crystal type over more stable forms—in fact, highly metastable polymorphs may often be produced in this way. In such cases T_H will not mimic the equilibrium liquidus line.

It is reasonable to suggest, on the basis of these considerations, that in the pressure range 0 to 2 kbar the local molecular order in water changes systematically with pressure in the direction of the ice III local structure. This is a multiple ring size (five-, six-, and eight-membered) arrangement of fully hydrogen bonded molecules in which the H–O–H angles are strongly distorted from the ideal tetrahedral value and the O–H···O bond angles are much less than 180° (*11*). Such a suggestion is quite consistent with the results of recent Raman spectral studies at high pressures (*12*) which show that volume decreases are not primarily associated with any increase in the proportion of broken hydrogen bonds, as two state models would suggest, but instead are accomplished by increased hydrogen bond distortion.

The fact that, above 2 kbar, T_H remains a constant 70° below the melting point shows that the low-pressure supercooling range of 38° is anomalously small. This premature termination of the supercooled state can be associated with the remarkable divergences in C_p, κ_T, and α (the heat capacity at constant pressure, compressibility, and expansivity) observed in low-pressure supercooled water (*2, 3, 6*) through the increase in nucleation probability which accompanies the implied increasing entropy and volume fluctuations ($\overline{\Delta S^2} = k_B C_p$, $\overline{\Delta V^2} = k_B T V \kappa_T$). The rapid increase in $(T_m - T_H)$ with increasing pressure therefore implies that these interesting thermodynamic anomalies are also washed out by increasing pressure [as already suggested by the disappearance of the density maximum above 2 kbar (*13*)]. Since we inferred above that the same pressure increases are systematically favoring bent bond topologies, we can conclude that the water anomalies all derive from the possibility, at low pressures, of forming bonds which on average approach the ideal 180° O–H···O angle (within a single network as in ice VII). It is this unusually open disposition of linked tetrahedral units, rather than tetrahedral coordination per se, which is the unique feature of low-pressure water. In support of this we note that SiO_2, whose crystalline polymorph crystobalite (high-temperature form) has the ice Ic structure with linear "oxygen bonds" (Si–O–Si) averages Si–O–Si bond angles of only 144° in the liquid at normal pressures and low temperatures [judged from the vitreous structure frozen in at about 1300°C (*14*)]. Although this network liquid does exhibit a volume minimum (*15, 16*) it is only a shallow one on both absolute and reduced bases of comparison with water, and is only encountered at large (200°) supercooling. For GeO_2, in which the analogous oxygen bond angle (Ge–O–Ge) in the vitreous state has decreased to an average value of 133° (*17*), no volume minimum is observed anywhere in the liquid state.

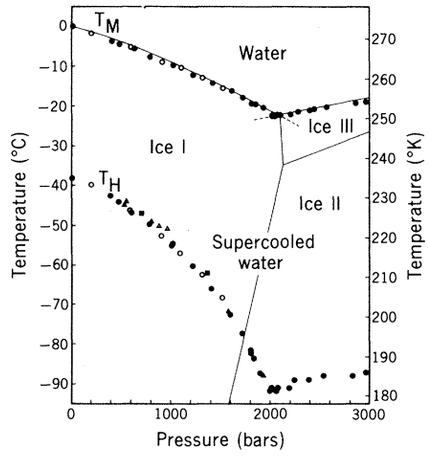

Fig. 1. Homogeneous nucleation and equilibrium melting temperatures for water in emulsion form as a function of pressure. Results obtained with different pressure cells and emulsion carrier fluids are distinguished as follows. Cell 1: (Δ) heptane, (■) pentane, (▲) methylcyclohexane. Cell 2: (○) heptane, (●) methylcyclopentane + methylcyclohexane. Solid lines are the accepted equilibrium phase boundaries.

H. KANNO
R. J. SPEEDY, C. A. ANGELL
Department of Chemistry,
Purdue University,
Lafayette, Indiana 47907

References and Notes

1. J. A. Schufle and M. Venugoplan, *J. Geophys. Res.* **72**, 3271 (1967).
2. B. V. Zhelezynyi, *Russ. J. Phys. Chem.* **43**, 1311 (1969).
3. D. H. Rasmussen, A. P. McKenzie, J. C. Tucker, C. A. Angell, *Science* **181**, 342 (1973); D. H. Rasmussen and A. P. McKenzie, *J. Chem. Phys.* **59**, 5003 (1973); C. A. Angell, J. H. Shuppert, J. C. Tucker, *J. Phys. Chem.* **77**, 3092 (1973).
4. K. I. Gillen, D. C. Douglas, M. J. R. Hock, *J. Chem. Phys.* **57**, 5117 (1972).
5. J. C. Hindman, A. Svirmickas, M. Wood, *ibid.* **59**, 1517 (1973); J. C. Hindman and A. Svirmickas, *J. Phys. Chem.* **77**, 2487 (1973).
6. R. J. Speedy and C. A. Angell, in preparation.
7. D. H. Rasmussen and A. P. McKenzie, in *Water Structure and the Water Polymer Interface*, H. H. G. Jellinek, Ed. (Plenum, New York, 1972), pp. 126–145. The subdivision of water involved in emulsification promotes supercooling by isolating foreign particles, on the surfaces of which crystallization normally commences, into a minute fraction of the sample. Crystallization of water samples of very different sources and initial purities then commences rather sharply at −38°C (*3*) only a little higher than the temperature observed with purified water in a cloud chamber [V. J. Schaeffer, *Ind. Eng. Chem.* **44**, 1300 (1952)] and in oil suspension [B. J. Mason, *Adv. Phys.* **7**, 221 (1958)]. Although a number of surfactants (Laurosyl L, Span 80, and Span 85) in various dispersant liquids lead to this same degree of supercooling with H_2O volume fractions as large as 70 percent, Span 65 in light hydrocarbon emulsions, described by Rasmussen and McKenzie, has the great advantage of long-term stability including the ability to survive numerous freeze-thaw cycles.
8. E. Williams and C. A. Angell, *J. Polym. Sci. Polym. Lett.* **11**, 383 (1973).
9. H. Yamada, *Rev. Sci. Instrum.* **45**, 640 (1974).
10. W. B. Hillig and D. Turnbull, *J. Chem. Phys.* **24**, 914 (1956); D. Turnbull, *J. Phys. Chem.* **66**, 609 (1962).
11. B. Kamb, *Acta Crystallogr.* **17**, 1437 (1964).
12. G. E. Walrafen, *J. Solution Chem.* **2**, 159 (1973).
13. P. W. Bridgman, *The Physics of High Pressure* (Bell, London, 1931).
14. R. L. Mozzi and B. E. Warren, *J. Appl. Crystallogr.* **2**, 164 (1969).
15. R. W. Douglas and J. O. Isard, *J. Soc. Glass Technol.* **35**, 206 (1951).
16. R. Bruckner, *J. Non-Cryst. Solids* **5**, 281 (1971).
17. A. J. Leadbetter and A. C. Wright, *ibid.* **7**, 37 (1972).
18. Supported by the Department of the Interior, Office of Water Resources Research, and by the National Science Foundation.

9 May 1975

Muscle Activation: Effects of Small Length Changes on Calcium Release in Single Fibers

Abstract. *In single muscle fibers, small (1 percent) changes of length have a marked effect of both the calcium activation and the tension elicited by a constant current stimulus. The decrease in tension with shortening is accounted for almost entirely by a decrease in calcium release, rather than by changes in mechanical factors, such as filament geometry.*

It was well established by the work of Gordon *et al.* (*1*) and others (*2*) that the active force a muscle can develop depends on the length of the muscle. Typically, for striated muscles, a plot of length against maximum active tension (force per unit area) shows a maximum near in situ rest lengths. At muscle lengths shorter or longer than the rest length, active tension decreases. Much of this decrease may be due to mechanical factors involving filament geometry. At long lengths, the decreased amount of overlap between the thick and thin force generating filaments is probably a major factor. At short lengths, the major mechanical factors are less well understood. However, evidence is accumulating that factors other than filament geometry—for example, changes in calcium activation—operate, particularly at short lengths (*3*).

In this report we examine the hypothesis that length changes directly affect muscle calcium "activation" (that is, the amount of calcium released by a constant electrical stimulus). Since calcium ions activate the force generating cross-bridges in muscle (*4*), reduced calcium release at shorter muscle lengths would result in a reduced force output. This hypothesis is tested by

observing length-induced changes in calcium activation and force simultaneously. Our method was to inject single giant barnacle muscle fibers with the calcium bioluminescent protein aequorin (*5*, *6*). This sensitive calcium indicator emits light in response to changes in intracellular calcium ion concentration: the calcium-mediated light response is called the calcium transient.

Our main conclusion is that length can affect the calcium activation of a muscle directly. Less calcium is released by a constant current stimulus at a short muscle length than at a longer one.

Large single muscle fibers from the barnacle *Balanus nubilus* were dissected and cannulated (*6*) and the fiber was injected with aequorin over its entire length by the replacement method (*7*). The fiber containing aequorin was then placed in a chamber with cold circulating saline (*8*). Light from the fiber was recorded with a photomultiplier tube (*6*) under conditions of constant current or constant voltage stimulation using single or dual axial electrodes (*9*) and appropriate electronics (*10*). The mechanical records were obtained using a chain and lever system similar to that described by Ashley and Ridgway (*6*). Force and length were measured while length was varied with a Shaker pot to which the force transducer (*11*), chain, and fiber were attached.

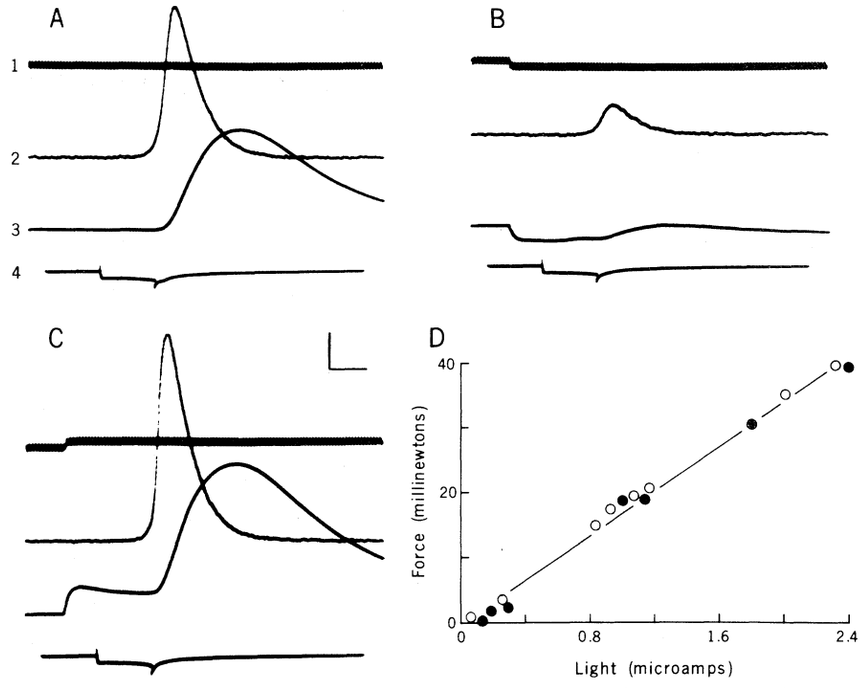

Fig. 1. Effect of prestretch and prerelease on the calcium transient and force responses under current clamp conditions. (A) Response at rest length, L_0. (B) Effect of a 0.2-mm (< 1 percent) release about 200 msec before the stimulus. (C) Effect of a 0.2-mm (< 1 percent) prestretch. Trace 1 is the change in muscle length recorded at 1 mm per calibration bar division; stretch is upward. Trace 2 is the calcium transient recorded at 200 na per division. Trace 3 is the isometric tension recorded at 6.5 millinewtons (mn) per division (note change in tension occurring just after change in length). Trace 4 is the stimulus artifact. Horizontal calibration, 200 msec per division; calibration bar, 1 division. Fiber length, 22 mm; fiber weight, 0.59 mn. Temperature, 12.5°C. (D) Plot of peak force against peak light output (peak calcium transient) for a series of responses in the same fiber. (Closed circles) Force was varied by varying muscle length at a constant depolarizing current. (Open circles) Force was varied by varying depolarizing current at a constant length. The relationship between calcium (light) and force remains fairly constant for these two methods of varying force.

Figure 1 illustrates the effect of a length change on the calcium transient and on tension. Figure 1A is the response at rest length (control). When the muscle is released by a small amount (Fig. 1B) immediately before the stimulus pulse, both activation and the tension responses are substantially reduced. Conversely, under stretch (Fig. 1C) the calcium transient and tension are substantially increased. A surprisingly small change in length has a marked effect: in Fig. 1B a decrease of less than 1 percent in muscle length causes an 80 percent decrease in maximum force, while in Fig. 1C an increase of less than 1 percent causes a 30 percent increase in force. The sensitivity to length change was somewhat variable from fiber to fiber but was present in all cases (*12*).

Three observations rule out the possibility that this effect is due to essentially mechanical factors [the steep lower branch of the classical active length-tension curve (*1*)]. First, we find not only a decrease in the maximum tension as a result of prerelease, but a decrease in the calcium transient as well. Second, the length depen-

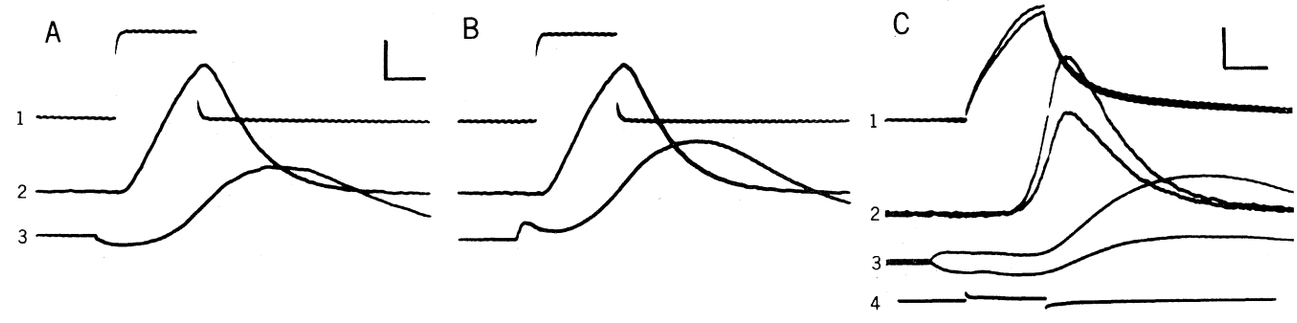

Fig. 2. (A and B) Responses to a 0.2-mm release and stretch, respectively, of a fiber under voltage clamp conditions. Trace 1 is the clamped membrane potential recorded at 10 mv per division. Trace 2 is the calcium transient recorded at 400 na per division. Trace 3 is the isometric force recorded at 6.5 mn per division. Horizontal calibration, 100 msec per division; calibration bar, 1 division. Fiber length, 22 mm; fiber weight, 0.592 mn. Temperature, 12.5°C. (C) Records of a 0.2-mm release from L_0 to L_r (released length) superimposed on a 0.2-mm stretch from L_r back to L_0. The upper trace of each pair was recorded during stretch, the lower during release. Trace 1 is the membrane potential recorded at 10 mv per division. Note that after a release the final depolarization level for a constant current stimulus is slightly less than that for a stretch. Trace 2 is the calcium transient recorded at 100 na per division. The smaller response occurred after the release. Trace 3 is isometric force recorded at 6.5 mn per division. The timing of the length change can be seen on the tension trace preceding the stimulus. Trace 4 is the stimulus current recorded at 50 μa per division. Horizontal calibration, 100 msec per division; calibration bar, 1 division. Fiber length, 22 mm; fiber weight, 0.59 mn. Temperature, 12.5°C.

dence of both force and calcium transients was much less (as a percentage) at larger stimulus currents than at smaller ones, although the mechanical-geometrical factors should have been the same for the two current intensities. Third, additional experiments argue that for these small length changes, the change in maximum active force is due almost entirely to the length dependence of the calcium activation.

Figure 1D shows the relationship between maximum force and light output when contractile force is varied in two different ways, by varying the depolarizing current at a constant length or by varying muscle length at a constant depolarizing current. The former involves variations in calcium activation. The question is whether the latter does also or whether it involves mainly mechanical factors. As can be seen in Fig. 1D, peak force seems to be uniquely related to the height of the calcium transient and depends on length only through the length dependence of the calcium transient. Since the total length change involved in the record in Fig. 1D is less than 5 percent of the muscle length, this does not imply that tension is not limited by mechanical factors or filament geometry at other lengths. In experiments with larger changes in length, deviations are observed from this unique relationship between maximum force and light, which are to be expected if geometric factors are important. For example, with large releases (decreases in muscle length) little isometric force develops, while the calcium transient is still substantial. Also, in some experiments the maximum active tension decreases if the muscle length is increased greatly, even though the calcium transient does not change. But, the main point illustrated by Fig. 1D is that the decrease in calcium activation accounts for the decrease in force without the necessity of invoking any argument involving filament geometry.

As to the timing of the length change, the most effective period (Fig. 1) is immediately before the stimulus pulse. If the length change is delivered some 30 seconds before the stimulus pulse, then the effect of the length change is reduced to roughly 40 percent of the value observed for a change delivered immediately before the stimulus. Visual examinations with a dissecting microscope of fibers during both stretch and release showed that the length change was accomplished almost instantly and that there was no subsequent "creep." Therefore, whatever process is responsible for the partial recovery of activation after a length change takes place at a submicroscopic level.

What are changes in length doing to affect calcium release? Do they affect the relationship between membrane potential and calcium release or do they affect membrane properties? Figure 2, A and B, demonstrates that under voltage clamp, rather than "constant" current conditions, when the membrane is depolarized by a constant amount, neither prerelease (Fig. 2A) nor prestretch (Fig. 2B) has an appreciable effect on the calcium or the force transient. In no experiment did the height of the calcium transient decrease more than 10 percent with decreases in length under voltage clamp conditions, whereas changes of as much as 80 percent were seen under constant current conditions. Therefore, controlling membrane voltage minimized or eliminated the length dependence of the calcium activation.

In contrast, Fig. 2C shows an experiment designed to test the effect of length change on the membrane potential during a constant depolarizing current pulse. The length record has been omitted; instead we have recorded the membrane potential (trace 1) using an intracellular electrode. Stretch (marked by upward deflection of the force trace) causes the membrane potential to be more polarized from the resting potential during the same constant current pulse than does release. The different response to constant current and constant voltage implies that the length dependence is not due to changes in the relationship between membrane voltage and Ca^{2+} release, but rather to changes in the amount of depolarization caused by a constant stimulating current.

The observations under voltage clamp conditions could most easily be accounted for by assuming that the length effect is due to a simple change in membrane resistance (a reduction in resistance or opening up of another path into the muscle on shortening). Stated as a working hypothesis, a decrease in membrane resistance on shortening would cause a constant current stimulus to give less membrane depolarization and thereby lead to a reduced calcium release. But a direct test of this hypothesis, using the voltage clamp to measure current-voltage (*I-V*) curves at different muscle lengths, did not consistently show an effect large enough to entirely account for the observed differences. Thus, a simple length-dependent resistance change is not the only mechanism involved. A second mechanism is suggested by observation of fibers during passive stretch and release. We usually saw small depolarizations (up to 2 mv) on stretch and hyperpolarizations (up to 1 mv) on release. These changes in potential are in the correct direction to contribute to the recorded changes in membrane potential with length. Together, these two mechanisms may account for the observed effects of length.

The decrease in calcium activation at decreased muscle lengths under constant current stimulating conditions is probably due to a decrease in Ca^{2+} release, rather than an increase in calcium binding to sites in the muscle (other than the activating sites, troponin c) or to an increased calcium uptake, for the following reasons. (i) Under voltage clamp conditions (Fig. 2, A and B), the change in calcium activation with length is virtually eliminated. This tends to rule out length-dependent Ca^{2+} binding. (ii) The calcium transient is decreased if the fiber is released at any time up to the end of the constant current pulse, but not after the pulse (even though the calcium transient is still near the peak, uptake is still high). In addition, the time constant of the decline in the calcium transient (which may mirror uptake) is not sensitive to changes in muscle length, even though the calcium transient is. These observations tend to rule out length-dependent Ca^{2+} uptake and leave effects on Ca^{2+} release as the most likely explanation.

Finally, it should be noted that the changes in force induced by changes in length, which are shown by these results, constitute negative feedback. A decrease in calcium release with shortening may be a more general process limiting force generation at short muscle lengths in a number of other muscles. It is of particular relevance that many muscles, like cardiac muscle, work predominantly in a region of muscle length where isometric force is declining with decreasing muscle length. How much of this decline in force at short muscle lengths seen in all striated muscle is due to decreased calcium activation? We have verified that in barnacle muscle a decrease in muscle length can cause a decreased calcium release under constant stimulating conditions (*13*).

E. B. RIDGWAY
Department of Physiology, Medical College of Virginia, Richmond 23298

A. M. GORDON
Department of Physiology and Biophysics, University of Washington School of Medicine, Seattle 98195

References and Notes

1. A. M. Gordon, A. F. Huxley, F. J. Julian, *J. Physiol. (Lond.)* **184**, 170 (1966).
2. R. W. Ramsey and S. F. Street, *J. Cell. Comp. Physiol.* **15**, 11 (1940); R. I. Close, *J. Physiol. (Lond.)* **220**, 745 (1972); B. C. Abbott and W. F. H. M. Mommaerts, *J. Gen. Physiol.* **42**, 533 (1959).
3. K. A. P. Edman and A. Kiessling, *Acta Physiol. Scand.* **81**, 182 (1971); R. Rudel and S. R. Taylor, *Science* **172**, 387 (1971); H. Gonzalez-Serratos, R. Valle, A. Cillero, *Nat. New Biol.* **246**, 221 (1973); M. Schoenberg and R. J. Podolsky, *Science* **176**, 52 (1972).
4. S. Ebashi and M. Endo, *Prog. Biophys. Mol. Biol.* **18**, 123 (1968).
→ O. Shimomura, F. H. Johnson, Y. Saiga, *J. Cell. Comp. Physiol.* **59**, 223 (1962); E. B. Ridgway and C. C. Ashley, *Biochem. Biophys. Res. Commun.* **29**, 229 (1967); P. F. Baker, A. L. Hodgkin, E. B. Ridgway, *J. Physiol. (Lond.)* **218**, 709 (1971).

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.

28 March 1975

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.

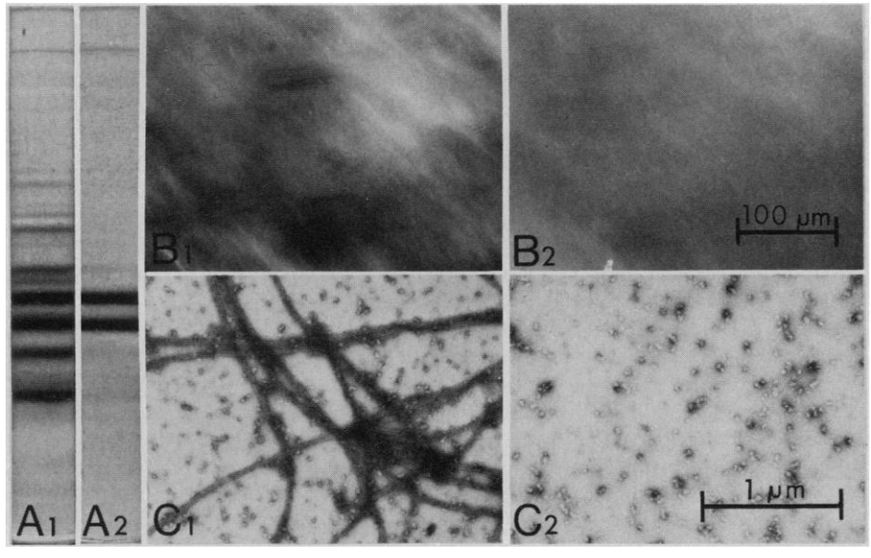

Fig. 1. (A) Electrophoretic analysis of tubulin solutions (*14*) in 25 m*M* tris-glycine, *p*H 8.3, sodium dodecyl sulfate 5 percent polyacrylamide gels. (A1) The supernatant after the first 40,000*g* centrifugation of the rabbit brain–buffer mixture; (A2) the final supernatant, after one cycle of polymerization-depolymerization 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