CYTOBIOLOGIE

European Journal of Cell Biology

Volume 13 · Number 1 · June 1976

WISSENSCHAFTLICHE VERLAGSGESELLSCHAFT MBH

BOOK REVIEWS

CYTOBIOLOGIE
13, 199–210 (1976)

The effect of microtubular inhibitors on secretion from liver into blood plasma and bile

Die Wirkung mikrotubulärer Hemmstoffe auf die Sekretion der Leber in das Blutplasma und die Galle

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Received April 10, 1976

Abstract

Liver – microtubular inhibitors – secretion - plasma – bile

Injection of either colchicine or vinblastine into rats in vivo reduced the levels of coagulation factors V and VII and triglycerides in blood plasma. In contrast, lumicolchicine, a structural isomer of colchicine which does not disrupt microtubules, had no effect on the levels of coagulation factors or triglycerides in blood plasma. The reduction in the levels of proteins and triglycerides in blood is accompanied by an accumulation of vesicles within the hepatocyte, as seen under a light microscope. These vesicles have been identified as Golgi-derived vesicles by electron microscopy. Golgi-derived secretory vesicles isolated from rat liver were found to contain more protein, coagulation factor V and triglycerides after injection of colchicine or vinblastine. These findings suggest an involvement of microtubules in secretion from liver into blood plasma. On the other hand, excretion of bilirubin-glucuronides and fluid into bile was not affected by these chemicals. This indicates that glucuronides are not transferred to the extracellular space by vesicular transport.

Introduction

The interference of microtubular inhibitors with the secretion of proteins and lipoproteins from the hepatocyte into the blood plasma studied in different laboratories [11, 12, 19, 22, 23] suggest an involvement of the microtubular system in this process. Colchicine and vinblastine reduces the serum triglyceride level in rats and concomitantly

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inhibits the release of proteins associated with serum very low density lipoproteins (VLDL) and serum high-density lipoproteins (HDL) [22]. As shown recently [19] microtubular inhibitors decrease the secretion of albumin and other unidentified proteins in vivo and in rat liver slices. Also, hepatocytes, incubated in suspension with colchicine, released fibrinogen at reduced rates [4]. Since these drugs do not impede the early steps in the secretory process, namely, the synthesis and the movement of secretory products from the rough endoplasmic reticulum to the Golgi complex, it was concluded that they inhibit discharge of proteins and lipids accumulated in Golgi-derived secretory vesicles [11, 12, 19, 22, 23]. These findings were further strengthened by morphological studies, since the accumulation of secretory vesicles filled with VLDL particles can be easily identified by electronmicroscopy [11, 12, 19, 22].

The antimitotic drugs colchicine and vinblastine are characterized by a specific binding to a protein, named tubulin, which in its polymeric state builds up microtubules. Therefore most of the effects evoked by these agents have been ascribed to microtubules.

The final steps of secretion, which seem to be impaired by microtubular inhibitors, involve the fusion of Golgi-derived vesicles with the plasma membrane. Therefore the interaction of the drugs colchicine and vinblastine with membranes is of particular interest. Nonspecific binding of colchicine has been reported for subcellular membranes of different tissues including liver (cf. [21]). Colchicine also seems to affect the distribution of intramembraneous particles in *Tetrahymena pyriformis* [25] as well as redistribution of lectin binding sites on the membrane of leukocytes [17].

To distinguish whether the effects of antimitotic drugs on the secretion of the liver are related to microtubules or are due to interaction with membranes, we used lumicolchicine which binds to membranes nearly as well as does colchicine [21] but lacks specific binding to tubulin [24]. The action of this structural isomer of colchicine on the secretion of triglycerides and proteins from rat liver was investigated and compared with that of colchicine and vinblastine. To study whether the secretion of proteins other than albumin [19], fibrinogen [4], or those associated with VLDL or HDL [22] are reduced after treatment of rats with microtubular inhibitors, we determined the levels of coagulation factors in the blood plasma. The accumulation of triglycerides and proteins inside the hepatocyte was followed by isolation of Golgi-derived secretory vesicles from rats injected with antimitotic drugs. To obtain information about the pathway leading from the hepatocyte to the bile, the excretion of fluid and bilirubin glucuronide was measured after injection of microtubular inhibitors.

Materials and methods

Female Sprague Dawley rats (180 to 220 g) were used throughout. The rats were fasted for 12 hours and anaesthetized with hexobarbital (100 mg/kg) by intraperitoneal injection. The general bile duct and the right jugular vein were cannulated with PVC tubing. To prevent hyperthermic alteration of the bile flow [10] the animals were warmed by means of a heating lamp. 0.9 ml blood was withdrawn every hour with a syringe containing 0.1 ml 0.1 M sodium citrate. In order to keep the blood volume constant, 0.9 ml 0.9 % sodium chloride was injected after each withdrawal. In separate experiments, after the first sampling the replacement solution contained colchicine (0.5 mg/100 g body weight, E. Merck, Darmstadt, Germany) or vinblastine (1 mg/100 g body weight, a gift from Eli Lilly GmbH, Giessen, Germany). The remaining replacement solutions contained only sodium chloride. After removal of cells by centrifugation plasma was assayed for prothrombin time, factor V, factor VII and triglycerides:

Prothrombin time was determined by adding to 0.1 ml of prewarmed plasma 0.2 ml of 1 : 1 mixture of 25 mM $CaCl_2$ and thromboplastin (Hoffmann-La Roche, Basel, Switzerland). Factor V activity was tested by mixing in prewarmed test tubes 0.1 ml of a solution of plasma deficient in factor V (Behring Werke AG, Marburg, Germany) with 0.1 ml plasma (diluted 1 : 20 in 0.05 M diethyl barbiturate-acetate buffer, *p*H 7.6) incubating for exactly 30 sec. at 37° C and then adding 0.2 ml prewarmed calcium thromboplastin solution (Behring Werke AG, Marburg, Germany). Factor VII-activity was determined in a similar way using factor VII-deficient plasma (obtained from Merz + Dade AG, Bern, Switzerland) and a 1 : 10 dilution of the plasma to be tested in a solution containing 2.8×10^{-2} M sodium barbital, 1.25 M $\times 10^{-1}$ M sodium chloride, buffered at *p*H 7.35. To start the reaction, 0.2 ml of 1 : 1 mixture of 25 mM $CaCl_2$ and thromboplastin (Merz + Dade AG, Basel, Switzerland) were added. The time of clot formation after the addition of Ca^{2+} was measured in a coagulometer according to SCHNITGER and GROSS (Heinrich Amelung, 492 Lemgo-Brake, Germany) and compared to a standard curve prepared by testing dilutions of normal pooled plasma (mixture of five plasmas) and plotting their clotting times on log-log paper. 100 units of coagulation factors were taken to be the amount present in 1 ml of pooled plasma.

Triglycerides were assayed by the enzymatic determination of glycerol obtained after alkaline hydrolysis [5].

Bile was collected in cooled and darkened polyethylene vessels containing 0.2 ml 10 mM EDTA. The vessels were changed every hour. Azopigments derived from conjugated bile pigments were analyzed by coupling with the diazonium salt of ethyl anthranilate [9].

Golgi-fractions were isolated from rat liver homogenates of control rats and of drug-treated rats four hours after intraperitoneal injection of 0.5 mg/100 g body weight colchicine or 1 mg/100 g body weight vinblastine. As a slight modification of the isolation procedure [6] we buffered all solutions with 0.01 M cacodylate at *p*H 7.4. Lumicolchicine was prepared by irradiation of colchicine with ultraviolet light [24]. Protein was determined as described [13] using crystalline bovine serum albumin as a standard. All chemicals not specified were of the purest grade commercially available.

For electron microscopic investigations, liver slices of both the control rats and those injected four hours previously with microtubular inhibitors were fixed at room temperature by treatment with 2 % glutaraldehyde in 0.25 M sucrose, 0.01 M cacodylate at *p*H 7.4 for 60 min. After postfixation in 1 % osmium tetroxide in the same buffer the slices were dehydrated with ethanol and embedded in Epon 812. Thin sections were cut with a Reichert ultramicrotome, stained with uranyl acetate and lead citrate in the conventional manner and

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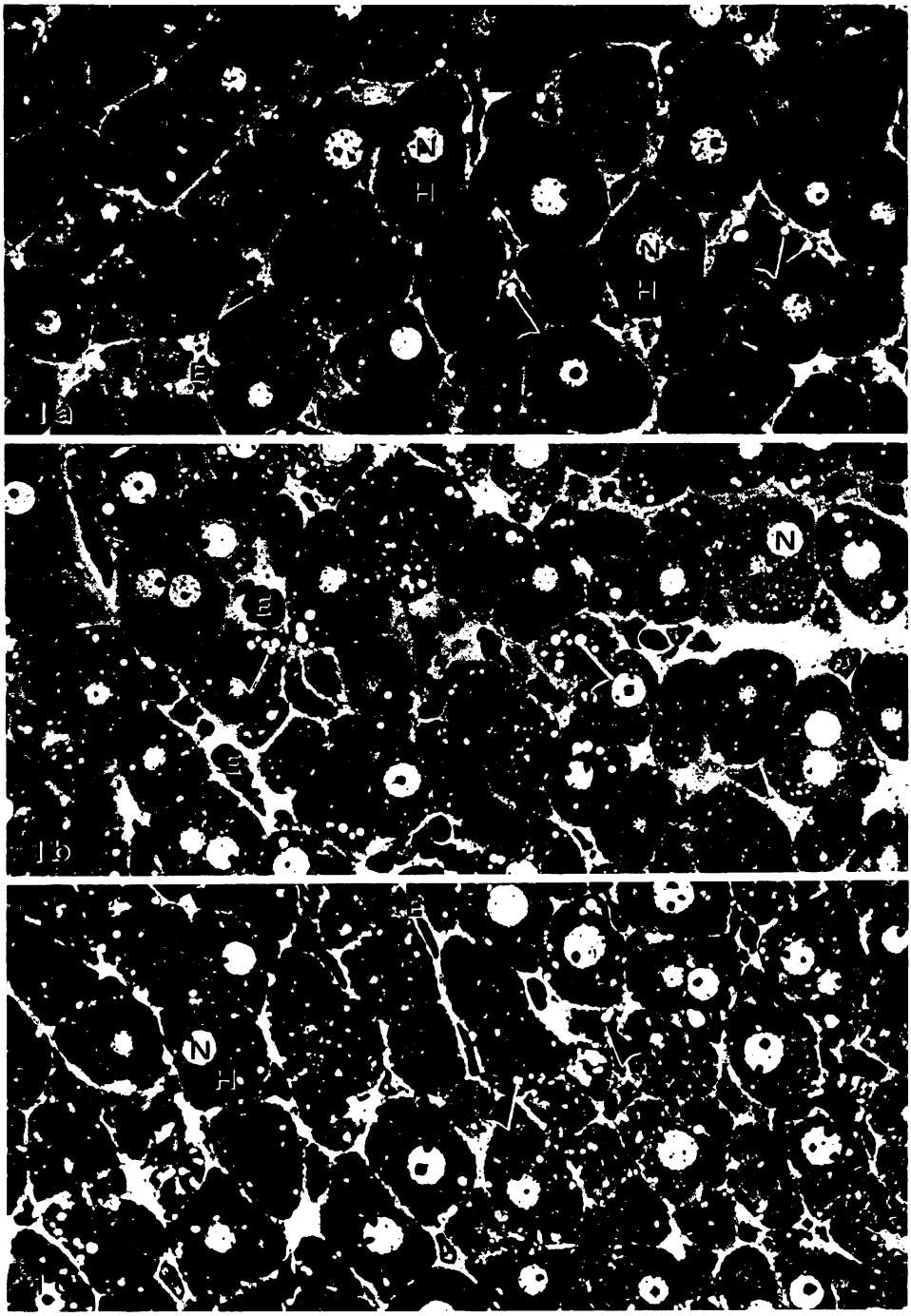
Figure 1 see page 202, Figure 2 see page 203.

Fig. 1. Semithin-sectioned rat hepatocytes observed under the light microscope. – H Hepatocyte. – N Nucleus. – E Erythrocytes. – **a.** Hepatocytes of control rats. Only a few lipid droplets (arrows) are visible in the dense homogenous cytoplasm. – 720 ×. – **b.** Hepatocytes after treatment with colchicine. Note the considerable increase of the number of the lipid droplets (arrows) in the cytoplasm of each cell. – 720 ×. – **c.** Hepatocytes after treatment with vinblastine. In these cells too the amount of lipid droplets (arrows) is higher than in control cells. – 720 ×.

Fig. 2. Two adjacent hepatocytes of vinblastine-treated rats. – **a.** Secretory vesicles *(arrows)* of varying size assembled in clusters can be demonstrated throughout the cytoplasm. – N Nucleus. – M Mitochondria. – ER Endoplasmic reticulum. – g Glycogen. – B Bile capillary. – 10 500 ×. – **b.** In higher magnification inside the secretory vesicles (V) beside the VLDL particles electron dense material *(arrows)* presumably secretory protein can be seen. – M Mitochondrion. – g Glycogen. – 50 000 ×.



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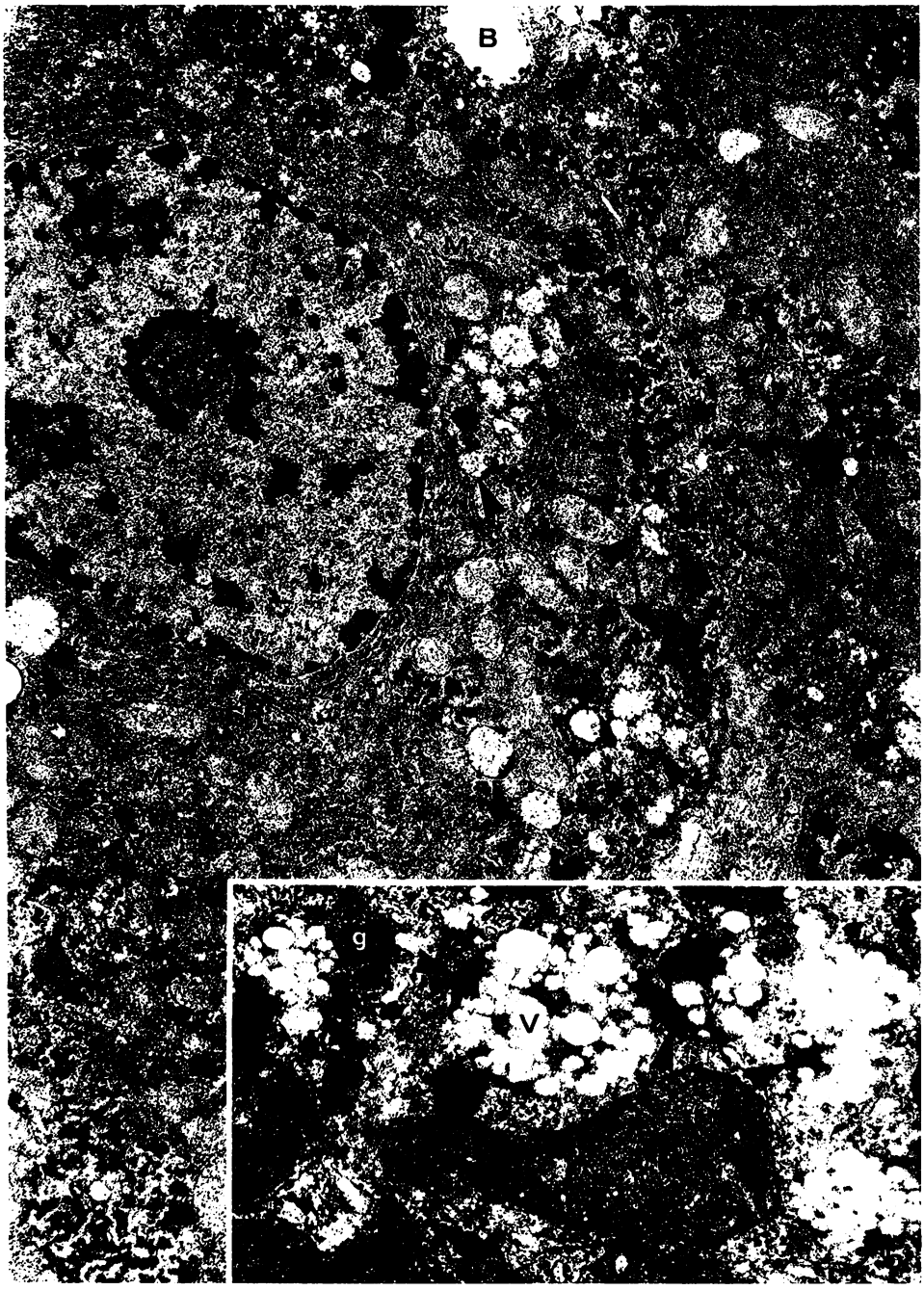

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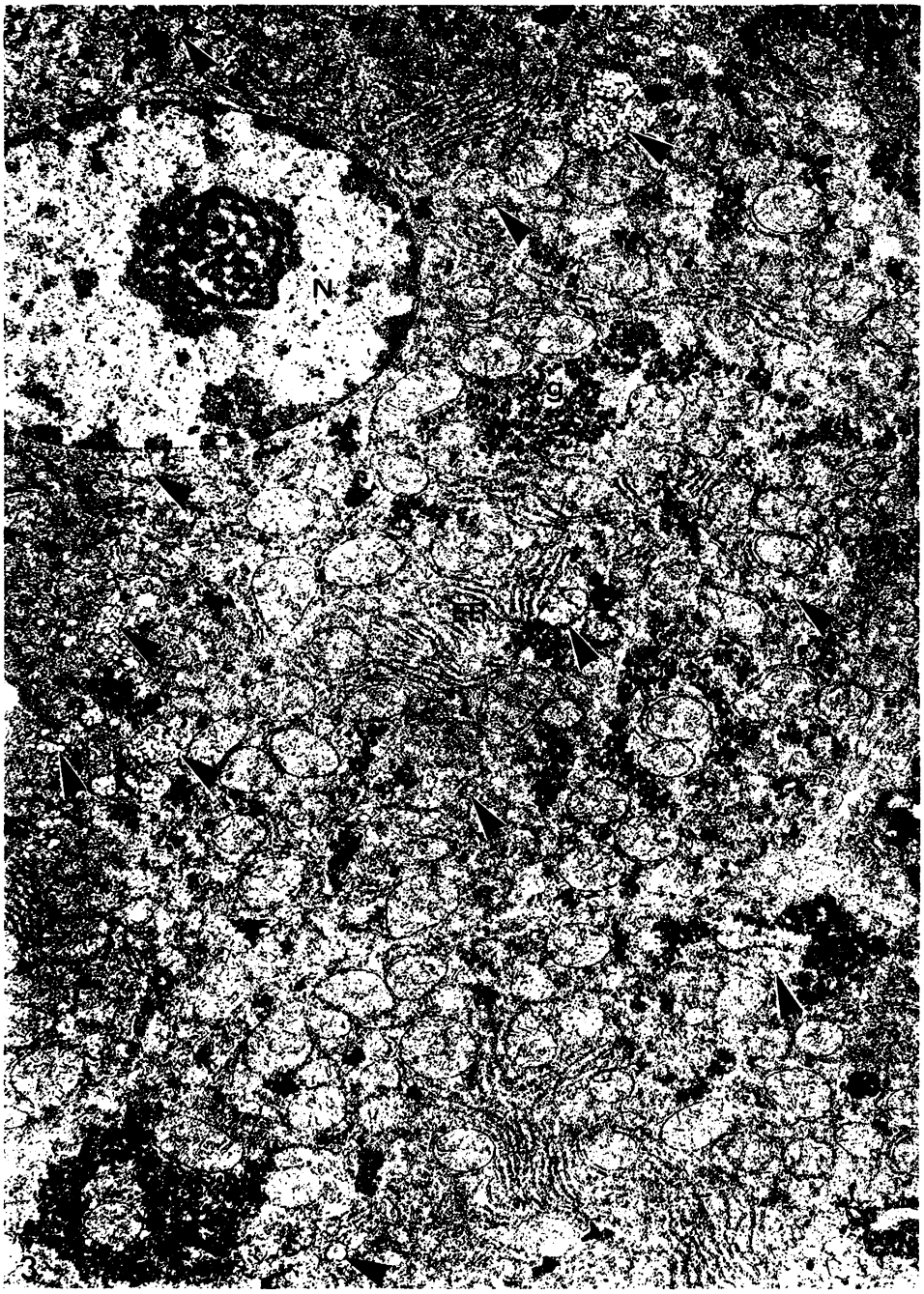


Fig. 3. Hepatocytes of colchicine-treated rats. Clusters of secretory vesicles *(arrows)* are scattered throughout the cytoplasm. – N Nucleus. – ER Endoplasmic reticulum. – g Glycogen. – 10 500 ×.

examined with a Siemens Elmiskop 101. For light microscopy epon embedded material was cut into sections of 1 µm and stained with methylene blue/azur according to RICHARDSON. Photographs were taken with a Zeiss Photomicroscope II on Agfapan 25 film.

Results

In comparison to control animals the injection of colchicine or vinblastine into rats led to an increase of lipid droplets within the hepatocyte as seen under a light microscope (Fig. 1 a to c). Electron microscopic examination of the same liver section revealed that most of these droplets contain VLDL-particles (Fig. 2, 3). In the untreated hepatocyte, secretory vesicles filled with VLDL particles are found in the Golgi-regions or scattered throughout the cytoplasm. Secretory vesicles discharge their content into the space of Disse and can easily be detected there in controls.

Microtubules were found in the peripheral cytoplasm of the hepatocytes as well as close to the nucleus. However, the number of identifiable microtubules was so small that changes in their amounts with or without drugs could not be determined.

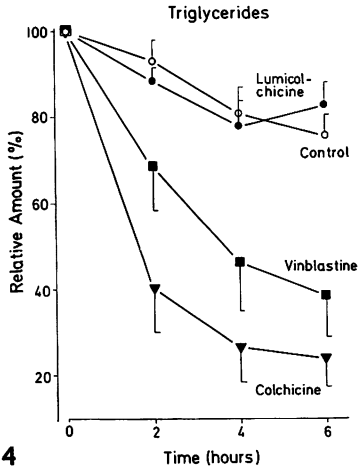
Vinblastine treatment leads to an apparent increase of secretory vesicles containing VLDL particles inside the hepatocyte (Fig. 2) and a concomitant decrease of VLDL particles in the space of Disse. These secretory vesicles vary in size and seem to contain more electron dense material than do those of the controls. Presumably this represents enclosed secretory proteins. This material was also seen in the livers of colchicine-treated rats, but was most prominent after treatment with vinblastine. These observations indicate an impairment in the transfer of secretory products from the intracellular to the extracellular fluid.

It has been demonstrated that vinblastine leads to a conversion of microtubules to paracrystals [1, 14]. In the present investigation paracrystals were observed occasionally in Kupffer cells but never in hepatocytes.

The ultrastructure of hepatocytes from rats treated with colchicine was similar to that observed in rats after injection of vinblastine (cf. Figs. 2, 3). The amount of secretory vesicles containing VLDL particles increased and very few VLDL-particles were seen in the space of Disse which often seems to be collapsed.

The VLDL-particles shown to accumulate within the hepatocyte after injection of microtubular inhibitors are rich in triglycerides [8]. As demonstrated in Figure 4 the increase of the amount of the secretory vesicles in the hepatocyte is accompanied by a decrease of plasma triglyceride levels in the intact rat. This finding accords with data presented by others [11, 12, 22, 23]. To investigate whether lumicolchicine, a drug known not to interfere with microtubules, exhibits the same effect, this structural isomer of colchicine was prepared and injected into rats. After injection of this drug (0.5 mg/100 g body weight) the levels of triglycerides did not differ from those of the control rats during the experiment (Fig. 4). The secretion of proteins was also unaffected by lumicolchicine as followed by assay of the coagulation factors (prothrombin time) in plasma (Fig. 5). Colchicine and vinblastine, in contrast, led to a drastic decrease in the levels of coagulation factors.

Assaying prothrombin time, the sum of several coagulation factors is determined. Those exhibiting the shortest half-life should decay rapidly if secretion is blocked. Therefore we assayed factor V and factor VII, which are known to fall to half in the plasma within hours [20] after administration of colchicine and vinblastine to rats (Figs. 6, 7). Compared to controls both drugs diminish the amount of these two coagulation factors present in blood plasma.



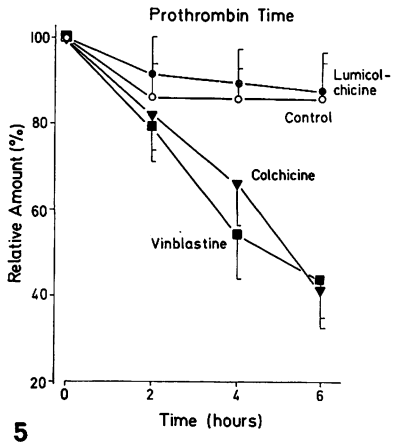
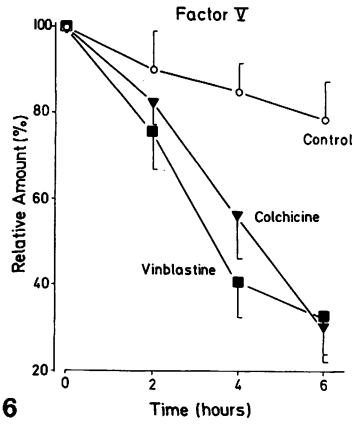


Fig. 4. Effect of colchicine, lumicolchicine or vinblastine on triglyceride levels in the plasma of rats in vivo. Where indicated microtubular inhibitors were injected after blood sampling at time 0. Each point is the mean value of 8 experiments (SEM).

Fig. 5. Effect of colchicine, lumicolchicine or vinblastine on the levels of coagulation factors (prothrombin time) in rat plasma. Where indicated, microtubular inhibitors were injected after blood sampling at time 0. Each point is the mean value of 6 experiments (SEM).



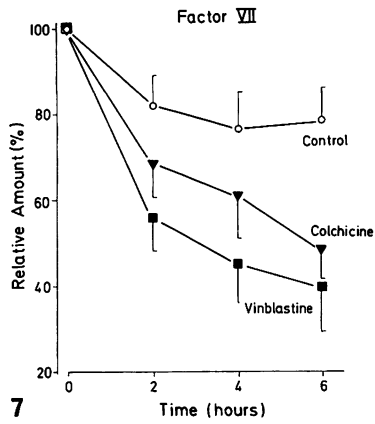


Fig. 6. Effect of colchicine or vinblastine on the level of factor V in rat plasma. Where indicated, microtubular inhibitors were injected after blood sampling at time 0. Each point is the mean value of 6 experiments (SEM).

Fig. 7. Effect of colchicine or vinblastine on the level of factor VII in rat plasma. Where indicated, microtubular inhibitors were injected after blood sampling at time 0. Each point is the mean value of 6 experiments (SEM).

To examine, whether the decreased levels of triglycerides and coagulation factors in plasma in fact are due to an impaired secretion, we isolated Golgi-derived secretory vesicles before and after treatment with microtubular inhibitors. Golgi fraction I represents primarily trans-Golgi elements from the secretory Golgi face [2, 6, 7] and these vesicles should accumulate inside the hepatocyte if secretion is blocked. Colchicine and vinblastine indeed cause an increase in the amount of secretory vesicles unable to discharge their content into the extracellular space (Tab. I). To analyze the intraluminal portion, which represents the material to be exported, the secretory vesicles were opened using a *p*H-jump method as follows: After titration of the suspension to *p*H 9.5 the vesicles were incubated for 20 min. at 37° C and than titrated back to *p*H 7.4. This represents a slight modification of a procedure already successfully applied to this fraction [6]. To remove Golgi membranes we centrifuged for 60 min. at 105 000 g and analyzed the supernatant for protein, triglycerides and coagulation factors. All secretory products were found to be at least doubled in isolated Golgi-derived secretory vesicles after treatment with colchicine or vinblastine (Tab. I). Unfortunately factor VII could not be determined in the extracted Golgi fraction because of the considerable inactivation of this protein during the incubation in alkaline medium.

Tab. I. Effect of colchicine or vinblastine (4 hours, intraperitoneally) on the recovery of Golgi I fraction and intraluminal compounds. Average of three experiments.

	Golgi I		Intraluminal		
	Triglycerides μ Mol/g liver	Protein mg/g liver	Protein mg/g liver	Factor V U/g liver	Triglycerides μ Mol/g liver
Control	0.037	0.049	0.030	0.43	0.031
Vinblastine	0.131	0.090	0.069	0.79	0.097
Colchicine	0.104	0.088	0.061	0.89	0.082

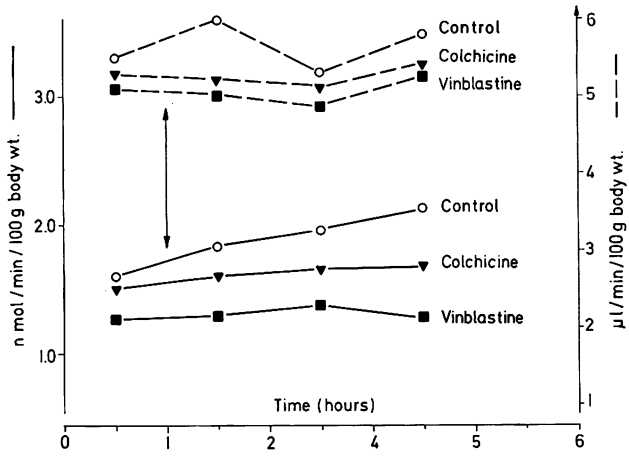


Fig. 8. Effect of colchicine or vinblastine on bile flow and bilirubin-glucuronide excretion. After a control period of one hour colchicine (0.5 mg/100 g body weight), vinblastine (1 mg/100 g body weight) or the solvent of these drugs, 0.9 % NaCl (control) were injected intravenously. Each point is the mean value of 4 experiments (SEM).

The lack of effect of colchicine on biliary excretion of phospholipids and cholesterol indicates that this transfer is not dependent on vesicular transport [22]. To find out whether this holds also for glucuronides we determined the excretion of fluid and bilirubin glucuronide into the bile. As shown in Figure 8 bile flow and transfer of bilirubin glucuronide is unchanged by microtubular inhibitors. Therefore, glucuronides, like biliary lipids, are not dependent on vesicular transport.

Discussion

The experiments here reported show that injection of microtubular inhibitors such as colchicine or vinblastine into rats in vivo decreases the secretion of coagulation factors from the liver. This decrease in secretion is accompanied by accumulation of secretory products within the hepatocyte. One of the coagulation factors tested could be traced back to its intracellular storage site. Together with undischarged triglycerides and proteins, coagulation factor V was found to have increased in the fraction of isolated secretory vesicles. Similar results were reported recently by others [19] who were able to show an increased accumulation of albumin inside secretory vesicles isolated from rat liver after injection of colchicine. From these studies and the observation of an increased protein content of isolated secretory vesicles as described [19] and as confirmed in this paper, it may be concluded that all plasma proteins except γ-globulins [15], accumulate in secretory vesicles inside the hepatocyte after administration of microtubular inhibitors. This conclusion is in accord with the observation of decreased secretion of fibrinogen by isolated hepatocytes [4] and of decreased levels of proteins associated with HDL and VLDL [22] in the plasma of rats treated with microtubular inhibitors. Also, it has been reported [11] that direct application of microtubular inhibitors to perfused mouse liver resulted in lowered release of albumin and other proteins into the perfusate. This study shows that the accumulation of secretory products can be detected by observations using a light microscope. However, under the higher magnification of an electron microscope, the nature of the vesicles observed is easily revealed by their load of VLDL-particles (cf. Figs. 1 b, c, 2, 3). The electron dense material observed inside the secretory vesicles in this study may represent accumulated proteins (Fig. 2 b).

In most of the recent studies, effects upon secretion observed after administration of colchicine or vinblastine were attributed to its effect on microtubules [11, 12, 22]. However, experiments concerning the binding of these drugs to subcellular membranes [21] cast some doubt on this concept especially since the release of proteins and lipids from the hepatocyte involves specific functions of the membrane of Golgi-derived vesicles as well as the plasma membrane. To examine the possible action of these drugs on subcellular membranes we used the technique of freeze-cleaving electron microscopy (G. DAHL and M. GRATZL, unpublished observations). Changes in the array of intramembranous particles as a function of temperature observed in membranes by others [25], or by interaction with colchicine or vinblastine [25] could not be observed in the contiguous surface of the plasma membrane. The microvillar surface, which would have been most interesting to observe, was unfortunately only exposed in small areas by this technique. Therefore the array of intramembranous particles in that part of the plasma membrane specialized for secretion could not be adequately determined. Thus, freeze-cleaving of hepatic tissue provided no evidence for the inhibitory effect of antimicrotubular drugs on secretion by interaction with membranes.

Lumicolchicine is a structural isomer of colchicine, which behaves like colchicine in its nonspecific binding to intracellular membranes but unlike colchicine lacks specific

binding to tubulin [21]. If the secretory process is affected by interaction of colchicine with membranes, comparable effects should occur with lumicolchicine. Since this was not the case our results favour the involvement of microtubules in the secretory processes studied.

The relationship between secretion and the microtubular system still remains to be clarified. Because of the relatively small number of morphologically identifiable microtubules in an untreated hepatocyte we could not relate the amount of microtubules after treatment with colchicine or vinblastine to the decrease in secretion as others have suggested [11, 12, 22]. In addition, the paracrystalline structures which appear if microtubules are treated with vinblastine [1, 14] were never observed in the liver parenchyma cells, although they were occasionally seen in Kupffer cells. Definitive proof for the existence of microtubules in the liver has been obtained from recent studies showning that tubulin, which binds antimicrotubular drugs is present in liver homogenates and can be purified therefrom [18].

The most striking observation of the present study is that drugs such as colchicine and vinblastine known to interfere with vesicular transport in the hepatocyte [11, 12, 19, 22, 23], do not inhibit fluid or bilirubin-glucuronide release into the bile. From the latency of the enzyme UDP-glucuronyl-transferase it has often been speculated that this enzyme is arranged in the endoplasmic membrane to allow the release of products into the intracisternal space. Due to the vectorial transfer during their formation, glucuronides could be carried to the secretory surface in membrane-bound vesicles (cf. [3]). Our results, however, clearly show that the transfer of glucuronides to the extracellular fluid is not dependent on vesicular transport. Studies concerning the permeability of glucuronides synthesized by isolated microsomal membranes [16] also suggest that these products are released into the cytoplasm. Another example of non-vesicular transport from the hepatocyte to the bile is the secretion of lecithin and cholesterol [22].

Acknowledgements. We thank Dr. G. Dahl for the performance of freeze-cleaving electron microscopy and its interpretation. The authors are indebted to Dr. P. Traylor for expert help during the preparation of this manuscript.

Parts of this paper have been presented at the 10th Meeting of the Federation of European Biochemical Societies, Paris, July 20–25, 1975 and the "International symposium on microtubules and microtubular inhibitors", Beerse (Belgium) September 2–5, 1975.

This work was supported by the Deutsche Forschungsgemeinschaft, mainly by the Sonderforschungsbereich 38 "Membranforschung".

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