

QUANTITATIVE ANALYSIS OF TUBULIN AND MICROTUBULE COMPARTMENTS IN ISOLATED RAT HEPATOCYTES

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ABSTRACT

A combined morphometric and biochemical approach has been used to identify and quantitate microtubules and tubulin in isolated hepatocytes. The total soluble pool of microtubule protein was estimated by specific high affinity binding to radiolabeled colchicine. Scatchard analysis of the data identified two populations of binding sites: high affinity-low capacity sites resembling tubulin and low affinity-high capacity sites believed to represent nonspecific colchicine-binding sites. Data from these studies indicate that tubulin represents 1% of the soluble protein of the cell, that 9.0×10^{-14} dimers of tubulin are present per microgram soluble hepatocyte protein, and that the average hepatocyte contains 3.1×10^7 tubulin dimers. Our calculations suggest that this amount of tubulin would form a microtubule 1.9 cm in length if totally assembled. However, stereological measurements indicate that the actual length of microtubules in the cytosolic compartment of the average hepatocyte is only 0.28 cm. Thus, these experiments suggest that only 15% of the available tubulin in hepatocytes of postabsorptive rats is assembled in the form of microtubules.

KEY WORDS tubulin · microtubules · isolated hepatocytes · ultrastructural morphometry · colchicine binding

Microtubule protein exists in cells in both assembled (microtubules) and an unassembled subunit form (tubulin). Evidence exists which indicates that these two forms of protein are functionally related, and it has been suggested that microtubules exist in cells in a state of dynamic equilibrium with the cellular pool of soluble tubulin (13). The possibility that the equilibrium between microtubule protein compartments could be shifted toward microtubules or tubulin (depending on the functional needs of the cell) has particular appeal when one considers possible mechanisms by which microtubules may participate in rapidly

changing secretory events (14, 17, 18, 24, 41). It has been of interest, therefore, to assess the proportion of assembled to unassembled microtubule protein in various secretory systems. Recently, two such reports were published in which the relative proportion of assembled to unassembled microtubule protein was estimated in mouse pancreatic islets (25) and liver (22). With somewhat similar approaches, these studies used glycerol as a microtubule-stabilizing agent, and assessed the proportion of the "total colchicine binding protein" present in assembled microtubules. The results of these studies indicate that a rather large proportion (35-40%) of the total colchicine binding protein of islet or liver tissue is assembled into microtubules under normal (unstimulated) conditions. However, the methods

used to provide these estimates do not take into account several important considerations. The first has to do with the fact that tissues from pancreatic islets or liver are structurally complex, being made up of several functionally different cell types (12, 21), and it is not at all certain that the ratio of assembled to unassembled microtubule protein in the whole tissue reflects the ratio present in the particular cells of interest. In addition, the colchicine binding protein estimated in these studies was not specifically identified as microtubule protein, an issue of some consequence as will be seen in the present report. Finally, the approach that has been used cannot provide a quantitative estimate of the total amount of microtubule protein present in a cell. Obviously, in order to evaluate the functional behavior of microtubule protein in various physiological situations, it is advantageous to be able to quantify the total size, as well as the relative size of the two forms of microtubule protein.

The present study was undertaken in an effort to obtain information on the content of the two forms of microtubule protein in a specific cell type. We have attempted to do this by using a combined morphometric and biochemical approach to identify and quantitate microtubules and tubulin in isolated rat hepatocytes. Hepatocytes were selected for study because they normally secrete large quantities of albumin and soluble lipoproteins each day, because colchicine and other antimicrotubule agents have been shown to interfere with the secretion of both these products from liver (18, 28, 30, 32), and because hepatocytes can be successfully isolated from other cells of the liver (4, 7, 8, 38), permitting tubulin and microtubule content to be specifically estimated in the parenchymal cell of this tissue. Using the isolated hepatocytes, we have quantified the total number of tubulin dimers (total soluble pool of microtubule protein) present per hepatocyte on the basis of specific high affinity binding to radiolabeled colchicine and have determined the microtubule content per cell by ultrastructural stereological techniques. We have then used the information provided by these measurements to estimate both the percent of the soluble protein of the cell which is tubulin and the ratio of assembled to unassembled microtubule protein.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats, weighing 250 ± 20 g,

were used for all experiments. The animals were fed standard rat chow and maintained on a 12-h light/dark (6 A.M./6 P.M.) cycle. All studies were begun between 9 and 10 A.M.

Preparation of Isolated Hepatocytes

Rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). Hepatocytes were obtained by *in situ* perfusion of livers with collagenase (50 mg/100 ml) in modified Swim's S77 medium (pH 7.4, 37°C, 95% O₂/5% CO₂) as described by Edwards (8). After perfusion, the liver tissue was gently minced, suspended in the above perfusate and incubated at 37°C for 10 min while being gassed with 95% O₂ and 5% CO₂. The resulting cell suspension was filtered through nylon, washed with Swim's medium and centrifuged at 50 g for 2 min at 22°C. Four additional washes followed: cells destined for electron microscopy were handled at 22°C throughout the washing procedure, whereas cells to be homogenized were maintained at 4°C. The isolation procedure and subsequent washings required 1-1.5 h. Efforts were made to minimize mechanical damage to the hepatocytes: viz, mixing and agitation of the cells was kept to a minimum and the cells were in contact with only plastic vessels and plastic pipettes.

Hepatocyte Viability, Number, and Size

Cells which had been washed in Swim's medium were immediately suspended in an equal volume of trypan blue (0.05%) solution and viewed with a light microscope. Those cells which excluded the dye were considered viable. Preparations in which cell viability was less than 90% were not used.

Cell counts were performed in a hemocytometer: duplicate counts (of 200-300 cells each) from each of two separate dilutions were made of each sample.

Cell diameter was determined in 100 viable cells per sample using an eyepiece micrometer which had been calibrated against a standard.

Preparation of Extracts from Isolated Hepatocytes and Chick Brain

Washed hepatocytes obtained from collagenase-perfused livers were suspended in 20 mM sodium phosphate buffer containing 100 mM sodium glutamate, pH 6.75 at 4°C and homogenized (Dounce homogenizer, tissue grinder B, Kontes Co., Vineland, N. J.) until no intact cells were visible with the light microscope: usually 90-130 strokes were required to homogenize 1.0-2.0 g cells in a total vol of 10 ml. (Preliminary experiments had determined that sonicated and homogenized samples had essentially the same colchicine-binding activity, so homogenized samples were used throughout the study.) Undiluted extracts were centrifuged at 100,000 g for 45 min in an International Model B-60 preparative

ultracentrifuge, with a no. 65 rotor (International Equipment Co., Boston, Mass.). The resulting soluble fraction contained a 6–8-mg protein/ml and was used for determination of colchicine-binding activity.

In order that we might be able to compare various aspects of the colchicine binding of isolated hepatocytes with the known characteristics of the colchicine binding of brain (3, 42), chick brain supernatant fractions were prepared in the following manner. Two brains from 13–15 day embryos were removed and homogenized in 10 ml of phosphate-glutamate buffer with a motor driven Teflon glass tissue homogenizer (Grenier & Co., Chicago Ridge, Ill., Model 7A; four strokes, setting no. 7). Brain homogenates were centrifuged as described above for hepatocytes: resulting soluble fractions contained 0.5 mg protein/ml and were used for measurements of colchicine-binding activity.

Colchicine-Binding Assay

Aliquots of cell supernates were incubated with [*methyl-³Hcolchicine (sp act = 0.5 Ci/mmol), and binding reactions were carried out in phosphate-glutamate buffer as previously described by Bamberg et al. (3), Wilson et al. (43), and Wilson and Bryan (44). Unless otherwise stated, incubations were carried out at 37°C for 2-h intervals, and the incubation mixtures contained 1×10^{-5} M vinblastine sulfate to help stabilize colchicine-binding activity (43, 44). Subsequently aliquots of the incubation mixture were passed through 1×13 cm columns of Biogel P10 to separate bound colchicine complex from free colchicine (43, 44). Radioactivity was determined by adding 1 ml of the bound fraction to 10 ml of scintillation fluid (2) and counted in a Nuclear-Chicago Mark II liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Efficiency was determined with tritiated water as an internal standard. Protein concentrations were determined by the method of Lowry et al (19).*

Ultrastructural Morphometric

Procedures

ISOLATED HEPATOCYTES: Freshly prepared hepatocytes that had been washed in Swim's medium (22°C) were gently centrifuged (50 g; 2 min), and the pellets were resuspended in 1.5% glutaraldehyde prepared in 0.1 M cacodylate buffer (mosmol = 380). Unless otherwise specified, the glutaraldehyde fixative was maintained at 22°C and buffered to pH 7.2. After 20 min, the cells were centrifuged at 10,000 g for 5 s in a Beckman microfuge (Model 152, Beckman Instruments, Inc., Fullerton, Calif.); the pellets were cut into 1-mm blocks and placed in fresh glutaraldehyde overnight. The following day, the cells were postfixed for 1 h in 1% osmium in Palade's veronal buffer (pH 7.0) and stained en bloc for 1 h with 0.5% uranyl acetate in veronal buffer (pH 5.5). The blocks were subsequently dehydrated and embedded in Epon-araldite plastic.

At the electron microscope level, low magnification

($\times 1,200$) photographs from each preparation were used to evaluate the preservation of large numbers of pelleted cells. In addition, three photographs were taken at higher magnifications ($\times 16,000$) of eight undamaged cells from each preparation: to avoid bias, photographs of each cell were taken at 12, 4, and 8 o'clock, without regard to the organelle composition of the region of the cell being photographed (other than to assure that perinuclear and plasma membrane regions of the cells were photographed with equal frequency).

Quantitative estimates of the fraction of the cytoplasmic volume occupied by microtubules and various other organelles were obtained by point-counting stereological techniques as outlined by Weibel (39, 40). In brief, photographic enlargements were made of each electron micrograph and microtubules and various other structures were identified on each print. Microtubules in both transverse and longitudinal profile were identified with the aid of a $2 \times$ magnifying glass on prints enlarged threefold (final print, $\times 48,000$). Longitudinal profiles of microtubules were identified as elongated, unbranched, gently curving or straight, cylindrical elements approx. 250 Å in diameter. At the final magnification used, a tubule of 2 mm or more in length could be recognized as a portion of a microtubule, and transverse sections of microtubules appeared as well-defined, hollow circles approx. 1 mm in diameter set apart from other cytoplasmic structures by a distance of 1 mm. Structures which did not clearly fit this description were not included in the assessment of microtubule volume density.

Transparent grids with lattice dimensions appropriate to the structures being examined¹ were placed over each print, and the number of points (P) of the lattice which fell on particular structures, relative to the number which fell on the cytoplasm, was recorded. The value for the fractional volume (volume density) of the structure within the cytoplasm was obtained from the ratio $\frac{P \text{ structure}}{P \text{ cytoplasm}}$ corrected for the dimensions of particular lattices used.

¹ Lattices in point-counting stereology generally provide a means of obtaining random points which are infinitely small relative to the structures being evaluated (39). In the construction of a millimeter grid with which to estimate microtubule content it was found that points or line-intersections, which were bold enough to be usable were relatively large compared to the size of the microtubules themselves. In using such a grid it was found that microtubule volume density was overestimated by a factor of 2.5. In order to compensate for this error, all estimates of microtubule volume density recorded in this report have been corrected by a multiplication factor of 0.4. Estimations of microtubule volume by this method appears to be highly reproducible from print to print; i.e., the coefficient of variation when individual prints are marked and re-estimated five times is less than 5%.

INTACT LIVER: In order to compare the microtubule content of isolated hepatocytes with liver cells preserved under the most optimal conditions, tissue was obtained from animals in which the livers had been fixed by glutaraldehyde perfusion. In these animals, as in the rats from which isolated hepatocytes had been obtained, blood was first removed from the tissue by a brief washout with Hanks' bicarbonate buffer (pH 7.4, 37°C, 95% O₂/5% CO₂). This was followed by 8 min of perfusion (15 ml/min) with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2, 22°C). All lobes of the liver blanched immediately, and the tissue became hard to the touch within 30 s of the onset of glutaraldehyde perfusion. Slices of the right lobe of the liver were obtained, cut into 1-mm blocks, and submerged in fresh fixative for 2-3 additional hours: subsequent tissue processing was identical to that described for isolated hepatocytes. It should be noted, however, that in glutaraldehyde-perfused livers, only periportal regions were sectioned and examined with the electron microscope.

Chemicals and Biological Materials

[methoxy-³H] Colchicine (Ring C) was obtained from New England Nuclear (Boston, Mass.) and was diluted to a specific activity of 0.5 Ci/mmol by addition of purified unlabeled colchicine (Sigma Chemical Co., St. Louis, Mo.). Podophyllotoxin was obtained from the Aldrich Chemical Co., Inc. (Milwaukee, Wis.). Vinblastine sulfate was a gift from Dr. R. M. Hosley (Eli Lilly and Company, Indianapolis, Ind.). Lumicolchicine was prepared from UV-radiation of colchicine as described by Mizel et al (20). Collagenase (type II) was obtained from Worthington Biochemical Corp. (Freehold, N. J.). Glutaraldehyde (8% purified) came from Polysciences, Inc., Warrington, Pa. D₂O (J. T. Baker Chemical Co., Phillipsburg, N. J.) was used at a pH meter reading of 7.0; actual pH of solution was presumed to be 7.3-7.4 (29).

Swim's media contained the following: 8.99% Swim's S77 (Grand Island Biological Co. [Gibco], Grand Island, N. Y.); 0.05 M tricine, 4% glucose, 0.03% L-glutamine and 1.5% bovine serum albumin. Hanks' buffer (10 ×, Ca⁺⁺- and Mg⁺⁺-free, Gibco) was used with 1 g/liter glucose and 25 mM bicarbonate at pH 7.4. Microtubule stabilizing medium (11) contained 50% (vol/vol) glycerol, 10% (vol/vol) dimethyl sulfoxide (DMSO), 5 mM MgCl₂, 0.1 mM ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), and 5 mM phosphate buffer pH 6.8. Glutamate-phosphate buffer contained 100 mM sodium glutamate and 20 mM sodium phosphate at pH 6.75.

RESULTS

General Characteristics of Isolated Cell Preparations

Examination of different batches of freshly prepared cells with light microscopy indicated that

hepatocytes varied in size: histogram analysis indicated that cell diameters ranged from 17 to 32 μm with a mode at 24.0 μm (cf. reference 7); nuclear diameter ranged from 6.7 to 11.1 μm with a mode at 9.0 μm. Low magnification electron micrographs of fixed cells, as illustrated in Fig. 1a, indicated that about 95% of the cells of each preparation were hepatocytes: of these, approx. 3% were considered to be dead and 25% were found to have discontinuous plasma membranes, vesiculation of smooth endoplasmic reticulum, swollen mitochondria, or some abnormality of distribution of organelles (most common being crowding of organelles around the nucleus of the cell). Such damaged cells were excluded from the stereological analysis reported below.

Stereological Analysis of Liver Cells

Isolated pelleted hepatocytes and hepatocytes from glutaraldehyde-perfused livers were subjected to extensive morphometric measurements (see Fig. 1b) in order to determine whether cells which had been exposed to collagenase treatment and to a lengthy isolation procedure were comparable to cells which had been rapidly fixed by glutaraldehyde perfusion. Analyses of four preparations of isolated cells and four perfused livers indicated that collagenase-separated cells survived the isolation procedure without significant morphological damage, i.e., values for the mean volume densities of mitochondria, lysosomes, peroxisomes, and rough and smooth endoplasmic reticulum of collagenase-isolated cells were identical to the values obtained for cells from glutaraldehyde-perfused livers.

The microtubule content of isolated hepatocytes and hepatocytes from glutaraldehyde-perfused livers was also found to be similar; specifically, the mean (±SD) volume density × 10² of microtubules in cells from seven collagenase-isolated preparations was 0.036 ± 0.011, whereas microtubule volume density of cells from seven glutaraldehyde-perfused livers was 0.030 ± 0.009. Analysis at the 95% confidence level indicates that the error (*E*) in the estimation of microtubules in isolated hepatocytes does not exceed 22% of the mean, i.e.,

$$\left(\frac{E}{\text{mean}} \right) 100 = 22\%,$$

when

$$E = \frac{2 \times SD}{\sqrt{n}}$$

(reference 31).

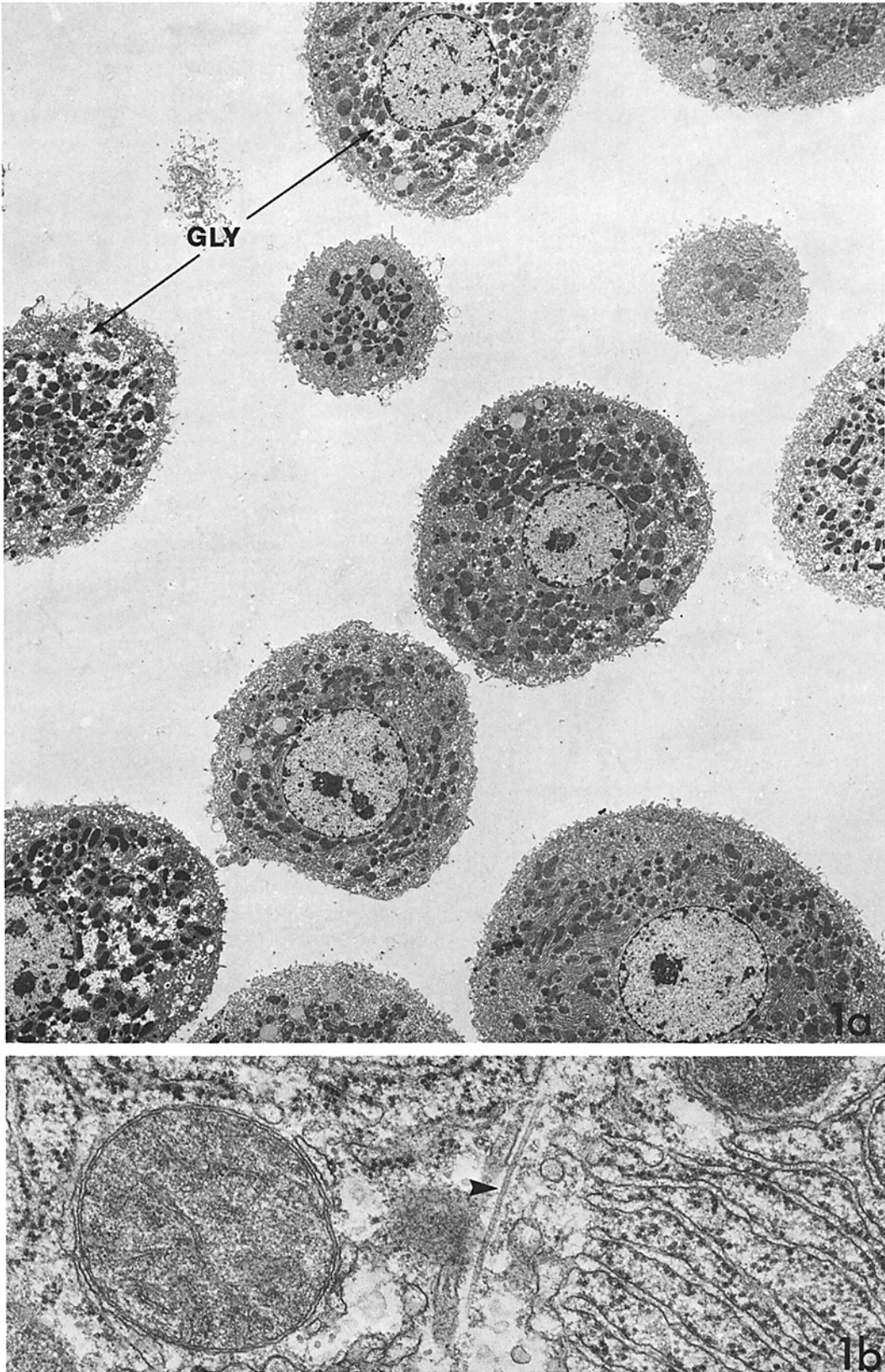


FIGURE 1 (a) Representative micrograph of portions of several isolated hepatocytes fixed with glutaraldehyde. Because of uranyl staining en bloc, pools of glycogen (*GLY*) are seen as clear areas. $\times 2200$. (b) A portion of a hepatocyte at the magnification used in the quantitation of cytoplasm organelles. The arrowhead identifies a longitudinal segment of a microtubule. $\times 48,000$.

Perusal of randomly obtained photographs of isolated hepatocytes indicated that microtubules are always located in the cytosolic compartment² of the cell cytoplasm, and that within that compartment, microtubules appeared to have no special localization or special association with other structures of the cell. Microtubule volume density as measured in hepatocytes of perfused or isolated cells was found to be quite low as compared to the cytoplasmic content of microtubules in other cells measured by the same techniques (26, 27).³ In an attempt to determine whether a larger number of microtubules could be preserved by altering the conditions of fixation, isolated hepatocytes were exposed to warm (37°C) glutaraldehyde or glutaraldehyde at pH 6.6 (24), as well as to agents such as D₂O (29, 37) and glycerol (29), before and during fixation. Measurements of microtubule content under these various conditions indicated that none of the variables introduced into the preparation of the hepatocytes had the effect of increasing the content of microtubules beyond that found under standard conditions.

Characteristics of Hepatocyte Colchicine-Binding Activity

The colchicine-binding activity of tubulin is unstable and decays according to apparent first-order kinetics; the half time for loss of binding activity varies with incubation conditions and the tubulin content of the preparation (42). Table I indicates that for hepatocyte supernate, the half time for loss of colchicine-binding activity is 0.8 h; colchicine-binding activity (at colchicine concentrations of 2×10^{-6} M) corrected for this decay is 3.5×10^{-14} mol of colchicine/ μ g protein. Table I indicates also that the rate of decay of colchicine binding in hepatocyte supernate is prolonged by the use of vinblastine sulfate (5×10^{-4} M), sucrose (1 M), and by low temperatures

² The portion of the cell represented by the cytosol is estimated to be 49% in collagenase-isolated hepatocytes. This figure is based on the finding that the nucleus and cytoplasm occupy 5.3% and 94.7% respectively of the whole cell and the noncytosolic fraction of the cytoplasm (that portion of the cytoplasm occupied by organelles) is shown to be 48%.

³ Reaven, E., R. Maffly, and A. Taylor. 1977. Evidence for involvement of microtubules in the action of vasopressin in toad urinary bladder. III. Morphological studies on the content and distribution of microtubules in bladder epithelial cells. *J. Membr. Biol.*

TABLE I
Decay Rates and Initial Binding Capacity (IBC) of Colchicine-Binding Protein in Soluble Fractions of Hepatocytes and Chick Brain

Tissue	Incubation conditions*	$t_{1/2}$ decay	
		h	mol colchicine/ μ g protein
Hepatocyte	37°C (control)	0.8	3.5×10^{-14}
	37°C + vinblastine sulfate (5×10^{-4} M)	2.9	4.5×10^{-14}
	37°C + vinblastine sulfate (5×10^{-4} M) + sucrose (1 M)	9.2	3.1×10^{-14}
	8°C + vinblastine sulfate (5×10^{-4} M)	10.3	5.6×10^{-14}
Chick Brain	37°C + vinblastine sulfate (5×10^{-4} M)	16.5	2.6×10^{-12}

* All samples were incubated with colchicine (2×10^{-6} M) for 2 h except for experiments at 8°C where 22 h incubations were used for each point.

(8°C). These data, together with the fact that large differences in decay times (induced by varying incubation conditions) do not significantly influence the initial binding capacity of the supernate (Table I), suggest that the colchicine-binding activity of hepatocyte supernate is due, at least in part, to the colchicine binding of the protein tubulin (42, 43).

On the other hand, the values obtained for both decay and binding activity in hepatocyte supernate are quite different than those observed in chick brain (3). In order to make certain that these differences are not due to methodological problems, colchicine binding to extracts of 13-day chick brain was also studied. These results appear in Table I and indicate that the decay rate of vinblastine-stabilized chick brain supernate is 16.5 h and the initial colchicine binding of chick brain is 2.6×10^{-12} mol colchicine/ μ g protein, when 2×10^{-6} M colchicine is used in binding experiments. These values are consistent with those obtained for developing chick brain in other laboratories (3, 43) and demonstrate that the low values for hepatocyte binding are not a function of technical problems in carrying out the colchicine-binding assay.

To further define the characteristics of the colchicine binding of the hepatocyte supernatant fraction, additional incubations were carried out with agents which have a known effect on the colchicine binding of tubulin. Incubations were

carried out for 2 h at 37°C with 2×10^{-6} M colchicine. The results showed that vinblastine sulfate (1×10^{-4} M) increased binding by 85% over control values; a fivefold excess of podophyllotoxin (1×10^{-5} M) reduced binding by 78%; a 10-fold excess of lumicolchicine (2×10^{-5} M) produced a minimal rise (10%) in colchicine binding. In addition, low temperatures (4°C) reduced colchicine binding by 84%. These various findings, when considered together, indicate that the soluble fraction of hepatocyte extracts contains a protein with the binding characteristics of tubulin.

Quantification of Total Tubulin

Content of Hepatocyte

Supernatant Protein

In order to determine how much of the colchicine binding of hepatocyte supernate was actually due to tubulin, additional experiments were carried out in which soluble extracts of hepatocyte preparations were incubated with various concentrations of colchicine under equilibrium conditions (6 h, 37°C). The amount of colchicine bound at various colchicine concentrations was corrected for decay of colchicine-binding activity which occurred during incubation; these corrected values are presented as a composite plot in Fig. 2. The binding curve is very steep at low ($<2-3 \times 10^{-6}$ M) concentrations of colchicine; and although it appears to flatten somewhat between 8×10^{-6} M and 1×10^{-5} M colchicine, it shows a further increase at higher colchicine concentrations. This lack of saturability with colchicine suggests the presence of more than one class of sites with different affinity for colchicine in hepatocyte supernatant fractions. Indeed, when these data are viewed as a Scatchard plot (Fig. 3) it is possible to separate at least two populations of binding sites: a straight line indicating a single affinity class of binding sites (*Site 1*) is obtained from incubation of hepatocyte supernates with colchicine between 8×10^{-7} – 8×10^{-6} M and a second straight line is obtained from incubation points at $>1 \times 10^{-5}$ M colchicine (*Site 2*).

In order to identify which of the colchicine binding sites represent tubulin, an additional experiment was carried out in which hepatocyte extracts were incubated with a sixfold excess of podophyllotoxin at various colchicine concentrations (Table II). These data indicate that podophyllotoxin (at this concentration) blocks approx. 75% of the total colchicine binding at very low colchicine concen-

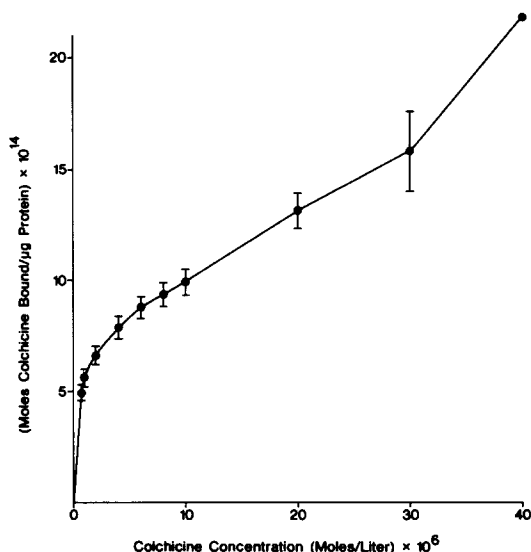


FIGURE 2 Colchicine binding to soluble hepatocyte protein at equilibrium. Each point represents a mean (\pm SE) of seven values. The binding reactions were allowed to proceed at 37°C for 6 h. Points are corrected for decay of colchicine-binding activity which occurred during the incubation periods. The binding curve is very steep at very low concentrations of colchicine, and, although it tends to flatten between 8×10^{-6} M and 1×10^{-5} M colchicine, it shows a further increase at higher colchicine concentrations. The lack of saturability with colchicine suggests the presence of more than one class of binding sites with affinity for colchicine.

trations. As the colchicine concentration is increased beyond 2.9×10^{-6} M, podophyllotoxin shows decreasing ability to compete for the colchicine binding. Since podophyllotoxin is believed to bind only to tubulin (43), these results suggest that the high affinity-low capacity (*Site 1*) sites of Fig. 3 are tubulin. This impression is strengthened by the fact that the binding constant calculated from the slope of the *Site 1* line in Fig. 3 was found to be 1.4×10^6 liters/mol, which is similar to the value obtained for the binding of colchicine to purified chick brain tubulin at 37°C (43).

Given these findings regarding the identity of the tubulin sites in liver cell extracts, it was now possible to correct each tubulin (*Site 1*) point on the Scatchard plot of Fig. 3 for the amount of contaminating nontubulin (*Site 2*) binding (10, 16); this resulted in a slight shift of the site 1 line to the left as indicated by the dashed line in Fig. 3.

Finally, the number of tubulin-related colchicine binding sites per microgram soluble hepatocyte protein could be directly determined from

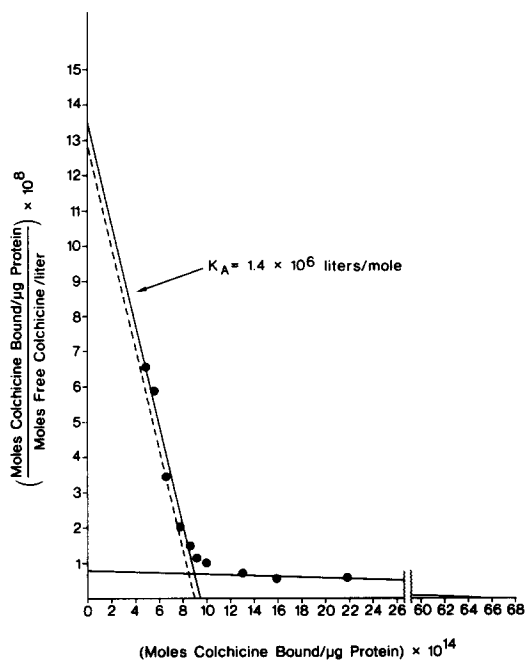


FIGURE 3 Scatchard plot of colchicine binding to hepatocyte tubulin. Data points are identical to those of Fig. 2. Solid line with intercept at 9.4×10^{-14} mol colchicine/ μg protein represents tubulin sites corrected for nontubulin binding. The colchicine-binding constant ($K_A = 1.4 \times 10^6$ liters/mol) for tubulin was calculated from the slope of the corrected (dashed) line. The low affinity-high capacity sites with approximate intercept at 65×10^{-14} mol colchicine/ μg protein probably represents nontubulin colchicine binding of soluble extracts.

TABLE II
Effect of Podophyllotoxin on Colchicine Binding of Soluble Hepatocyte Protein*

Colchicine concn	Inhibition of colchicine binding
M	%
8.5×10^{-7}	76
1.7×10^{-6}	77
2.9×10^{-6}	75
8.5×10^{-6}	64
1.7×10^{-5}	45
2.9×10^{-5}	39

All samples were incubated at 37°C for 6 h and included vinblastine sulfate (1×10^{-4} M) during incubation. Each experimental point was corrected for decay.

* [Podophyllotoxin] = 6 × [colchicine].

the corrected (dashed line) intercept on the abscissa of the Scatchard plot shown in Fig. 2: this value was found to be 9.0×10^{-14} mol colchicine/ μg protein. Based on the assumption that one

colchicine binding site represents one tubulin dimer (5, 43), this value represents 9.0×10^{-14} mol of tubulin or 5.5×10^{10} ($9.0 \times 10^{-14} \times$ Avogadro's number) dimers of tubulin per microgram protein.

The minimum (23) tubulin content of hepatocytes can be calculated by two separate methods resulting in identical answers. In the first, the number of moles of hepatocyte tubulin/gram protein multiplied by the mol wt of tubulin (110,000 [34]) indicate that tubulin represents 1.0% of the soluble cell protein. In the second, less direct method, tubulin content is determined by comparing the values for initial colchicine-binding capacity (IBC, see Table I) of hepatocyte extracts with that of 13-day chick brain extracts known to contain 42% of tubulin (3, 44). In using the equation:

[% tubulin in hepatocyte supernate

$$= \frac{\text{IBC hepatocyte supernate}}{\text{IBC chick brain supernate}} \times 42\%,$$

one can determine that the tubulin content of hepatocyte supernate is 1.0% of the soluble protein of the extract.

Quantification of Assembled Tubulin in Hepatocytes

By knowing the number of tubulin dimers (5.5×10^{10}) and the average number of cells (1,800) per microgram soluble protein, it is possible to estimate that the average hepatocyte contains 3.1×10^7 dimers of tubulin. If all this hepatocyte tubulin were assembled, the average hepatocyte would contain a microtubule $1.9 \times 10^4 \mu\text{m}$ in length: this calculation is based on the assumption that the length of a tubulin dimer is $80 \times 10^{-4} \mu\text{m}$ (1, 33), and that there are 13 dimers (protofilaments) per microtubule cross section (1, 33, 36) in hepatocytes.

However, the combined length of all microtubules actually present in a single hepatocyte is somewhat less than calculated above, as can be determined from stereological measurements. Microtubule volume (V_{MT}) in a cell is equal to the volume of the cell compartment containing microtubules (cytosol) times the percent of microtubules (MT) within that compartment. Thus,

$$V_{MT} = \left(\frac{4}{3} \pi R^3 \right) \times (\% \text{ of cell which is cytosol}) \times (\% MT).$$

Since R = mean radius of hepatocytes = $12 \mu\text{m}$, then

$$V_{MT} = \left(\frac{4}{3}\pi 12^3\right) \mu\text{m}^3 \quad (1)$$

$$\times (49\%) \times (0.036\%).$$

However, the volume of a microtubule is also the volume of a cylinder with a radius, r , and length, l ; thus,

$$V_{MT} = \pi r^2 l, \quad (2)$$

where $r = 0.012 \mu\text{m}$.

Since Eq. 1 = Eq. 2, one can solve for l as follows:

$$\left(\frac{4}{3}\pi 12^3\right) \mu\text{m}^3 \times (49\%)$$

$$\times \frac{(0.036\%)}{\pi \times (0.012)^2 \mu\text{m}^2} = 2.8 \times 10^3 \mu\text{m},$$

which is equal to the microtubule length per average hepatocyte.

Given the value for the assembled length of tubulin (microtubule) per cell ($2.8 \times 10^3 \mu\text{m}$ or 0.28 cm) and the length of the theoretical microtubule which could be assembled from the total available tubulin dimers of the average cell ($1.9 \times 10^4 \mu\text{m}$ or 1.9 cm) it is possible to calculate ($0.28 \text{ cm}/1.9 \text{ cm}$) that the assembled tubulin of hepatocytes of normal rats represents 15% of the total available tubulin of the cell.

DISCUSSION

Results from these studies have characterized the colchicine-binding protein present in the soluble fraction of homogenates from rat hepatocytes, and have identified and quantitated that portion of the total colchicine binding which can be attributed to tubulin. It appears that hepatocyte supernate is comprised of at least two populations of colchicine-binding sites: *Site 1* is a population of high affinity-low capacity sites which resembles tubulin in every characteristic measured; *Site 2* is a low affinity-high capacity class of sites which appears when colchicine concentrations greater than $1 \times 10^{-5} \text{ M}$ are used in binding experiments. *Site 2* does not saturate with colchicine and does not competitively bind with podophyllotoxin and most probably represents nonspecific, nontubulin binding. Patzelt et al. (22) have observed similar nonspecific colchicine binding (at 10-fold higher colchicine concentrations) in mouse liver. Although the nature of these secondary sites is

unknown, in rat liver they bind with colchicine at such low colchicine concentrations, that one must consider the possibility that they may participate in colchicine binding in vivo and perhaps contribute to the functional effects of colchicine on liver. On the other hand, the high affinity colchicine-binding sites (tubulin) appear to represent only 1.0% of the soluble protein of hepatocytes. As such, the content of hepatocyte tubulin is far less than the 42% found in developing chick brain (3, 44) but is comparable to the 4-5% found, by use of the same techniques, in soluble supernatant fractions of toad bladder epithelium (35), and in sea urchin eggs (23).

Calculations derived from measurements of the microtubule and tubulin content of hepatocytes suggest that only a small proportion (15%) of the soluble tubulin of hepatocytes may be actually assembled in the form of microtubules. This value is based on the observations that 3×10^7 tubulin dimers are present in the cytosol of the average hepatocyte and on the calculation that these tubulin dimers would, if totally assembled, form a microtubule 1.9 cm in length. Since the actual length of microtubules in the cytosolic compartment of the average hepatocyte is estimated by stereological measurements to be 0.28 cm, it appears that only 15% of the available tubulin is in the form of microtubules at the time of fixation.

Obviously, the usefulness of this technique for estimating the microtubule content of cells depends on the validity of various assumptions: first, we have assumed that microtubules are not altered (either increased or decreased in amount) during the preparation or fixation of the isolated hepatocytes. In this regard, our stereological analysis has offered assurance that the microtubule content of isolated hepatocytes is identical to that found in ideally fixed hepatocytes from glutaraldehyde-perfused livers. Second, we have assumed that the method for estimating the percent of microtubules per volume cytoplasm gives accurate values. At best, stereological methods provide only estimates of the volume concentration of structures within cells. For microtubules, certain technical problems relating to the small diameter of the microtubule probably lead to a systematic overestimation of this value (9, 26), although the extent to which this overestimation distorts the actual values for microtubule volume cannot be determined. However, in this study it would have the effect of overestimating the percentage of micro-

tubule protein which is assembled into microtubules: thus, the value for this figure is probably somewhat less than the calculated 15%. Beyond this systematic error one has 95% confidence that the error in microtubule estimation does not exceed 22% of the mean, i.e., that the estimation of the length of microtubules in hepatocytes could range from 0.22 to 0.34 μm . This potential deviation from the microtubule mean (0.28 μm) could alter the calculation of the percent of assembled microtubule protein of the hepatocyte by approx. 2%. Third, we have assumed that the 13 protofilaments/80 Å dimer model for microtubule structure is correct. Recently, Tilney et al. (36) obtained direct evidence that microtubules from a wide variety of sources all typically contain 13 subunits. Although the number 13 is apparently not invariant, microtubules which reportedly have fewer (or more) protofilaments appear to be exceptions from the norm. A number of studies have suggested an 80 Å axial repeat (see reference 33 for review) within the protofilament which is thought to be a dimer of two 40 Å globular monomers tilted slightly with respect to the protofilament lattice. However, the precise arrangement of monomers to each other and to the protofilament lattice has not been established for most microtubule systems. It is possible, therefore, that the calculated length of our hypothetical hepatocyte microtubule may have to be adjusted when the precise length of the tubulin dimer is known. However, it should be noted that in our mathematical construction of the proportion of tubulin assembled in hepatocytes, a 10% change in the length of tubulin dimer, would alter the amount of assembled tubulin by only 10% of the existing figure: thus, a 10% reduction in the length of the tubulin dimer would increase the amount of assembled tubulin from the estimated 15% to 16.5% of the total available tubulin of the cell; and an unlikely reduction of 50% in the length of the tubulin dimer would increase the amount of assembled tubulin from the estimated 15% to 22.5% of the tubulin of the cell. Finally, our data assume that all the hepatocyte tubulin which is competent to assemble into microtubules is in a form which can be measured by the colchicine-binding assay. A number of recent studies (see reference 33 for review) have shown, however, that intermediate polymeric forms of tubulin exist in extracts of cells at low temperatures. If present in hepatocyte homogenates, such aggregated tubulin could become part of the solu-

ble protein of the supernatant fraction, but it may bind only minimal amounts of colchicine (15). However, if a significant amount of tubulin did exist in this form, it would have the effect of increasing the size of the hepatocyte tubulin pool and, as such, would decrease the relative proportion of assembled microtubules. It should be noted that these data say nothing about the tubulin content of membranes and other structures which might also be found within the insoluble (particulate) fraction of liver cells.

Thus, a consideration of the various potential errors in the method does not appear to alter the finding that hepatocytes from normal postabsorptive rats contain only a small pool of microtubule protein in the assembled form. However, the estimate of 15% differs substantially from the 40% figure reported by Patzelt et al. (22) for mouse liver. Whether the difference in results between the two studies stems from the use of a different animal species, or from organ vs. parenchymal cell analysis, or whether it is related to differences in methodology, remains to be seen.

In conclusion, this report describes a combined biochemical and ultrastructural approach for the quantitation of tubulin and microtubules in hepatocytes: in this initial study, tubulin and microtubules have been described in cells from normal, postabsorptive animals, but it is anticipated that the methodology will be useful in quantitating changes in hepatocytes from animals in altered physiological states.

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