COLCHICINE-BINDING PROTEIN OF THE LIVER

Its Characterization and Relation to Microtubules

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ABSTRACT

Colchicine-binding activity of mouse liver high-speed supernate has been investigated. It has been found to be time and temperature dependent. Two binding activities with different affinities for colchicine seem to be present in this high-speed supernate, of which only the high-affinity binding site (half maximal binding at 5×10^{-6} M colchicine) can be attributed to microtubular protein by comparison with purified tubulin. Vinblastine interacted with this binding activity by precipitating it when used at high concentrations (2×10^{-3} M), and by stabilizing it at low concentrations (10^{-5} M). Lumicolchicine was found not to compete with colchicine.

The colchicine-binding activity was purified from liver and compared with that of microtubular protein from brain. The specific binding activity of the resulting preparation, its electrophoretic behavior, and the electron microscope appearance of the paracrystals obtained upon its precipitation with vinblastine permitted its identification as microtubular protein (tubulin).

Electrophoretic analysis of the proteins from liver supernate that were precipitated by vinblastine indicated that this drug was not specific for liver tubulin.

Preincubation of liver supernate with 5 mM EGTA resulted in a time-dependent decrease of colchicine-binding activity, which was partly reversed by the addition of Ca^{++}. However, an in vitro formation of microtubules upon lowering the Ca^{++} concentration could not be detected.

Finally, a method was developed enabling that portion of microtubular protein which was present as free tubulin to be measured and to be compared with the total amount of this protein in the tissue. This procedure permitted demonstration of the fact that, under normal conditions, only about 40% of the tubulin of the liver was assembled as microtubules. It is suggested that, in the liver, rapid polymerization and depolymerization of microtubules occur and may be an important facet of the functional role of the microtubular system.

Recent studies from these laboratories have shown that the secretion of very low density lipoproteins (VLDL), and of proteins such as albumin (11, 12, 18), by perfused livers requires an intact microtubular system. These findings have been corroborated by similar experiments carried out in vivo (23, 24), thus clearly indicating that the microtubular system of the liver may be of great

physiological importance, and could be a site at which regulation of these various secretory processes occurs. One should stress that there is only very limited knowledge of the colchicine-binding property of the liver (5, 22). Since colchicine binding may represent tubulin, the microtubular protein, the biochemical characterization of this protein is a prerequisite for the understanding of microtubular function in the liver.

In the present work, we have therefore characterized the microtubular protein of mouse liver, comparing it to the rather well-defined (10, 21, 28, 29) tubulin of the brain. We have also attempted to determine the relative quantity of free tubulin and of the tubulin assembled as microtubules in livers since the dynamic equilibrium between these two portions may be an important clue in the understanding of microtubular function.

MATERIALS AND METHODS

Animals

6-wk old male Swiss albino mice weighing about 30 g, fed ad libitum with UAR laboratory chow (Villemoisson, Epinay/Orge, France), and bred in these laboratories, have been used throughout these studies.

Preparation of Liver Homogenates

Animals were anesthetized by intraperitoneal injection of pentobarbital (100 mg/kg body weight). In all experiments livers were washed free of blood by rapid portal perfusion with cold 0.9% NaCl solution, and homogenized with 2 vol (wt/vol) of either PMG buffer[1] (28) or MES-MEG buffer (21). Homogenization was done at 0°C in a Sorvall Omni-mixer three times for 30 s at maximal speed. For preparation of high speed supernates, homogenates were centrifuged in a Beckman L 50 preparative ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 100,000 *g* for 60 min. When the measurement of the relative quantity of free tubulin and of tubulin assembled as microtubules was carried out, homogenization was modified as follows: mice were anesthetized and the livers were washed free of blood by rapid portal perfusion with MES-MEG buffer (4°C), half of them in the presence, the other half in the absence of 4 M glycerol. Livers were homogenized at 4°C with 2 vol (wt/vol) of MES-MEG buffer, again with or without 4 M glycerol, by 10 strokes of a motor-driven glass homogenizer. The time that elapsed between perfusion of livers, as just mentioned, and the beginning of homogenization never exceeded 30 s. Homogenates were then stirred in an icebath for 30 min, all of them equalized to 4 M glycerol, and centrifuged (100,000 *g*, 4°C) for 60 min to sediment microtubules, and the supernate was used to measure colchicine-binding activity. This methodology is further discussed and justified in Results.

Colchicine-Binding Assay

The assay for binding of [^{3}H]colchicine was that of Weisenberg (27), and was modified as follows: the various samples used were incubated with 10^{-4} M [^{3}H]colchicine (50 mCi/mmol) at 30°C unless indicated otherwise. Blanks containing labeled colchicine but no protein were carried out in each single experiment. At time 0 and usually after 45 min 0.2 ml of the sample was withdrawn, transferred to 1 ml of packed cold DEAE-Sephadex equilibrated with PMC-buffer, stirred, and added after 10 min with 5 ml of cold PMC buffer. After 5 min and ocasional stirring, the resin was sedimented by centrifugation (5 min, 1,000 *g*), then freed of unbound colchicine by five successive washes and centrifugations with cold PMC buffer. At the end of this procedure, no release of labeled colchicine from the DEAE-Sephadex could be detected upon incubation of the samples for 2 h in cold PMC buffer. Bound colchicine was directly eluted from DEAE-Sephadex by addition of 10 ml of Bray's solution (6), and counted in a liquid scintillation spectrometer (Packard Tri-Carb, Model 3380) (Packard Instruments, Inc., Downers Grove, Ill.). Quenching correction was done by using internal standards or an external standard channels ratio method.

Purification of Microtubular Protein from Mouse Liver and Brain

Microtubular protein (tubulin) was prepared by the method of Weisenberg et al. (28, 29) with the following minor modifications. In each experiment, 30 brains and livers were rapidly removed and homogenized with 2 vol of PMG buffer as mentioned above. The microtubular protein was precipitated from 100,000 *g* supernates between 35% and 45% ammonium sulfate saturation. The elution from DEAE-Sephadex was done with PMG-buffer containing 0.6 M KCl instead of 0.8 M. The final precipitation step was carried out with 50 mM $MgCl_2$ according to the original procedure (29).

Reassembly of Brain Microtubules In Vitro

Reassembly of microtubules from brain was adapted from a method described previously (21). Mouse brains

[1] *Abbreviations used in this paper:* EGTA, ethylene glycol bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; GTP, guanosine-5′-triphosphate; MES, morpholinoethane sulfonic acid; MES-MEG buffer, 10^{-1} M MES, pH 6.5, 5×10^{-4} M $MgCl_2$, 10^{-3} M EGTA, 10^{-3} M GTP; PMB buffer, 10^{-2} M sodium phosphate, pH 6.5, 5×10^{-3} M $MgCl_2$, 10^{-4} M GTP; PMC buffer, 10^{-2} M sodium phosphate, pH 6.5, 5×10^{-3} M; MCl_2, 10^{-4} M colchicine; SDS, sodium dodecyl sulfate.

were homogenized with 1 vol of MES-MEG buffer and centrifuged at 4°C for 60 min at 100,000 *g*. To the supernate was added 1 vol of MES-MEG buffer containing 8 M glycerol, and the resulting mixture was incubated for 30 min at 37°C. The microtubules thus obtained were sedimented by a centrifugation at 100,000 *g* and 25°C for 60 min. They were suspended in 0.25 of the original volume of cold MEG buffer and depolymerized by 30 min of incubation at 0°C. After an additional ultracentrifugation, the supernate was used to study the polymerization cycle of microtubules. The kinetics of this process were measured by light scattering in a Beckman double beam spectrophotometer at 460 nm and 37°C. The reference cuvette contained, in addition, colchicine at 10^{-4} M.

Preparation of Lumicolchicine

Lumicolchicine was prepared by exposure of a 10^{-3} M solution of colchicine in 95% ethanol to UV light for 30 min. Actual conversion of colchicine to lumicolchicine was controlled by measuring the shift in their respective spectra, and purity was estimated by thin-layer chromatography (31).

Electron Microscope Studies

The formation of brain microtubules was verified by electron microscopy after negative staining of the samples with 1% uranyl acetate. In other experiments, aliquots of purified liver or brain tubulin were precipitated with 2×10^{-3} M vinblastine, centrifuged at 8,000 *g* for 45 min, and the supernate was discarded. Pellets thus obtained were fixed in the tube with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h. In order to prevent disruption of the pellets upon further manipulation, they were first embedded in 7% agar. The agar blocks containing the pellets were then postfixed in 2% phosphate-buffered osmium tetroxide, dehydrated in increasing ethanol concentrations, and embedded in Epon (13). Thin sections, contrasted with uranyl acetate and lead citrate, were examined in a Philips EM 300 microscope.

Polyacrylamide Gel Electrophoresis

Electrophoresis was performed according to the method described previously (7). Buffers and gels contained 0.1% SDS, and gels contained 8 M urea where indicated. A current of 3 mA was applied on each gel for about 4 h. The gels were stained for 5 h in 1% amido black 10 B in 7.5% acetic acid and destained by soaking for 48 h in 7.5% acetic acid.

Chemicals

[^{3}H]colchicine was purchased from the Radiochemical Centre (Amersham, England). Vinblastine was kindly supplied by Eli Lilly & Co. (Indianapolis, Ind.) and Trasylol was a gift of Bayer (Leverkusen, West Germany). DEAE-Sephadex was purchased from Pharmacia (Uppsala, Sweden) and guanosine-5'-triphosphate from C. F. Boehringer and Sons (Mannheim, West Germany). The other reagents were secured from Merck A. G. (Darmstadt, West Germany), or Fluka A. G. (Basel, Switzerland). Materials for polyacrylamide gel electrophoresis were purchased from Serva (Heidelberg, West Germany).

RESULTS

Colchicine-Binding Activity in Liver High-Speed Supernate

In the first series of experiments, the characteristics of colchicine binding by high-speed supernates of liver were investigated. As can be seen in Table I, most of the colchicine-binding activity was present in the soluble fraction. However, about 23% of the binding activity was found in the pellet, from which it could not be extracted even by repeated washings or by treatment with 1% Lubrol (I. C. I. America Inc., Stamford, Conn.) The colchicine-binding activity of liver high-speed supernates was clearly temperature and time dependent, little binding being seen at 0°C. Equilibrium was attained after about 15–20 min (Fig. 1). This binding activity was linearly related to the protein concentration of the high-speed supernate (Fig. 2). Furthermore, at all protein concentrations tested, the colchicine binding disappeared, i.e. was precipitated, upon addition of a high concentration (2×10^{-3} M) of vinblastine to the supernate (Fig. 2). It

TABLE I

Distribution of the Colchicine-Binding Activity in the Liver as Measured by Fixation to DEAE-Sephadex

	Bound [^{3}H]colchicine		
	pmol/mg protein	*pmol/ml*	*% of total*
Total homogenate	6.22	1,258	100
1st supernate	32.07	955	79
2nd supernate	21.32	117	9
Pellet	5.35	297	23

Homogenates were prepared with PMG buffer and ultracentrifuged as described in Materials and Methods. The pellet of the first centrifugation was resuspended in PMG buffer and centrifuged once more, resulting in the second supernate and final pellet. Supernates and pellets were made up to the original volume with PMG buffer to be comparable on a volume basis.

Each value represents the mean of three experiments.

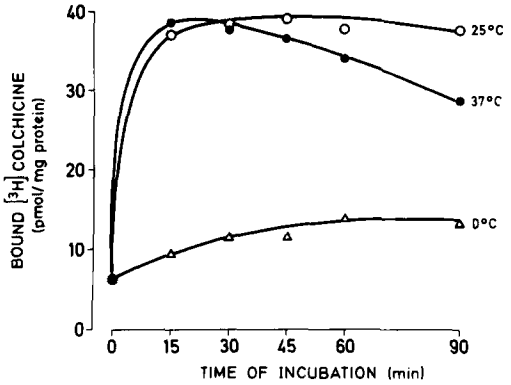

FIGURE 1 Time and temperature dependency of the colchicine-binding capacity of liver high-speed supernate. 100,000-*g* supernate of liver homogenate prepared with PMG buffer was incubated with 10^{-4} M [^{3}H]colchicine at 0°C, 25°C, and 37°C. At various time intervals, samples were taken, washed, and counted as described in Materials and Methods. Each value represents the mean of three experiments.

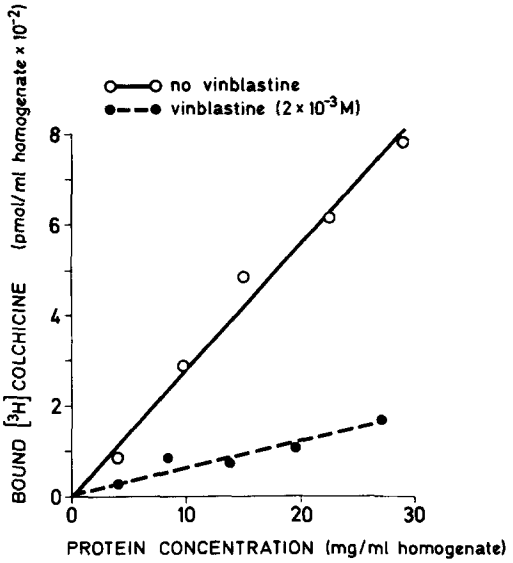

FIGURE 2 Protein dependency of the colchicine-binding activity in liver high-speed supernate and its precipitation by vinblastine. Supernates containing various amounts of protein were preincubated with or without vinblastine (2×10^{-3} M) for 30 min at 37°C and centrifuged at 10,000 *g* for 20 min. Thereafter, they were incubated in a total volume of 0.5 ml with labeled colchicine for binding measurements. Each value represents the mean of three experiments.

was of further interest to observe that, when used at considerably lower concentration, the presence of vinblastine in the high-speed supernate had a stabilizing effect on the colchicine-binding activity, as shown in Table II. Thus, colchicine binding on high-speed supernates was not altered by preincubation at 0°C. However, preincubation of high-speed supernate at 37°C caused a loss of binding activity that was partly prevented by the addition of vinblastine. Similar observations have been made with brain preparations (30). In the next series of experiments, the specificity of colchicine binding to high-speed supernate was studied. It is evident that unlabeled lumicolchicine did not compete with labeled colchicine, while unlabeled colchicine used at equimolar concentration resulted in a marked decrease in the binding of [^{3}H]colchicine (Table III).

Characterization of Microtubular Protein from Liver: Comparison with That of Brain

It was of considerable importance to determine how liver microtubular protein, of which almost nothing is known, compares with another microtubular protein that has been more thoroughly investigated, i.e. that of brain. It was found that there was considerably less colchicine binding activity in the 100,000 *g* supernate of liver than in that of brain (liver bound [^{3}H]colchicine, 38.71; brain, 577.14, pmol/mg protein). However, when microtubular proteins of liver and brain were purified and analyzed for colchicine binding, their respective binding activities were found to be identical, as further discussed below (cf. Fig. 5), indicating that the purified tubulin of liver was likely to be similar to that of brain. This conclu-

TABLE II

Effect of Low Concentrations of Vinblastine on the Colchicine-Binding Activity of Liver Supernate

	Bound [^{3}H]colchicine	
	Incubation temperature	
	0°C	37°C
	pmol/mg protein	*pmol/mg protein*
None	25.66	14.90
Vinblastine 10^{-7} M	25.79	14.63
Vinblastine 10^{-6} M	25.57	14.49
Vinblastine 10^{-5} M	22.33	16.82
Vinblastine 10^{-4} M	22.25	19.22

Liver high-speed supernate (prepared with PMG buffer) was preincubated for 40 min at 0° or 37°C, with and without various concentrations of vinblastine. Thereafter the binding of colchicine was measured.

Each value represents the mean of three experiments.

TABLE III

Effect of Lumicolchicine on the Binding of Colchicine by Liver High-Speed Supernate

Addition	Bound [^{3}H] colchicine
	dpm/ml × 10^{-3}
None	266
5×10^{-4} M colchicine	41
5×10^{-4} M lumicolchicine	235

All samples were incubated with 10^{-4} M [^{3}H]colchicine (50 mCi/mmol) as described in Materials and Methods.

Each value is the mean of three experiments.

sion was strengthened by the electrophoretic behavior of purified microtubular proteins of both liver and brain since, as previously described for rat brain tubulin (10), mouse brain tubulin and liver tubulin exhibited a fast-migrating band corresponding to the subunit and other more slowly migrating fractions corresponding to the dimeric and further polymeric states (Fig. 3 *a* and *b*). When SDS (0.1%) was added to the buffer and gels, only the fast-migrating fraction was obtained electrophoretically, in a way that was similar for both liver and brain microtubular proteins (Fig. 3 *c* and *d*). Finally, this fraction from both liver and brain could be split into α- and β-tubulin upon addition of 8 M urea to the gels (Fig. 3 *e*, and *f*). Electron microscope examination of purified microtubular protein from both tissues after precipitation with vinblastine was of additional interest. In the preparation obtained from liver tubulin (Fig. 4 *a*), a pattern of ordered structures could be found which displayed an appearance and spacing similar to those formerly described for vinblastine-treated brain tubulin (3, 14). The mouse brain tubulin presented analogous morphological appearance (Fig. 4 *b*).

The kinetics of colchicine binding to liver high-speed (100,000 *g*) supernate, to purified liver microtubular protein, or to a purified brain microtubular protein were then studied and compared, as illustrated by Fig. 5. Colchicine binding of liver high-speed supernate reached a plateau at 10^{-4} M colchicine and showed a further increase at higher colchicine concentrations, suggesting the presence of another binding activity with lower affinity for the ligand. This activity at high colchicine concentrations was not precipitated by vinblastine. Therefore, it remains questionable whether this activity can be attributed to the microtubular system. Of further interest was the realization that the dose-response curves for the binding of labeled colchicine to either purified liver or brain tubulin were almost identical and corresponded only to the high-affinity binding site of the supernate, half maximal binding occurring at about 5×10^{-6} M and saturation at 10^{-4} M (Fig. 5). Assuming a molecular weight of 110,000 for dimeric tubulin (10, 28), a specific binding activity of about 0.44 mol colchicine per mol tubulin can be calculated. The deviation from the theoretical value of 1 mol colchicine per mol tubulin (28) may result from the instability of the binding per se and/or from partial alteration of the binding site during the purification procedure.

Studies on the Specificity of Vinblastine for Liver Microtubular Protein

Aliquots of 100,000 *g* liver supernates prepared with PMG buffer were incubated for 30 min at 37°C with vinblastine (2×10^{-3} M). The resulting precipitate was centrifuged (10,000 *g*, 10 min) and washed twice with PMG buffer containing the

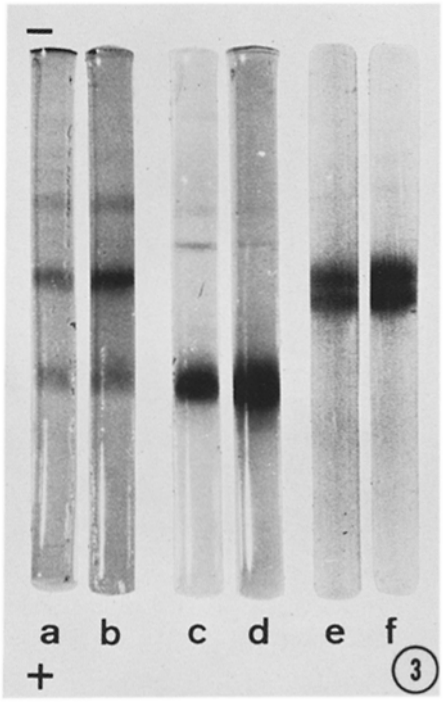

FIGURE 3 Electrophoresis of purified microtubular protein from liver and brain. Native tubulin from liver (*a*) and brain (*b*). Liver (*c*) and brain (*d*) tubulin in gels containing 0.1% SDS. Liver (*e*) and brain (*f*) tubulin in gels containing 0.1% SDS plus 8 M urea.

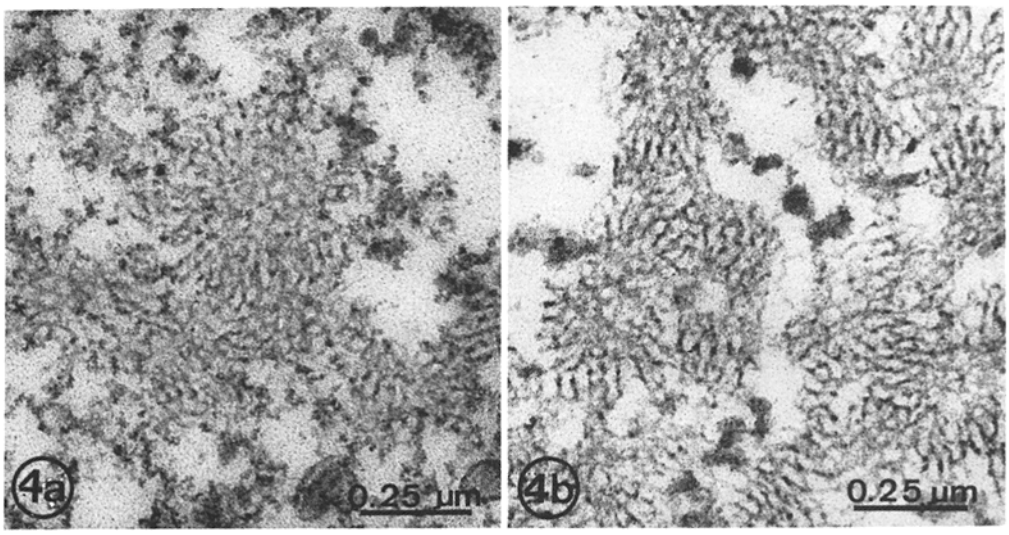

FIGURE 4 Thin sections of pellets from vinblastine-precipitated mouse liver microtubular protein (*a*) and (*b*) mouse brain microtubular protein. Patterns of ordered structures showing similar aspects and sizes can be seen in both images (× 60,000).

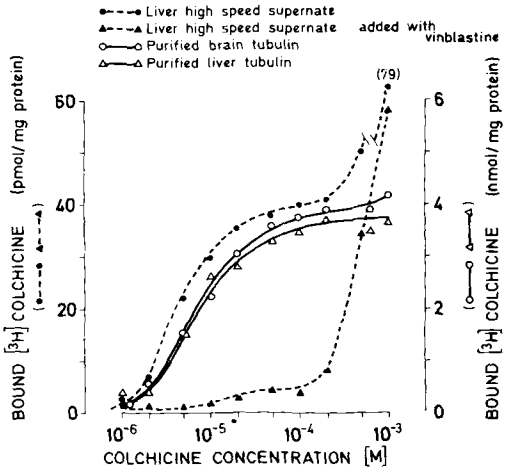

FIGURE 5 Dependency of the colchicine-binding activity of liver high-speed supernate and of purified liver and brain tubulins upon colchicine concentrations. Liver 100,000-*g* supernate was prepared with PMG-buffer and part of it precipitated with vinblastine (2×10^{-3} M). The supernate and purified tubulins were incubated with various concentrations of labeled colchicine as described in Materials and Methods. Each value is the mean of three experiments.

same concentration of vinblastine. The final pellet was dissolved in 1% SDS and compared by disc gel electrophoresis to purified liver microtubular protein (Fig. 6 *a–c*). For further comparison, high-speed (100,000 *g*) supernate and purified tubulin from brain were processed and analyzed the same way (Fig. 6 *d–f*). It is evident from such experiments that vinblastine interacted not only with liver tubulin (Fig. 6 *c*) but also with several undetermined proteins separable from microtubular proteins on disc gel electrophoresis (Fig. 6 *a* and *b*).

Effect of EGTA and Ca^{++} on the Colchicine-Binding Activity of the Liver

As shown in Fig. 7, preincubation of a liver high-speed supernate (prepared with PMG buffer) in the presence of 1 mM EGTA had little effect on the colchicine-binding activity. However, when EGTA was increased to 5 mM, a marked decrease in colchicine-binding activity occurred that reached a maximum after 80 min. The addition of Ca^{++} (10 mM, as $CaCl_2$) partially restored the colchicine-binding activity of the sample that had been initially preincubated in the presence of EGTA. One should stress the fact that the colchicine binding of purified microtubular protein from liver was unmodified by the presence of EGTA.

Measurement of the Respective Amounts of Microtubules and Free Tubulin in the Liver

Since liver tubulin appears from the present experiments to be quite similar to that of brain, it

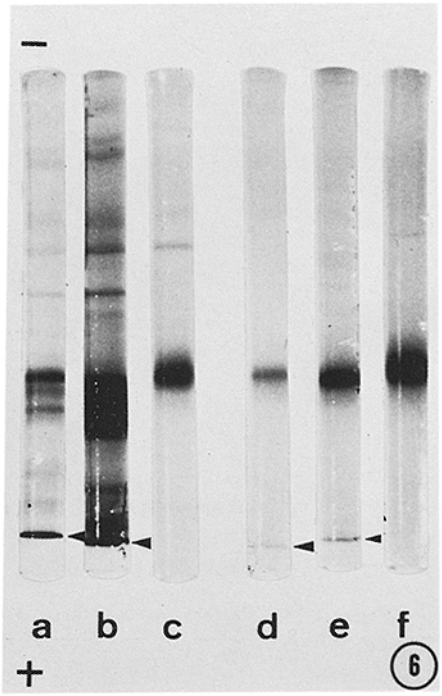

FIGURE 6 Electrophoresis of proteins from high-speed supernate of liver and brain precipitated by vinblastine: comparison with liver and brain purified tubulin. (*a* and *b*) 200 and 400 μg of protein precipitated from liver supernate. (*c*) Purified liver tubulin. (*d* and *e*) 200 and 400 μg of protein precipitated from brain supernate. (*f*) Purified brain tubulin. The arrows indicate the position of the marker dye running in front of the electrophoresis.

was thought that the respective amounts of free tubulin and of tubulin polymerized as microtubules could be measured in the liver by applying to that organ some of the methodology used previously for studying brain microtubules in vitro (17, 21, 26). The formation of brain microtubules resulted in vitro in an increase in turbidity that could be followed by changes in the optical density (measured at 460 nm). This turbidity reached a plateau after about 30 min, at which time typical microtubules were detected electron microscopically. As shown in Table IV, the optical density of this preparation decreased rapidly upon cooling from 37° to 4°C, to become almost nil within 30 min. Concomitantly, microtubules could no longer be observed. In marked contrast, when glycerol (4 M) was present, cooling did not alter optical density even after 120 min and microtubules remained present (Table IV). This indicated that, in the presence of 4 M glycerol and at 4°C, polymerization and depolymerization of microtubules were completely halted. This methodology, the use of glycerol as stabilizing agent in particular, was then extended to the liver, on the basis of the rationale that the properties of liver and brain tubulin were, as described above, very similar. As schematized by Fig. 8 and described in detail in Materials and Methods, two series of liver homogenates were prepared. The first series of homogenates was made in 4 M glycerol and at 0°C, it was ultracentrifuged to sediment microtubules thus stabilized, and the supernate was used to measure residual (free) colchicine-binding activity (i.e. not assembled as microtubules). The homogenization was started within seconds and rapidly completed (see Materials and Methods). With this experimental approach, the spontaneous depolymerization of microtubules that would artifactually increase the amount of free tubulin was probably kept to a minimum, since the depolymerization process, as observed in vitro (Table IV), was shown to require

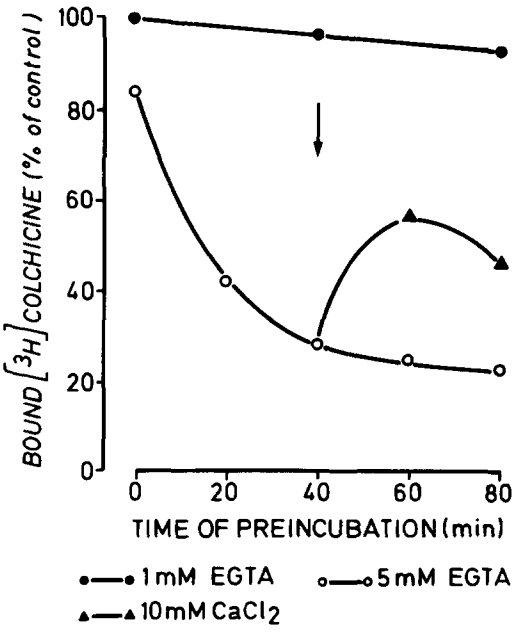

FIGURE 7 Effect of preincubation with EGTA on the colchicine-binding activity of liver high-speed supernate. 100,000-*g* supernate prepared with PMG-buffer was preincubated with EGTA as indicated. At various intervals of preincubation, samples were withdrawn and analyzed for their cholchicine binding. The arrow indicates time of addition of $CaCl_2$ of (10 mM). Each point is the mean of three values.

TABLE IV

Stabilizing Effect of Glycerol on Brain Microtubules in the Cold as Measured by Changes in Turbidity

	No addition		4M glycerol	
Time at 4°C*	Optical density	Microtubules in electron microscope	Optical density	Microtubules in electron microscope
min	*460 nm*		*460 nm*	
0	0.120	Present	0.120	Present
2	0.115			
5	0.085			
10	0.025			
30	0.010		0.120	
60	0.010	Absent	0.120	
120			0.120	Present

* Microtubules from mouse brain were first polymerized at 37°C, as described in Material and Methods (protein concentration of the tubulin preparation, 2.81 mg/ml). The actual experiment started upon cooling the preparation containing polymerized microtubules.

Each value is the mean of two experiments.

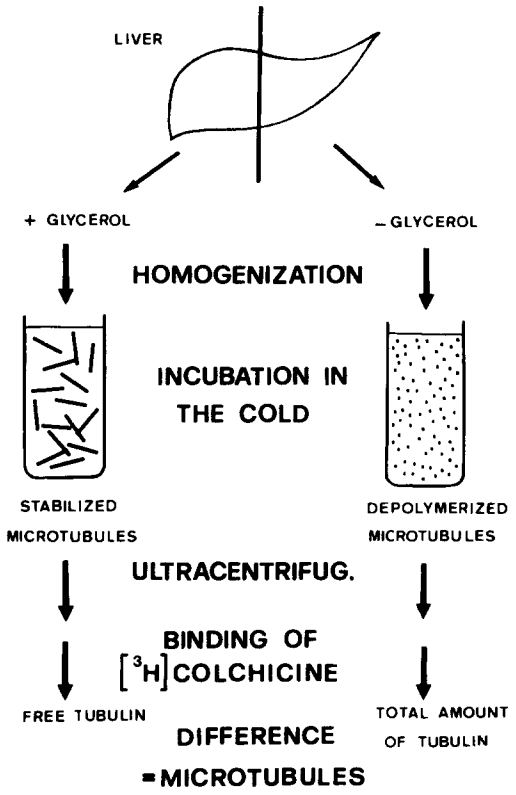

FIGURE 8 Schematic representation of the method used to estimate the respective proportion of microtubules and of free (residual) tubulin in the liver (see section on Materials and Methods).

several minutes to be initiated. The second series of liver homogenates (Fig. 8) was made under the same conditions, at the same temperature in particular, but in the *absence* of glycerol, so that all microtubules were presumably depolymerized. The supernate of these homogenates was then used to measure colchicine binding, which was taken as an index of the *total* amount of tubulin present in the preparation. The binding of colchicine per se was shown not to be modified by the presence of glycerol.[2] The results of these experiments are summarized in Table V. It is evident that, under normal conditions, i.e. when livers from fed animals were used, about 60% of the total tubular protein measured in the liver was present as free tubulin, indicating that a rather small proportion of this material was actually assembled as microtubules at the time of the experiment. To substantiate further the assumption that stabilization and destruction of microtubules were analogous in vivo and in vitro (an assumption upon which the results of Table V are based), an aliquot of a mixture of in vitro polymerized microtubules

[2] The absence of modification of this colchicine-binding assay by glycerol was shown as follows; High-speed liver supernates were preincubated for 30 min with or without 4 M glycerol. After equalization of glycerol concentrations, a colchicine-binding assay was carried out as usual and found to be 40.7 (no glycerol) and 41.0 (glycerol) pmol [^{3}H] colchicine per milligram protein, mean of three experiments.

TABLE V
Colchicine-Binding Activity in Supernates of Liver Homogenates Prepared with or without Glycerol

Experimental conditions	Bound [^{3}H]colchicine	
	pmol/mg protein	%
Without glycerol (total tubulin)	38.71* ± 6.43	100
With glycerol (4 M) (free tubulin)	23.17 ± 3.33	59.9

Experimental design, see Materials and Methods and Fig. 8. Each value is the mean of eight experiments ± standard deviation.

* Significance of the difference between the two groups, $P < 0.001$.

and free tubulin from brain was added to liver samples just before the homogenization of the latter. The respective amounts of total tubulin (no glycerol added) and free tubulin (glycerol added) were then tested separately for the brain preparation, the liver homogenates, and for the mixture of both. As shown in Table VI, calculated values were very close to measured ones, thus confirming the validity of the technique used. One should add that substances with the same viscosity as 4 M glycerol but without its stabilizing effect on brain microtubules in vitro, e.g., 2% dextran, were ineffective when used in liver homogenates.

DISCUSSION

This study is the first attempt to characterize in some detail the microtubular system of the liver. It represents therefore an important initial step that should contribute to an eventual understanding of microtubular function in this organ.

These experiments indicate that the colchicine-binding activity of liver high-speed supernate has several characteristics in common with that of the brain (2, 4, 10, 29, 30). It is time and temperature dependent, it is precipitated by a high concentration of vinblastine, and stabilized by low concentration of this vinca alkaloid. It is not affected by lumicolchicine, a finding that is in keeping with the observation of the lack of any effect of lumicolchicine on triglyceride secretion by perfused livers while an equimolar concentration of colchicine is markedly inhibitory.[3] The purification of liver tubulin and its subsequent comparison with brain tubulin indicates that these two purified tubulin preparations must have several properties in common. Both have similar specific binding activities for colchicine (Fig. 5) and identical electrophoretic behavior, suggesting that liver tubulin may have a similar molecular weight and subunit composition as well as a propensity to form polymeric aggregates in the native state (Fig. 3). Both have a similar appearance in the electron microscope after precipitation by vinblastine (Fig. 4) (3, 14). Liver and brain differ from each other, however, by the fact that microtubules can easily be reassembled from high-speed supernate of the latter in vitro (21, 26, Table IV), while attempts to polymerize liver tubulin into microtubules have so far been unsuccessful. This suggests that the actual process of polymerization may be somewhat different in the liver than in the brain, although the nature of this difference is not understood at

TABLE VI
Recovery of a Mixture of Brain Microtubules and Tubulin Added to Liver during Homogenization

Experimental designs	Bound [^{3}H]colchicine		
	Glycerol		
	Absent (A) (total tubulin)	Present (B) (free tubulin)	(A)-(B) (microtubules)
	(*cpm/mg protein*)		
I. Brain*	9,776	5,564	4,212
II. Liver‡	968	617	351
III. Brain + Liver§ (experimental)	2,133	1,148	985
IV. Brain + Liver‖ (calculated)	1,734	872	862

* I. Brain refers to a mixture of brain tubulin and polymerized microtubules obtained in vitro as described in Materials and Methods.

‡ II. Liver refers to livers obtained from anesthetized animals homogenized as described in Materials and Methods.

§ III. Brain + Liver (experimental) refers to a mixture of brain tubulin and microtubules, obtained in vitro, which was homogenized together with livers taken from anesthetized animals as described above.

‖ IV. Brain + Liver (calculated), from the colchicine binding data I and II, the theoretical value for a mixture of both was calculated. Since the samples from brain (I) show a high, and those from liver (II) a low specific binding activity, the mixture of both resulted in an intermediary value.

[3] Le Marchand, Y., Ch. Patzelt, and B. Jeanrenaud. Unpublished observations.

present. The failure to demonstrate in vitro polymerization of tubulin in liver supernate could be related to the lower concentration of microtubular protein present in the liver, since in vitro formation of microtubules has been found to be critically dependent upon the concentration of tubulin (17). The high proteolytic activity of hepatic tissue may also be a causal factor in this respect, although, in our hands, the presence of Trasylol (25) did not favor in vitro liver microtubule formation. The lack of some unidentified factor(s) might finally be responsible for the present difficulty in polymerizing liver microtubules from high-speed supernate in vitro.

The decrease in colchicine-binding activity of liver 100,000 *g* supernate produced by the absence of calcium (i.e. presence of EGTA), and its reversal by addition of calcium, cannot yet be explained simply, as in brain (8, 21, 26), by successive polymerization and depolymerization of microtubules, due to our failure to visualize microtubules in the electron microscope under those conditions. These changes could, however, be related to the formation of some unidentified precursors of microtubules endowed with a decreased affinity for colchicine in a way possibly similar to what was suggested for the 30–36S subunit of brain microtubules (9, 20).

One should stress the fact that the minimal (10^{-6} M) and optimal (10^{-4} M) concentrations of colchicine which have been shown to be inhibitory on both VLDL and protein secretion by the liver (11, 12) are in excellent agreement with the dose response curve of colchicine binding in either high-speed liver supernate or purified liver tubulin (Fig. 5). Although vinca alkaloids have been found to be effective, over a similar concentration range, in inhibiting VLDL or protein secretion by perfused livers (11, 12), it is clear from this study that these drugs are not specific for liver tubulin, and that vinblastine, at least at high concentration, obviously precipitates proteins other than the colchicine-binding protein (Fig. 6). The specificity of low concentrations (10^{-5} M) of vinblastine for liver tubulin cannot be assessed since no protein precipitation occurs under these conditions. Vinblastine at this concentration is likely to interfere with microtubular protein, as illustrated by the stabilizing effect of the drug on the binding of colchicine (Table II).

Cytoplasmic microtubules are usually considered to be organelles that are in a dynamic equilibrium with a pool of free tubulin, implying that polymerization and depolymerization occur continuously (16). Colchicine is assumed to bind not to microtubules per se, but only to free tubulin (8), thus preventing the cycle of polymerization and depolymerization. The remarkable difference in the sensitivity of various organs to colchicine may well be related to the relative concentration of tubulin, i.e. to the rapidity of the turnover of the polymerization-depolymerization cycle. Thus, a low sensitivity to colchicine may reflect a situation in which microtubules are relatively stable, tubulin being scant, and vice versa. Of note in this respect is the existence of a marked insensitivity of the chromatophores (melanophores) of *Fundulus heteroclitus* to colchicine, linked with that of apparently stable microtubules (15), and the much greater sensitivity of the chromatophores (erythrophores) of *Holocentrus ascensionis* to this drug, which is apparently associated with rapid turnover of microtubules (19). The wide variation in the sensitivity of various organs to colchicine, a variation actually ranging, depending upon the tissues, from 0.5 to 1,000 μM (1), may thus reflect widely different microtubular systems: some may be represented by stable microtubular elements, while others may be the site of rapid polymerization and depolymerization of these organelles. It is of great interest, therefore, to recall that the liver is very sensitive to colchicine, since 10^{-6} M of the drug resulted in marked decrease of VLDL or protein secretion within 30 min (11, 12). This observation is consistent with the present data (Table V) demonstrating that, under basal conditions, about 60% of the total amount of tubulin present in the liver appeared to exist as free tubulin, while only 40% was presumably assembled microtubules. The validity of the method used to reach such conclusions has been extensively discussed in Results section (Tables IV and VI, Fig. 8). This method takes into account the two main states of the system, i.e. microtubules and solubilized tubulin. It cannot, therefore, evaluate possible intermediary step(s) such as the formation, observed for brain in vitro, of 30–36S rings of tubulin filaments (9, 20), the possible importance of which cannot therefore be assessed.

Thus, the microtubular system of liver might well be one in which the polymerization-depolymerization cycle is very rapid. This would be consistent with our previous observation that, in the electron microscope, microtubular structures are

rather difficult to detect (11). More importantly, it may be hypothesized that, in the liver, a directional assembly-disassembly of microtubules could be intimately linked with the actual intracellular translocation of particles that are eventually secreted. Further studies presently being undertaken will show whether the ratio of total to free tubulin (i.e. tubulin left once microtubular assembly is completed) may be altered by various agents or hormones, or by various metabolic conditions, and whether such changes in this ratio are related to simultaneous changes in the secretory process of the liver.

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