

COLCHICINE INHIBITION OF PLASMA PROTEIN RELEASE FROM RAT HEPATOCYTES

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ABSTRACT

Colchicine, both in vitro and in vivo, inhibits secretion of albumin and other plasma proteins. In vitro, secretion by rat liver slices is inhibited at 10^{-6} M with maximal effect at 10^{-5} M. Inhibition of secretion is accompanied by a concomitant retention of nonsecreted proteins within the slices. Colchicine does not inhibit protein synthesis at these concentrations. Vinblastine also inhibits plasma protein secretion but lumicolchicine, griseofulvin, and cytochalasin B do not. Colchicine also acts in vivo at 10–25 $\mu\text{mol}/100$ g body weight. Inhibition of secretion is not due to changes in the intracellular nucleotide phosphate levels. Colchicine, administered intravenously, acts within 2 min and its inhibitory effect lasts for at least 3 h. Colchicine has no effect on transport of secretory proteins in the rough or smooth endoplasmic reticulum but it causes these proteins to accumulate in Golgi-derived secretory vesicles.

Plasma proteins, produced by the liver, are synthesized on polysomes attached to the membrane of the endoplasmic reticulum (ER)¹ (12, 14, 29, 30, 37) and are then transported, stepwise, from the lumen of the rough ER (RER) to the smooth ER (SER) and to the Golgi complex. Finally, Golgi-derived vesicles, which contain the secretory proteins, migrate to the sinusoidal cell surface, fuse with the plasma membrane, and empty their contents in the space of Disse (13, 25, 26). While this intracellular pathway has now been well established, there is still a paucity of information

¹ *Abbreviations used in this paper:* ER, endoplasmic reticulum; Golgi I, II, and III, light, intermediate, and heavy Golgi fractions, respectively, KRB, Krebs-Ringer bicarbonate medium; RER, rough endoplasmic reticulum or rough microsomal fraction; SER, smooth endoplasmic reticulum or smooth microsomal fraction; TKM, 10 mM Tris-HCl pH 7.4, 25 mM KCl, and 5 mM MgCl_2 ; TSH, thyroid-stimulating hormone; VLDL, very low density lipoproteins.

on how the secretory proteins are moved from one cellular compartment to another. The use of drugs, which inhibit the secretory process at distinct locations in the cell, may prove valuable for more detailed studies of specific steps in secretion and may lead eventually to an understanding of the molecular basis of the mechanism involved in intracellular transport.

It has been demonstrated that certain alkaloids, such as colchicine, affect secretion in a variety of tissues. Some examples are the inhibition of the secretion of insulin (16), thyroxine (41, 42), salivary gland mucin (32), hepatic very low density lipoproteins (VLDL) (17, 36), that of the conversion of procollagen to collagen (10) (which is assumed to be dependent on secretory discharge), that of the epinephrine-stimulated release of amylase from the parotid (5), and that of the acetylcholine-stimulated release of catecholamine from the adrenal medulla (7, 27). Colchicine has also been shown to inhibit the release of lysosomal enzymes

from human phagocytes (45) and this is another instance in which colchicine affects discharge. Exceptions have been recorded, however, since glucagon secreted by isolated pancreatic islets (guinea pig) is enhanced, rather than depressed, by colchicine (8) and since the drug does not affect the secretion of immunoglobulins by mouse plasmacytomas (24), the potassium-stimulated release of catecholamines from the adrenal medulla (7), nor the thyroid-stimulating hormone (TSH) secretion by the pituitary (38).

This paper reports the results of a detailed study of the action of colchicine and other functionally related agents in inhibiting, *in vitro* and *in vivo*, the secretion of albumin and other plasma proteins by rat liver. We show that this drug does not impede the early steps in the secretory process, namely, the synthesis and the movement of secretory proteins from the RER to the Golgi complex, but that it inhibits late events which occur after the filling of Golgi-derived secretory vesicles with secretory protein but before the fusion of the vesicles with the sinusoidal plasma membrane.

A preliminary report of some of this work has appeared in abstract form (31).

MATERIALS AND METHODS

Animals

Young male rats (120–175 g) were starved overnight before the experiments. Ethanol was given by stomach tube (0.6 g ethanol as a 50% solution per 100 g body weight). Colchicine or radioactive amino acid was administered by intravenous injection into the femoral vein. Serial blood samples were obtained from the tail vein or by heart puncture when larger quantities were needed.

Preparation and Incubation of Liver Slices

Slices, approximately 0.5 mm in thickness, were prepared with a Stadie-Riggs hand slicer. About 0.3 g of slices were incubated in 5 ml of Krebs-Ringer bicarbonate (KRB) saline supplemented with 0.21 M glucose and a mixture of 20 amino acids (0.1 mM) minus leucine which was introduced as L-[U-¹⁴C]leucine, 0.25 μ Ci/ml, (sp act 348 mCi/mmol). After a pulse incubation of 7–9 min at 37°C, the slices were recovered by filtering out the medium; they were washed with 0.7 mM nonradioactive L-leucine in 0.154 M NaCl and reincubated at 37°C in 5 ml of KRB containing 0.21 M glucose and a complete 0.1 mM mixture of 21 nonradioactive amino acids. The secreted proteins were recovered from the incubation medium by precipitation with TCA or by immunoprecipitation, and the proteins retained within the tissue were obtained from slices washed free of medium by three successive treatments with 0.154 M NaCl.

Cell Fractionation Procedures

The livers were homogenized in 5 vol of ice-cold 0.25 M sucrose with five to eight strokes of a Potter-Elvehjem homogenizer with a Teflon pestle, motor driven at 1,000 rpm. The homogenate was cleared of cell debris, nuclei, and mitochondria by centrifugation at 10,000 g for 10 min. A total microsomal fraction, i.e. a mixture of microsomes and Golgi elements was obtained by centrifuging the ensuing supernate of 105,000 g for 90 min. The pellet was suspended in a small volume of 0.25 M sucrose and sufficient 2.1 M sucrose was added to obtain a 1.17 M sucrose concentration. All sucrose solutions were checked in a refractometer to assure correct final sucrose concentrations. Three Golgi fractions were isolated from this suspension by the procedure of Ehrenreich et al. (9) with a few modifications: an additional sucrose layer (1.15 M) was introduced between the load and the rest of the discontinuous gradient and centrifugation was carried out for 3 h at 81,750 g_{avg} . To obtain SER and RER fractions, the load fraction and the pellet were further processed by pooling and diluting them with TKM buffer (10 mM Tris-HCl pH 7.4, 25 mM KCl, and 5 mM MgCl₂) to a sucrose concentration of 0.25 M and by harvesting them as a pellet by centrifugation at 50,000 rpm (Spinco 50 Ti) for 1 h; this mixture of RER and SER was then further fractionated by the procedure of Ragland et al. (28).

Preparation of Samples for

Immunoprecipitation Assays

The three Golgi fractions and the RER and SER fractions were removed from discontinuous gradients and were diluted with TKM to obtain a final sucrose concentration of 0.25 M. Particles were pelleted by centrifugation at 50,000 rpm (Spinco 50 Ti) for 1 h. The pellets were resuspended in 0.154 M NaCl with TKM and sodium deoxycholate was added to a final concentration of 0.5%. At this concentration, the detergent releases all the radioactive albumin and ~90% of the total acid-insoluble radioactivity sequestered within, or associated with, ER and Golgi vesicles. The concentration was chosen for convenience: it causes extensive solubilization of membranes, disperses the contents, and does not interfere with the antibody-antigen reaction. The samples were then cleared of nonsolubilized material by centrifugation at 50,000 rpm (Spinco 50 Ti) for 1 h. When determining the amounts of plasma protein in the homogenate, a sample corresponding to 0.1 g liver was diluted with 0.154 M NaCl in TKM to 10 ml and was then treated with 0.5% sodium deoxycholate and cleared of insoluble material by centrifugation. The soluble cytoplasmic fraction was not treated with sodium deoxycholate but it was buffered with 0.01 M Tris-HCl pH 7.4 and 0.154 M NaCl. The medium in which slices had been incubated was used without further treatment. Blood samples were heparinized and the plasma obtained by

centrifugation was diluted 200 times with 0.154 M NaCl in TKM.

Immunoprecipitation Assays

Radioactivity in albumin and in other plasma proteins was determined as previously described (29, 30). Recently it was reported that intracellular albumin obtained by immunoprecipitation is chemically different from plasma albumin and may represent an albumin precursor (15, 33). Antibodies to rat plasma proteins other than albumin were prepared by absorbing out the anti-rat albumin from an antibody preparation to total rat plasma protein, using the method of Avrameas and Ternynck (1).

Other Procedures

Radioactive samples, either TCA-precipitated protein or antibody-antigen complexes, were dissolved in 1 ml of Soluene plus 0.1 ml of glacial acetic acid, and 10 ml of toluene scintillation phosphor were added before counting in a Packard scintillation radiometer.

Protein was assayed by the method of Lowry et al. (18), with bovine plasma albumin as a standard.

The specific activity of nucleotide phosphates was determined by adsorption of the nucleotides from a 5% TCA-soluble fraction on charcoal and hydrolysis of the adsorbed material in 1 N HCl for 10 min at 100°C as described by Crane and Lipmann (6). Phosphorus analysis was performed by the method of Bartlett (2).

Electron Microscopy

Liver samples from control or treated rats were fixed either in 1% OsO₄ in 0.1 M cacodylate buffer or in 2.5% formaldehyde-2% glutaraldehyde in the same buffer followed by 1% OsO₄. The tissues were stained in block in uranyl acetate before dehydration in graded ethanols and finally embedded in Epon. Thin sections stained with uranyl acetate and lead citrate were examined in a Siemens Elmiskop 102. Golgi fractions were processed for electron microscopy as previously described (9).

Sources of Material

L-[U-¹⁴C]Leucine (348 Ci/mmol) was purchased from Amersham/Searle Corp., Arlington Heights, Ill.; [*methoxy*-¹⁴C]colchicine (15 Ci/mmol) was obtained from New England Nuclear, Boston, Mass. Vinblastine sulfate was a kind gift from Eli Lilly and Company, Indianapolis, Ind. Colchicine was bought from Sigma Chemical, Co., St. Louis, Mo.; griseofulvin from Aldrich Chemical Co., Inc., Milwaukee, Wis., and cytochalasin B from Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N. Y. Lumicolchicine was prepared and given to us by Dr. M. Rifkin of The Rockefeller University, New York.

RESULTS

Effect of Colchicine and Vinblastine on the Secretion of Plasma Protein (Albumin) by Liver Slices

The initial observations, which showed that colchicine and vinblastine inhibit secretion, were performed on liver slices pulse labeled with L-[¹⁴C]leucine for 7–9 min, then transferred to a “chase” medium containing nonradioactive leucine and 5×10^{-5} M colchicine or vinblastine. In control slices, radioactive TCA-precipitable protein began to appear in the medium after a 15–20-min chase and continued to accumulate for 2 h. In the presence of colchicine or vinblastine, there was a 40–70% inhibition at all time points of the amount of radioactive protein secreted by the slices (Fig. 1). The inhibitory effect appeared to be unique to colchicine and vinblastine: lumicolchicine, a structural isomer of colchicine, was five times less effective at 10^{-5} M (which is the concentration at which colchicine showed maximal inhibitory activity). Lumicolchicine is contaminated by 3% colchicine, as shown by spectral analysis, and this may explain, at least in part, the effect observed. Cytochalasin B at 4.8×10^{-6} – 4.8×10^{-4} M and griseofulvin at 10^{-8} – 10^{-4} M did not inhibit secretion (Table I).

The inhibitory effect of colchicine was noticed at concentrations as low as 10^{-6} M, with a maximal effect at 10^{-5} M. Fig. 2 shows an experiment in which secretion into the medium as well as retention of albumin within the slices was measured at

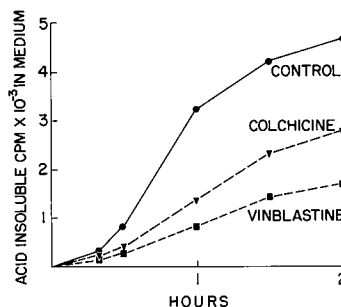


FIGURE 1 Time-course of protein secretion by rat liver slices: effects of colchicine and vinblastine. Rat liver slices were pulse labeled for 9 min with L-[¹⁴C]leucine and were then chased with nonradioactive amino acid for the times indicated. Details are presented in Materials and Methods. Colchicine and vinblastine were introduced in the chase medium at a final concentration of 5×10^{-5} M.

TABLE I
Effect of Colchicine, Lumicolchicine, Cytochalasin B, and Griseofulvin on Plasma Protein Secretion by Rat Liver Slices

Additions to chase medium		Plasma protein secreted (cpm/mg slice protein)	Inhibition %
<i>M</i>			
Exp 1			
Control		316	—
Dimethyl sulfoxide		304	4
Cytochalasin B	4.8×10^{-6}	312	1
Cytochalasin B	2.4×10^{-5}	315	0
Cytochalasin B	4.8×10^{-5}	285	10
Colchicine	5.0×10^{-5}	108	66
Exp 2			
Control		120	—
Lumicolchicine	5×10^{-6}	110	8
Lumicolchicine	10^{-5}	108	10
Lumicolchicine	2.5×10^{-5}	101	16
Lumicolchicine	3.0×10^{-5}	95	21
Colchicine	10^{-5}	44	63
Colchicine	2.5×10^{-5}	36	70
Exp 3			
Control		155	—
Griseofulvin	10^{-6}	141	9
Griseofulvin	10^{-5}	156	0
Griseofulvin	10^{-4}	173	0
Colchicine	10^{-5}	56	64

Rat liver slices were pulse labeled with L-[14 C]leucine for 9 min and then chased with nonradioactive medium containing the above concentration of drugs for 180 min in exp. 1 and 60 min in exps. 2 and 3. The proteins secreted into the chase medium were determined by immunoprecipitation. Dimethyl sulfoxide was used as a solvent for cytochalasin B and griseofulvin. The final concentration was 0.028 M. These are representative experiments from a series of three for each drug concentration.

different concentrations of colchicine and vinblastine: the inhibition of secretion was accompanied by a concomitant retention of albumin within the slices, presumably in the hepatocytes.

The colchicine and vinblastine effect on secretion was not the result of an inhibition of protein of protein synthesis. When liver slices were incubated in the presence of L-[14 C]leucine and 5×10^{-5} M colchicine or vinblastine, there was no effect on the initial rates of protein synthesis as measured by the

incorporation of radioactivity into TCA-precipitable protein. Vinblastine at 10^{-4} M showed a small inhibitory effect at 1 h of incubation but colchicine did not (Fig. 3).

In Vivo Effects of Colchicine

Colchicine, given intravenously, (7–25 μ mol/100 g of body weight) inhibited the secretion of albumin in the plasma, and caused concomitant accumulation of albumin within the liver in intact animals (Fig. 4). The rats tolerated doses of 25 μ mol/100 g of body weight quite well, but doses higher than 30 μ mol/100 g body weight occasionally caused death, especially if the rats had been fed alcohol.

Radioactive albumin appears in the blood 15–20 min after the injection of L-[14 C]leucine. Colchicine inhibited secretion from the onset and the effect persisted for 3 h (Fig. 5). Albumin was not

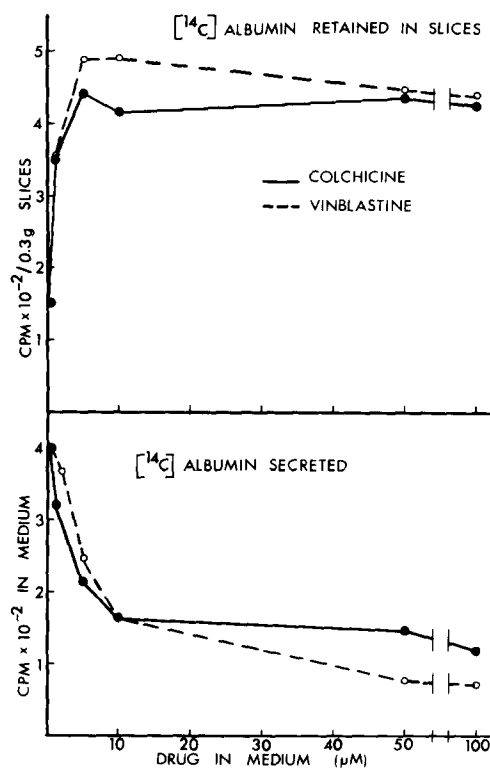


FIGURE 2 Effect of various concentrations of colchicine and vinblastine on albumin secretion by rat liver slices. Rat liver slices were incubated as in Fig. 1. The chase incubation was carried out for 90 min. Radioactive albumin, secreted into the medium or retained within slices, was determined as described in Materials and Methods.

the only protein affected by colchicine. The secretion of all the other plasma proteins was similarly inhibited. This was determined by immunoprecipitation using two different antisera; one which

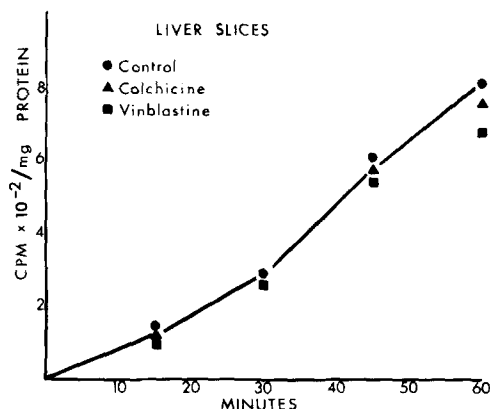


FIGURE 3 Incorporation of L-[¹⁴C]leucine into TCA-precipitable protein by rat liver slices. Rat liver slices were incubated with L-[¹⁴C]leucine as described in Materials and Methods. Colchicine or vinblastine (5×10^{-5} M) was added at zero time and the slices were incubated for varying times up to 1 h. At the end of incubation the slices and the incubation medium were homogenized and total TCA-precipitable radioactivity was determined. Thus the points represent radioactivity (cpm $\times 10^{-2}$) per milligram total protein in both medium and tissue.

reacted with rat albumin and another which reacted with all the rat plasma proteins except albumin. Since colchicine has also been shown to inhibit hepatic secretion of VLDL (17, 36), it appears to act as a general inhibitor of the secretion of all plasma proteins by the liver.

The effect of colchicine on secretion is prompt as shown by the results of the experiment in Fig. 6. Colchicine ($25 \mu\text{mol}/100 \text{ g}$ body weight) was given 33 min after an initial injection of L-[¹⁴C]leucine, a time at which there is linear secretion of albumin. Within 2 min after the administration of the drug there was a noticeable reduction in the rate at which albumin was secreted into the plasma. An equally prompt effect was obtained when colchicine was injected at 42 min after the administration of L-[¹⁴C]leucine (not shown) at a time when secretion of radioactive albumin begins to subside.

Lack of Effect of Colchicine on the Nucleotide Phosphate Levels

In a previous experiment it was shown that colchicine did not affect albumin secretion by inhibiting protein synthesis (Fig. 3). Secretion may be inhibited, however, by drug interference with cellular energetics, primarily a decrease in the intracellular concentration of ATP. To determine

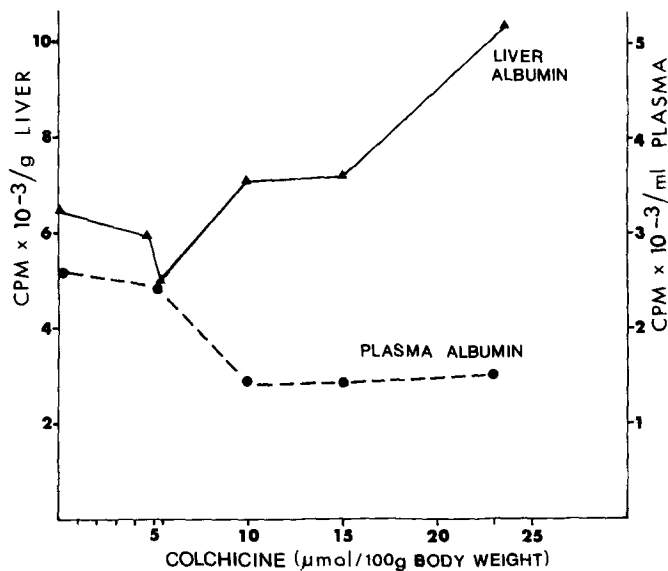


FIGURE 4 In vivo inhibition of albumin secretion by colchicine. Varying amounts of colchicine (in a 0.15 M saline solution) were injected intravenously into rats. This was followed immediately with a second injection of $7.5 \mu\text{Ci}/100 \text{ g}$ body weight of L-[¹⁴C]leucine. 45 min after the administration of the radioactive leucine, a sample of blood was taken from the rat by heart puncture and the livers were removed. The amount of radioactive albumin in the liver and in plasma was determined as described in Materials and Methods.

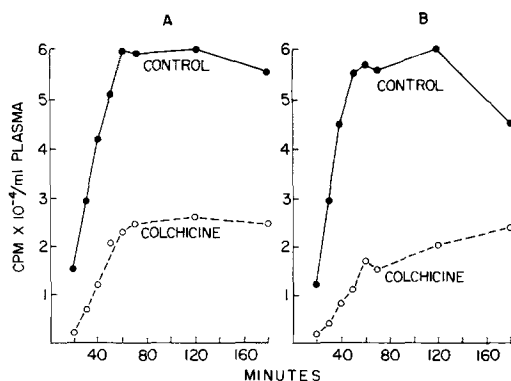


FIGURE 5 In vivo secretion of albumin and other serum proteins: effect of colchicine. Colchicine (25 $\mu\text{mol}/100$ g body weight) was injected into the femoral vein of a rat, followed closely by a second injection of 12.5 $\mu\text{Ci}/100$ g body weight of L-[^{14}C]leucine. Control rats received an equal volume of saline instead of colchicine. At specified times the rats were bled from the tail vein and 75 μl of blood were placed in 3 ml of 0.154 M NaCl in TKM. The blood cells were removed by centrifugation and the supernate was used to determine radioactive albumin or other serum proteins as described in Materials and Methods. Fig. 5 A describes the appearance of radioactive albumin in plasma and Fig. 5 B that of the other proteins.

if this is the case, radioactive inorganic phosphate was injected into control and colchicine-treated rats and at various times both the entry of $^{32}\text{P}_i$ into the liver and its conversion into nucleotide phosphates were determined. Neither the specific activity of the $^{32}\text{P}_i$ nor its conversion to nucleotide phosphate was affected by colchicine (Fig. 7).

Intracellular Localization of the Colchicine Effect

Since colchicine causes an inhibition of secretion and an accumulation of the secretory products within the cell (Fig. 2), it was of interest to find out in what intracellular compartment the accumulation occurs. To this intent, rats were injected intravenously with L-[^{14}C]leucine and at various time intervals after the onset of radioactive protein synthesis, the livers were removed, homogenized, and fractionated. In some experiments the rats were fed alcohol since this has been shown to increase the yield and purity of Golgi fractions (9). Table II shows the cell fractions isolated in these experiments and the average yields from control and from colchicine-treated rats. The yield of Golgi fractions varied from experiment to experiment but in all cases larger amounts of the three

Golgi fractions could be obtained from alcohol-fed rats than from normal rats. With or without ethanol pretreatment of the animals, colchicine caused a general increase in the yield of hepatic Golgi fractions. The morphology of the fractions isolated from ethanol-treated or colchicine-treated rats was almost the same, except that the vesicles of the light and intermediary Golgi fractions were smaller and had a lower load of VLDL particles after colchicine than after ethanol. Fig. 8-10 show that the Golgi fractions obtained from colchicine-treated animals are reasonably homogeneous and consist essentially of recognizable Golgi elements.

The effect of colchicine in increasing the amount of radioactive albumin within the Golgi fractions is demonstrated in Figs. 11-14. This experiment shows that the amount of radioactive albumin per gram liver and per milligram of cell fraction protein is greatly enhanced in the three Golgi fractions, but that colchicine does not alter the nascent albumin concentration in the other cell fractions. Using the data on Table II and Figs. 11-14 we can calculate that there is little leakage of albumin from the cell fractions, since only 2-3% of radioactive albumin is recovered in the final supernate; and we can also conclude that there is good drainage of newly synthesized albumin from the ER into the Golgi elements since 38-58% of its total can be recovered at this time in the Golgi

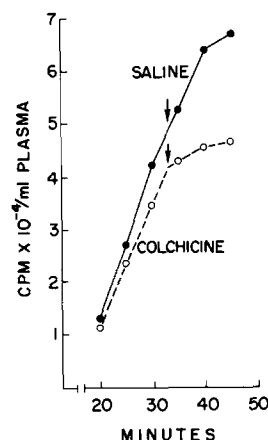


FIGURE 6 Time-course of the inhibition of albumin secretion by colchicine in vivo. 12.5 $\mu\text{Ci}/100$ g body weight of L-[U- ^{14}C]leucine was injected into the femoral vein of rats at zero time. Saline or colchicine (25 $\mu\text{mol}/100$ g body weight) was injected at 33 min after the administration of L-[U- ^{14}C]leucine (arrows). Blood samples were taken from the tail at the specified times and the amount of radioactive albumin in the plasma was determined by immunoprecipitation.

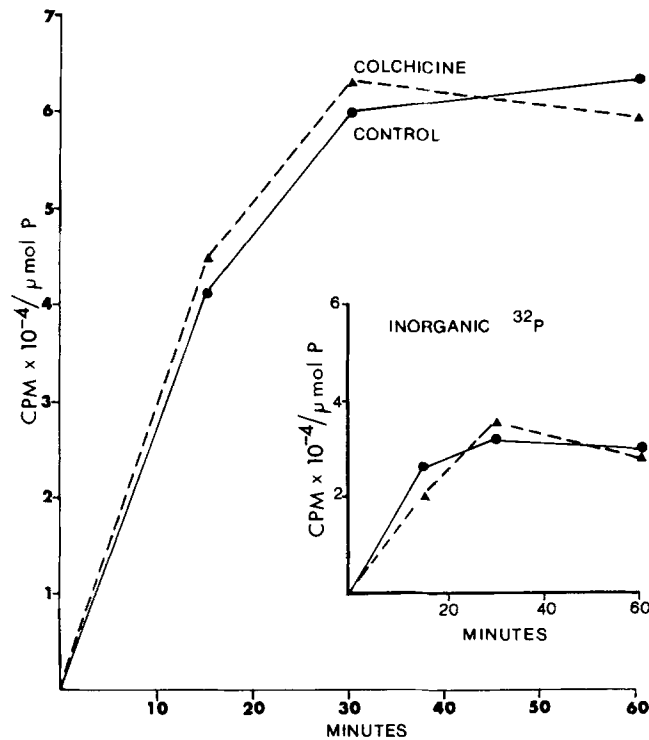


FIGURE 7 Specific radioactivities of intracellular nucleotide phosphates in response to colchicine. Experimental animals received intravenously (femoral vein) $25 \mu\text{mol}/100 \text{ g}$ body weight colchicine and 2 min after it $^{32}\text{P}_i$ ($25 \mu\text{Ci}/100 \text{ g}$ body weight) in saline. Control rats received saline instead of colchicine. At the specified times, the livers were removed, rinsed in 0.25 M sucrose, and homogenized in ice-cold 5% TCA. The specific radioactivities of the nucleotide phosphates and of the intracellular inorganic phosphate were determined from the TCA-soluble fraction (6).

fractions, with the remainder in the smooth and rough microsomes. This is in contrast to the recoveries of TCA-precipitable radioactivity where 58–64% is found in the soluble cytoplasmic fraction and only 9–14% in Golgi elements.

A time-course study showed that there was peak albumin radioactivity at 10 min after the administration of radioactive leucine (to the rat) in the rough microsomes and at 30 min in the smooth microsomes and the Golgi fractions (Fig. 15). Colchicine treatment did not affect the movement of albumin radioactivity in the RER or SER nor the rate of entry of radioactive albumin into the Golgi fractions (see 10- and 20-min time points); it did affect, however, the rate of release of albumin from the Golgi fractions. In control rats, there was a gradual loss of radioactive albumin from the Golgi fractions after 30 min but the colchicine-treated rats retained the radioactive albumin in these fractions for the duration of the experiment (90 min). The radioactive albumin from the light

Golgi fraction (Golgi I) is not shown in Fig. 15 because at some of the time points in these experiments alcohol-fed rats were not used and insufficient and often variable yields of Golgi I were obtained. We do, however, have information on the amounts of radioactive albumin in Golgi I at 45, 60, and 90 min, and at these times the colchicine effect is similar to that seen in the intermediate Golgi fraction (Golgi II).

Since colchicine does not affect the transport of nascent albumin in the ER, nor does it affect the filling of the Golgi vesicles with albumin, then it must be acting at a stage in secretion after the Golgi-derived secretory vesicles have been filled but before their fusion with the plasma membrane.

Hepatocyte Morphology

By comparison with their corresponding controls, the hepatocytes of colchicine-treated animals ($25 \mu\text{mol}/100 \text{ g}$ body weight) have Golgi elements

TABLE II
Recovery of Liver Cell Fractions from Colchicine-
and Alcohol-Treated Rats

	Proteins in cell fractions (mg/g liver)			
	Control	Colchicine	Alcohol	Alcohol and colchicine
Homogenate	190.5	193.2	196.7	199.7
Fractions				
Soluble	39.5	37.8	36.5	40.8
RER	6.31	5.60	7.07	5.95
SER	5.49	5.29	5.44	5.70
Golgi I	0.08	0.11	0.12	0.17
Golgi II	0.28	0.33	0.35	0.54
Golgi III	1.18	1.22	1.39	1.61

Average of four experiments. RER, rough microsomal fraction; SER, smooth microsomal fraction; Golgi I, II, and III, light, intermediate, and heavy Golgi fractions, respectively. Cell fractionation was performed as described in Materials and Methods. The low protein recovery (about 25% of the homogenate proteins recovered in the sum of the fractions listed) reflects heavy losses of microsomes and Golgi elements at the first centrifugation of the homogenate.

with a higher load of VLDL particles (Fig. 16) and a large population of VLDL-containing secretion vacuoles (Figs. 16–18). The latter, besides being concentrated along the sinusoidal front of the cells (Fig. 17), are also found scattered throughout the cytoplasm (Fig. 18). The secretion vacuoles show considerable variation in size and compaction of content and in many of them the VLDL particles appear embedded in a homogeneous dense matrix. In ethanol-treated rats, the VLDL load of Golgi elements is more pronounced and the concentration of secretion vacuoles higher in Golgi regions than in the rest of the cytoplasm (9).

In the hepatocytes of control animals, microtubules are found in the Golgi regions and in the peripheral cytoplasm but their concentration is low and their distribution is apparently random, without any indication of special relationship with secretion vacuoles. Neither the concentration nor the arrangement of microtubules seems to be affected by colchicine treatment at a time (1 h) when inhibition of plasma protein secretion is already maximal. In fact, microtubules are still detected in Golgi regions, along bile capillaries, and along the sinusoidal front of the hepatocytes 3 h after the administration of the drug (Figs. 19 and 20).

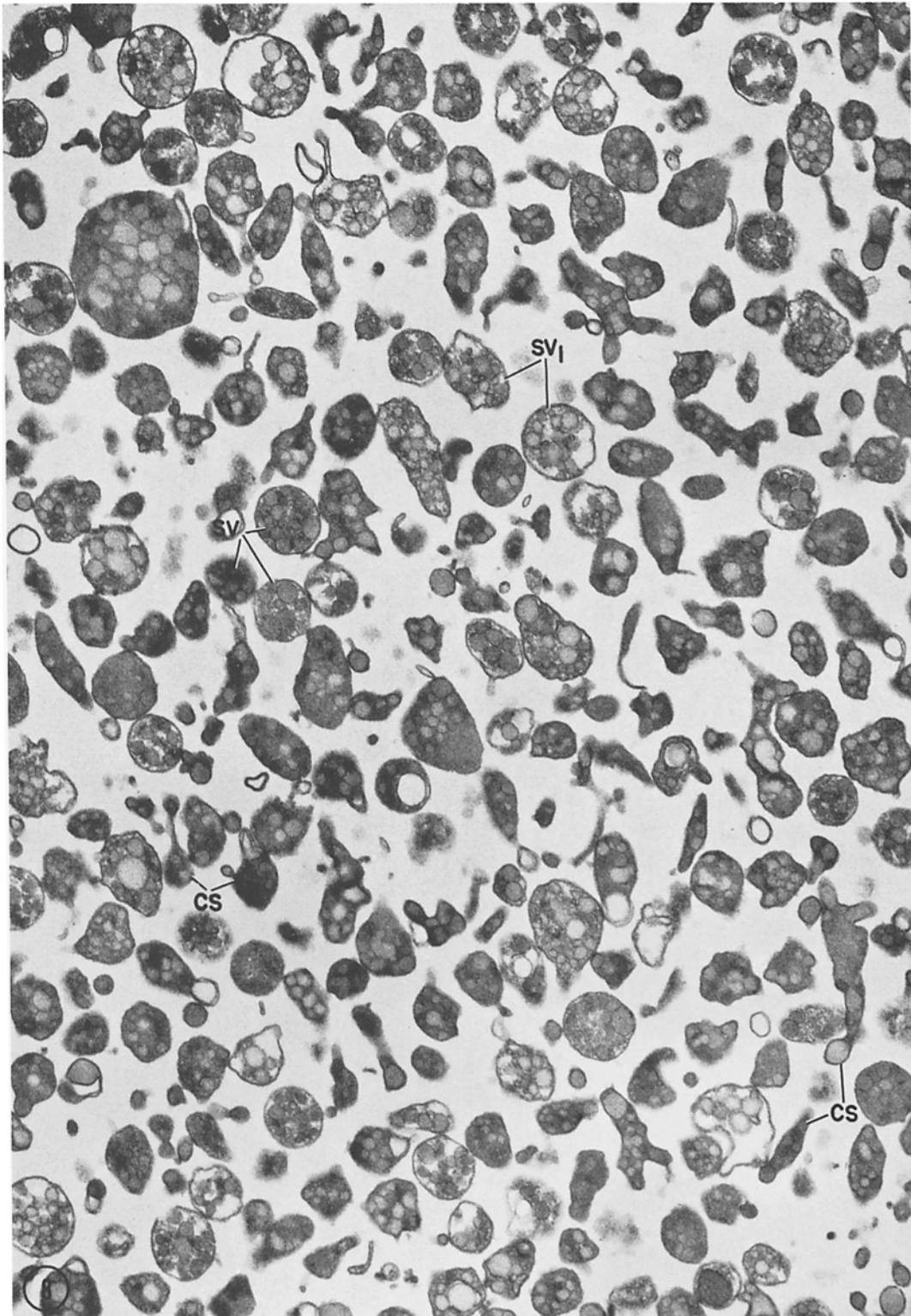
DISCUSSION

The effect of colchicine on the general physiology of the liver has been studied in some detail in perfused preparations. The results indicate that colchicine at 10 μ M concentration does not affect glucose production, intracellular ATP levels, oxygen consumption, protein and lipid synthesis, nor ureogenesis but causes a marked inhibition of VLDL release (17). Colchicine has also been shown to inhibit VLDL release to the extent of 50–80% in intact rats pretreated for 3 h with 0.25–2.5 μ mol of drug/100 g body weight (36).

Our studies show that colchicine and vinblastine inhibit the secretion of plasma proteins by liver cells both in vivo (Figs. 4 and 5) and in vitro (Figs. 1 and 2). Concomitant with reduced secretion, these drugs caused an accumulation of the nonsecreted proteins within the cell (Figs. 2 and 4). In the intact animal the colchicine effect is prompt; it is detected within minutes (Fig. 4), and it is durable (Fig. 5). The sites of accumulation of secretory proteins, as shown by cell fractionation and electron microscopy of intact liver cells, are the Golgi complex and Golgi-derived secretory vacuoles. Intracellular transport of secretory proteins in liver proceeds from RER to SER, to the Golgi complex (26). The time-course presented in Fig. 15 confirms this progression except that a time interval between smooth microsomes and heavy Golgi fraction (Golgi III) is not resolved. The data suggest that the transit from SER to the Golgi complex is so rapid that kinetic differences can be obscured by individual variations among animals and possibly by some cross-contamination between the corresponding cell fractions. The experiments show, however, that colchicine does not affect the movement of secretory proteins in the RER or the SER, nor does it affect filling of the Golgi vesicles; it impedes instead discharge and leads to accumulation of secretory protein in all Golgi elements (Figs. 8–18).

The mechanism by which colchicine elicits this response is not known. We have shown that the effect is due neither to a decrease in the intracellular level of nucleotide phosphates (Fig. 7) nor to an impairment of protein synthesis (Fig. 3).

Since colchicine has a high specific affinity for tubulin and inhibits tubulin polymerization into microtubules (3, 4), the effects of this drug on several secretory systems have been ascribed to its interaction with microtubules (19, 23). The data show that the effect of colchicine on secretion, like



FIGURES 8-10 Golgi fractions isolated from colchicine-treated rats 1 h after the administration of the drug (25 $\mu\text{mol}/100\text{ g}$ body weight). Representative fields in sectioned pellets. At 3 h after colchicine the appearance of the fraction is the same except for the VLDL load which is heavier.

FIGURE 8 Golgi I. The preparation is homogeneous and consists of numerous rounded secretion vacuoles (sv) and relatively few cisternae (cs), all filled and marked by VLDL particles embedded in an amorphous dense matrix; (sv₁) marks secretion vacuoles which have lost part of their VLDLs. $\times 37,000$.

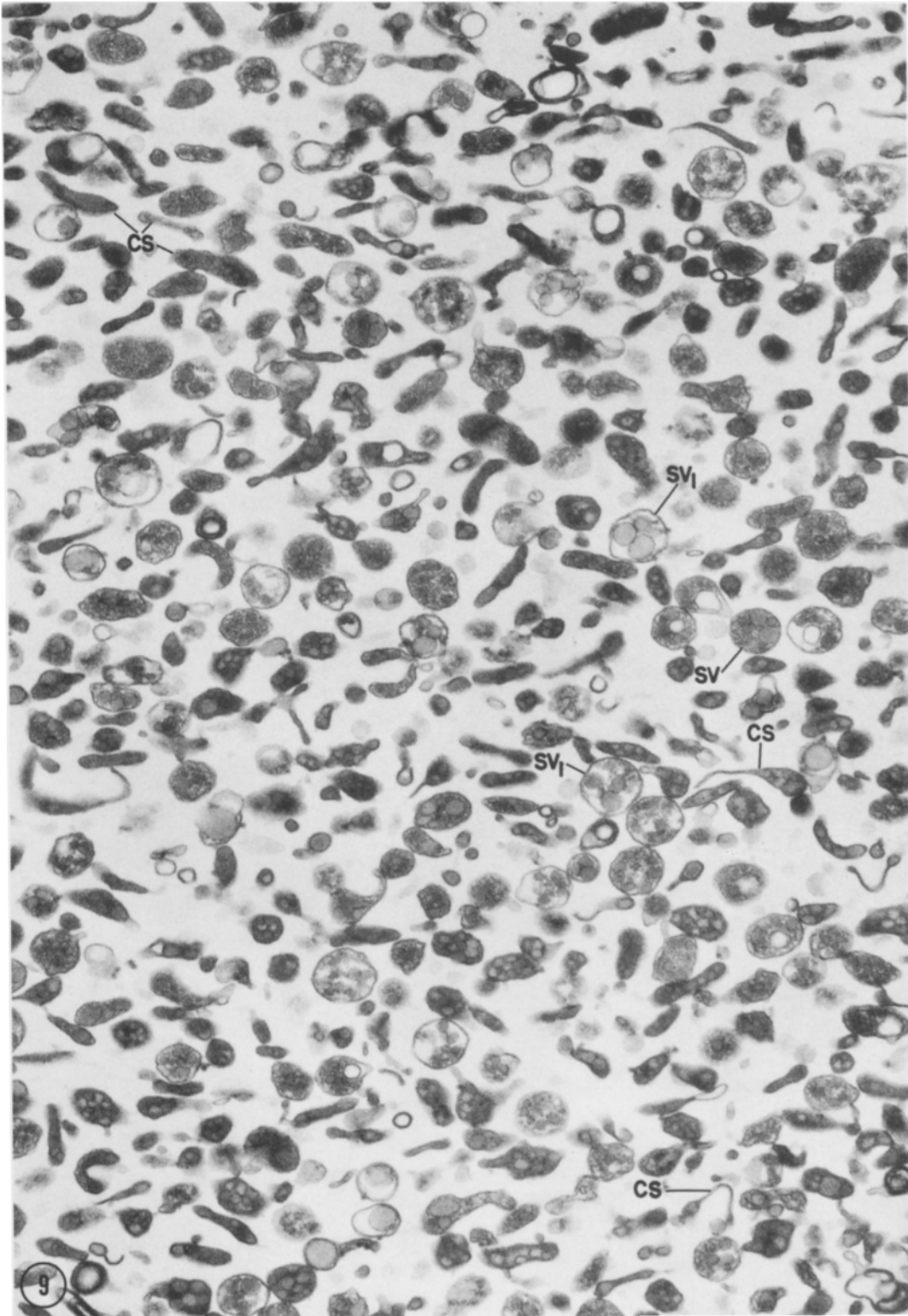


FIGURE 9 Golgi II. The predominant elements are still secretion vacuoles (*sv*) and cisternal elements (*cs*). They are of generally smaller size than in Golgi I and many appear to have lost in part their VLDL content (*sv₁*). $\times 37,000$.

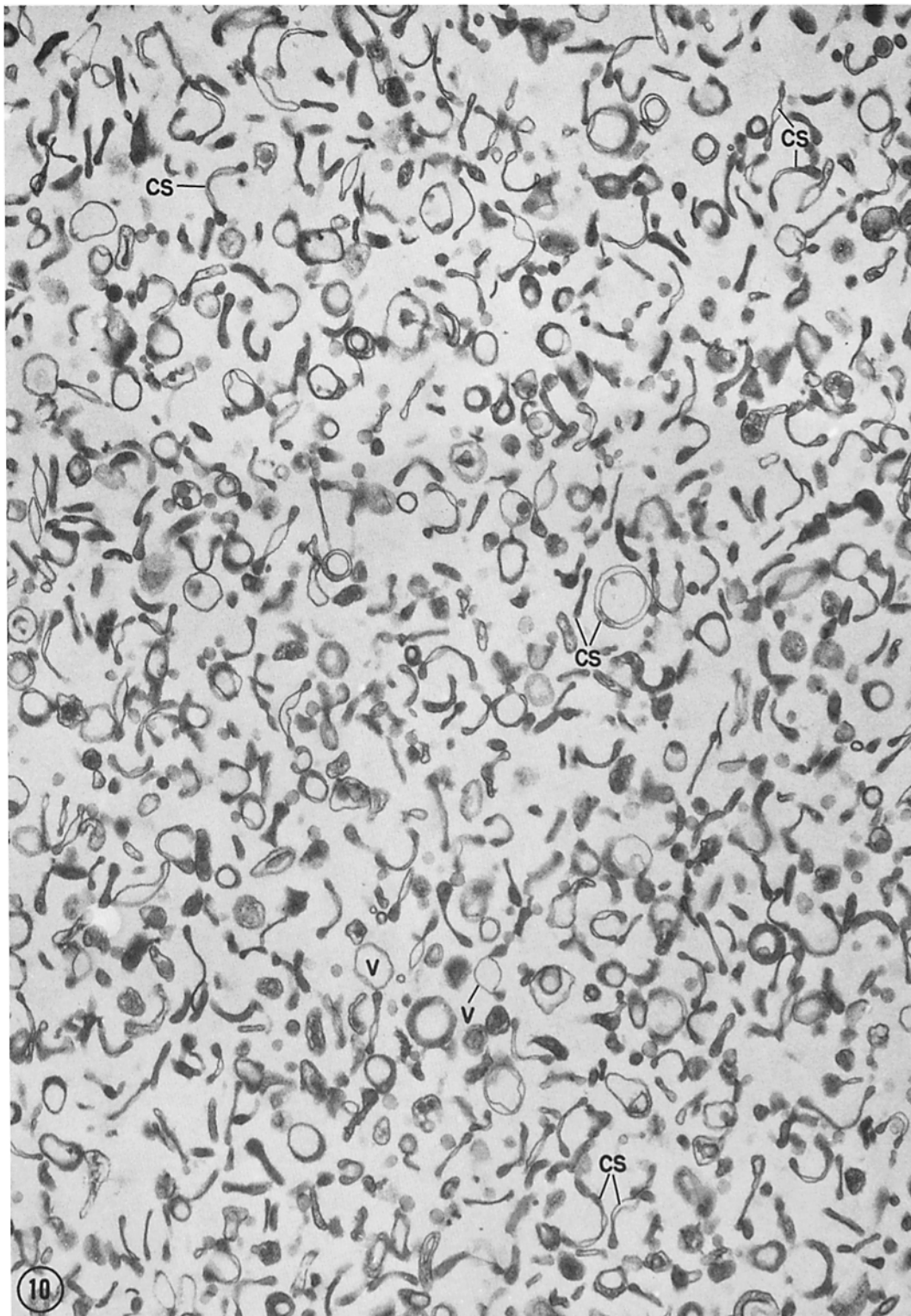
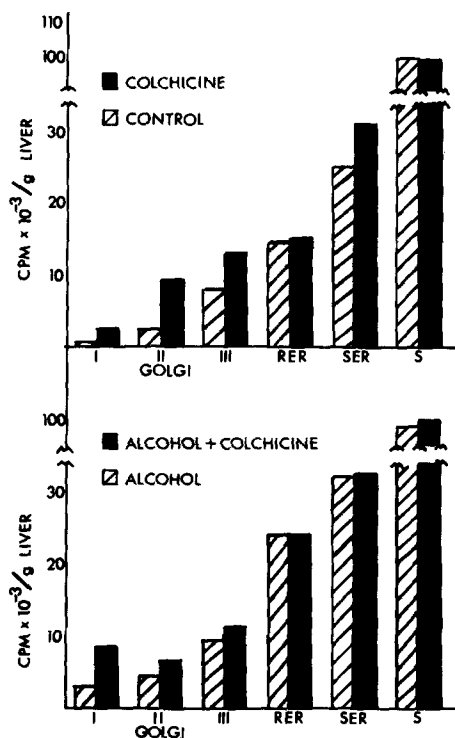


FIGURE 10 Golgi III. The dominant elements in this fraction are flattened Golgi cisternae (*cs*) with slightly distended rims occasionally marked by VLDL particles. The intracellular origin of the large empty vacuoles (*v*) is uncertain. $\times 36,000$.



FIGURES 11 and 12 Intracellular distribution of radioactive TCA-precipitable protein in rat liver 1 h after the administration of L-[U-¹⁴C]leucine. Rats were injected with 25 μ mol/100 g body weight of colchicine; control rats received an equal volume of saline. Both colchicine and saline were followed by the administration of 12.5 μ Ci/100 g body weight of L-[U-¹⁴C]leucine. All injections were given in the femoral vein. 1 h after administration of L-[¹⁴C]leucine the livers were removed and cell fractionation was performed as described in Materials and Methods. Rats were fed alcohol by stomach tube 30 min before the administration of L-[¹⁴C]leucine.

FIGURE 11 Describes the results in terms of radioactivity of TCA-precipitable protein in the cell fractions obtained from 1 g of liver. The homogenate fraction of control animals contained 454,800 cpm/g liver and that of colchicine-treated rats contained 497,500 cpm/g liver.

its effect on tubulin, is "specific," since plasma protein secretion is not inhibited by: (a) lumicolchicine, which does not bind to tubulin, (b) griseofulvin, an antimetabolic agent which does not block the *in vitro* polymerization of tubulin (43), and (c) cytochalasin B which does not bind to tubulin but inhibits glucose uptake (21) and is thought to disorganize microfilaments (40).

There is, however, little evidence in normal rat

hepatocytes of microtubule concentration and special orientation at the discharge sites, where we have localized the colchicine effect; and in our *in vivo* experiments microtubules are still recognizable in the Golgi region, in the vicinity of bile capillaries, and along the sinusoidal front of the hepatocyte, after colchicine treatment (see, however, reference 17 for opposite findings.). There is, therefore, no consistent correlation between microtubule depolymerization and secretory inhibition, but more subtle effects limited to the point of interaction of microtubules with the plasma membrane cannot be ruled out.

Besides tubulin binding, colchicine has, however, multiple cellular effects which may be involved in the secretory inhibition described. It has been claimed that it binds to isolated membrane fractions (11, 35), it seems to affect the distribution

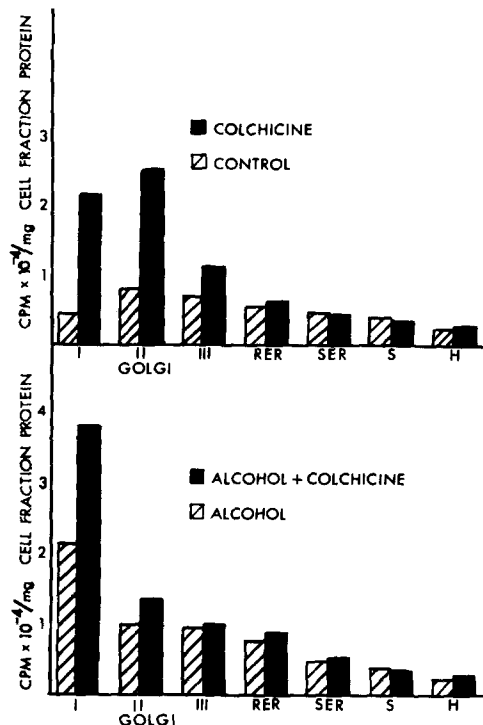
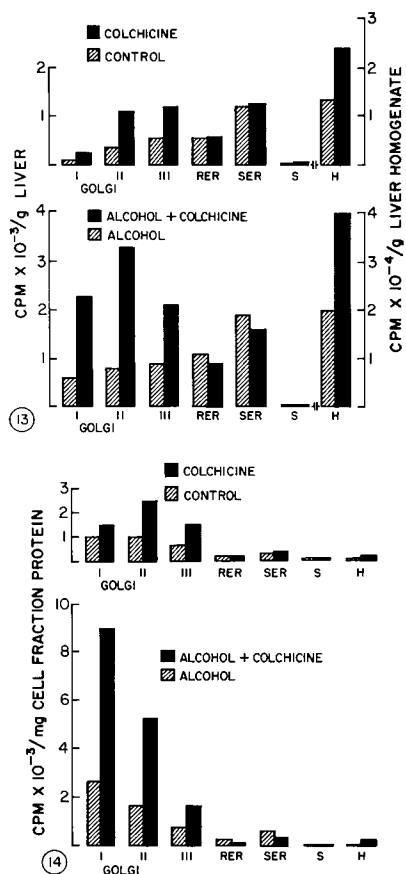


FIGURE 12 Gives the same results as TCA-precipitable radioactivity per milligram of protein from the isolated cell fraction. The recoveries of protein in cell fractions isolated from 1 g of liver are shown in Table II. The solid bars represent colchicine (or alcohol + colchicine)-treated rats; the cross bars represent control (or alcohol-treated) rats. RER, rough microsomal fraction; SER, smooth microsomal fraction; S, final supernate; H, homogenate.



FIGURES 13 and 14 Distribution of nascent albumin in rat liver 1 h after L-[U-¹⁴C]leucine administration: effect of colchicine. The experimental protocol was the same as for Figs. 11 and 12 except that radioactivity was determined in the albumin present in the various cell fractions, instead of in TCA-precipitable proteins. Solid bars refer to colchicine (or alcohol + colchicine)-treated rats, and the cross bars represent control (or alcohol-treated) animals. As in Figs. 11 and 12 the data in the top figure were obtained from normal rats and the bottom figure from alcohol-fed rats. In Fig. 13 the data are normalized per gram liver, whereas in Fig. 14 the

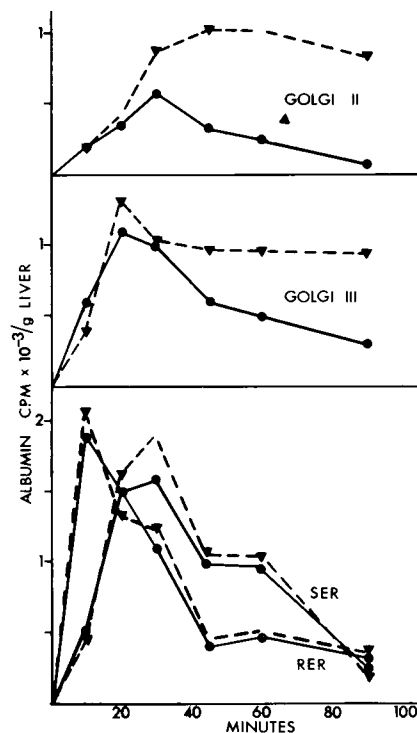


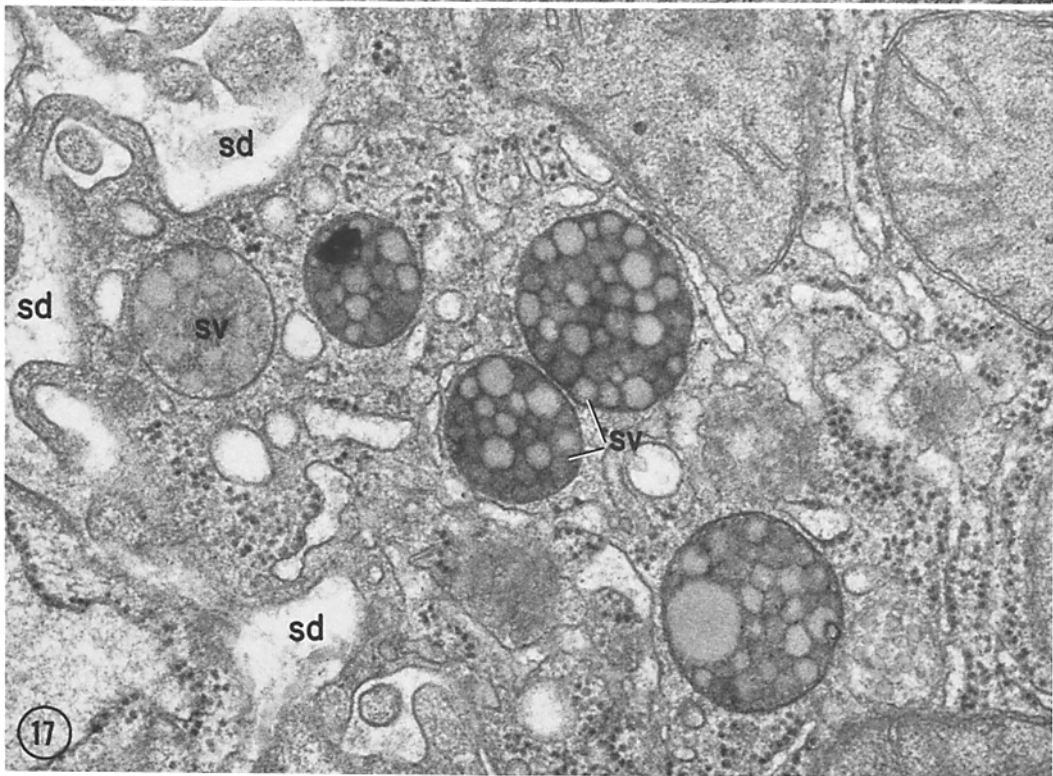
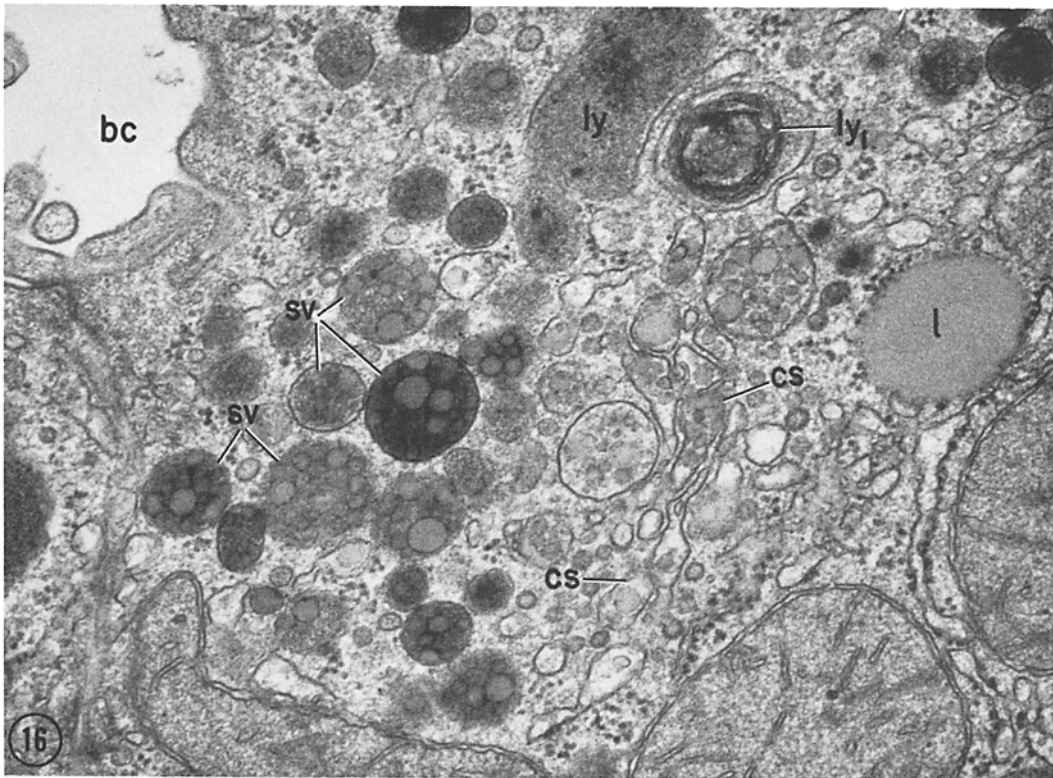
FIGURE 15 Time-course of distribution of nascent albumin in various liver cell fractions in control and in colchicine-treated rats. Rats were treated as described in the legend of Figs. 11 and 12, and the livers were removed at the specified times after the injection of L-[U-¹⁴C]leucine. Each time point represents a different experiment. The dotted lines represent the amounts of nascent albumin in colchicine-treated rats and the solid lines represent nascent albumin in the control animals. For the designation of the cell fractions, see the legend of Figs. 11 and 12.

results are expressed per milligram cell fraction protein. For the designation of the cell fractions, see the legend of Figs. 11 and 12.

FIGURES 16-18 Hepatocytes 1 or 3 h after administration of colchicine in vivo (25 μ mol/100 g body weight). All specimens were fixed by perfusion with glutaraldehyde-formaldehyde and postfixed in OsO₄ as indicated in Materials and Methods.

FIGURE 16 Golgi region in the vicinity of a bile capillary (*bc*). The cisternal elements (*cs*) of the Golgi complex are partially filled with VLDL particles. The rest of the Golgi region is occupied by numerous VLDL-containing secretion vacuoles (*sv*). Lysosome, *ly*; lysosome formed in connection with an autophagic vacuole, *ly*₁; lipid droplet, *l*. \times 41,000.

FIGURE 17 Cell periphery on the sinusoidal front of the cell. Cluster of VLDL-containing secretion vacuoles, *sv*; space of Disse, *sd*. \times 49,000.



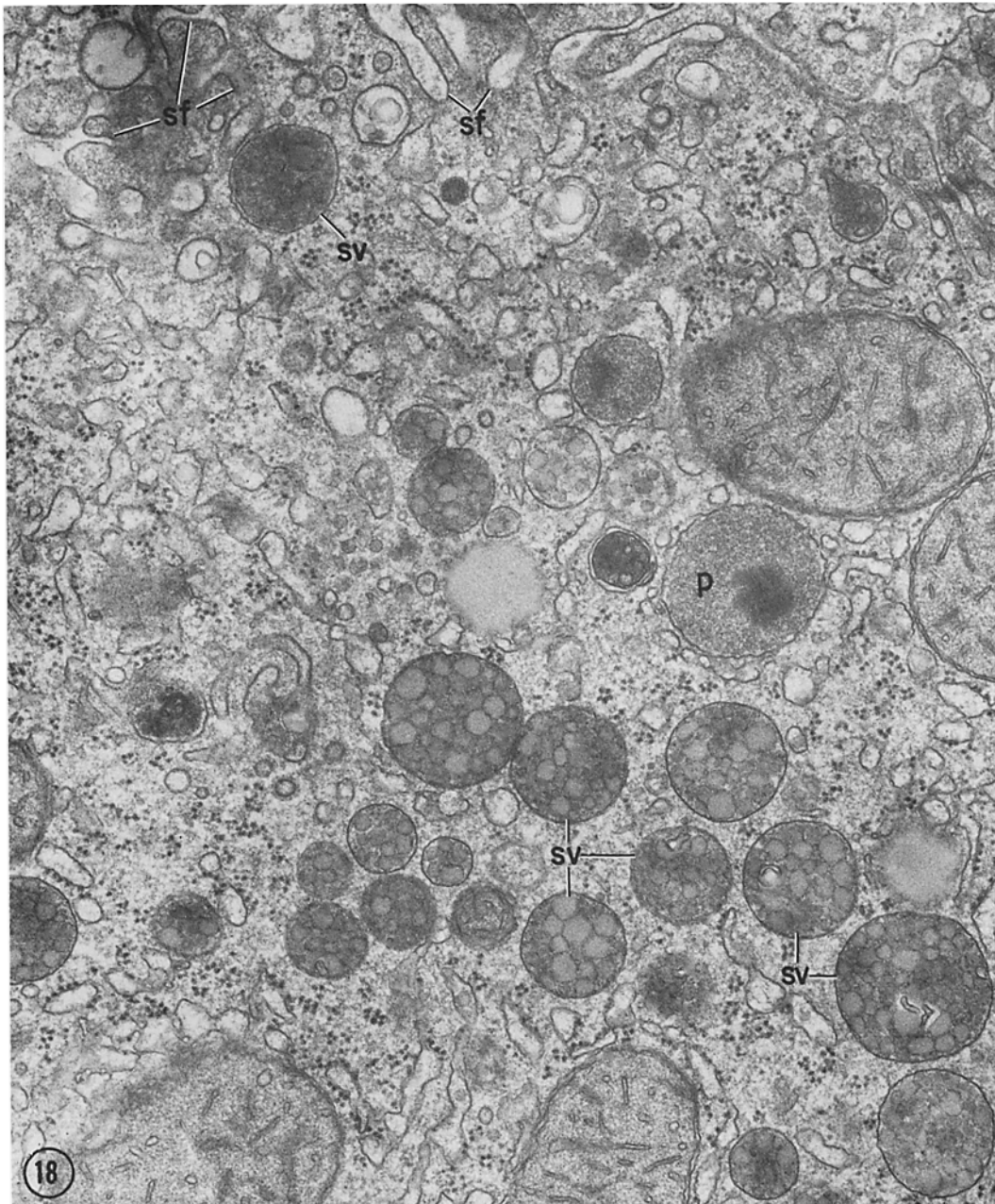


FIGURE 18 Large field in the cytoplasm of a hepatocyte containing numerous secretion vacuoles (sv) filled with VLDL particles. Sinusoidal front of the cell, sf; peroxisome, p. $\times 32,000$.

of intramembrane particles in *Tetrahymena pyriformis* (44), it causes a redistribution of lectin-binding sites on the membrane of leukocytes (22), and it has been shown to affect nucleoside transport promptly and drastically (20, 39). Vinblastine

modifies the behavior of erythrocytes in hypotonic hemolysis presumably by accumulating in, and expanding, their plasmalemma (34).

With the evidence available, we cannot ascribe the inhibitory effect of colchicine on plasma pro-

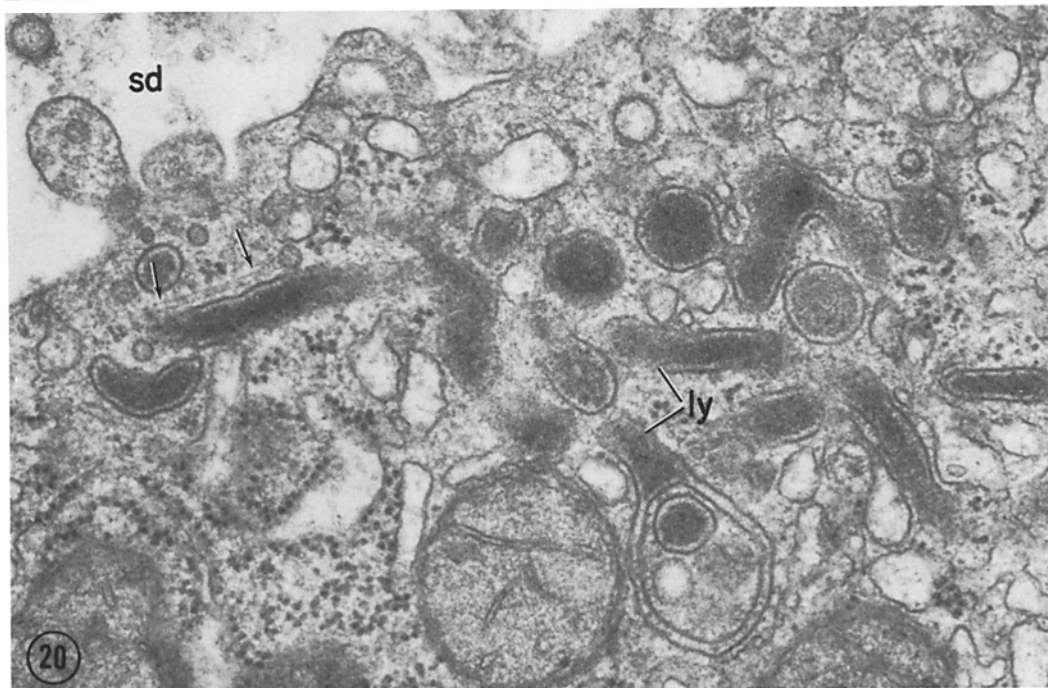
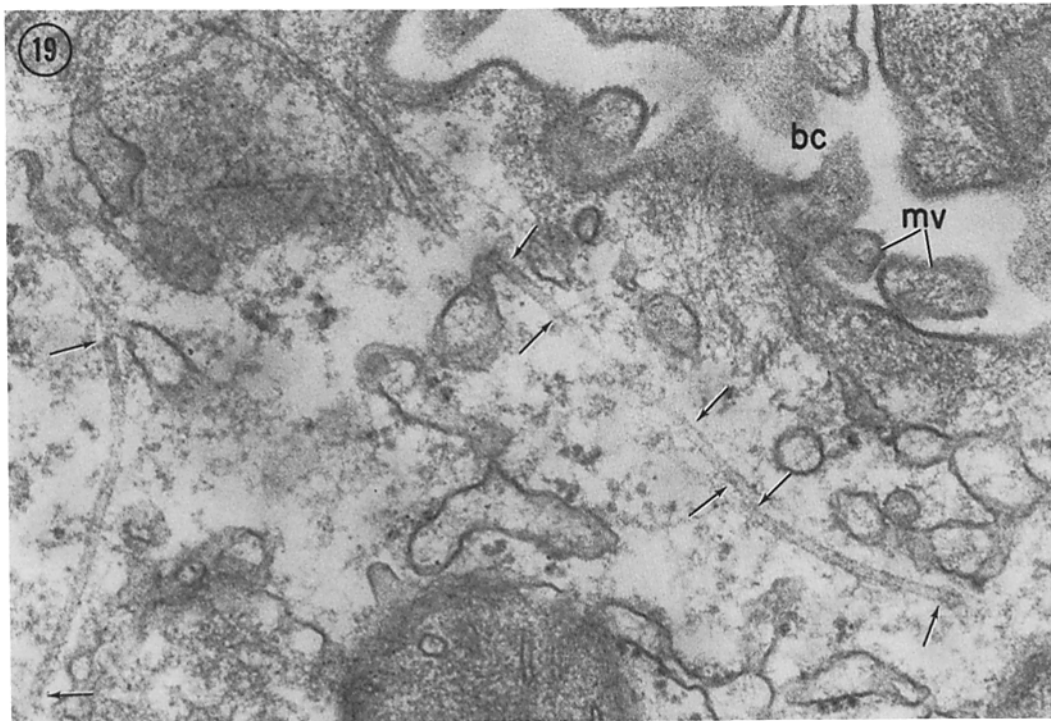


FIGURE 19 Golgi region and vicinity of a bile capillary (*bc*) in a hepatocyte 3 h after colchicine administration ($25 \mu\text{mol}/100 \text{ g}$ body weight). Four microtubules (arrows) traverse the region at various angles; microfilaments appear in bundles at some distance from the cell surface, or as a feltwork immediately under the plasmalemma. Microvilli in the bile capillary, *mv*. $\times 80,000$.

FIGURE 20 Periphery of a hepatocyte along the sinusoidal front. Space of Disse, *sd*; lysosomes, *ly*. Lysosomes of the type illustrated in this field as well as autophagic vacuoles appear to increase in frequency in colchicine-treated animals. A microtubule running parallel to the cell surface is marked by arrows. $\times 50,000$.

tein secretion to microtubule depolymerization, or to a direct effect on the metabolism of the hepatocyte. The inhibitory mechanism remains to be explained by further studies. The experiments which we have described, however, localize the colchicine effect at a post-Golgi stage and indicate that the drug acts by hampering discharge of secretory proteins from Golgi-derived secretory vacuoles into the space of Disse.

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