6. C. C. Ashley and E. B. Ridgway, *J. Physiol. (Lond.)* **209**, 105 (1970).
7. A. L. Hodgkin and R. D. Keynes, *ibid.* **131**, 592 (1956).
8. G. Hoyle and T. Smyth, Jr., *Comp. Biochem. Physiol.* **10**, 291 (1963).
9. A. L. Hodgkin, A. F. Huxley, B. Katz, *J. Physiol. (Lond.)* **116**, 424 (1952).
10. S. Hagiwara, K. Takahashi, D. Junge, *J. Gen. Physiol.* **51**, 157 (1968).
11. D. C. Hellam and R. J. Podolsky, *J. Physiol. (Lond.)* **200**, 807 (1969).
12. As a general observation for fibers at, or near, rest length, the muscle is more sensitive to a small decrease in length than to an equally small increase.
13. A brief report of this work was presented at the Biophysical Society Meetings, Philadelphia, 18 to 21 February 1975 [A. M. Gordon and E. B. Ridgway, *Biophys. Abstr.* **15**, 151a (1975)].
14. We thank the director, A. O. D. Willows, and the staff at Friday Harbor Laboratories, Friday Harbor, Washington, where this work was done. Supported by NIH grant NS10919 to E.B.R. and NIH grant NS08384 to A.M.G.

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Pressure-Induced Depolymerization of Brain Microtubules in vitro

Abstract. *Microtubules, assembled in vitro from tubulin extracted from rabbit brain, were subjected to changes in hydrostatic pressure (200 to 10,000 pounds per square inch) and temperature (37° to 0°C). Increased pressure, like cooling, reversibly depolymerizes microtubules, as measured by changes in either turbidity, birefringence, or the number of microtubules seen in electron micrographs. The characteristic response of brain microtubules in vitro to pressure is similar to that of mitotic spindle microtubules in vivo.*

Investigations of the assembly and disassembly of mitotic spindle microtubules in vivo have indicated that these labile microtubules are in a dynamic equilibrium with a cellular pool of subunits (*1*). Having the same effect as low temperature and colchicine, hydrostatic pressure from 3500 to 7000 pounds per square inch (psi) (at normal physiological temperatures) rapidly and reversibly depolymerizes spindle microtubules (*2*) and other labile cytoplasmic microtubules in vivo (*3*). The reversible depolymerization of brain microtubules in vitro by low temperature and colchicine has been examined (*4–7*), and the characteristics of the assembly-disassembly process are similar to those observed for labile microtubules in vivo. We now report that brain microtubules in vitro can also be reversibly depolymerized by hydrostatic pressure and that the characteristics of the polymerization-depolymerization reaction to pressure in vitro is similar to that of spindle microtubules in vivo.

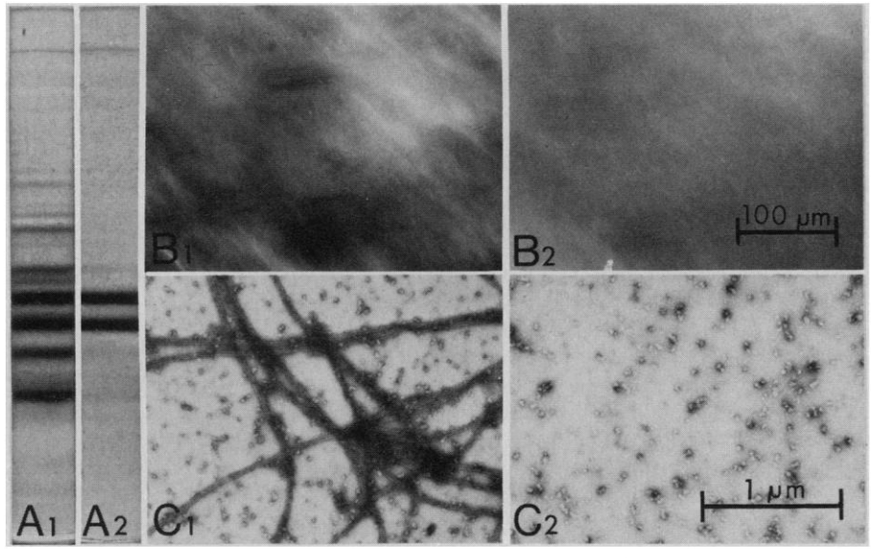


Fig. 1. (A) Electrophoretic analysis of tubulin solutions (*14*) in 25 m*M* tris-glycine, *p*H 8.3, sodium dodecyl sulfate 5 percent polyacrylamide gels. (A1) The supernatant after the first 40,000*g* centrifugation of the rabbit brain–buffer mixture; (A2) the final supernatant, after one cycle of polymerization-depolymerization and centrifugation at 100,000*g* and 4°C. The α and β tubulins comprise more than 75 percent of the total protein as determined by densitometry of the gels stained with fast-green. (B) Intensity and distribution of birefringence retardation after the initial 40,000*g* supernatant (see above) was incubated at 35°C for 2 hours and then placed in the microscope pressure chamber at 29°C and viewed with polarization microscopy (B1) at 200 pounds per square inch (psi) and (B2) 10 seconds after returning to 200 psi from 9600 psi held for 3 minutes. Areas of dark and light contrast in (B1) are produced principally by densely packed, aligned microtubules. (C) Electron micrographs of a purified tubulin solution at 22°C. (C1) Tubulin polymer solution fixed before pressurization, and (C2) the same solution pressurized at 10,000 psi for 5 minutes and fixed about 2 minutes after the pressure was released. Negative staining shows many microtubules in (C1). Depolymerization is essentially complete in (C2). Of the original absorbance at 37°C, 10 percent remains after depolymerization by pressure or low temperature. The irreversible absorbance (A_i) appears to represent nonfilamentous aggregates of tubulin that remain after the microtubules have disassembled.

Rabbit brain tubulin was extracted by a modification of the procedure of Shelanski *et al.* (*8, 9*); it consistently yielded about 11 mg of protein, more than 75 percent being pure tubulin (Fig. 1A), from 8 g of raw, minced brain. Microtubules were assembled by incubating the tubulin in buffer solution at 37°C for 15 minutes. The degree of microtubule assembly was assessed simply and quantitatively by measuring changes in turbidity (absorbance, *A*), since these changes in absorbancy are proportional to changes in the amounts of polymerized microtubules (*5, 6*). Turbidity (*A*) was monitored at 403 nm with a Beckman DB spectrophotometer or a high-pressure, temperature-controlled spectrophotometer cell system (*10*). Microtubule assembly was also assessed qualitatively by measuring birefringence retardation with polarization microscopy (Fig. 1B) and by examining negatively stained (1 percent uranyl acetate) grids with an electron microscope (Fig. 1C).

Pressure induces rapid, reversible depolymerization of the brain microtubules in vitro (Figs. 1 and 2). At 21°C, 9000 psi completely depolymerizes the microtubules within 1.5 minutes. When pressure is released (returned to 200 psi), repolymerization is slow, but at 30°C the absorbance returns to more than 90 percent of its original value within 15 minutes. Increasing magnitudes of pressure induce progressively increasing rates of depolymerization, as well as lower equilibrium levels of microtubule assembly (Fig. 2). The kinetics of depolymerization induced by pressure appear to be first-order, similar to the kinetics of depolymerization in vitro induced by rapid cooling (*6*). At 30°C, the time constant for the kinetics of depolymerization is about 0.4 minute; for repolymerization, about 3.0 minutes. These time constants are nearly identical to those for the pressure-induced disassembly of spindle microtubules in vivo measured by monitoring changes in spindle size and birefringence retardation (*2*).

The equilibrium level of microtubule polymerization depends on temperature as well as the magnitude of pressure (Fig. 3A). The effects of pressure and low temperature are synergistic. Although 10,000 psi will not completely depolymerize the microtubules at 37°C, at 16.5°C disassembly is complete with only 3000 psi. The range of pressure and temperature

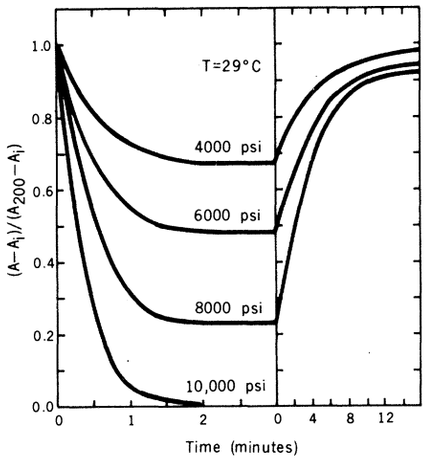
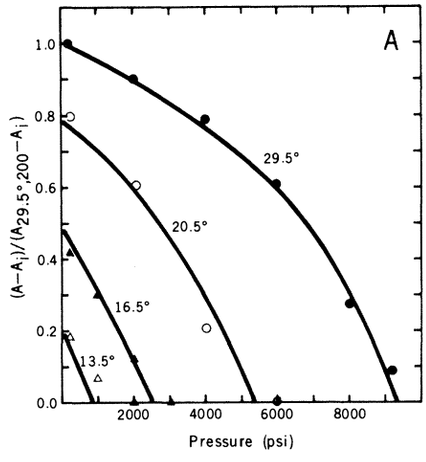


Fig. 2. Kinetics of the depolymerization and repolymerization of microtubules: changes in turbidity as a function of pressure. The data were taken from repetitive pressurizations of a single preparation at 29°C, and replotted from the original graphic chart recordings. Pressure was increased rapidly ($\simeq$ 2 seconds required for 10,000 psi), held for 3 minutes, and returned to 200 psi; the absorbance was then allowed to recover. For pressures that did not produce complete depolymerization, recovery began immediately after the pressure was released, and the absorbance returned to within 10 percent of its prepressurization value before another cycle was started. Because the absorbance did not recover completely after the pressure was released, the difference between the measured absorbance (A) and the irreversible absorbance (A_i, as defined in Fig. 1) has been normalized by the absorbance at 200 psi before pressurization. Time is set at zero for the time of pressurization and the time of pressure release. The recovery time scale has been compressed. After release of 10,000 psi, the chamber was opened, and samples were taken for electron microscopy.



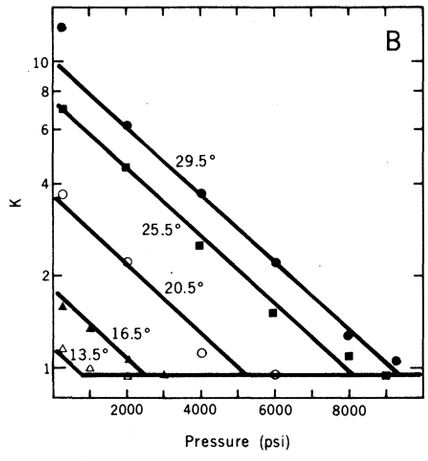


Fig. 3. (A) Equilibrium level of polymerization (measured by absorbancy) as a function of pressure and temperature. The data were taken from a single experimental preparation, but they are representative of results from four other experiments. Microtubules were assembled by incubating the tubulin solution at 37°C, then the temperature was reduced to 29.5°C. At a constant temperature, pressure was increased in steps and held for 3 minutes at each level. When depolymerization was complete, the pressure was returned to 200 psi and the temperature returned to 29.5°C. After about 20 minutes for repolymerization, the temperature was lowered, time was allowed for reequilibration, and sequential pressurizations were repeated. The data have been normalized by the absorbancy at 29.5°C and 200 psi before each pressurization sequence to eliminate variations caused by the less than 100 percent repolymerization. The solid lines were calculated from an equation based on a nucleated condensation model for microtubule assembly, with $\Delta\bar{V}$ = 90 ml/mole, as described in the text. (B) The dependence of log $K_{T,P}$ with pressure at five experimental temperatures. Except for the data at 25.5°C, the equilibrium constant values were all derived from light scattering measurements for a single preparation (as in A). The solid lines have approximately the same slopes that were determined from the equation, $d\log K/dP = \Delta\bar{V}/2.3\,RT$ with $\Delta\bar{V}$ = 90 ml/mole. The normalized value of A_{37} used for the calculations was 1.06.

that effects depolymerization of microtubules in vitro is essentially the same as that which induces disassembly of spindle microtubules in vivo (*1, 2, 11*).

It now appears that microtubules are assembled in vitro by a nucleated condensation mechanism (*6, 7*). Theoretical curves derived from an equation based on nucleated condensation polymerization do predict the equilibrium data remarkably well (Fig. 3A) (*16*). The equation for the theoretical curves was generated as follows. For nucleated condensation polymerization, polymerization occurs only at the ends of the microtubules, and the equilibrium constant is equal to the reciprocal of the concentration of the unpolymerized tubulin (*6, 7*). In terms of the turbidity measurements, increases in absorbance are proportional to the mass of assembled microtubules (*5, 6*). Since absorbance increases insignificantly between 30° and 37°C, it was assumed that the reversible absorbance at 37°C measures the total amount of polymerizable tubulin. At a given pressure, P, and temperature, T, the concentration of unpolymerized tubulin will be proportional to the difference ($A_{37} - A_{T,P}$) and the equilibrium constant will be proportional to the reciprocal of this value:

$$K_{T,P} \sim 1/(A_{37} - A_{T,P}) \qquad (1)$$

A plot of the dependence of log $K_{T,P}$ on pressure at the constant experimental temperatures (Fig. 3B) is fitted with a family of straight lines which have a constant slope corresponding to a value for the partial molar volume change of polymerization of $\Delta\bar{V} \simeq$ 90 ml per mole of polymerizing subunit. The solid lines through the original data points in Fig. 3A were derived from the corresponding straight lines in Fig. 3B, from Eq. 1. Because of the assumptions made in the analysis, the value $\Delta\bar{V} \simeq$ 90 ml/mole should be considered a maximum estimate of the partial molar volume change, but it is nearly identical to the value of $\Delta\bar{V}$ estimated for the polymerization (by a nucleated condensation model) of spindle microtubules in vivo (*11*).

Table 1. Percentage decrease in turbidity of tubulin solutions at 10,000 psi for different percentages of glycerol (by volume) in the reassembly buffer at various temperatures.

Temperature (°C)	Decreases in absorbance at glycerol concentrations of:		
	0%	15%	30%
35	85		
29	100	40	0
21	100	65	5
14	100	100	
8	100	100	30

Increasing concentrations of glycerol in the reassembly buffer increasingly stabilize microtubules against depolymerization by pressure (Table 1) as well as by low temperature (*8*). The stabilization of microtubules in vitro by glycerol is not surprising, since glycerol preparations are used to isolate stable mitotic spindles (*12*).

During the time these experiments were being conducted, O'Connor *et al.* (*13*) reported that neuronal microtubules were unaffected at 37°C by pressures as high as 10,000 psi, both in vivo and in vitro. The reported insensitivity of neuronal microtubules in vitro apparently conflicts with my results. The nucleotide chosen for the reassembly buffer, however, has a significant effect on the stability of the microtubules. O'Connor *et al.* used guanosine triphosphate (GTP) in their buffer. I used adenosine triphosphate (ATP) for both physiological and economic reasons since 1 m*M* ATP has been reported to be as effective as GTP in promoting microtubule polymerization in purified tubulin solutions (*7*). In brief, at any given temperature, depolymerization of microtubules by pressure will be less when the buffer

contains GTP than when it contains ATP. In a GTP reassembly buffer the dependence of equilibrium microtubule polymerization on pressure is similar to that in Fig. 3A, but for correspondingly lower temperatures. For example, the decreases in equilibrium absorbance induced by pressure at 21°C in 0.5 m*M* GTP buffer is nearly equivalent to the decrease at 29.5°C in 1.0 m*M* ATP buffer, and the decrease at 16°C in GTP buffer is nearly equivalent to the decrease at 21°C in ATP buffer.

It should also be noted that little high-molecular-weight protein copolymerizes with the microtubules prepared in the 1.0 m*M* ATP buffer (Fig. 1A). Copolymerizing, high-molecular-weight proteins account for 15 to 20 percent, by weight, of microtubules purified in 0.5 m*M* GTP buffer (*6, 7*). The significance of these copolymerizing proteins, however, is not yet clear.

As is shown above, pressure depolymerizes brain microtubules in vitro, and this process is reversible. The results are quantitatively consistent with those of experiments on the depolymerization of microtubules by various agents in vivo. The similar behavior of microtubules in response to pressure in vitro and in vivo further substantiates the conclusion (*11*) that pressure affects microtubule assembly in vivo directly through the large positive volume change associated with polymerization.

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References and Notes

1. S. Inoué and H. Sato, *J. Gen. Physiol.* **50** (Suppl.), 259 (1967).
2. A. M. Zimmerman and D. Marsland, *Exp. Cell Res.* **35**, 293 (19 → E. D. Salmon, *J. Cell Biol.* **65**, 603 (1975); *Ann. N.Y. Acad. Sci.* **253**, 383 (1975).
3. D. Marsland, in *High Pressure Effects on Cell Processes*, A. M. Zimmerman, Ed. (Academic Press, New York, 1970), pp. 260–312; L. Tilney, in *Origin and Continuity of Cell Organelles*, J. Reinert and H. Urspring, Eds. (Springer, New York, 1971), pp. 222–260.
4. R. Weisenberg, *Science* **177**, 1104 (19 → J. B. Olmsted and G. G. Borisy, *Biochemistry* **12**, 4282 (1973); *Annu. Rev. Biochem.* **42**, 507 (1973).
5. Y. C. Lee, F. E. Samson, Jr., L. L. Houston, R. H. Himes, *J. Neurobiol.* **5**, 317 (1974).
6. F. Gaskin, C. R. Cantor, M. L. Shelanski, *J. Mol. Biol.* **89**, 737 (1974).
7. J. B. Olmsted, J. M. Marcum, K. A. Johnson, C. Allen, G. G. Borisy, *J. Supramol. Struct.* **2**, 429 (1974); G. G. Borisy, J. B. Olmsted, J. M. Marcum, C. Allen, *Fed. Proc.* **33**, 167 (1974).
8. M. L. Shelanski, F. Gaskin, C. R. Cantor, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 765 (1973).
9. Tubulin was purified from rabbit brain in a reassembly buffer composed of 0.1 *M* MES [2-(*N*-morpholino)ethane sulfonic acid; *p*H 6.4], 0.1 m*M* DDT (dithiolthreitol), 1 m*M* EGTA [ethylene-bis-(oxyethylenenitrilo)tetraacetate], 1 m*M* $MgCl_2$, and 1 m*M* ATP. Approximately 8 g of brain was minced and washed in buffer. Cold buffer (10 ml) was added, the brain was homogenized, and the mixture was centrifuged at 40,000*g* for 1 hour at 4°C. Glycerol was added to the supernatant to make a solution 25 percent glycerol by volume, and this solution was incubated at 37°C for ½ hour. After centrifugation at 100,000*g* for 1 hour at 30°C, the pellets were either used immediately or stored on ice overnight. Before an experiment a pellet was suspended in cold buffer and cleared by centrifugation at 100,000*g* for 1 hour at 4°C. Microtubules were assembled by incubating a solution of approximately 2.5 mg of protein per milliliter at 37°C for 15 minutes. The resulting change in absorbancy upon polymerization was about 0.2 at 403 nm. Although it is possible that small amounts of glycerol remain attached to the tubulin even when no glycerol is present in the experimental reassembly buffer, the consequent effects on the observed polymerization of microtubules are probably very small.
10. A temperature-controlled pressure spectrophotometer cell, similar to the microscope pressure chamber of Salmon and Ellis (*15*), was constructed with a 1-cm internal space between the optical glass windows. The temperature control and pressure generating equipment was the same as described in (*15*). The chamber was mounted in its temperature control stage on a Zeiss WL microscope equipped with two Leitz UM 20× objectives, one used as a condenser. The aperture diaphragms were adjusted to give a 2-mm diameter beam, significantly smaller than the 3-mm apertures of the chamber ports. A tungsten lamp (line voltage regulated) with 403-nm interference filter provided light. Voltage changes in the output of a silicon solar cell placed above the ocular were proportional to changes in light intensity and were read directly into a Beckman 10-inch (1 inch = 2.54 cm) Lin-Log potentiometric recorder.
→ E. D. Salmon, *J. Cell Biol.* **66**, 114 (1975).
12. A. Forer and A. M. Zimmerman, *J. Cell Sci.* **16**, 481 (1975).
13. T. M. O'Connor, L. L. Houston, F. Samson, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4198 (1974).
14. J. Bryan, *Fed. Proc.* **33**, 152 (1974).
→ E. D. Salmon and G. W. Ellis, *J. Cell Biol.* **65**, 587 (1975).
16. E. D. Salmon, in preparation.
17. Supported by NIH grant GM20644-01.

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Inhibition of the Growth and Development of a Transplantable Murine Melanoma by Vitamin A

Abstract. *Vitamin A, given either orally or intraperitoneally, has a dramatic inhibitory effect on the growth and development of a highly immunogenic murine melanoma. This effect may be due to enhancement of antitumor immunity by vitamin A.*

Dresser (*1*) and others (*2*) have shown that vitamin A is capable of acting as an immune adjuvant. This and work with other immune stimulants suggests that vitamin A might also be active in inhibiting tumor growth. Vitamin A has been capable of decreasing tumor incidence following administration of certain viruses (*3*) and chemical carcinogens (*4*). To date, however, multiple attempts to show that vitamin A inhibits the growth of transplanted tumor cells has been unsuccessful. Specifically, Bollag (*5*) was unsuccessful in six transplantable tumor systems in preventing tumor growth by pretreatment with vitamin A in mice. Meltzer and Cohen (*6*) were able to show enhancement of bacillus Calmette-Guérin (BCG) effects against transplantable mouse tumors with vitamin A but found no effect of vitamin A alone on the growth and development of a transplanted methylcholanthrene-induced murine sarcoma. We now describe experiments demonstrating that vitamin A given either orally or intraperitoneally has a dramatic inhibitory effect on the growth and development of a highly immunogenic murine melanoma.

In all experiments S-91 (Cloudman) melanoma was used. This murine melanoma arose spontaneously in the tail of a DBA mouse and has subsequently been passed in BALB/c mice. This tumor grows progressively in untreated BALB/c mice. The tumor was maintained by serial transplantation with single cell suspension. In each experiment, a single cell suspension was prepared, the cells were counted, and 10^5 or 5×10^5 cells were injected between the scapulae in the subcutaneous tissue of each mouse. Tumors developed during the third to fifth week after inoculation, and grew progressively in size to cause death of the affected animals. No tumors developed during the subsequent observation period of 3 to 6 months.

A series of experiments were performed to determine the value of pretreatment with oral vitamin A. Ten- to 12-week-old male BALB/c mice were divided into two groups. The experimental group received vitamin A palmitate (Aquasol A, USV Pharmaceutical, Tuckahoe, N.Y.) in their drinking water (625 units per milliliter) with an average daily intake of 5 ml of water, or approximately 3100 units of vitamin A palmitate. The control group received ordinary drinking water, which was freely available. All animals in these experiments received NIH Open Formula rat and mouse ration, which contains 15 international units of vitamin A palmitate per gram of food. After 2 weeks of vitamin A palmitate or normal drinking water, each group was challenged with either 10^5 or 5×10^5 tumor cells. Mice were maintained

Table 1. Comparison of the inhibition of tumor development by varying doses of intraperitoneal vitamin A. The significance of the differences was measured by the chi-square test. For the saline (S) versus the A_2 group, $P < .01$. For the saline versus the A_3 group, $P < .005$.

Group	Dose of vitamin A (units/day × 5 days)	No. of mice with tumor/ No. of mice inoculated
S	0	20/25
A_1	2500	16/28
A_2	3500	9/22
A_3	5000	4/17