Evidence for a Role of the Microtubular System in the Secretion of Newly Synthesized Albumin and Other Proteins by the Liver

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ABSTRACT Livers of normal mice were prefused *in situ* and the secretion of newly synthesized (i.e. labeled) proteins into the perfusate were measured. In control livers, the secretion of newly synthesized proteins was found to be linear with time. In marked contrast, when livers were perfused with vinblastine, vincristine, or colchicine, drugs known to interfere with the hepatic microtubular system, the release of newly synthesized proteins was either strongly inhibited or completely suppressed although total hepatic protein synthesis (estimated by the incorporation of labeled amino acids into hepatic plus perfusate proteins) remained unaltered. Chromatographic separation of the various secreted proteins showed that the release of albumin, globulins, and small polypeptides was decreased to a similar extent by vincristine or colchicine. In the particular case of albumin, it was further observed that total (i.e. liver plus perfusate) labeled amino acid incorporation into albumin was not altered by either vincristine or colchicine, whereas the incorporation of these amino acids into liver albumin was markedly increased but incorporation into perfusate albumin was decreased, suggesting that the translocation of this particular protein from the liver to the perfusate had been affected by the presence of these drugs. It is proposed that the functional integrity of microtubules is necessary for the intracellular movement and eventual release of albumin and other proteins by the liver, and suggested that microtubules might possibly be a site of regulation of hepatic protein secretion.

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INTRODUCTION

Previous studies carried out either in an isolated system, the perfused livers of normal mice (1–3), or in vivo using normal rats (4) have shown independently that an intact microtubular system was necessary for the release of very low density lipoproteins (VLDL) by the liver. The experiments performed with the perfused mouse livers demonstrated in particular that the presence of colchicine or vincristine, drugs known to interfere with the microtubular system (5, 6), resulted in a marked decrease in the secretion of VLDL into the perfusate and an accumulation of triglycerides in the liver at a time when electron microscopy revealed that microtubules had virtually disappeared from the hepatocytes and VLDL-like particles had accumulated within the parenchyma (2, 3). This suggested an inhibition of the intracellular translocation of VLDL particles. The specificity of the action of vincristine or colchicine on microtubules was further substantiated by the observations that these drugs did not alter basic functions of the liver (oxygen consumption, maintenance of normal ATP levels, of normal glucose or urea production), and did not alter lipogenesis or protein synthesis (3). Another important function of the liver is the synthesis and subsequent secretion into the blood of proteins such as albumin, α- and β-globulins, and fibrinogen (7–10). Such proteins are synthesized by the rough endoplasmic reticulum and most, if not all, are transferred to the Golgi apparatus, subsequently packaged in cytoplasmic vacuoles which are finally extruded into the space of Disse (11–14). However, the mechanisms by which these proteins are transported intracellularly before be-

ing released are unknown. Since it has been suggested that albumin may be transported within the hepatocyte as a granular material that appears to follow a path similar to that followed by VLDL particles (13), we have hypothesized that the hepatic microtubular system might play a role not only in the secretion of VLDL but also in the secretion of albumin and perhaps other proteins. The present experiments indicate that interference with the integrity of liver microtubules by vinblastine, vincristine, or colchicine is accompanied by a marked inhibition in the secretion of albumin and at least two other protein and polypeptide species.

METHODS

Perfusion technique and medium. Livers of 6-wk-old male Swiss mice were perfused *in situ* as previously described (3, 15), using 50 ml of a Krebs-Ringer bicarbonate buffer containing 10 mg unlabeled amino acid mixture, 10–20 μCi randomly labeled [^{14}C]amino acid mixture, 20% washed bovine erythrocytes, and either 3% defatted bovine albumin or 25% charcoal-treated bovine serum plus 1.5% bovine albumin bound to 1.7 mM oleate as indicated for each particular experiment. The unlabeled amino acid mixture was added to the stock albumin or serum solutions 15 h before perfusion to prevent subsequent adsorption of labeled amino acids onto perfusate proteins, an adsorption which occurred if such a precaution was not taken. The complete medium was filtered through a Sartorius filter (0.45 μm pore size, Sartorius Company, Göttingen, Germany) before use and before addition of the [^{14}C]amino acid mixture. Vinblastine, vincristine, or colchicine was dissolved in 0.5 ml isotonic saline and added to the medium at zero time.

Analysis of perfusate and tissues. Samples of 3–4 ml of the perfusion medium were taken at time 0 and at 30-min intervals. Ketone bodies, free fatty acids, triglycerides, urea, and glucose were measured as previously described (3). Total labeled proteins were determined as follows: 1 ml of 10% trichloroacetic acid (TCA) was added to 0.5 ml of perfusate. The resulting precipitate was washed twice with 5% TCA, heated for 15 min at 90°C to prevent albumin solubilization in organic solvents (16), washed once successively with ethanol, diethyl ether-ethanol (1:1), diethyl ether, and twice with 5% TCA. The final precipitate was dissolved in 1 ml NaOH (1 N) and a sample (0.2 ml) was counted for its radioactive content in 10 ml of toluene-ethanol (7:3) containing 4 g 2,5-diphenyl-oxazole and 0.04 g paraphenylene bis-methyl-phenyloxazol/liter. The radioactivity was determined in a liquid scintillation spectrometer (model 3380, Packard Instrument Co., Inc., Downers Grove, Ill.). Incorporation of label into hepatic proteins was determined by the same procedure, after small pieces (about 50 mg) of livers had been sonicated (Sonifier B 12, Branson Sonic Power Co., Danbury, Conn.) in 10% TCA. Label in newly synthesized albumin was determined using a modification of the previously described TCA-alcohol procedure (16, 17). 0.5-ml samples of perfusate, or fragments of livers (about 150 mg) obtained at the end of 2 h of perfusion, were sonicated in 10% TCA after the addition of 0.1 ml bovine serum used as protein carrier. The respective precipitates so obtained were washed three times with 5% TCA, and then the albumin was extracted three times into 1% TCA in 96% ethanol according to the method of Schwert (16). Albumin was then reprecipitated from the combined extracts by the addition of 3 vol diethyl ether at 4°C (17). The precipitated albumin was redissolved in 0.1 N NaOH, precipitated again with 10% TCA, and counted as previously described after dissolution in 1 N NaOH. Correction for quenching was made using an internal standard.

Separation of labeled proteins released into the perfusate was performed by column chromatography. Samples (2 ml) of perfusate obtained at the end of 2 h of perfusion were applied to a Sephadex G-100 column (1.4 × 75 cm) and eluted at room temperature with 1 M NaCl in 0.1 M Tris-HCl buffer (pH 8.0). Fractions (1.2 ml) were collected and counted for their radioactive content in a scintillation solution containing 15% of solubilizer (Biosolv, Beckman Instruments, Inc., Fullerton, Calif.). Results have been expressed as counts per minute per fraction since the counting efficiency, assessed by the use of internal standard, was constant for all the fractions. The surface area of each peak was estimated by calculating the surface area of the triangle drawn by eye to include the peak from the base line to the highest value. The area of each particular peak was then expressed as the percentage of the summed surface area of the relevant peaks, i.e. peaks 1, 2, and 3 (Fig. 2). Polyacrylamide gel electrophoresis (18) was carried out on some of the fractions from peaks 1 and 2. The possibility that radioactivity detected in the protein fractions represented not protein synthesis, but nonspecific adsorption of amino acids to the proteins was investigated as follows: (*a*) A chromatographic separation of medium proteins before perfusion was performed: the maximal value for radioactive content was observed in peak 2, a value which corresponded to less than 1% of the value obtained for peak 2 when measured in medium obtained after perfusion of livers with the labeled amino acids. (*b*) Livers were perfused for 2 min with labeled amino acids and then extracted for their labeled protein content as described above: the values observed were only 1.5% of those obtained after 2 h of perfusion. (*c*) When medium containing labeled amino acids was added to nonperfused, sonicated livers (20%, vol/wt) which were then washed and extracted for their labeled protein content, only background values were obtained. These controls are important since we have found that unless the precaution previously mentioned was taken, i.e. addition of unlabeled amino acids to the perfusing medium 15 h before the addition of the radioactive amino acids, then significant adsorption of amino acids to perfusate proteins can occur.

Chemicals. All organic and inorganic reagents were purchased from E. Merck AG (Darmstadt, West Germany) or Fluka AG (Basel, Switzerland) and were of analytical grade. Labeled compounds were secured from the Radiochemical Centre (Amersham, Buckinghamshire, England). Colchicine was obtained from E. Merck AG. Vincristine and vinblastine were a gift of Eli Lilly & Company (Indianapolis, Ind.). Amino acid mixture (TC amino acids HeLa × 100) was obtained from the Difco Laboratories (Detroit, Mich.).

RESULTS

As illustrated by Fig. 1 the release of newly synthesized (i.e. labeled) proteins by control livers was linear for 90 min, after a lag period of 30 min. In marked contrast, when livers were perfused in the presence of vinblastine (100 μM) the secretion of labeled proteins was completely abolished. Although not abolished, newly synthe-

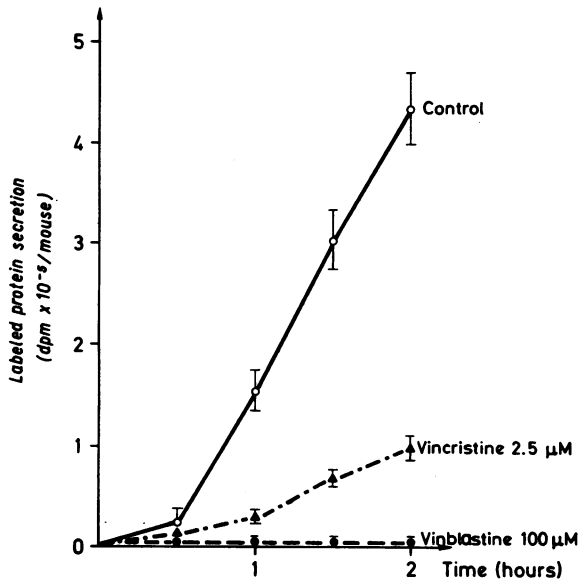

FIGURE 1 Effect of vinblastine (100 μM) or vincristine (2.5 μM) on the secretion of newly synthesized (i.e. labeled) proteins by perfused mouse livers. Livers from fed mice were perfused with 50 ml recirculating Krebs-Ringer bicarbonate buffer containing initially, 20% bovine erythrocytes, 1.5% bovine albumin bound to 1.7 mM oleate, 25% charcoal-treated bovine serum, 10 mg amino acid mixture, and 10 μCi [U-^{14}C]amino acids. Vinblastine or vincristine was added at time 0. Each point is the mean value of 10 (control), 6 (vincristine), or 4 experiments (vinblastine) ±SEM.

TABLE I

Effects of Vinblastine on Perfused Mouse Livers*

Measurements	Control livers	Vinblastine-treated livers	*P* values
Cellular ATP content‡	1.83±0.15	1.92±0.10	NS
Glucose production§	561±48	715±66	NS
Urea production§	105±5	76±3	<0.0025
Fatty acid uptake§	183±3	185±6	NS
Ketone body production§	266±19	123±5	<0.0025
Triglyceride secretion§	27.9±1.2	2.3±0.8	<0.0005
Hepatic triglycerides‡	11.05±0.39	16.0±1.03	<0.0025
Labeled amino acid incorporation into hepatic triglycerides‖	1.65±0.15	3.19±0.40	<0.01
Labeled amino acid incorporation into total proteins‖	31.6±2.3	27.3±1.0	NS

Livers were perfused for 2 h as described in Fig. 1.
Values are presented as means of four experiments ±SEM and are compared by *t* test.
* Vinblastine, 100 μM.
‡ Expressed as micromoles/gram wet liver wt.
§ Expressed as μmoles/100 g body wt.
‖ Expressed as dpm/mouse × 10^{-5}.

sized protein secretion was also markedly inhibited by vincristine used at a much lower concentration (2.5 μM). As the absence of labeled proteins in the perfusate of vinblastine-treated livers could be related to an unspecific toxic effect of the drug or to a marked inhibition of protein synthesis, a spectrum of metabolic parameters was measured, as shown in Table I. Cellular ATP content, glucose production, and fatty acid uptake were not modified by the drug. Of further interest was the finding that vinblastine, even when used at such a high concentration, did not inhibit total (i.e. hepatic plus perfusate) amino acid incorporation into proteins, making it unlikely that the cessation of protein release was secondary to a general inhibition of protein synthesis. In keeping with previous experiments carried out with vincristine (2, 3), vinblastine (100 μM) also markedly affected lipid metabolism, i.e., triglyceride secretion was nearly abolished at a time when hepatic lipogenesis (measured by labeled amino acid incorporation into triglycerides) and hepatic triglyceride content were increased. These findings are consistent with our earlier hypothesis which postulates a blockade of the translocation of lipoprotein particles in the presence of unaltered synthesis of lipoproteins (3). In contrast with the results previously obtained with 2.5 μM vincristine (3), 100 μM vinblastine markedly inhibited ketone body production and somewhat decreased urea production (Table I). These apparently nonspecific toxic effects of vinblastine led us to use two other compounds in our investigation of the role of microtubules in protein release. Thus we have used the vinblastine analogue vincristine at 2.5 μM, and colchicine at 10 μM, two concentrations of inhibitors that have been demonstrated to interfere with the microtubular system of the perfused mouse liver, without affecting unspecifically other parameters (3). As shown in Fig. 1 and Table II, when livers were perfused with either vincristine or colchicine, a marked (75%) reduction in the appearance of labeled proteins in the perfusate was observed. More importantly, *total* (liver plus perfusate) amino acid incorporation into proteins was not altered by either of these inhibitors, whereas *hepatic* amino acid incorporation into proteins was significantly higher in either vincristine- or colchicine-treated livers than in control livers (Table II). This suggested that the lesser appearance of labeled proteins in the perfusate of treated livers was not related to a decrease in protein synthesis but represented a decrease in the secretory process per se perhaps due to a blockade in the translocation of proteins associated with their accumulation and continued synthesis within the hepatocytes. It should be emphasized that the accumulation of total labeled proteins in livers treated with vincristine or colchicine was not very marked, a finding to be expected since only about

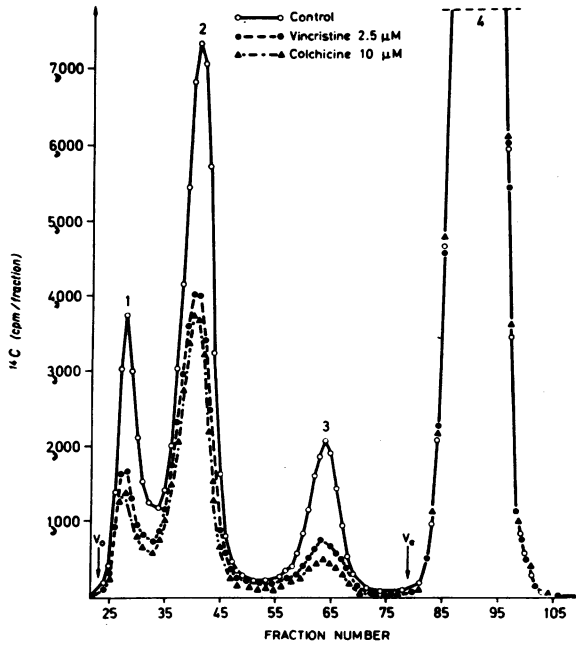

FIGURE 2 Effect of vincristine (2.5 μM) or colchicine (10 μM) on the chromatographic pattern of labeled proteins secreted by perfused mouse livers. Livers were perfused as described in Table II. After 2 h of perfusion, samples of perfusate (2 ml) were applied to a column of Sephadex G-100 (1.4 × 75 cm, 13.5 ml/h flow rate). The column was eluted with 1 M NaCl in 0.1 M Tris-HCl buffer (pH 8.0) at room temperature, 1.2-ml fractions were collected and counted for their radioactive content. Peak 1 corresponded to globulins > 150,000 mol wt, peak 2 to albumin, peak 3 to polypeptides of low mol wt, peak 4 to amino acids. Control values for nonspecific adsorption of labeled amino acids to proteins: see *Analysis of perfusate and tissues* in Methods.

20% of the total protein synthesized by perfused mouse livers is secreted into the medium, a finding that is similar to that reported in rat liver slices (19). The metabolic changes observed in the presence of vincristine or colchicine became much more marked when the main secreted protein, i.e. albumin, was studied (Table II). As may be seen, more than 50% of the newly synthesized (i.e. labeled) albumin was secreted into the perfusate of control livers. In the presence of vincristine or colchicine, the newly synthesized albumin that appeared in the perfusate was drastically reduced and the accumulation of labeled albumin within the liver now became quite obvious although total labeled albumin synthesis remained unchanged (Table II).

To determine whether the inhibition of protein secretion observed in livers perfused with vincristine or colchicine was specific for one particular group of secreted proteins, chromatographic separation of various secreted proteins was carried out on Sephadex G-100 (Fig. 2). The first peak eluted with the void volume and corresponded to globulins of high molecular weight (> 150,000), a fraction which could be shown to correspond on polyacrylamide gel electrophoresis with several different bands, as yet unidentified. Peak 2 cochromatographed with bovine albumin and its position corresponded exactly, in separate experiments, to that of mouse serum albumin. This fraction migrated as a single band on polyacrylamide gel electrophoresis to the same position as a bovine serum albumin standard. Peak 3, which eluted after hemoglobin, consisted of polypeptides of low molecular weight. Peak 4 represented labeled amino acids. As suggested by the results illustrated in Fig. 2, the inhibition of protein secretion produced by vincristine or colchicine affected each of the three different protein peaks to a similar degree. This finding was confirmed in a series of quantitative analysis summarized in Table III which demonstrated that in 14 separate perfusions, the secretion of individual proteins was indeed reduced proportionately by vincristine or by colchicine.

It was somewhat unexpected to observe that the secretion of small polypeptides would require a functioning microtubular system as it was thought that they would rather simply diffuse out of the liver. It was therefore hypothesized that peak 3 might be a degradation product derived from either peak 1 or 2, and not an actual secreted polypeptide. To test this hypothesis the following

TABLE II

Effect of Vincristine or Colchicine on Labeled Amino Acid Incorporation into Proteins by Perfused Mouse Livers

Additions	No. of experiments	Incorporation of labeled amino acids: Into hepatic proteins	Into perfusate proteins	Into total proteins
		dpm/mouse/2 h × 10⁻⁵		
None	7	45.2±1.7	12.0±0.9	57.2±5.7
Vincristine, 2.5 μM	4	60.0±5.8*	4.3±0.7§	64.3±6.4
Colchicine, 10 μM	6	53.9±2.3‡	3.7±0.4§	57.6±2.7
		Into hepatic albumin	Into perfusate albumin	Into total albumin
		dpm/mouse		
None	7	5.4±0.3	6.7±0.3	12.1±0.5
Vincristine, 2.5 μM	4	8.2±0.9‡	2.7±0.3§	10.9±1.3
Colchicine, 10 μM	3	9.2±0.3§	2.9±0.2§	12.1±0.6

Livers were perfused for 2 h with 50 ml recirculating Krebs-Ringer bicarbonate buffer containing initially 20% washed bovine erythrocytes, 3% defatted bovine albumin, 10 mg amino acid mixture, and 20 μCi ^{14}C randomly labeled amino acids. Vincristine or colchicine was added at time 0. Values are presented as means ±SEM, and are compared by *t* test.

* Significantly different from controls at $P < 0.025$.

‡ Significantly different from controls at $P < 0.0125$.

§ Significantly different from controls at $P < 0.0005$. (All other differences from controls: NS.)

TABLE III

*Labeled Proteins Secreted by Mouse Livers Perfused with or without Vincristine or Colchicine**

Additions	No. of experiments	Secreted proteins	Surface area of Peak 1	Peak 2	Peak 3
		cpm/2 ml/ 2 h × 10^{-3}	*% of respective total surface area*		
None	6	64.9±4.7	20.1±0.7	61.8±1.2	18.1±0.9
Vincristine, 2.5 μM	4	23.5±2.8	21.9±2.1	61.6±4.5	16.5±2.3
Colchicine, 10 μM	4	21.1±1.6	20.4±2.4	62.2±3.9	17.4±2.3

Livers were perfused as described in Table II and 2-ml samples of perfusate were chromatographed as described in Fig. 2. Total surface area is surface of peaks 1 plus 2 plus 3, peak numbers being the same as those indicated in Fig. 2. Values are presented as means of four to six chromatograms obtained from four to six different perfusions±SEM.

* Relative surface areas of peaks obtained by chromatography on Sephadex G-100 column.

experiment was performed. Livers were first perfused for 2 h with labeled amino acid mixture as described above. The perfusate was then taken, dialyzed to remove remaining labeled amino acids, and aliquots of it chromatographed to show that labeled proteins (i.e. peaks 1, 2, and 3) had been synthesized during this 2 h perfusion time (Table IV). The dialyzed medium containing the newly synthesized proteins was then used again to perfuse a new series of livers, and to test the existence of possible alteration of these newly synthesized proteins by the livers. It was reasoned that if peaks 1 or 2 would be degraded into small polypeptides during the perfusion, then radioactivity collected in the peak 3 would be increased. It may be seen in Table IV that this was not the case: total labeled proteins in the perfusate decreased only little after 2 h of perfusion, in keeping with a slight decrease in peak 1. Peak 2 was barely altered and, more importantly, peak 3 did not increase thus ruling out the possibility that it might mainly be a degradation product of other larger proteins.

TABLE IV

Lack of Degradation of Prelabeled Protein Species into Small Polypeptides by Perfused Livers

	Labeled protein	Surface area of Peak 1	Peak 2	Peak 3
	cpm/2 ml × 10^{-3}	*% of respective total surface area*		
Initial perfusate A*	42.0	15.35	61.25	23.4
Perfusate A after 2 h perfusion	38.2±1.2	12.6±0.3	63.6±0.4	23.8±0.6

* Livers were perfused for 2 h with 50 ml recirculating perfusate A containing 20% washed bovine erythrocytes. Before these experiments, perfusate A was obtained by collecting the medium after perfusion of normal livers in the presence of labeled amino acids for 2 h as described in Table II. Perfusate A was then dialyzed for 2 h against five changes of 2 liters Krebs-Ringer bicarbonate buffer to remove remaining labeled amino acids. After addition of bovine erythrocytes to perfusate A, aliquots of it were centrifuged and chromatographed as described in Fig. 2 and Table III, and shown to contain labeled globulins (peak 1), albumin (peak 2), and small polypeptides (peak 3). After 2 h of perfusion with this perfusate 2-ml samples were rechromatographed and the pattern compared with that seen with the initial perfusate A. Values for initial perfusate A are means of two chromatograms. Values for perfusate A after 2 h of perfusion are presented as means from three chromatograms obtained from three separate experiments±SEM.

DISCUSSION

The most striking observation of the present study is that drugs such as vinblastine, vincristine, or colchicine, known to interfere specifically with the hepatic microtubular system (2, 3), strongly inhibit or completely suppress the release of newly synthesized proteins by the perfused mouse liver. Of further importance is the finding that, at any of the concentrations tested, these agents did not interfere with total protein synthesis as estimated by labeled amino acid incorporation into hepatic plus perfusate proteins (Tables I and II). This observation rules out the possibility that the observed decrease in protein secretion might be secondary, as is the case with drugs such as puromycin or actinomycin D (20), to decreased protein synthesis. Furthermore, it could be observed that when added to perfused liver at appropriate concentrations, vincristine (2.5 μM) or colchicine (10 μM) brought about a marked decrease in labeled amino acid incorporation into total proteins or into albumin released into the perfusate, at a time when labeled amino acid incorporation into either total hepatic proteins or hepatic albumin was actually increased. This observation is consistent with the concept that the decrease in total protein secretion, or in albumin secretion produced in livers treated by vincristine or colchicine is likely to be related to an alteration in hepatic microtubular activity which results in a blockade of the intracellular translocation of the proteins to be secreted, together with their continuing synthesis and eventual accumulation within the hepatocytes. The present study further indicates that the microtubular system appears

to play a role in the secretion of all of the major proteins secreted by the liver (Fig. 2, Table III). Of particular significance is the demonstration that albumin, the major protein produced by the liver, is markedly dependent upon the microtubular system for its secretion (Table III). It is of additional interest to note that even small polypeptides appear to be secreted by the liver via a functioning microtubular system and do not represent either products released by simple diffusion or degradation products derived from larger proteins (Fig. 2, Tables III and IV).

It is not yet established whether all proteins or polypeptides are transported intracellularly as vesicle-enclosed products. One may spectulate that, since the secretion of all proteins and polypeptides is affected by vinblastine, vincristine, or colchicine, these various proteins might possibly be segregated within vesicles which are moved in a similar fashion towards the vascular pole of the hepatocyte by the microtubular system. However, the precise nature of the relationship between secretory products and the microtubular system still remains to be clarified. Finally, this study raises the possibility that variations in the hepatic microtubular system may play a more important role in the physiological regulation (and perhaps also in pathology) of secretion of albumin and globulin than has previously been suspected.

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