Rabbani, L. D., Pagnozzi, M., Chang, P., & Breslow, E. (1982) *Biochemistry 21*, 817–826.
Rausch, R., Hollenberg, M. D., & Hope, D. B. (1969) *Biochem. J. 115*, 473–479.
Rholam, M., Nicolas, P., & Cohen, P. (1982) *Biochemistry 21*, 4968–4973.
Ross, P. D., & Subramanian, S. (1981) *Biochemistry 20*, 3096–3102.
Steinhardt, J., & Beychok, S. (1964) *Proteins (2nd Ed.) 2*, 140–304.
Sturtevant, J. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2236–2240.
Sur, S. S., Rabbani, L. D., Libman, L., & Breslow, E. (1979) *Biochemistry 18*, 1026–1036.
Tellam, R., & Winzor, D. J. (1980) *Arch. Biochem. Biophys. 201*, 20–24.
Virmani-Sardana, V., & Breslow, E. (1983) *Int. J. Pept. Protein Res. 21*, 182–189.
Whittaker, B., & Allewell, N. M. (1984) *Arch. Biochem. Biophys. 234*, 585–590.

Formation of Microtubules at Low Temperature by Tubulin from Antarctic Fish†

Robley C. Williams, Jr.,* and John J. Correia
Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee 37235
Arthur L. DeVries
Department of Physiology and Biophysics, University of Illinois, Urbana, Illinois 61801
Received September 5, 1984; Revised Manuscript Received December 10, 1984

ABSTRACT: Tubulin was isolated from two species of antarctic fish, *Pagothenia borchgrevinki* and *Dissostichus mawsoni*, by cycles of temperature-dependent assembly, centrifugation, disassembly, and centrifugation. The preparations were found to consist almost entirely of tubulin and to contain negligibly small amounts of microtubule-associated proteins. This tubulin polymerized to make microtubules of ordinary dimensions. The formed microtubules appear to be in labile equilibrium with free tubulin dimer at all temperatures observed. In a buffer consisting of 0.1 M 1,4-piperazinediethanesulfonic acid, 2 mM dithioerythritol, 1 mM $MgSO_4$, 2 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, and 1 mM guanosine 5′-triphosphate, pH 6.9, the tubulin of *P. borchgrevinki* has a critical concentration for assembly of 0.046 (±0.008) mg/mL at 35 °C and 0.74 (±0.15) mg/mL at the habitat temperature of the fish, −1.8 °C. The critical concentration measured at the lower temperature is quite small relative to the critical concentration for formation of mammalian microtubules from pure tubulin at the same temperature, which must be at least 2 orders of magnitude larger. The antarctic fish microtubules may thus be called "cold stable" by comparison with mammalian microtubules. They do not fully dissociate at temperatures near 0 °C because they are composed of tubulin that assembles more readily at these temperatures than does mammalian tubulin. There is no evidence for the presence of a cold-stabilizing factor in association with the tubulin. These findings suggest that alteration of tubulin may be a means by which some poikilotherms can adapt to a cold environment. A van't Hoff plot of the apparent association constants measured between −1.2 and 35 °C was linear and yielded an apparent $\Delta H°$ of +13.7 kcal/mol and an apparent $\Delta S°$ of +74 eu for the addition of a fish tubulin subunit to the end of a growing microtubule.

Microtubules formed in vitro from the tubulin of mammals and birds are observed to disassemble to yield free tubulin dimers at temperatures near 0 °C. The free dimers can reassemble spontaneously to form microtubules at temperatures near 37 °C. [For reviews, see Timasheff & Grisham (1980) and Correia & Williams (1983).] In contrast, the microtubules present in the tissues of poikilotherms that live in cold habitats must be assembled from tubulin dimers at low temperatures, and they must remain assembled under those conditions. The fishes of the polar seas present an extreme example of such organisms: the two species dealt with in this paper, *Pagothenia borchgrevinki* and *Dissostichus mawsoni*, live perennially at temperatures near −1.8 °C. This paper describes the temperature dependence of assembly of tubulin from these fishes.

Microtubules that are "cold stable" have been described both in mammals and in cold-living poikilotherms. In mammalian cells, there appear to be different populations of cytoplasmic microtubules that can be differentiated on the basis of their resistance to depolymerization at low temperatures. Brinkley & Cartwright (1975), working with PtK_2 cells, have shown that at metaphase the kinetochore to pole microtubules do not depolymerize at temperatures near 0 °C although the adjacent interpolar microtubules do. Jones et al. (1980), in an electron microscopic study of neurons of rat brain, found that numerous intact microtubules were present after incubation of tissue slices for 1 h at 0 °C. A series of experiments with isolated microtubles led them to hypothesize that resistance to disassembly at low temperatures is conferred on tubulin by the presence of a non-tubulin factor of unknown identity. When microtubules were isolated from mouse brain by a cycle of

† This work was supported by Research Grant GM 25638 from the National Institutes of Health (to R.C.W.), by grants from the Vanderbilt University Research Council and the Vanderbilt University Natural Sciences Committee (to R.C.W.), and by Grant DPP81-16917 of the National Science Foundation (to A.L.D.).

0006-2960/85/0424-2790$01.50/0

Ca^{2+}-induced disassembly and ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA)[1]-induced assembly (Webb & Wilson, 1980), a fraction of the microtubules that were formed at 30 °C in the absence of Ca^{2+} was not caused to disassemble by subsequent incubation at 0 °C. These cold-stable structures were found to be composed of tubulin and a "cold-stabilizing factor" that appeared to be dialyzable. It was concluded that their resistance to disassembly is not an inherent property of the tubulin in the structures but rather resides in the factor. Margolis and co-workers (Job et al., 1981, 1982; Margolis & Rauch, 1981; Pirollet et al., 1983) have also studied similar cold stable microtubules isolated from brain of rat and sheep and have concluded that their cold stability resides not in the tubulin but in several auxiliary proteins associated with the microtubules. One is called the "switch polypeptide", and its presence in microtubules in a dephosphorylated state appears to prevent their disassembly at low temperatures. Another set of proteins, called the "stable tubule only polypeptides", appears to have a similar activity when incorporated into the microtubule. Thus, there appears to be general agreement in the literature that a non-tubulin factor or factors conveys cold stability (which means resistance to disassembly at low temperatures) upon mammalian microtubules. Cold-stable microtubules are made from tubulin that is thought to be identical with the tubulin that composes "cold-labile" microtubules.

The property of cold stability in these microtubules is not thought to be one of their physiologically relevant ones. Rather, it is taken to be an incidental result of a difference in composition that also conveys other properties, such as an inhibition of opposite-end assembly (Job et al., 1982) or other subtler differences (Webb & Wilson, 1980), on some microtubules. Nonetheless, one can ask whether the presence of a cold-stabilizing factor accounts for the evident stability of microtubules in cold-living poikilotherms, or whether these organisms have evolved an altered tubulin which has a relatively large association constant, in the absence of auxilliary factors, at low temperatures.

The small amount of literature on cold stability of microtubules from poikilotherms indicates that the second possibility may be likelier than the first. A quantitative electron microscopic study of microtubules in the spinal ganglia and dorsal roots of the lizard *Lacerta muralis* (Pannese et al., 1982) showed no significant difference in the number or disposition of microtubules between neurons which came from lizards that had spent 90 days at 30 °C and others which came from lizards that had spent 90 days at 0 °C. It thus appears that the neuronal microtubules of this organism may be resistant to disassembly at low temperature. Lopez & Bertini (1982) found that the quantity of assembled microtubules that can be isolated from the brain of the toad *Bufo arenarum* decreased by only 30% below the value obtained at 20 °C when the animals were first held for 17 h at 0 °C. Tubulin isolated from the unfertilized eggs of the sea urchin *Strongylocentrotus purpuratus* (Suprenant & Rebhun, 1983) is devoid of microtubule-associated proteins (MAPs) and shows a critical concentration of 0.81 mg/mL at the approximate habitat temperature of 15 °C. Tubulin from the eggs of the surf clam (*Spisula solidissima*) also lacks MAPs and has a critical concentration near 0.36 mg/mL at 22 °C (Suprenant & Rebhun, 1984). Hence, each protein will assemble into microtubules at the habitat temperature of the organism from which it comes. In both cases, the critical concentrations near 37 °C are small (approximately 0.2 and 0.09 mg/mL, respectively), and assembly is apparently reversible at the habitat temperatures. At temperatures below 25 °C, the MAP-free tubulin ordinarily isolated from mammalian brain has a critical concentration in excess of 10 mg/mL under the buffer conditions employed in the above studies (Lee et al., 1978; R. C. Williams, Jr., unpublished results). Consequently, it appears that these poikilotherms may have evolved modified tubulins that possess appropriately large association constants at their habitat temperatures instead of evolving MAPs that would make the microtubules cold stable. In order to obtain a clear test of the idea that the cold stability of some microtubules may lie in the tubulin rather than in the MAPs, one should study tubulin from an organism whose internal temperature is greatly different from that of mammals. The antarctic fishes fulfill this requirement. We have isolated essentially MAP-free tubulin from brains of two species of antarctic fish and have established by a number of criteria that it is authentic tubulin and that it assembles spontaneously into microtubules at several temperatures. We have measured its critical concentration as a function of temperature over the range −1.2 to 37 °C. The results of these experiments are compatible with the notion that the cold stability of the microtubules of antarctic fish is largely caused by alterations in the tubulin which result in an association constant roughly 100-fold higher at 37 °C than that of a typical mammalian tubulin. The association constant of the fish tubulin at −1.2 °C is approximately as large as that of bovine tubulin at 37 °C.

Materials and Methods

Reagents. GTP (type II-S), EGTA, DTE, colchicine, and Pipes buffer were purchased from Sigma Chemical Co. Phosphocellulose was Whatman P11. Ultrapure urea was purchased from Schwarz/Mann. Other materials were reagent grade.

Buffers. Buffer 1 was 0.1 M Pipes–KOH, 1 mM EGTA, 2 mM $MgSO_4$, and 2 mM DTE adjusted to pH 6.9 This buffer, with additions and modifications as noted, was used in all experiments. Buffer 2 was identical with buffer 1 except that EGTA was absent.

Preparation of Tubulins. Tubulin was prepared at McMurdo Station from the brains of two species of antarctic fish by a modification of the assembly/disassembly method of Shelanski et al. (1973). Operations were carried out at 0–4 °C except where noted. Fish were caught with hook and line in the southern end of McMurdo Sound. Brains were dissected from a number of fish, washed in ice-cold 0.25 M NaCl, blotted, and weighed. (Typically, 15 g of tissue was obtained from 100 specimens of *Pagothenia borchgrevinki* and 5 g from 3 specimens of *Dissostichus mawsoni*.) Preliminary experiments indicated that inclusion of glycerol in the buffers employed for assembly did not increase the yield of microtubule protein, so buffers without glycerol were employed throughout. Brains were homogenized in buffer 1 (1.1 mL/g of tissue) in a Sorvall blade homogenizer operated for 20 s at speed 10. The homogenate was centrifuged for 15 min at 10000*g* (12000 rpm) in a Beckman type 40 rotor. The supernatant was removed and centrifuged for 45 min at 68000*g* (32000 rpm). The supernatant, designated high-speed supernatant, was re-

[1] Abbreviations: C_c, critical concentration; CLC, colchicine; TB, tubulin; DTE, dithioerythritol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; GTP, guanosine 5'-triphosphate; MAPs, microtubule-associated proteins; $NaDodSO_4$, sodium dodecyl sulfate; PC-tubulin, tubulin purified by passage through a column of phosphocellulose; Pipes, 1,4-piperazinediethanesulfonic acid; 1×MT, microtubule protein purified by one cycle of assembly/disassembly; 2×MT, microtubule protein purified by two cycles of assembly/disassembly; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

moved, brought to 1 mM GTP, and incubated at 37 °C for 45 min allow microtubules to assemble. It was then subjected to centrifugation for 45 min at 68000*g* (32000 rpm) and approximately 32 °C. The pellet was resuspended in half the original volume of cold buffer 1 by gentle treatment with a Dounce homogenizer of 5-mL capacity, incubated for 45 min on ice, and centrifuged for 45 min at 68000*g*. The resulting supernatant protein was designated once-cycled microtubule protein (1×MT). A second cycle of assembly, centrifugation, disassembly, and centrifugation was initiated by adding sufficient GTP to the 1×MT to bring the solution to 1 mM, incubating it for 45 min at 37 °C, and proceeding as described above. The solution obtained as supernatant from the second cold centrifugation was designated twice-cycled microtubule protein (2×mT). Chromatography on phosphocellulose (Weingarten et al., 1975) was carried out by applying 2×MT (typically 3 mg) to a 1 × 10 cm column that had been previously equilibrated with buffer 1 + 0.1 mM GTP. Tubulin was eluted in this buffer and was termed phosphocellulose-purified tubulin (PC-tubulin). Enough concentrated $MgCl_2$ was added to the eluted protein to bring it to 1 mM Mg^{2+} (Williams & Detrich, 1979). Other proteins were eluted by application of buffer 1 + 0.1 mM GTP + 0.8 M NaCl. Proteins were stored in the frozen state at −20 °C at McMurdo Station, were shipped to the United States on dry ice, and were stored at −70 °C subsequently.

Protein Determination. Protein concentrations were estimated by the method of Bradford (1976), calibrated against bovine serum albumin. Separate calibrations were performed at McMurdo Station and in the United States and were found to be closely similar.

Microtubule Assembly. Polymerization of tubulin was monitored by measurement of the change in the apparent A_{350}. At McMurdo Station, a Perkin-Elmer Model Lambda 3A spectrophotometer was employed, and in the United States, a Cary Model 118 spectrophotometer was used. Both instruments recorded absorbance as a function of time, and both were equipped with thermostated cell holders. In measurements of critical concentration as a function of temperature, the temperature inside the cuvette was measured directly with a Bailey Model BAT-4 thermocouple thermometer, which had been calibrated against an NBS-certified mercury thermometer. Microtubule assembly was also assessed by measurement of viscosity in a capillary viscometer having an outflow time for water of approximately 60 s (Olmsted & Borisy, 1973). The viscometer was charged with the solution to be measured and immersed in a constant-temperature bath at 35 °C for approximately 30 min. The solution was then drawn up into the upper reservoir of the viscometer, and the outflow time was measured.

Colchicine Binding Assay. Colchicine was added to a solution of tubulin to produce a 10-fold molar excess of drug over tubulin dimer. The solution (0.3 mL) was incubated for 90 min at 4 °C and then applied to a 1 × 20 cm column of Sephadex G-25. The column was previously equilibrated with buffer 1 + 0.1 mM GTP, and it was eluted with the same buffer. Fractions of 0.5 mL were collected, and the absorption spectrum of each was measured. The peak containing the protein was clearly separated from the trailing peak containing the unbound colchicine. From the absorption spectrum of any of the protein-containing fractions, the molar ratio of bound colchicine to tubulin was determined from the following expression:

$$C_{CLC}/C_{TB} = (\epsilon_{353}^{TB}A_{279} - \epsilon_{279}^{TB}A_{353})/(\epsilon_{279}^{CLC}A_{353} - \epsilon_{353}^{CLC}A_{279}) \quad (1)$$

where A_{279} and A_{353} are the observed absorbances at 279 and 353 nm, respectively, ϵ_{279}^{CLC} and ϵ_{353}^{CLC} are the molar extinction coefficients of colchicine at the two wavelengths, and ϵ_{279}^{TB} and ϵ_{353}^{TB} are the molar extinction coefficients of the tubulin dimer at the two wavelengths. The value of ϵ_{353}^{CLC} in buffer 1 was measued to be 1.60×10^4 M^{-1} cm^{-1}, and that of ϵ_{279}^{CLC} was measured to be 4.56×10^3 M^{-1} cm^{-1}. The value of ϵ_{279}^{TB} was taken to be 1.23×10^5 M^{-1} cm^{-1} on the basis of the weight-based extinction coefficient (Detrich & Williams, 1978) and a molecular weight of 100000 for the tubulin dimer (Krauhs et al., 1981; Ponstingl et al., 1981; Valenzuela et al., 1981). The value of ϵ_{353}^{TB} was found to be negligibly small when calculated on the basis of ϵ_{279}^{TB} and the observed absorption spectrum of tubulin. Control measurements carried out on bovine PC-tubulin by this technique gave values of 0.96–1.06 mol of CLC bound per mole of tubulin, in good agreement with the accepted value of 1.0.

Determination of Critical Concentrations. A value of the critical concentration for tubulin polymerization (Oosawa & Kasai, 1962; Lee & Timasheff, 1977) was measured at 37 °C by monitoring, at each of several concentrations of protein, the change in the apparent A_{350} until it reached a plateau. The maximum values of A_{350} were plotted against tubulin concentration, and the value of the critical concentration was taken to be the concentration at which the extrapolated straight line that best fit the data crossed the abscissa. At the higher measured concentrations, the turbidity reached a peak somewhat higher than the final plateau value. In such cases, the apparent A_{350} at the peak was used in estimation of the apparent critical concentration. The uncertainty introduced by this procedure is small, since the plateau was reached at the lower concentrations without an observable peak in turbidity. To estimate critical concentrations at other temperatures, the following method was employed. A concentrated suspension (sufficiently concentrated that microtubules were still present after it was cooled to −1.6 °C) of microtubules was produced by assembly and centrifugation at 37 °C. The suspension was introduced into a thermostated 100-μL cuvette, and its apparent A_{350} was measured in the Cary 118 spectrophotometer at differing temperatures over the range −1.2 to 37 °C. The critical concentration was then estimated according to the expression:

$$C_c = C_{tot} - A_{350}/\alpha \quad (2)$$

where C_c is the desired critical concentration and C_{tot} is the total initial concentration of tubulin. The coefficient α is the change of absorbance with concentration of assembled microtubules, found to be 0.21 (A_{350} mg^{-1} mL) in a separate experiment. The value of α was assumed to be independent of temperature. Critical concentrations were also measured, at a series of temperatures, by a centrifugal technique (Johnson & Borisy, 1977). The supply of driving air to a Beckman Airfuge was guided through a length of copper tubing immersed in a constant-temperature bath so that the steady operating temperature of the rotor could be controlled. A solution of tubulin was then placed in the rotor, incubated at the desired temperature for 50 min, and then centrifuged at full speed (165000*g*) at the same temperature for 15 min. The concentration of the supernatant was measured and taken to be equal to C_c (Johnson & Borisy, 1977). This technique is sensitive to the potential presence of nonpolymerizable tubulin. For this reason, a check was performed with TB that had been given an extra cycle of assembly and disassembly just prior to the experiment.

Electron Microscopy. Samples were prepared for routine electron microscopy (Williams, 1981) by mixing them gently

with one-tenth of their volume of fresh 8% glutaraldehye (Polysciences Corp.) After 10 min, a drop of the sample was applied to a freshly glowed, carbon-coated 400-mesh grid. The drop was allowed to stand for 10 s and then removed by aspiration. The grid was rinsed with 4 drops of 0.005 M ammonium acetate buffer, pH 6.5. A drop of stain was applied, allowed to stand for 15 s, and then aspirated. The grid was air-dried. This procedure was carried out both in the warm and in the cold. When the procedure was done in the cold, great care was taken not to allow the sample to become warmed at any time during the process. All apparatus and solutions were prechilled in the cold room. They were kept on ice where possible. The experimenter wore gloves to avoid transferring heat from his hands to the sample-bearing grid by conduction through the intervening forceps.

To allow accurate measurement of dimensions, the electron microscope (JEOL Model 180) was calibrated, at the same magnifications as those used for measurement, with polystyrene beads of known diameter (Pelco, Tustin, CA). Dimensions were measured directly on the original electron micrographs by means of a Nikon microcomparator.

Electrophoresis and Isoelectric Focusing. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed on 8% slab gels according to the method of Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R-250. Isoelectric focusing was carried out in 16-cm-long cylindrical gels of 4.3% acrylamide by a modification of the method of George et al. (1981). The tubulin solution was mixed directly with solid urea to give final concentrations of 9.16 M urea, 5% β–mercaptoethanol, 2% Nonidet, 0.03% ethanolamine, and 2% LKB Ampholine, pH range 4–6. The gels were run for 72 h at 300 V at a temperature near 15 °C.

Results

Preparation of Tubulin. Protein resembling tubulin was selectively purified by cycles of temperature-dependent assembly/disassembly from brain tissue of the two species of antarctic fish investigated. Typically, 1 g of brain tissue yielded approximately 0.6 mg of 1×MT, 0.15 mg of 2×MT, and 0.12 mg of PC-tubulin. The final amount is quite comparable to the typical yield from bovine brain: approximately 0.16 mg of PC-tubulin per gram of brain tissue (Williams & Lee, 1982). Figure 1A shows a $NaDodSO_4$ gel of microtubule protein from *P. borchgrevinki* at four stages in the preparation. One can see that the PC-tubulin (lane 3), heavily loaded to reveal minor bands, contains very little protein of molecular weight different from that of tubulin. The sole extra proteins are a pair with molecular weights near 93 000, one with a molecular weight near 45 000, and one with a molecular weight near 40 000. The total amount of these non-tubulin proteins is a minute fraction of the mass of the PC-tubulin sample, even though the high-speed supernatant of brain (lane 6) appears to have large fractions of them. The 2×MT and 1×MT (lanes 4 and 5, respectively) contain only slightly greater fractions of these minor proteins, indicating that they are efficiently removed in the assembly/disassembly cycles of the preparation. In contrast, one can see the large fractions of MAPs (M_r >200 000) present in 2×MT from bovine brain (lane 8), which are removed (lane 7) by passage through a phosphocellulose column. It may be concluded that no significant amount of MAPs copurifies with the tubulin from the brains of these fish. Tubulin that is effectively MAP free is also obtained when 2×mT are prepared from *D. mawsoni* (lane 1).

Figure 1B shows the central regions of isoelectric focusing gels of 2×MT from *P. borchgrevinki* (lane 1) and from cow (lane 2). The two patterns are clearly different in detail,

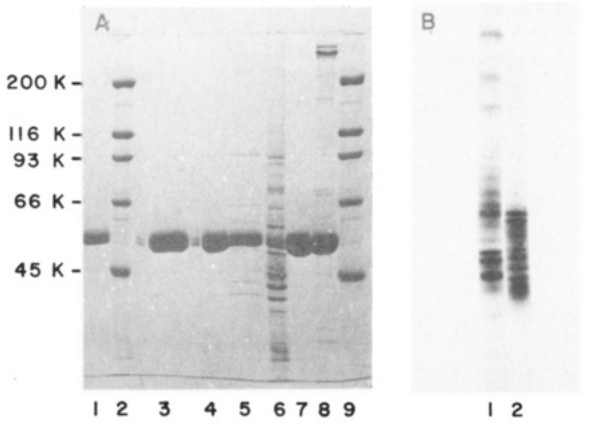

FIGURE 1: Electrophoresis and isoelectric focusing of microtubule protein from antarctic fish. (A) $NaDodSO_4$–PAGE slab gel of microtubule protein at several stages of purification. Lane 1, once-cycled microtubule protein isolated from brain of *D. mawsoni*; lane 2, standards; lane 3, phosphocellulose-purified tubulin from brain of *P. borchgrevinki*; lane 4, twice-cycled microtubule protein from brain of *P. borchgrevinki*; lane 5, once-cycled microtubule protein from brain of *P. borchgrevinki*; lane 6, high-speed supernatant of brain of *P. borchgrevinki*; lane 7, phosphocellulose-purified bovine tubulin; lane 8, twice-cycled microtubule protein from bovine brain; lane 9, standards. The standard proteins employed were ovalbumin, bovine serum albumin, phosphorylase *b*, β-galactosidase, and myosin. (B) Isoelectric focusing pattern of phosphocellulose-purified tubulin. Negative electrode is at the top. Gel 1, tubulin from brain of *P. borchgrevinki*; gel 2, bovine tubulin. In the original gel, 17 or 18 bands could be counted. (With slightly longer gels, 21 or 22 bands can be resolved.)

although the major proteins from the fish are located in the same region of the gel as those from the cow, and the major four bands appear to have possible homologues in the bovine pattern. The number of minor bands is comparable between the two species. When a similar isoelectric focusing experiment was conducted with PC-tubulin, a similar pattern was seen, except that the three bands visible near the top of the gel were absent.

Formation of Microtubules. Figure 2 shows an electron micrograph of the polymers formed when the tubulin from fish brain is warmed to 37 °C. They resemble microtubules. The mean apparent diameter of the rods was found to be 31.3 (±0.6) nm. In regions where the subunits were clearly visible, their apparent longitudinal spacing could be measured and was found to be 4.21 (±0.13) nm. These values are close to those expected for 13-protofilament or 14-protofilament microtubules (see Discussion).

A value of 300 (±50) cm^3/g was obtained for the reduced specific viscosity (η_{sp}/C) of a solution of 1×MT in buffer 1 + 1 mM GTP that had been incubated for approximately 30 min at 35 °C. Such a large viscosity is consistent with formation of microtubules. Its magnitude is quite comparable to the values observed in other microtubule-forming systems with similar viscometers (Olmsted & Borisy, 1973).

When its behavior was examined by measurement of turbidity, the protein was found to be capable of cycles of temperature-dependent assembly and disassembly, as shown in Figure 3. The value of the turbidity rises when the solution is warmed and returns almost to the zero-time value when the temperature is reduced to 5 °C. The peak turbidity reached in the second treatment at 36 °C was 96% of that attained in the first. Also shown in Figure 3 is the fact that the addition of 5×10^{-5} M colchicine (approximately a 10-fold excess over tubulin dimer) abolishes the turbidity change. The moderate decline in turbidity, evident after the peak, was most prominently observed at high protein concentrations (above 0.3

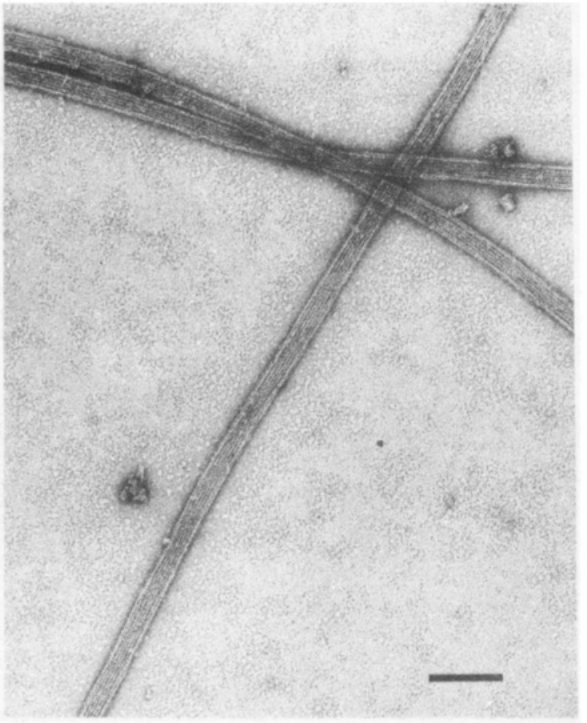

FIGURE 2: Microtubules formed in vitro from phosphocellulose-purified tubulin from *P. borchgrevinki*. Note the characteristic uniform diameter and rows of protofilaments. Bar = 0.1 μm. The lack of MAPs is evident from the smooth outer profiles of the tubules.

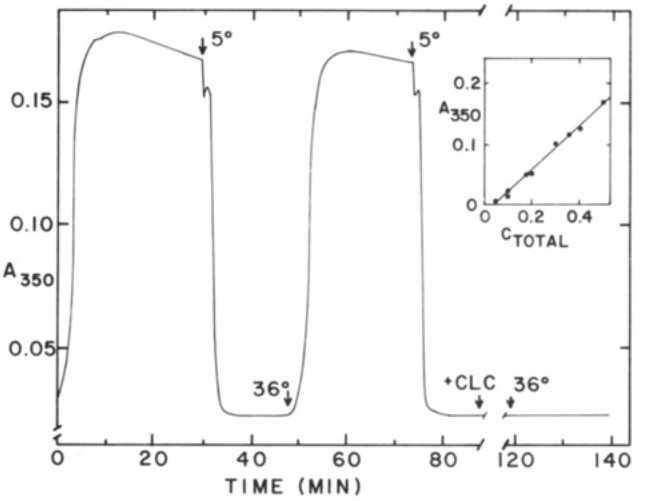

FIGURE 3: Assembly of fish microtubules at elevated temperatures. Turbidity (measured as apparent A_{350}) of a solution (0.36 mg/mL) of twice-cycled microtubule protein of *P. borchgrevinki*, in buffer 1 made 1 mM in GTP, is shown through two cycles of temperature-induced assembly and disassemby. The cold solution was introduced into a prewarmed cuvette at zero time. The temperature of the water circulating through the thermostated cuvette holder was lowered to 5 °C, raised to 36 °C, and again lowered to 5 °C at the times indicated by the arrows. The small bumps in the curve observed during cooling were the result of thermal convection in the rapidly cooling solution. At the time indicated by the arrow, a small aliquot of a concentrated stock solution of colchicine was added to bring the solution to 5×10^{-5} M colchicine. The solution was incubated 35 min on ice and then replaced in the warmed (36 °C) cuvette holder of the spectrophotometer and observed for 20 min. Inset: Measurement of the critical concentration of tubulin of *P. borchgrevinki* at 36 °C in buffer 1 plus 1 mM GTP. Polymerization of a series of dilutions of each of two preparations (indicated by squares and circles) of twice-polymerized tubulin was carried out until a steady plateau of the turbidity was reached. The solution was then cooled on ice for 20 min, and its turbidity was again measured. The value of the maximal turbidity minus the final turbidity of the cold solution is shown as the ordinate in the inset. The total concentration is shown as the abscissa. The line represents the best fit obtained by the method of least squares.

mg/mL). It is seen in other assembly reactions of MAP-free tubulin when they are monitored by turbidity (Herzog & Weber, 1977; Detrich & Wilson, 1983; Suprenant & Rebhun, 1983). It apparently results from a rearrangement of the assembled structures rather than from degradation of the protein or depletion of GTP. Similar experiments (not shown) demonstrate that the presence of GTP in the buffer is required for assembly to occur and that a cold-reversible change in turbidity occurs even in the presence of 1 mM Ca^{2+}.

Colchicine Binding. PC-tubulin (1.25×10^{-5} M dimer) and colchicine (1.25×10^{-4} M) were incubated together for 90 min at 0 °C. The mixture was gel filtered as described above, and the peak fraction of the excluded material was taken for spectral measurement. The values of $A_{278.5}$ and $A_{352.5}$ (corresponding to the tubulin peak and the colchicine peak) were 0.298 and 0.0365, respectively. The corresponding value of the ratio of colchicine to tubulin (eq 1) was found to be 0.99 ± 0.08. This value is taken to be the ratio of colchicine bound per tubulin dimer.

Measurement of Critical Concentrations. The inset to Figure 3 shows the maximal turbidities of solutions of 2×MT as a function of protein concentration, at 35 °C. The results of two separate experiments are shown. The data appear to fit well to a straight line, consistent with a simplified description of the assembly process as a nucleated polymerization reaction (Oosawa & Kasai, 1962). By extrapolation of these data to zero turbidity, the critical concentration under the conditions of measurement was found to be 0.046 (± 0.008) mg/mL. The presence of a preplateau peak in the turbidity at high concentrations, and the likelihood that the assembly process reaches a steady state rather than a true equilibrium, complicates the interpretation of this number slightly (see Discussion).

Temperature Dependence of Assembly. Microtubules were formed from PC-tubulin by incubation of a concentrated solution at 34 °C for 20 min. At room temperature, an aliquot of this material was immediately fixed, applied to an electron microscope grid, and stained as described. The tube containing the remainder of the tubulin was immersed in a stirred ice–water bath. Samples were taken at intervals and were similarly treated for electron microscopy, except that all manipulations were carried out in the cold room at 4 °C with thermally equilibrated equipment and solutions. Figure 4A shows a representative area of a grid made at room temperature. Figure 4B shows a representative area of a grid made at 4 °C after the solution had been incubated 60 min at 0 °C. Figure 4C,D represents equivalent controls made with bovine microtubule protein. It is clear that numerous microtubules assembled from fish tubulin remain in the assembled state after an hour at 0 °C, whereas microtubules made from bovine tubulin become completely disassembled after similar treatment. Microtubules were apparent in a solution of fish tubulin that was kept at 0 °C for 24 h (data not shown). These results are qualitative: they do not specify what fraction of the microtubules remains assembled. They do, however, show that the primary assembled structure present at 0 °C is the microtubule rather than some other polymer.

Figure 5 shows measurements of the critical concentration obtained at temperatures between 35 and −1.2 °C. Data from two separate experiments carried out by the turbidity method described under Materials and Methods are shown (squares and circles) in addition to three measurements made by the centrifugal technique described there (triangles) and the single

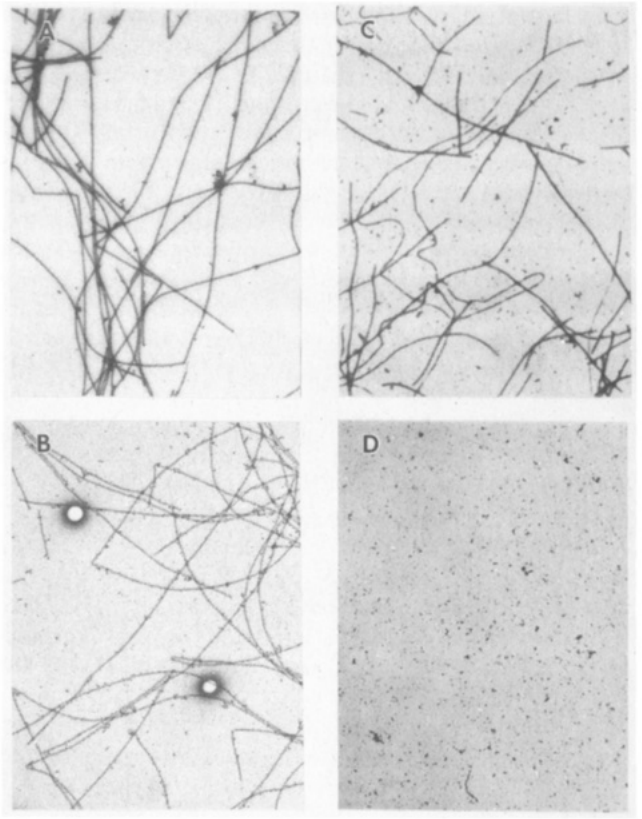

FIGURE 4: Electron micrographs of samples of tubulin solutions incubated at 24 and 0 °C. (A) Phosphocellulose-purified tubulin of *P. borchgrevinki* (approximately 1 mg/mL) incubated for 20 min at 34 °C. Grid prepared at 23 °C. (B) The same sample incubated a further 60 min at 0 °C. Grid prepared at 4 °C. (C) Bovine 2×MT (approximately 1 mg/mL) incubated for 20 min at 34 °C. Grid prepared at 23 °C. (D) Same sample incubated a further 5 min at 0 °C. Grid prepared at 4 °C.

point from the inset to Figure 3 (star). It can be seen that the critical concentration increases monotonically with decreasing temperature over the range observed. In the first set of turbidity measurements (squares), the tubulin was thawed and used directly after equilibration with buffer. In the second set (circles), the thawed tubulin was carried through a cycle of assembly, centrifugation, disassembly, and centrifugation before its final equilibration with buffer and its use in the experiment. The agreement between the two sets of data indicates that there was little influence of denatured or assembly-incompetent tubulin on the data. The inset to Figure 5 shows the same data as a van't Hoff plot. Here the assumption has been made (see Discussion) that the apparent association constant corresponding to the addition of a tubulin dimer to the end of a growing microtubule is equal to the reciprocal of the critical concentration (Lee & Timasheff, 1977). The value of the corresponding apparent enthalpy change, $\Delta H°$, obtained from the best-fitting straight line is 13.7 kcal mol^{-1}. The value of the corresponding apparent standard-state entropy change, $\Delta S°$, accompanying addition of a dimer to the tubule, was +74 eu. The assembly reaction is therefore decidedly entropy driven.

The extrapolated value of the critical concentration of this essentially MAP-free tubulin at the habitat temperature of the antarctic fish (−1.8 °C) is seen to be 0.74 (±0.15) mg/mL.

Discussion

Characteristics of Tubulin and Microtubules. The tubulin prepared from the brain of *P. borchgrevinki* by cycles of temperature-dependent assembly and disassembly comigrates

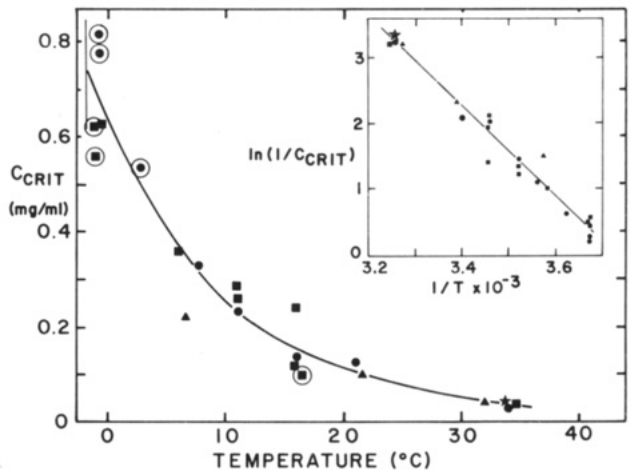

FIGURE 5: Critical concentration of fish tubulin as a function of temperature. Four distinct sets of measurements are shown: (squares) results for phosphocellulose-purified tubulin thawed and used directly; (circles) results for phosphocellulose-purified tubulin thawed and then carried through an additional cycle of assembly/disassembly before the experiment; (triangles) measurements made by centrifugal technique; (star) measurement shown in the inset to Figure 3. The encircled points represent measurements in which the temperature of interest was approached from above. The curve represents the best-fitting description of the data to the equation $C_c = \beta \exp(\Delta H°/RT)$. Values of the fitting constant β and of $\Delta H°$ were obtained from a van't Hoff treatment of the data. Inset: The same data represented as a van't Hoff plot. The straight line represents the best fit by the method of least squares.

with authenic bovine tubulin on $NaDodSO_4$ gels and is resolved into two bands in the same fashion. Upon isoelectric focusing in a urea-containing buffer, it forms an appropriate number of focused bands at appropriate positions. The protein binds, within error, one molecule of colchicine per 100 000 daltons, as does authentic tubulin (Shelanski & Taylor, 1967; Wilson et al., 1974), and its assembly into polymers is inhibited by the binding of colchicine, depends on the presence in solution of GTP, and is essentially fully reversible by means of a change in temperature. The fact that isolation by cycles of temperature-dependent assembly and disassembly yields almost no MAPs stands in contrast to the situation in mammals. Apparently MAP-free tubulin has, however, been isolated by assembly/disassembly techniques from dogfish brain (Langford, 1978), from sea urchin eggs (Kuriyama, 1977; Detrich & Wilson, 1983; Suprenant & Rebhun, 1983), from yeast (Kilmartin, 1981), and from outer doublet microtubules of sperm (Kuriyama, 1976; Binder & Rosenbaum, 1978; Farrell & Wilson, 1978). In all of these cases, the isolated tubulin polymerizes well in vitro, demonstrating that MAPs are not essential. However, this fact does not imply that MAPs are absent or unnecessary in vivo (Caplow et al., 1982; Vallee & Bloom, 1983).

The elevated value of the reduced specific viscosity and the electron microscopic evidence (Figure 2) obtained at temperatures near 35 °C indicate that microtubules are formed upon assembly. Their mean apparent diameter (31.3 nm) is somewhat greater than the values near 25 nm typically found by electron microscopy of embedded and sectioned microtubules, but within the range of 27–35 nm commonly observed in negatively stained specimens [cf. Amos (1979)]. The apparent subunit spacing of 4.2 nm is comparable to values obtained with mammalian microtubules [cf. Erickson (1974)]. Close examination of the image shows that the protofilaments lie at a slight angle to the longitudinal axis of the microtubule (visible by following a single protofilament near the edge of the tubule) and that a periodic moiré pattern produces diagonal

striations at intervals of approximately 96 nm along the axis. These features have been shown by Langford (1980) to be characteristic of a 14-protofilament microtubule with a discontinuous surface lattice, commonly observed in microtubules purified by assembly/disassembly in vitro [cf. McEwen & Edelstein (1980)]. The electron micrograph of Figure 4B demonstrates that the polymers that remain in solution at temperatures near 0 °C are microtubules.

Temperature Dependence of Microtubule Formation. The measurements of critical concentration cannot be interpreted within a strict thermodynamic framework because of the fact that hydrolysis of GTP occurs in concert with the assembly process, together with a flux of subunits through the assembled microtubule (Wegner, 1976; Margolis & Wilson, 1978; Caplow et al., 1982). The assembly reaction is thus not strictly reversible in the thermodynamic sense, since it involves a step (the nucleotide hydrolysis) that is not reversible. Equilibrium is not strictly attained. The plateau of turbidity observed at long times after the main burst of assembly is the result of the achievement of a steady state between the assembly and disassembly reactions. The value of the critical concentration is given by

$$C_c = (k_2^+ + k_2^-)/(k_{-1}^+ + k_{-1}^-) \quad (3)$$

where k_2^+ and k_2^- are the second-order rate constants for addition of a subunit at the two distinguishable ends (+ and −) of the microtubule and k_{-1}^+ and k_{-1}^- are the first-order rate constants for dissociation of a subunit. As discussed by Wegner (1976) for the case of opposite-end assembly, by Oosawa & Asakura (1975) for the case of assembly at identical ends involving nucleotide hydrolysis, and by Carlier & Pantaloni (1982), the apparent equilibrium constant, K_{app}, calculated as $K_{app} = 1/C_c$, is an average of the values obtained at the two ends and reflects not only the free energy of association of the protein subunits but also the free energy of hydrolysis of nucleotide. It is nonetheless a useful number, a constant under a given set of conditions at a given concentration of nucleotide. It is comparable to the numerous other apparent constants already reported. Knowledge of its value allows one to predict, for instance, the effect of dilution on the mass of assembled tubules. The free energy, enthalpy, and entropy derived from it are likewise apparent values, but they are useful in characterizing the concentrations of microtubules and monomers present in solution.

The data of Figures 4 and 5, taken together, indicate that microtubules made of fish brain tubulin can be present, at accessible concentrations of protein, at temperatures as low as −1.2 °C. The critical concentration differs by a factor of about 16 between 37 and −1.2 °C. It is this difference that makes possible the reversible assembly and disassembly shown in Figure 3. The protein concentration there was 0.36 mg/mL, large enough to be substantially in excess of the C_c of 0.046 mg/mL at 37 °C, but much smaller than the C_c of 0.45 mg/mL that obtains at 5 °C. Hence, a majority (about 90%) of the protein would be in the form of microtubules at 37 °C, while essentially none would be expected to be assembled at 0 °C. Doubtless, the assembly/disassembly purification entails substantial loss of tubulin. Microtubules initially present may be discarded in the first (cold) centrifugations, and tubulin present at concentrations greater than about 0.5 mg/mL will be discarded in subsequent cold centrifugations.

One can ask if there is reason to believe that the microtubules are not in equilibrium with free tubulin dimer over the range of temperatures measured. A good test of this point is to ask whether the data of Figure 5 show a systematic trend in the measured critical concentration that depends on whether the temperature of measurement was approached from above or below (see legend). Although approaches both from above and from below were not made at many temperatures, it is evident that there is no large effect of direction of approach. The magnitudes of the steps between temperatures were also varied over a large range, and no systematic effect of the size of the step was apparent in the data. Thus, the presence of microtubules in a solution that has been cooled from a higher temperature is solely a result of the apparent equilibrium that obtains at that temperature and is not the result of a kinetic effect: the microtubules are not present simply because they are slow to dissociate when cooled. The fish tubulin possesses a large tendency to associate at low temperatures (a property of the apparent equilibrium that obtains between the tubulin and the microtubule) rather than a simple resistance to disassembly at low temperatures (a property of the rates of association and dissociation of tubulin and microtubule).

Thus, microtubules formed from the tubulin of this fish are comparatively stable in the cold as a result of the fact that, at a given temperature, the apparent association constant ($1/C_c$) for addition of a dimer of fish tubulin to the ends of a microtubule is substantially larger than the apparent association constant for the addition of a dimer of mammalian tubulin under similar conditions. The observed cold stability is thus purely an effect of the apparent equilibrium in this case.

One can also ask if the microtubules that are present at low temperatures are formed from a subpopulation of the tubulin. The good agreement between the values of C_c obtained when PC-tubulin was used directly and those obtained when the tubulin was cycled an extra time before the experiment (Figure 5) argues strongly that this is not the case. If cold-stable tubules were formed only from a subpopulation, a substantial fraction of that subpopulation would have been discarded, during the extra cycle, in the pellet that results from the cold centrifugation, and the apparent critical concentration would have been raised greatly. Since there is no significant evidence of such an effect, one can conclude that it is unlikely that there is a subpopulation of uniquely cold-stable tubulin. Likewise, if the cold stability were conferred on an otherwise cold-dissociable microtubule by the presence of stabilizing MAPs, one would expect differences between the two tubulin preparations, since the MAPs would have been discarded in the cold pellet. Hence, the presence of such MAPs (and their amounts would have to be small, as shown by the electrophoresis results) is not a likely explanation of the comparatively small critical concentration observed at −1.8 °C.

A Modified Tubulin. Evidently, then, the tubulin of *P. borchgrevinki* has been altered in such a way as to increase its apparent association constant at any given temperature above the values observed for mammalian tubulins. The differences in the isoelectric focusing pattern revealed in Figure 1B are certainly compatible with this idea. The apparent association constant of the tubulin at the habitat temperature of the fish (−1.8 °C) is $(1.3 \pm 0.2) \times 10^5$ M^{-1} (Figure 5). In comparison, the critical concentration of MAP-free bovine tubulin in buffer 1 + 0.5 mM GTP at 37 C is about 2.4 mg/mL (R. C. Williams, Jr., and J. J. Correia, unpublished results), corresponding to an association constant of 0.42×10^5 M^{-1}. MAP-free tubulin from the eggs of *S. purpuratus* has an association constant of 1.2×10^5 M^{-1} at its approximate habitat temperature of 15 °C in a buffer similar to buffer 1 (Suprenant & Rebhun, 1983), and that from the eggs of *Spisula solidissima* has an association constant of 0.36×10^5 M^{-1} at 22 °C (Suprenant & Rebhun, 1984). Thus, it appears that these different organisms have modified their tubulins in

such a way as to keep the critical concentrations approximately the same (within, for instance, an order of magnitude) at the internal temperature of the organism. In an extensive comparative study of muscle actins from 15 vertebrates adapted to different temperatures, Swezey & Somero (1982) found that each actin has been modified so that the association constant for addition of a monomer to the end of a growing actin filament is roughly invariant (within an order of magnitude) when measured in vitro at the habitat temperature of the organism from which the protein came. The results reported here suggest that the same adaptive strategy may apply in the case of microtubules. Modifications of tubulin would be the fundamental means of adaptation. Modifications of MAPs or other auxiliary factors would then be supposed to play a secondary role, if any, in adaptation to low temperatures.

The apparent values of $\Delta H° = +13.7$ kcal/mol and $\Delta S° = +74$ eu at -1.2 °C show the overall assembly reaction of the tubulin of *P. borchgrevinki* to be entropy driven, as are the assembly reactions of other tubulins. The values obtained can only be compared to figures obtained with MAP-free tubulins, since the binding of MAPs is expected to contribute significantly to the observed thermodynamic quantities. Lee et al. (1978) found a curved van't Hoff plot in measurements made with bovine tubulin in 0.01 M sodium phosphate, 0.1 mM GTP, 1 mM EGTA, 16 mM $MgCl_2$, and 3.4 M glycerol. They measured values of $\Delta H° = +2.15$ kcal/mol at 37 °C and $\Delta H° = +22.8$ kcal/mol at 23 °C. Robinson & Engelborghs (1982), making measurements of tubulin in a different solvent (0.05 M Mes, 1 mM EGTA, 0.5 mM $MgCl_2$, 70 mM KCl, 1 mM NaN_3, and 1 mM GTP, pH 6.4), found a linear van't Hoff plot over the range 10–35 °C, characterized by a $\Delta H° = +6.33$ kcal/mol. Values of $\Delta S° = +30$ and +44.4 eu, respectively, were found by the two groups. Although the comparative data are few, it may be tentatively concluded that the apparent enthalpy of polymerization of antarctic fish tubulin is roughly comparable to the values for mammalian tubulins and that the apparent entropy of polymerization is somewhat greater.

Acknowledgments

We thank Kate Welch for excellent technical assistance. We are grateful to Drs. H. W. Detrich, Jane Baxandall, and L. Wilson for much helpful discussion. The Division of Polar Programs of the National Science Foundation, ITT Antarctic Services, and the Antarctic Air Development Squadron (VXE-6) of the U.S. Navy provided invaluable logistic support.

References

Amos, L. A. (1979) in *Microtubules* (Roberts, K., & Hyams, J. S., Eds.) Academic Press, New York.

Binder, L. I. & Rosenbaum, J. L. (1978) *J. Cell Biol. 79*, 500–515.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Brinkley, B. R. & Cartwright, J., Jr. (1975) *Ann. N.Y. Acad. Sci. 253*, 428–439.

Caplow, M., Langford, G. M., & Zeeberg, B. (1982) *J. Biol. Chem. 257*, 15012–15021.

Carlier, M.-F., & Pantaloni, D. (1982) *Biochemistry 21*, 1215–1224.

Correia, J. J., & Williams, R. C., Jr. (1983) *Annu. Rev. Biophys. Bioeng. 12*, 211–235.

Detrich, H. W., III, & Williams, R. C., Jr. (1978) *Biochemistry 17*, 3900–3907.

Detrich, H. W., III, & Wilson, L. (1983) *Biochemistry 22*, 2453–2462.

Erickson, H. P. (1974) *J. Mol. Biol. 60*, 153–167.

Farrell, K. W., & Wilson, L. (1978) *J. Mol. Biol. 121*, 393–410.

George, H. J., Misra, L., Field, D. J., & Lee, J. C. (1981) *Biochemistry 20*, 2402–2409.

Herzog, W., & Weber, K. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1860–1864.

Job, D., Fischer, E. H., & Margolis, R. L. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4679–4682.

Job, D., Rauch, C. T., Fischer, E. H., & Margolis, R. L. (1982) *Biochemistry 21*, 509–515.

Johnson, K. A., & Borisy, G. G. (1977) *J. Mol. Biol. 117*, 1–31.

Jones, D. H., Gray, E. G., & Barton, J. (1980) *J. Neurocytol. 9*, 493–504.

Kilmartin, J. (1981) *Biochemistry 20*, 3629–3633.

Krauhs, E., Little, M., Kempf, T., Hofer-Warbinek, R., Ade, N., & Ponstingl, H. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4156–4160.

Kuriyama, R. (1976) *J. Biochem. (Tokyo) 80*, 153–165.

Kuriyama, R. (1977) *J. Biochem. (Tokyo) 81*, 1115–1125.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Langford, G. M. (1978) *Exp. Cell Res. 111*, 139–151.

Langford, G. M. (1980) *J. Cell Biol. 87*, 521–526.

Lee, J. C., & Timasheff, S. N. (1977) *Biochemistry 16*, 1754–1764.

Lee, J. C., Tweedy, N., & Timasheff, S. N. (1978) *Biochemistry 17*, 2783–2790.

Lopez, L. A., & Bertini, F. (1982) *J. Exp. Zool. 221*, 9–12.

Margolis, R. L., & Wilson, L. (1978) *Cell (Cambridge, Mass.) 13*, 1–8.

Margolis, R. L., & Rauch, C. T. (1981) *Biochemistry 20*, 4451–4458.

McEwen, B., & Edelstein, S. J. (1980) *J. Mol. Biol. 139*, 123–145.

Olmsted, J. B., & Borisy, G. G. (1973) *Biochemistry 12*, 4282–4289.

Oosawa, F., & Kasai, M. (1962) *J. Mol. Biol. 4*, 10–21.

Oosawa, F., & Asakura, S. (1975) *Thermodynamics of the Polymerization of Protein*, Chapter 2, Academic Press, New York.

Pannese, E., Arcidiacono, G., Rigamonti, L., Procacci, P., & Ledda, M. (1982) *J. Ultrastruct. Res. 79*, 18–30.

Pirollet, F., Job, D., Fischer, E. H., & Margolis, R. L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1560–1564.

Ponstingl, H., Krauhs, E., Little, M., & Kempf, T. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2757–2761.

Robinson, J., & Engelborghs, Y. (1982) *J. Biol. Chem. 257*, 5367–5371.

Shelanski, M. L., & Taylor, E. W. (1967) *J. Cell Biol. 34*, 549–554.

Shelanski, M. L., Gaskin, F., & Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 765–768.

Suprenant, K., & Rebhun, L. I. (1983) *J. Biol. Chem. 258*, 4518–4525.

Suprenant, K. A., & Rebhun, L. I. (1984) *J. Cell Biol. 98*, 253–266.

Swezey, R. R., & Somero, G. N. (1982) *Biochemistry 21*, 4496–4503.

Timasheff, S. N., & Grisham, L. M. (1980) *Annu. Rev. Biochem. 49*, 565–592.

Valenzuela, P., Quiroga, M., Zaldivar, J., Rutter, W. J., Kirschner, M. W., & Cleveland, D. W. (1981) *Nature (London) 289*, 650–655.

Vallee, R. B., & Bloom, G. S. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6259–6263.

Wegner, A. (1976) *J. Mol. Biol. 108*, 139–150.
Weingarten, M. D., Lockwood, A. H., Hwo, S.-Y., & Kirschner, M. W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1858–1862.
Williams, R. C., Jr., & Detrich, H. W., III (1979) *Biochemistry 18*, 2499–2503.
Williams, R. C., Jr., & Lee, J. C. (1982) *Methods Enzymol. 85*, 376–385.
Williams, R. C., Sr. (1981) *J. Mol. Biol. 150*, 399–408.
Wilson, L., Bamburg, J. R., Mizel, S. B., Grisham, L. M., & Creswell, K. M. (1984) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33*, 158–166.

Trehalose-Containing Lipooligosaccharides from Mycobacteria: Structures of the Oligosaccharide Segments and Recognition of a Unique *N*-Acylkanosamine-Containing Epitope†

Shirley Wu Hunter,‡ Ian Jardine,§ Donna L. Yanagihara,‡ and Patrick J. Brennan*,‡

Department of Microbiology, Colorado State University, Fort Collins, Colorado 80523, and Department of Pharmacology, Mayo Clinic, Rochester, Minnesota 55905

Received August 14, 1984; Revised Manuscript Received December 4, 1984

ABSTRACT: The structures of the oligosaccharide segments of nine trehalose-containing lipooligosaccharides (LOS) of *Mycobacterium kansasii* have been established by positive and negative fast-atom bombardment mass spectrometry, acetolysis, partial acid hydrolysis, methylation analyses, and nuclear magnetic resonance. Upon acetolysis, all produce the α,α-trehalose-containing tetraglucose (Glc_4) "core" -β-D-Glc*p*(1→3)-β-D-Glc*p*(1→4)-α-D-Glc*p*(1→1)-α-D-Glc*p*. The simplest (LOS I′) contains an additional α1→3-linked 3-*O*-methyl-L-rhamnopyranose (3-*O*-Me-L-Rha*p*) unit; those of intermediate complexity (LOS I–III) contain an additional D-xylopyranose (D-Xyl*p*) residue or xylobiose in β1→4 linkage; and those of ultimate complexity (LOS IV–VIII) contain further D-Xyl*p* residues and the distal *N*-acylkanosamine- (Kan*N*Acyl) and fucopyranosyl- (Fuc*p*) containing disaccharide Kan*N*Acyl(1→3)Fuc*p*. Thus, the structure of the oligosaccharide from LOS VII is Kan*N*Acyl(1→3)Fuc*p*(1→4)[-β-D-Xyl*p*(1→4)]$_6$-α-L-3-*O*-Me-Rha*p*(1→3)Glc_4. Polyclonal rabbit and murine monoclonal antibodies react only with the more complex Kan*N*Acyl-Fuc*p*-containing lipooligosaccharides, indicating that the Kan*N*Acyl distal end, not the trehalose end, contains the antibody binding site unique to *M. kansasii* and is responsible for the serological distinctiveness of *M. kansasii* among mycobacterial species.

The precise serological differentiation, most of the overt antigenicity, and features of the pathogenesis of many species within the *Mycobacterium* genus are due to a family of rather extraordinary trehalose-containing lipooligosaccharides (LOS)[1] (Brennan, 1984). In them, trehalose is found glycosidically linked as part of a tri- or a tetraglucosyl "core", which in turn is glycosidically modified in distinct fashion to confer species-specific antigenicity (Hunter et al., 1983; unpublished observations).[2] The sugar appendages unique to *Mycobacterium kansasii* are Kan*N*Acyl, Fuc, Xyl, and 3-*O*-Me-Rha (Hunter et al., 1983, 1984b). No other trehalose-containing lipooligosaccharide antigens have been examined in detail, although Saadat & Ballou (1983), working in a different context, found a dipyruvylated pentasaccharide, 4,6-(1-carboxyethylidene)-3-*O*-Me-β-D-Glc*p*(1→3)-4,6-(1-carboxyethylidene)-β-D-Glc*p*(1→4)-β-D-Glc*p*(1→6)-α-D-Glc*p*(1→1)-α-D-Glc*p*, and a related monopyruvylated tetrasaccharide, in *Mycobacterium phlei*, and these were lipid in origin. In this paper, a combination of chemical analyses and antibody reactivity using murine monoclonal antibodies was used to arrive at structures for the component oligosaccharides of the LOS family of *M. kansasii* and which allowed recognition of the segment of the oligosaccharide within the highly reactive polar lipooligosaccharides responsible for the specific antigenicity of *M. kansasii*.[3]

Experimental Procedures

Strain of M. kansasii. Previously we reported that the strain under examination was *M. kansasii* strain Forbes [No. 1201 in the Trudeau *Mycobacterial* Culture Collection (1980)]. In fact, the strain was Florisse, which has not been acceeded to the Trudeau mycobacterial collection. However, TMC strains 1201, 1214, 1217, and 1204 did contain the same array of LOS, both polar and apolar, seen in Florisse. *M. kansasii* was grown as described (Hunter et al., 1983). The entire sus-

† Supported by the National Institutes of Health (Grant AI-18357 to P.J.B.) and the Mayo Foundation (support of I.J.). The CSU Chemistry Department Regional NMR Center was supported by National Science Foundation Grant CHE 78-18581.

‡ Department of Microbiology, Colorado State University.
§ Department of Pharmacology, Mayo Clinic.

[1] Abbreviations: LOS, lipooligosaccharide; Ose, nonreducing oligosaccharide; FAB, fast-atom bombardment; MS, mass spectrometry; EI, electron ionization; GC, gas chromatography; TLC, thin-layer chromatography; Kan*N*Acyl, *N*-acylkanosamine [4,6-dideoxy-2-*O*,3-*C*-dimethyl-4-(2-methoxypropionamido)-α-L-*manno*-hexopyranose and -β-L-*manno*-hexopyranose]; Glc*p*, glucopyranose; Fuc*p*, fucopyranose; Rha*p*, rhamnopyranose; Xyl*p*, xylopyranose; Glc_4, tetraglucose "core"; Me, methyl or *O*-methyl; TMC, Trudeau mycobacterial collection.

[2] Work conducted with Virginia L. Barr.

[3] A preliminary account of this work has appeared (Hunter et al., 1984a).

0006-2960/85/0424-2798$01.50/0