

# PHYSIOLOGICAL REGULATION OF TOTAL TUBULIN AND POLYMERIZED TUBULIN IN TISSUES

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## ABSTRACT

Polymerized and depolymerized forms of tubulin were measured in rat and mouse liver, rat islets, human lymphocytes, and platelets. The percent of the total tubulin present in the polymerized form varied from  $30.3 \pm 1.5\%$  in the liver of the fed rat to  $89.2 \pm 0.2\%$  in human platelets. Fasting decreased the total tubulin and to a greater extent the polymerized form of tubulin in both rat and mouse liver. Glucose feeding increased the polymerized tubulin without affecting the total tubulin content in rat liver. Phytohemagglutinin-stimulated lymphocytes exhibited at least a three-fold increase in total tubulin (expressed in terms of DNA content), which during the initial 48 h of incubation was accounted for *in toto* by an increase in polymerized tubulin. It is suggested that the lectin not only accelerates tubulin synthesis but also stimulates the polymerization process. Storage of platelets at  $4^{\circ}\text{C}$  for 6 days resulted in a marked decrease in total tubulin and an even greater reduction in the polymerized form. It is concluded that both the total tubulin content and its degree of polymerization can be modulated independently by a wide variety of physiological factors.

The existence of a dynamic equilibrium between the polymerized and depolymerized forms of tubulin and its possible role in microtubule-dependent functions has been suggested by many investigators (7, 18). To determine whether physiological factors might exert their action on microtubule-dependent processes through alterations in this equilibrium, a rapid and sensitive assay was developed in our laboratory which allows quantitation of the polymerized and depolymerized forms of tubulin in tissues. The present study reports the application of this method to various tissues under experimental conditions presumed to influence their microtubule-dependent activities.

## MATERIALS AND METHODS

### Materials

Dextran (clinical grade, Sigma Chemical Co., St.

Louis, Mo.), hypaque sodium 50% (Winthrop Laboratories, New York.) and Ficoll 400 (Pharmacia, Inc., Piscataway, N. J.) were used in the isolation of human lymphocytes. Purified E-phytohemagglutinin (E-PHA) was a gift from Dr. S. Kornfeld. [ $^3\text{H}$ ]thymidine (6.7 Ci/mmol) was purchased from New England Nuclear (Boston, Mass.). All tissue culture media were obtained from Grand Island Biological Co. (Grand Island, N.Y.). The chemicals used for the colchicine-binding assay have been described previously (19).

### Animals

Male Sprague-Dawley rats (300–350 g) had free access to regular Purina rat chow (Ralston Purina Co., St. Louis, Mo.). In the fasting studies, food was withheld for 60–72 h. Glucose-fed animals were maintained *ad libitum* for 72 h exclusively on a 30% dextrose drinking solution in addition to normal drinking water. Previous studies (2, 3) in our laboratory have demonstrated that these animals do not ingest sufficient calories to maintain

weight, but despite weight loss the high carbohydrate intake stimulates hepatic lipoprotein synthesis and maintains the "fed state" for the hepatic enzyme glycolytic-gluconeogenic profile. Thin and obese mice (C57 and BL/6J) were obtained from the Jackson Laboratories (Bar Harbor, Maine.)

### Quantitation of Polymerized and Depolymerized Forms of Tubulin

The experimental procedure for quantifying the polymerized and depolymerized forms of tubulin in tissues is described in the companion paper (19). The technique involves the homogenization of tissues in a microtubule-stabilizing solution (MTS), using a tissue/buffer ratio ranging from 1:13 to 1:80. After centrifugation at room temperature, the precipitated microtubules were resuspended and centrifuged in a depolymerizing solution (TS). For high tissue dilutions, Trasylol, 300 U/ml, was added to MTS, and 0.1% albumin to TS. Both depolymerized tubulin, which is recovered in the first supernatant fraction SN-I, and polymerized tubulin, which is recovered in the second supernatant fraction SN-II, were measured by a colchicine-binding assay. The polymerized and depolymerized forms of tubulin are expressed in terms of tissue wet weight, DNA content (9), and protein content (14). The statistical significance of differences was calculated according to the Student's *t* test.

### Preparation of Tissue Samples

**LIVER TISSUE:** After decapitation, 0.5 g of liver tissue was removed rapidly from the right anterior lobe and quickly immersed in 6.0 ml of MTS at room temperature. After homogenization with a motor-driven Teflon pestle, 4 ml of the homogenate was centrifuged at 100,000 *g* for 45 min at room temperature. The pelleted fraction was resuspended and centrifuged in 2 ml of ice-cold TS, and aliquots of the SN-I and SN-II fractions were assayed for colchicine-binding activity.

**ISLETS OF LANGERHANS:** Islets of Langerhans were prepared by the collagenase technique (12). All media used were kept at room temperature. 200 isolated islets were homogenized in 125  $\mu$ l of MTS by three passes of a loosely fitting Teflon pestle. The homogenate was transferred with a siliconized pipette into a Sorvall polycarbonate tube (DuPont Instruments, Sorvall Operations, Newtown, Conn.) and centrifuged for 10 min at 8,000 *g*. The pelleted fraction was resuspended by sonication in 100  $\mu$ l of ice-cold TS and centrifuged at 8,000 *g* for 10 min at 4°C.

**LYMPHOCYTES:** Human peripheral blood lymphocytes were obtained in >95% purity by Ficoll-Hypaque gradient separation (27). Cells ( $1-4 \times 10^6$ ) were suspended in 2 ml of TC-199 supplemented with 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml). Incubations were carried out at 37°C in an atmosphere of 5% CO<sub>2</sub>-95% O<sub>2</sub> for periods up to 96 h. Mitogenic stimulation was induced by 5  $\mu$ g/ml

E-PHA; control cells were incubated for identical time periods without additions of lectin. At various intervals, cell samples were homogenized in 150  $\mu$ l of MTS and processed as described for islets. [<sup>3</sup>H]thymidine incorporation was measured by the method of Smith et al. (25).

**PLATELETS:** Platelets were isolated from 60 ml of human blood as described by Tollefsen et al. (29).  $4 \times 10^8$  cells were homogenized in 300  $\mu$ l of MTS and processed as described for islets. Out-dated platelets were prepared from platelet-rich plasma stored at 4°C.

## RESULTS

### Polymerized and Depolymerized Forms of Tubulin in Various Tissues

Both the polymerized and depolymerized forms of tubulin were present in the four tissues studied, but their relative proportions differed markedly (Fig. 1). Only 30-35% of the total tubulin was present in the polymerized form in rat liver and islets, whereas much higher levels were present in lymphocytes (~60%) and platelets (~90%). The lability of the cytoplasmic microtubules of these tissues to cold was demonstrated in two ways. When the tissues were homogenized in cold TS, the colchicine-binding activity in the SN-II fraction was reduced to background levels and the depolymerized tubulin measured in the SN-I fraction was increased correspondingly. Pre-exposure of the tissues to 4°C for 30 min before homogenization in MTS also decreased the polymerized tubulin with a concomitant increase in the depolymerized fraction. The total tubulin content was not altered by these experimental manipulations. The total tubulin content in platelets stored for 6 days at 4°C ranged from 3.0 to 3.5  $\mu$ g/ $10^9$  cells, representing

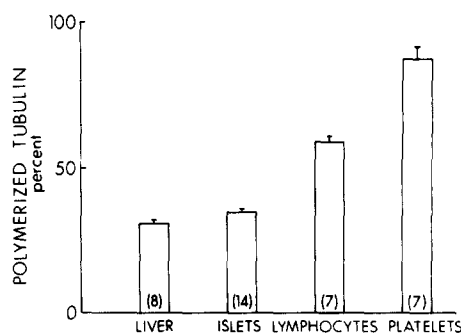


FIGURE 1 Polymerized tubulin content of rat liver, rat pancreatic islets, human lymphocytes, and human platelets. Number of experiments are indicated in parentheses. Values represent mean  $\pm$  SEM and are expressed as percent of total tubulin content.

less than a third of the values obtained in freshly prepared platelets ( $10.1 \pm 1.1 \mu\text{g}/10^9$  cells). The decrease in the polymerized tubulin in stored platelets was even more impressive:  $0.7\text{--}1.2 \mu\text{g}/10^9$  outdated platelets versus  $7.9 \pm 0.9 \mu\text{g}/10^9$  freshly prepared platelets.

#### Regulation of Total Tubulin and Polymerized Tubulin in Liver Cells

Fasting resulted in an  $\sim 35\%$  decrease in the total tubulin content of liver, a fall somewhat less than that observed in the total soluble protein (Table I). Comparable values were obtained when tubulin was expressed in terms of DNA content (Fig. 2). Furthermore, the effect of fasting was more marked on the polymerized tubulin fraction than on the total tubulin content (Fig. 2). Glucose feeding prevented the decrease in polymerized tubulin; indeed, the percent of the total tubulin pool present in this form was increased  $\sim 50$  and  $\sim 80\%$  above that in the fed and fasted rat, respectively.

Both forms of tubulin were also demonstrable in the livers of lean and obese C57 BL/6J mice (Fig. 3). Although the total tubulin content was markedly increased ( $\Delta \sim 140\%$ ) in the livers of the obese littermates (Table II), the concentration per cell (expressed in terms of DNA) was only slightly elevated ( $P < 0.02$ , Fig. 3). Fasting resulted in an  $\sim 15\%$  ( $P < 0.02$ ) and  $\sim 30\%$  ( $P < 0.02$ ) decrease in the total hepatic tubulin content of thin and obese littermates, respectively (Table II). Polymerized tubulin was decreased to an even greater extent, averaging  $\sim 25\%$  ( $P < 0.01$ ) in the thin and  $\sim 35\%$  ( $P < 0.01$ ) in the obese mouse (Fig. 3).

#### Changes in Total Tubulin and Polymerized Tubulin during the Cell Cycle

E-PHA increased the [ $^3\text{H}$ ]thymidine incorporation by human lymphocytes incubated for 72 h,

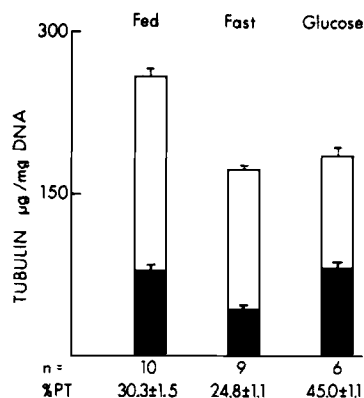


FIGURE 2 Effect of fasting and glucose feeding on polymerized (closed column), depolymerized (open column), and total tubulin (combined column) content of rat liver. Values represent mean  $\pm$  SEM, n indicates number of experiments, and % PT the percent of total tubulin in the polymerized form.

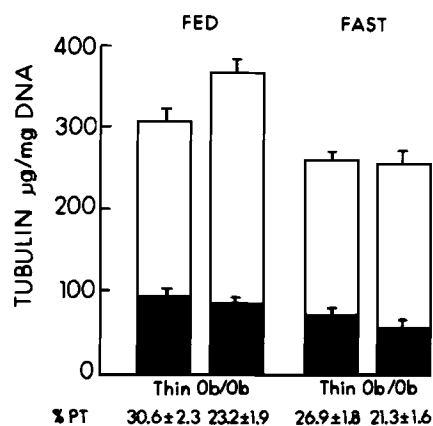


FIGURE 3 Effect of fasting on polymerized (closed column), depolymerized (open column), and total tubulin (combined column) content of the liver of thin and obese (Ob) littermates of the C57 BL/6J mouse. Values represent mean  $\pm$  SEM of six experiments and % PT the percent of total tubulin in the polymerized form.

TABLE I  
Effect of Fasting and Glucose Feeding on Total Tubulin and Polymerized Tubulin in Rat Tissue

Exp condition	$\Delta$ Weight	Liver weight	Protein	Bound colchicine		Tubulin	
				SN-I	SN-II	Total	Polymerized
				nmol/liver		mg/liver	
Control	+14.7 $\pm$ 0.3	11.0 $\pm$ 0.7	2.1 $\pm$ 0.2	43.9 $\pm$ 3.7	19.1 $\pm$ 0.9	6.8 $\pm$ 0.6	2.1 $\pm$ 0.1
Fast	-44.8 $\pm$ 1.9	7.1 $\pm$ 0.2	1.5 $\pm$ 0.1	30.6 $\pm$ 1.6	10.1 $\pm$ 0.8	4.4 $\pm$ 0.2	1.1 $\pm$ 0.1
Glucose-fed	-23.6 $\pm$ 2.9	8.7 $\pm$ 0.5	1.2 $\pm$ 0.1	24.3 $\pm$ 1.2	19.1 $\pm$ 0.9	4.8 $\pm$ 0.2	2.2 $\pm$ 0.1

Values represent mean  $\pm$  SEM of six to ten experiments.

TABLE II  
Effect of Fasting on Total Tubulin and Polymerized Tubulin of the Liver of the C57 BL/6J Mouse

Exp. condition	Mouse	Age	Body weight		Liver weight		Total tubulin content	
			mo	g	g	g	$\mu\text{g/g liver wt}$	$\mu\text{g/liver}$
Fed	Thin	2	23.2 $\pm$ 0.3	1.26 $\pm$ 0.06	415 $\pm$ 22	483 $\pm$ 31		
	OB/OB		41.5 $\pm$ 1.1	3.36 $\pm$ 0.23	335 $\pm$ 24	1,225 $\pm$ 139		
Fed	Thin	8	35.2 $\pm$ 0.7	1.90 $\pm$ 0.10	439 $\pm$ 16	875 $\pm$ 24		
	OB/OB		67.8 $\pm$ 2.5	5.27 $\pm$ 0.03	393 $\pm$ 7	2,068 $\pm$ 98		
Fast	Thin	8	23.6 $\pm$ 0.5	0.89 $\pm$ 0.03	815 $\pm$ 24	734 $\pm$ 25		
	OB/OB		54.6 $\pm$ 1.9	2.55 $\pm$ 0.18	556 $\pm$ 19	1,440 $\pm$ 65		

Values represent mean  $\pm$  SEM of six experiments. OB, Obese.

indicating its mitogenic effectiveness in the system used (Table III). The total DNA levels increased  $\sim$ 70% during this incubation period, suggesting that the synchronized cells completed almost one cell cycle. Incubation with E-PHA resulted in a significant increase ( $P < 0.01$ ) in both total tubulin ( $\sim$ 66%) and polymerized tubulin ( $\sim$ 100%) within 24 h (Fig. 4). By 96 h, the polymerized tubulin had increased sevenfold ( $P < 0.001$ ) and the total tubulin fivefold ( $P < 0.001$ ). The marked increase in total tubulin content of lectin-stimulated cells was also evident when expressed in terms of DNA: after 72 h of incubation, non-stimulated cells contained  $30.8 \pm 2.8$  ng tubulin/ $\mu\text{g}$  DNA whereas E-PHA-stimulated cells exhibited levels of  $92.4 \pm 6.3$  ng tubulin/ $\mu\text{g}$  DNA. As is evident in Fig. 4, all of the increase in tubulin content during the initial 48 h of incubation was accounted for by polymerized tubulin. Thus, the percent of tubulin present in the polymerized form was increased from initial levels of  $58.3 \pm 5.8\%$  to  $67.2 \pm 6.2\%$  at 24 h and  $70.9 \pm 5.1$  at 48 h. Subsequently, depolymerized tubulin did increase approx. twofold after 96 h of incubation. In non-stimulated cells, the total tubulin content remained constant for the initial 48 h of incubation and then fell  $\sim$ 35% after 96 h, with apparently similar decreases in both the polymerized and depolymerized forms of tubulin—although only the decrease in depolymerized tubulin was statistically significant ( $P < 0.02$ ).

#### Purification of Liver Tubulin

The ability to precipitate and depolymerize cytoplasmic microtubules quantitatively provides a useful technique for the isolation and purification of tubulin from tissues which are less rich in tubulin than is brain. Livers from glucose-fed animals, characterized by an increased microtubule content, were homogenized in MTS and centrifuged

TABLE III  
Effect of E-PHA Stimulation on DNA Synthesis in Human Lymphocytes

Exp condition	$^3\text{H}$ Thymidine incorporation	
	$\text{cpm} \times 10^3$	DNA content $\mu\text{g}$
Control	43 $\pm$ 17	1.23 $\pm$ 0.21
E-PHA	597 $\pm$ 43	2.11 $\pm$ 0.22

Values represent mean  $\pm$  SEM of four experiments in which  $10^6$  cells were incubated for 72 h.

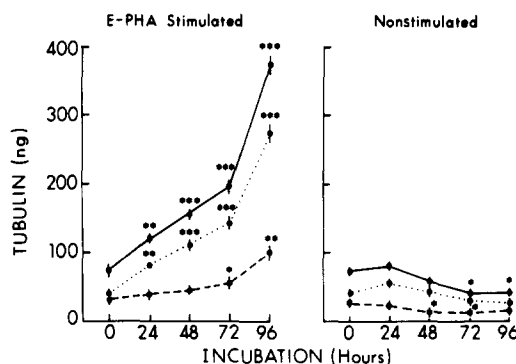


FIGURE 4 Effect of E-PHA stimulation on total (—), polymerized (---), and depolymerized (...) tubulin levels in human lymphocytes incubated in the presence and absence of E-PHA (5  $\mu\text{g}/\text{ml}$ ) for varying periods. Values represent mean  $\pm$  SEM of five to seven experiments, and are expressed as ng of tubulin per incubation flask (see Materials and Methods). The statistical significance of differences compared to 0-time values were calculated according to the Student's *t* test: \* $P < 0.02$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

at room temperature at 40,000 g for 30 min. The pelleted fraction was then resuspended and centrifuged in cold depolymerizing TS (1 mg/g initial wet weight). The SN-II fraction, after the addition of 1 M sucrose, 1 mM EGTA, and 1 mM GTP, was incubated for 60 min at 37°C—conditions

favoring tubulin polymerization (24). After centrifugation at 100,000 g for 45 min at 30°C, the pellet was resuspended in cold TS and eluted on a Biogel A 5-m column with TS (Fig. 5). Peak no. II, which coeluted with <sup>125</sup>I-rat brain tubulin, was dialyzed against distilled water and demonstrated >90% purity when multiple samples were subjected to polyacrylamide gel electrophoresis (Fig. 5).

## DISCUSSION

The demonstration that colchicine, a drug known to disrupt and inhibit the formation of mitotic spindles (18), also inhibits various functions of nondividing cells, led to the suggestion that cytoplasmic microtubules are involved in such diverse processes as secretion (5, 11), intracellular transport (1, 15), membrane movement (17), and the maintenance of cell shape (28). If polymerized tubulin is required for these cellular events, a reduced level of activity would be anticipated when polymerized tubulin is decreased. Conversely, an increase in polymerized tubulin might be expected to gear cells for a more rapid and complete response. The remarkably high degree to which tubulin is polymerized in freshly prepared platelets is consistent with this view, although the role of microtubules in the platelet release mechanism is still controversial (4, 31). The observation that tubulin is not only depleted but also mostly depolymerized in stored platelets

raises the question whether the altered responsiveness of stored platelets (16) is related to impaired microtubule function. The grossly different levels of tubule polymerization seen in platelets and lymphocytes in contrast to liver appear to reflect the cell type under study rather than experimental differences in the stabilization of tubules in cell suspensions vs. tissues. For example, preliminary studies of tubule polymerization in hepatocyte suspensions have demonstrated levels comparable to those seen in hepatic tissue.

The role of microtubules in the mechanism of release of albumin and lipoprotein from liver cells has been demonstrated more convincingly (13, 26). Although the release of lipoprotein from hepatocytes has not been thought of as a model for exocytotic secretion, it may represent a useful system for examining the role of microtubules in the intracellular transport of secretory granules. Transport from the endoplasmic reticulum to the cell membrane probably constitutes an important component in the secretion of lipoprotein and albumin, particularly in view of the high rate of turnover of these secretory products (13) and the absence of an identifiable storage compartment. Since it is known that lipoprotein release is decreased by fasting (6) and markedly stimulated by glucose feeding (2, 22), the alterations in polymerized tubulin observed during these conditions support the hypothesis that changes in polymerized tubulin influence intracellular transport and/or secretory responsiveness. Similar results have also been recently reported for pancreatic islets (20), in which it was demonstrated that alterations in the level of polymerized tubulin were accompanied by parallel changes in the insulin secretory response to a glucose stimulus. An increase in microtubule content in response to a secretory stimulus has also been noted in parathyroid glands treated with phosphorus when assessed by electron microscopy (23).

These studies further support the concept that the distribution of tubulin between the polymerized and depolymerized forms represents a dynamic equilibrium which can be modulated by a variety of physiological factors. Variations in the percent of tubulin present in the polymerized form were demonstrated independent of equivalent changes in the total tubulin content. For example, fasting reduced the polymerized tubulin in the liver to a far greater extent than the total tubulin in both the rat and C57 mouse. Glucose feeding overcame completely the effect of fasting on the

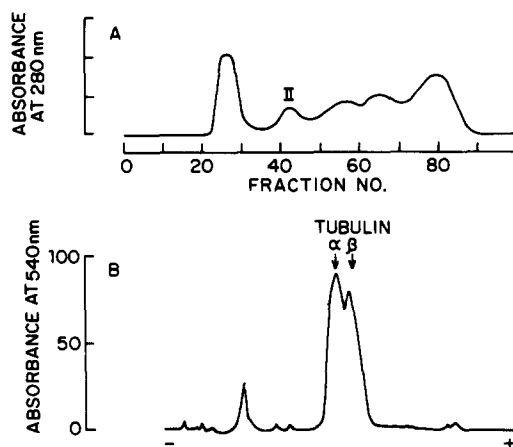


FIGURE 5 Purification of liver tubulin. (A) Elution profile of a tubulin-rich fraction from liver on a Biogel A 5-m column eluted with TS; tubulin is contained in peak II. (B) Densitometric tracing of peak II subjected to SDS-polyacrylamide gel electrophoresis.

degree of tubulin polymerization, but did not affect the decrease in the total tubulin content. The fact that fasting altered the total tubulin and the polymerized tubulin to a far greater extent in the obese mouse than in its thin littermate also raises the interesting possibility that genetic factors might influence the responsiveness of the tubulin system to physiological modulation.

The present experiments indicate that the tissue levels of colchicine-binding protein as well as its degree of polymerization can be modulated by a wide variety of physiological conditions. These changes may be a consequence of the altered rates of synthesis, degradation, and polymerization of tubulin. The possibility exists that physiologic factors may influence the specific colchicine-binding activity of tubulin, but such changes could only be detected when techniques become available for the preparation of purified (de)polymerized tubulin from tissues other than brain. In this context, it has been suggested, on the basis of studies with brain tubulin, that this protein may be present in tissue in a form(s) which is not detected by colchicine binding (8, 30). However, under the conditions of our assay procedure, i.e., tissue dilution >1:13 and the depolymerization solution used, the tubulin present in brain which does not bind colchicine but is demonstrated by chromatography and gel electrophoresis represents <3% of the total tubulin present. It is of interest that the quantitative and qualitative changes in the hepatic microtubular system observed during fasting and glucose feeding are remarkably similar to those noted in pancreatic islets under identical conditions (20). Recent studies in our laboratory (21) have indicated that tubulin synthesis is markedly depressed in islet cells after fasting and is stimulated by glucose feeding. It is possible, therefore, that the variations in the total tubulin content of hepatocytes associated with fasting and glucose feeding may reflect similar changes in tubulin synthesis. Furthermore, the increased degree of tubulin polymerization observed in liver during glucose feeding may represent another instance of the polymerizing effect of glucose as originally observed in pancreatic islets (20).

The marked increase in tubulin levels in PHA-stimulated lymphocytes is probably due to increased tubulin synthesis, although the possibility of concomitant changes in the rate of degradation cannot be excluded. In this context, it is of interest to note that a recent report describes an increased rate of tubulin synthesis in differentiating *Nae-*

*gleria gruberi* (10). Since the E-PHA-induced increase in total tubulin content during the initial 48 h of incubation is accounted for *in toto* by an increase in polymerized tubulin, it would appear that E-PHA not only accelerates tubulin synthesis but also stimulates the polymerization process.

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