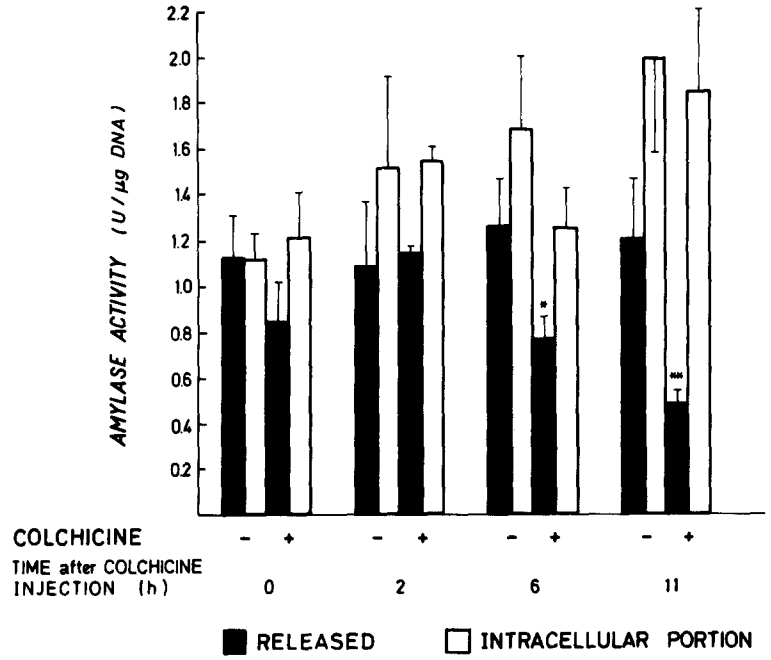

FIGURE 2 Effect of pretreatment with colchicine in vivo upon amylase secretion by subsequently isolated rat parotid glands (experimental design, see Fig. 1). Slices of parotid glands were all incubated with D,L-isoproterenol (3×10^{-5} M). After incubation (1 h), the amylase released into the medium or present in the tissue was measured. Each bar is the mean of 4 experiments ± SEM *: $p < 0.05$; **: $p < 0.01$.

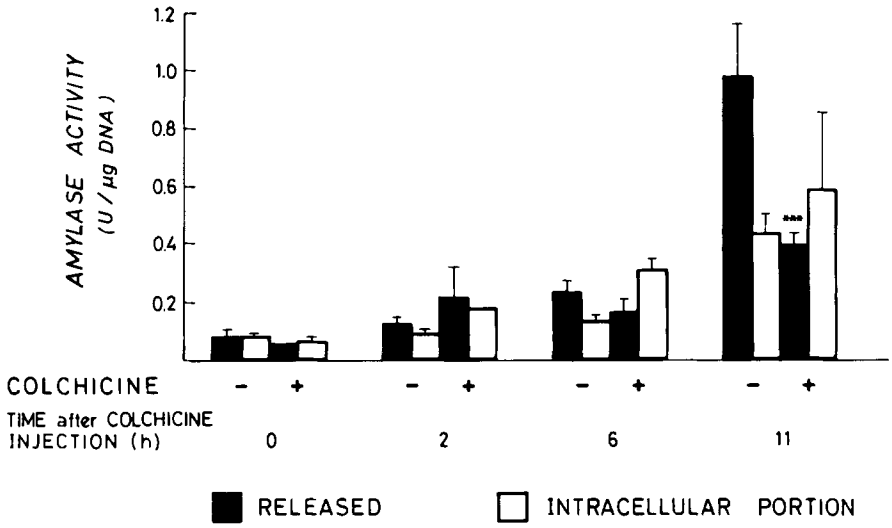

FIGURE 3 Effect of pretreatment with isoproterenol and colchicine in vivo upon amylase secretion by subsequently isolated rat parotid glands (experimental design, see Fig. 1). Slices of parotid glands were all incubated with D,L-isoproterenol (3×10^{-5} M). After incubation (1 h), the amylase released into the medium or present in the tissue was measured. Each bar is the mean of 4 experiments ± SEM. ***: $p < 0.001$.

6*b*). Microtubules in control tissues were seen in most of the Golgi regions examined, an observation which was made more striking by their comparative scarcity in the rest of the cytoplasm. Longitudinally sectioned microtubules ran along the length of the cisternae, whereas those seen in transverse section were usually located at the end of cisternae. In addition, microtubules were often seen in close proximity to condensing vacuoles. Microtubules essentially disappeared in glands from colchicine-treated animals.

Effect of Colchicine In Vitro on Amylase Synthesis and Secretion by Parotid Glands

As shown in Fig. 7, the presence of colchicine during a prolonged (330 min) in vitro incubation did not influence basal or isoproterenol-induced amylase secretion. Tissue ATP and potassium content remained relatively stable throughout the 330-min incubation time (Table I), indicating, together with the observed stimulatory effects of isoproterenol, that tissues were satisfactorily preserved.

Experimental conditions similar to those indicated in Fig. 7 were used for measurement of the incorporation of labeled leucine into labeled tissue proteins and of labeled proteins secreted into the medium. It was observed that total (i.e. tissue plus medium) incorporation of leucine into proteins increased almost linearly with the duration of incubation, whatever the experimental conditions used (Fig. 8). In control tissues (i.e. no added colchicine), both the basal- and isoproterenol-induced release of newly synthesized proteins increased with time. The basal release of newly synthesized proteins was not significantly altered by incubation with colchicine (Fig. 8). In the presence of the drug, isoproterenol-induced amylase secretion was not modified by colchicine up to 150 min of incubation. However, from 210 min of incubation onwards, the stimulatory effect of isoproterenol upon release of newly synthesized proteins was markedly curtailed in colchicine-treated glands. To overcome the problem of fluctuations of absolute values from experiment to experiment, these results were expressed as percentages and further extended for newly synthesized amylase secretion in Fig. 9. As mentioned above, colchicine did not influence the secretion of stored amylase but was effective in inhibiting the secretion of newly synthesized protein in the presence of isoproterenol. Moreover, the specific activity of amylase released into the medium decreased, whilst it increased in the tissue, an indication that the intracellular movement of amylase had been interfered with.

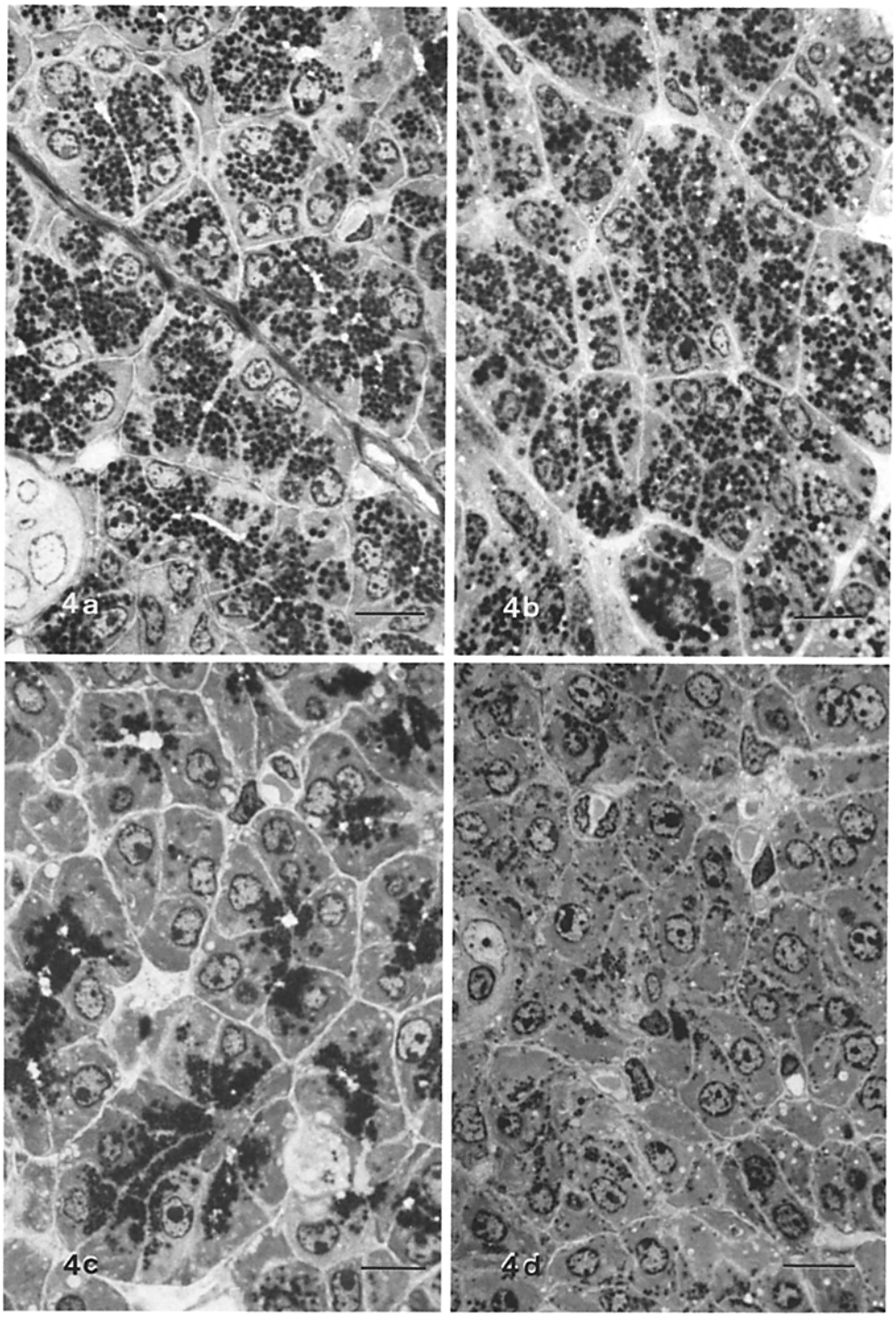

FIGURE 4 Light microscopy of rat parotid glands examined under different experimental conditions (experimental design, see Fig. 1). Fig. 4*a*: Appearance of control glands following injection of saline alone in vivo. Dense population of secretory granules. Fig. 4*b*: Appearance of glands 12 h after injection of colchicine in vivo. Dense population of secretory granules together with the presence of a few lipid droplets. Fig. 4*c*: Appearance of glands 12 h after injection of D,L-isoproterenol in vivo. The number of granules has not returned to that of controls although granules are conspicuous, located principally at the apical pole of the cells. Fig. 4*d*: Appearance of gland 12 h after injection of D,L-isoproterenol plus colchicine in vivo. Cells are very poorly granulated and contain several lipid droplets. × 1,000. Scale bar, 10 μm.

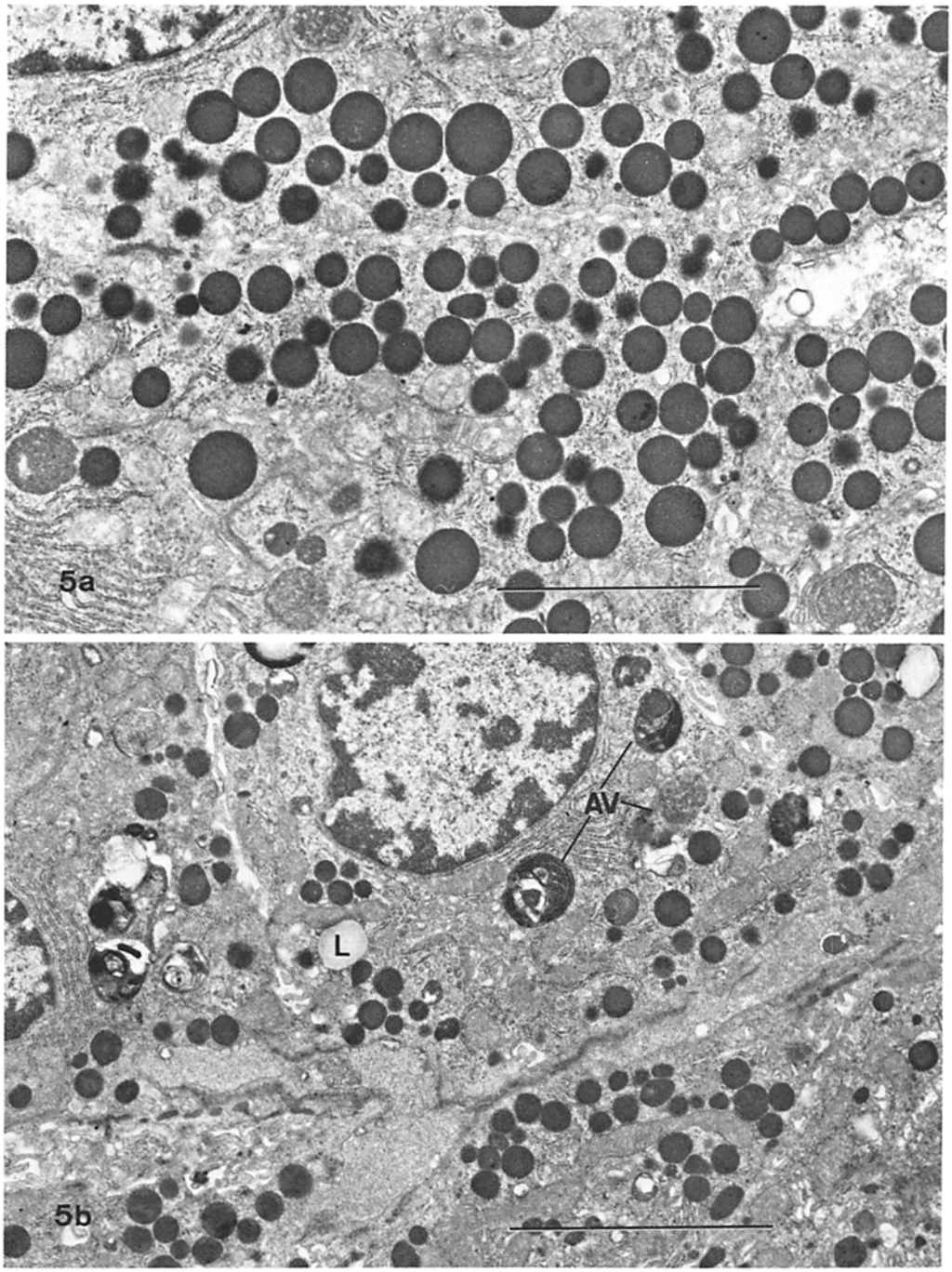

FIGURE 5 Electron micrographs of parotid glands from rats 12 h after in vivo injection of D,L-isoproterenol (Fig. 5*a*), or of D,L-isoproterenol plus colchicine (Fig. 5*b*) (experimental design, see Fig. 1). Fig. 5*a* shows the typical appearance of cells 12 h after isoproterenol-induced amylase discharge. A large number of mature secretory granules and condensing vacuoles are present. Fig. 5*b* shows cells following treatment with isoproterenol and colchicine. There are fewer granules, and they are smaller and often irregularly-shaped. Condensing vacuoles are scarce. Lipid droplets (*L*) and autophagic vacuoles (*AV*) are present in increased numbers. × 7,500. Scale bars, 5 μm.

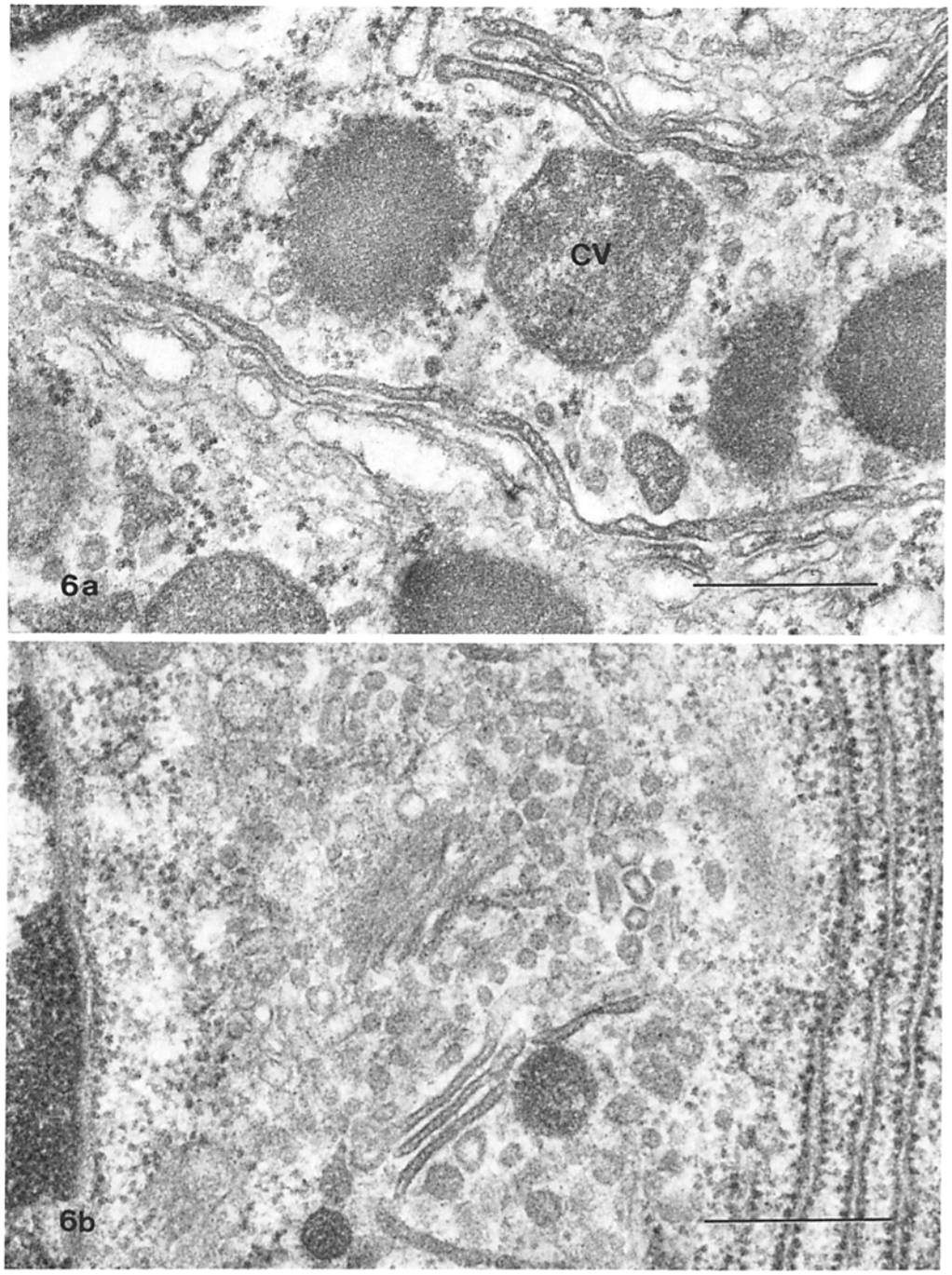

FIGURE 6 Electron micrographs of parotid glands from rats 12 h after in vivo injection of D,L-isoproterenol (Fig. 6*a*), or of isoproterenol plus colchicine (Fig. 6*b*). Fig. 6*a* Golgi area from isoproterenol-treated gland showing large condensing vacuoles (*CV*). Fig. 6*b* Golgi area from isoproterenol- and colchicine-treated glands. Cisternae are much reduced in size and surrounded by small vesicles. Condensing vacuoles are virtually absent. × 51,600. Scale bars, 0.5 μm.

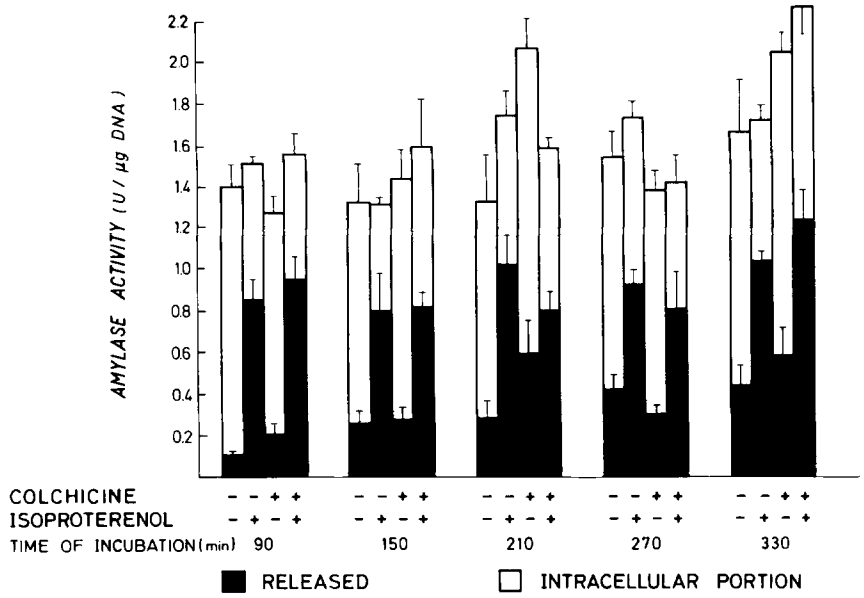

FIGURE 7 Lack of effect upon amylase secretion of prolonged exposure of rat parotid glands to colchicine in vitro. Parotid gland slices were incubated for various durations, as described in Materials and Methods, with or without colchicine (1×10^{-4} M), with or without D,L-isoproterenol (3×10^{-5} M). Following incubation, the amylase released into the medium or present in the tissue was measured. Each bar is the mean of 4 experiments ± SEM.

TABLE I

Potassium and ATP Concentrations in Incubated Parotid Gland Slices

Time of incubation	Intracellular concentration	
	Potassium	ATP
min	*µeq/mg DNA*	*µmol/mg DNA*
0	12.7 ± 1.1	76.48 ± 5.57
90	11.8 ± 1.9	81.99 ± 3.77
150	12.0 ± 1.9	73.37 ± 3.42
210	14.1 ± 2.5	89.99 ± 3.58
270	10.0 ± 0.5	73.04 ± 6.53
330	8.8 ± 0.8	58.53 ± 4.29

Slices of parotid glands were incubated as described in Materials and Methods. At time intervals, tissues were collected and homogenized for the measurements indicated. Time 0 refers to tissues taken directly, without incubation.

Values are means of four experiments ± SEM.

Binding and Uptake of Colchicine by Parotid Glands

The binding characteristics of tritiated colchicine were studied in this tissue with a MES-MEG buffer, since this buffer has been shown to improve the preservation of the colchicine-binding site of isolated tubulin (11). Table II shows that the binding activity was present in both 100,000 *g* supernates and washed particulate fractions of parotid gland homogenates. The binding activity measured in the particulate fraction was quite marked, being actually greater than the sum of the amounts found in the two supernates. No release of binding activity could be observed after treatment of the pellet with a mild detergent (0.5% Nonidet P 40) (5).

Binding of colchicine as a function of colchicine concentration was measured in 100,000 *g* supernates of parotid gland homogenates as well as in washed pellets. The binding kinetics as obtained according to Scatchard's plot (33) were compared to those of purified rat brain tubulin (40). The binding characteristics of isolated brain tubulin and the soluble fraction of parotid glands were found to be linear in the range of 10^{-7} to 10^{-4} M colchicine (Fig. 10), while the binding characteristics of the particulate fraction were not, suggesting the presence of two binding sites with different affinities for colchicine. The slopes of purified brain and parotid gland supernates were not markedly different, the latter being almost identical to the higher affinity binding activity of the particulate fraction (Fig. 10).

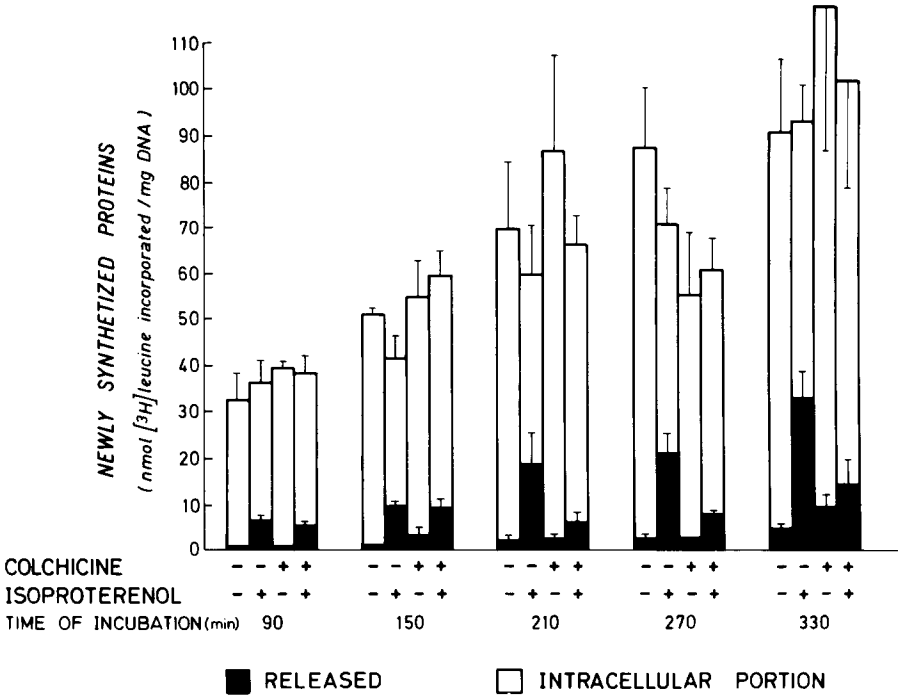

FIGURE 8 Effect of prolonged exposure of rat parotid glands to colchicine in vitro upon proteins newly synthesized from labelled leucine, and released into the medium. Parotid gland slices were incubated for various durations, as described in Materials and Methods, in the presence of tritiated leucine (1 mM), with or without colchicine (1×10^{-4} M), with or without D,L-isoproterenol (3×10^{-5} M). Following incubation, the incorporation of tritiated leucine into proteins released into the medium or present in the tissue was measured. Each bar is the mean of 4 experiments ± SEM.

The uptake of [^{3}H]colchicine by parotid glands was rapid, since intracellular and extracellular levels were equalized within 15 min (Fig. 11). Similarly, the binding of colchicine to both soluble and particulate fractions of homogenates of parotid gland slices also reached a plateau after 15 min of exposure to labeled colchicine (Fig. 11*a*). When tissue samples were preincubated for 30 min with unlabeled colchicine (10^{-4} M) and then transferred to [^{3}H]colchicine-containing medium, the uptake of the latter was not altered. However, binding to either soluble or particulate fractions was almost completely abolished (Fig. 11*b*). This suggests a low rate of exchange of colchicine at these binding sites, in agreement with observations made on purified tubulin (12).

Free and Total Tubulin in Parotid Glands

Assuming that the soluble colchicine-binding activity of parotid homogenate represents tubulin which might be partially assembled as microtubules in the tissue, an attempt was made to measure this microtubular portion in relation to total tubulin, using a method described previously (28). It was observed that in parotid glands the difference between free and total tubulin was very small, indicating that, in this tissue, only about 14% of total tubulin was assembled as microtubules (Table III). This difference completely disappeared upon cooling, markedly decreased upon incubation with colchicine, and disappeared upon incubation with vinblastine (Table III). In the latter case, the amount of tubulin was also reduced, presumably due to its precipitation as paracrystals which were observed in glands incubated for one hour with vinblastine (10^{-5} M) (D. Brown, unpublished data). The validity of the method used to obtain these results was supported by the observation of a satisfactory rate of recovery of isolated brain microtubules added to parotid gland homogenates, as shown in Table IV.

DISCUSSION

The present data show that colchicine was effective in inhibiting amylase secretion from parotid

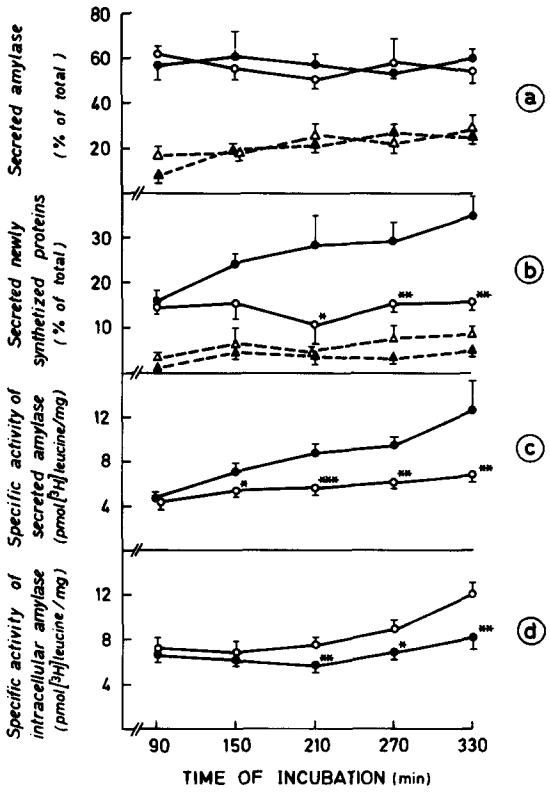

FIGURE 9 Synopsis of the in vitro effects of colchicine upon secretory processes in rat parotid glands. Parotid gland slices were incubated as described in Materials and Methods, and measurements a-b-c-d were performed at various time intervals. ●: addition of D,L-isoproterenol (3×10^{-5} M). ○: addition of D,L-isoproterenol plus colchicine (1×10^{-4} M). ▲: no addition. △: addition of colchicine. Each point is the mean of 4 experiments ± SEM. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

glands only when the glands were exposed to the drug in vivo for 6 h or more. In contrast, colchicine was completely ineffective in vitro, even after prolonged (330 min) incubation, in inhibiting either basal or isoproterenol-induced release of stored amylase. Since colchicine uptake and binding were very rapid, this delayed effect could not be the result of the inability of the drug to reach its reaction site. Following depletion of amylase by prior isoproterenol treatment, it was observed that colchicine prevented the replenishment of parotid glands as evidenced by the scarcity of granules that were, in addition, small and irregular. This suggested that colchicine was likely to exert an effect at a site more proximally located and related to granule formation. Such a hypothesis was strengthened by two additional observations: (*a*) colchicine was found to inhibit newly synthesized protein and amylase secretion in vitro without altering total protein synthesis. Such an inhibitory effect was relatively rapid (i.e., occurring within 3 h), being thus in agreement with the known time course of the secretory process in parotid glands, since labeled amino acids have been shown to appear in mature secretory granules after 2 h, and to reach a peak 3–4 h after stimulation (7). Furthermore, the inhibition of the secretion of newly formed material was accompanied by an intracellular accumulation of labeled amylase: This was probably due to an interference, brought about by the drug, with some step of the intracellular translocation of this secretory material; (*b*) the blockade of the secretory activity observed in parotid glands obtained from colchicine-treated rats was accompanied by marked changes in the morphology of the Golgi region and the complete disappearance of the microtubules in this region. The small, often irregular granules seen in isoproterenol-stimulated glands treated with colchicine were probably formed before the full inhibitory action of the drug was exerted, since in another tissue, the exocrine pancreas, new granules which formed 30 min after stimulation were also reportedly small and irregular (15).

The discrepancy between the in vivo and in vitro effects of colchicine (compare Figs. 3 and 8) on "newly-synthesized" material is probably due to the manner in which such material was detected in the two systems. In vitro, the release of labeled,

TABLE II

Distribution of the Colchicine-Binding Activity in Rat Parotid Glands as Measured by Fixation to DEAE-Sephadex

	Bound [³H] colchicine	
	pmol/mg protein	*pmol/ml supernate*
Total homogenate	6.77 ± 0.23	310.78 ± 22.6
1st supernate	11.14 ± 0.65	114.83 ± 5.93
2nd supernate	26.23 ± 0.99	36.00 ± 2.35
Pellet A	7.93 ± 0.60	313.38 ± 31.78
3rd supernate	9.71 ± 2.26	13.99 ± 1.21
Pellet B	4.78 ± 1.49	305.49 ± 94.60

Homogenates of fresh parotid glands were prepared with MES-MEG buffer in an all-glass-homogenizer, and ultracentrifuged (100,000 × *g*, 60 min, 4°C). The pellet was resuspended and centrifuged once more, resulting in the second supernate and pellet A. This pellet was resuspended in buffer containing 0.2% Nonidet p-40 at 4° for 30 min (5) and centrifuged again (Pellet B). From the final supernate, proteins were separated by a 60% ammonium sulfate precipitation and redissolved in MES-MEG-buffer (3rd supernate). All fractions were made up to the original volume for comparison. Each value represents the mean of 3 experiments ± SEM.

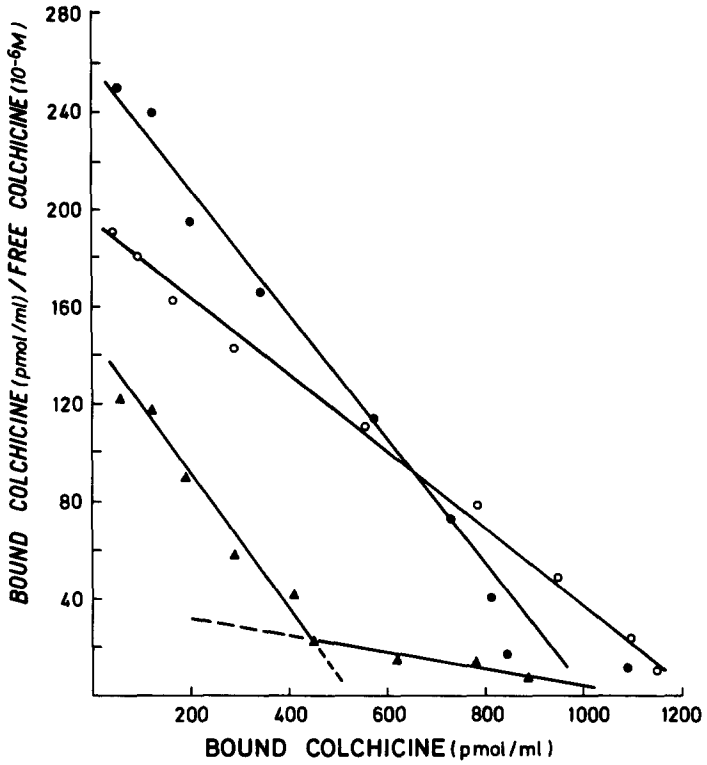

FIGURE 10 Colchicine-binding characteristics of isolated brain tubulin (○), and of 100,000 *g* supernates (●) or particulate fractions (▲) of rat parotid gland homogenates.

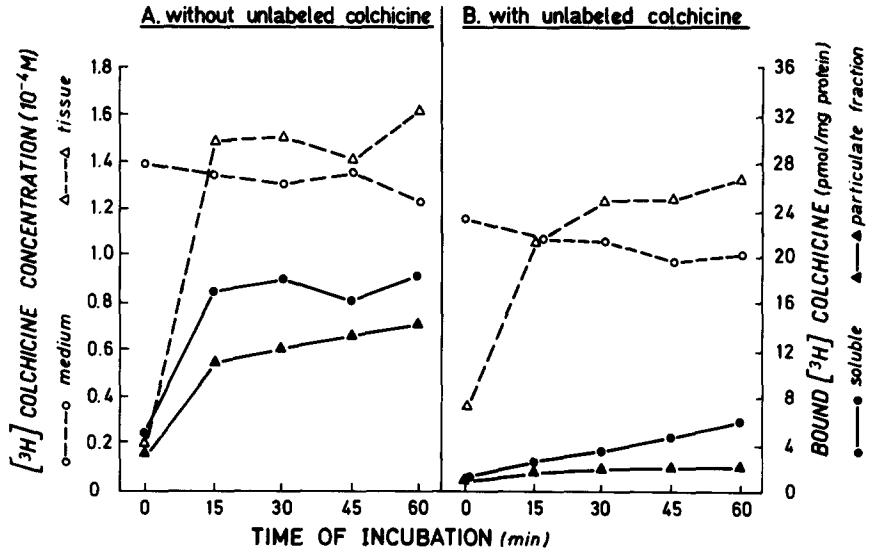

FIGURE 11 Uptake of [^{3}H]colchicine by slices of rat parotid glands, and its binding to soluble and particulate fractions of tissue homogenates. Experiment A: No preincubation with unlabeled colchicine. Experiment B: 30 min preincubation with unlabeled colchicine (10^{-4} M). Each point is the mean of 2 values.

i.e. newly synthesized protein (*not* total protein) was the parameter used, whereas in vivo the total amylase was assayed, i.e. newly synthesized plus residual amylase. Such material is present in the endoplasmic reticulum, the Golgi complex and the remaining secretory granules following isoproterenol stimulation. This material would be released on subsequent stimulation if packaged into exportable granules before the full inhibitory action of colchicine took effect. The presence of this material would mask the difference in the amount of newly-synthesized material secreted from the colchicine-treated and control tissue. Indeed, when release of total, and not labeled, amylase was measured in vitro, no inhibitory effect of colchicine could be detected, probably due to the large amount of stored amylase present in the tissue (Fig. 7).

TABLE III

Experimentally Produced Changes in the Portion of Microtubular Protein Assembled as Microtubules in Rat Parotid Glands

Experimental conditions	Bound [^{3}H]colchicine (*pmol/mg protein*)		
	Total tubulin (A)	Free tubulin (B)	Microtubules (A)-(B)
Controls*	19.89 ± 0.56	16.93 ± 0.68	2.96‡
Low temperature§	19.51 ± 0.31	19.76 ± 0.44	–
Colchicine‖	19.77 ± 0.50	19.07 ± 0.46	0.70
Vinblastine**	14.21 ± 0.29	14.65 ± 0.13	–

* Incubated for 1 h at 37°C as described in Materials and Methods.
‡ $p < 0.01$.
§ Incubated for 40 min at 37°C and subsequently for 20 min at 0°C.
‖ Incubated for 1 h in the presence of 10^{-4} M colchicine.
** Incubated for 1 h in the presence of 10^{-6} M vinblastine.
Values represent means of 4 experiments ± SEM.

A further difference between the in vivo and in vitro results was that after a 5.5 h incubation period, total amylase was similar in control and colchicine-treated tissue (Fig. 8), whereas 11 h following in vivo colchicine treatment the total amylase was lower in colchicine-treated tissue than in the controls (Fig. 2). This could possibly be explained by a partial exhaustion of the storage (due to continuous saliva production) that could not be compensated for by replenishment of the gland due to the proximal inhibitory effect of colchicine suggested above. However, the fact that in this situation no intracellular accumulation of amylase could be observed (Fig. 2) points to an additional effect of colchicine in vivo, i.e. an inhibitory effect on protein synthesis. Alternatively, such a reduced amylase content of colchicine-treated tissue could reflect increased degradation since there was a large accumulation of autophagic vacuoles after 11 h of colchicine treatment.

Taken together, the findings in this study make it unlikely that microtubules are directly involved in the hormone-stimulated release of the stored material of parotid glands. In contrast, a blockade of the secretory pathway in the Golgi region may represent the underlying mechanism for all the observed effects of colchicine on the secretory processes of these glands. It should be noted that a similar though not complete dissociation of colchicine effects on the release of stored and newly synthesized material has been reported for the exocrine pancreas (35). One should also stress that both parotid and the pancreatic acinar cells are polarized cells with a large capacity for storage of their secretory products. The question therefore arises as to whether or not our conclusions can be

TABLE IV

Recovery of Isolated Brain Microtubules Homogenized together with Parotid Gland Slices

	Bound [^{3}H]colchicine (*pmol/ml*)		
	Total tubulin (A)	Free tubulin (B)	Microtubules (A)-(B)
Isolated microtubules*	279.77 ± 19.11	118.77 ± 18.69	161.00
Parotid homogenate‡	105.47 ± 6.42	91.00 ± 3.08	14.47
Isolated microtubules + Parotid homogenate§ (experimental)	382.40 ± 35.74	238.92 ± 8.98	143.58
Isolated microtubules + Parotid homogenate‖ (calculated)	386.24	209.77	175.47

* Isolated brain microtubules (36) in equilibrium with free tubulin.
‡ Parotid gland slices incubated for 1 h and homogenized as described in Materials and Methods.
§ Isolated brain microtubules mixed with parotid gland slices before homogenization.
‖ Calculated values for the mixture of isolated microtubules and parotid gland homogenate.

extended to nonpolarized cells and/or to secretory cells with small or no storage capacity.

In this study, the characterization of the colchicine-binding activity of parotid glands was also attempted. As the determination of the association constants from such binding kinetics may lead to erroneous results because of the instability of the colchicine-binding site of the microtubular protein (4), this problem was circumvented by the use of purified brain tubulin as a standard. The soluble colchicine-binding activity of parotid gland homogenates showed an affinity for colchicine that was similar to that of brain tubulin. It can therefore be considered as representing microtubular protein, which agrees with similar findings in practically all mammalian tissues. The nature of the particle-bound colchicine-binding activity of parotid gland homogenates is less clear. The portion of such colchicine-binding activity with the highest affinity for colchicine may well represent tubulin, although the specificity of this binding remains to be established.

Within the limits of the biochemical method used for measuring the portion of soluble tubulin assembled as microtubules, the striking observation made was that only about 14% of the total soluble tubulin of parotid glands could be attributed to the microtubular portion. The few analogous measurements reported for other tissues showed higher values such as, for instance, 40% in the liver (28) and 30–50% in the endocrine pancreas (31). The microtubular portion of the total tubulin in parotid glands disappeared under conditions which are known to destroy cytoplasmic microtubules (29). Since colchicine is assumed to interfere with the assembly-disassembly cycle of microtubules (28, 29), this effect of the drug suggests that such a cycle is probably operative in parotid glands.

The precise role of the microtubular system in the parotid glands, as assessed by the action of colchicine upon the secretion of amylase and by the characteristics of colchicine-binding activity, is not clear as yet. Taken as a whole, the present observations are consistent with colchicine's having four possible effects on the secretory process. (*a*) colchicine could interfere with the microtubular assembly-disassembly cycle that may be associated functionally with the transfer of secretory material towards or away from the Golgi region. (*b*) Colchicine could react with microtubular protein that would be necessary for the actual function of the Golgi complex, or for the maintenance of normal three-dimensional morphological relationships in the Golgi region. Indeed, it has been reported in other cell types that microtubules may play a structural role in this region (23) and, in the parotid gland, microtubules were seen in close proximity to most Golgi cisternae. (*c*) Colchicine could interfere with membrane-bound tubulin, the function of the latter being possibly unrelated to microtubules per se but conceivably involved in membrane fusion processes. (*d*) Colchicine might bind unspecifically to membranes, thus altering the function of organelles such as the Golgi complexes.

Whether the perturbation by colchicine of a single process or a combination of these processes results in the observed changes remains to be established. Furthermore, the presence in the parotid glands of a microtubular "system" represented mostly by its unpolymerized protein and the postulated restricted role of this system to a specific intracellular process indicate that some current hypotheses of microtubule function may need reappraisal.

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