Are microtubules cold-stable in the Atlantic cod, *Gadus morhua*?

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The amount of axonally transported proteins in the nervus splanchnicus of cod (*Gadus morhua*) was found to be temperature-dependent in vitro, with an optimum at 8°C. The transport was markedly reduced at 2 °C, probably caused by decreased protein synthesis rather than disassembly of microtubules. Microtubules were isolated from cod brain by cycles of assembly-disassembly. These microtubules were cold-labile, had a low amount of microtubule-associated proteins and a high critical concentration for assembly. The possibilities exist that a cold-stabilizing factor or cold-stable microtubules are lost during the preparation, or that cold-stable microtubules are components of the peripheral axons only.

Microtubule *Cold stability* *Cold lability* *Axonal transport* *Microtubule-associated protein*

1. INTRODUCTION

Microtubules play a role in many cell activities, such as cell division and axonal transport [1]. Microtubules have hitherto mainly been characterized from microtubule proteins prepared by a temperature-dependent assembly-disassembly method from mammalian and bird brains. The method takes advantage of the fact that these microtubules break down at low temperature and reassemble with increasing temperature.

Evidence of the presence of cold-stable microtubules in addition to the cold-labile microtubules in the mammalian brain has been presented recently [2,3]. However, little attention has been focused on animals living at low temperatures, animals which must have microtubules that remain assembled at the low temperature of their normal environment. In order to investigate temperature effects on microtubules in cod, an animal living in water close to or even below 0°C, two different systems were used. Studies were performed on a microtubule-dependent process, axonal transport in the splanchnic nerve and on microtubules isolated from the brain.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

GTP (type II-S) was purchased from Sigma (USA) and [^{3}H]leucine from Amersham (England). All other chemicals were of reagent grade.

2.2. *Axonal transport*

Nervus splanchnicus with ganglion coeliacum attached was dissected out and placed in an incubation chamber containing cod Ringer's solution [4] and sucrose (1 g/l). The incubation chamber was divided into two compartments by a wall containing a slide to allow the ganglion to be placed in the smaller compartment (3 ml) and the nerve in the other (10 ml). The two parts of the chamber were separated from each other by silicone grease [5]. Both compartments were continuously bubbled

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with O_2/CO_2 (97:3%). [^{3}H]Leucine was added to the ganglion compartment to study the transport of proteins. The incubation was interrupted according to [5]. The nerve segments were dissolved by addition of 250 μl soluene, 10 ml Instagel added, and the pH adjusted to neutral followed by counting in an LKB Wallac scintillation counter.

The effect of temperature was determined by varying the incubation temperatures; 20, 8 and 2°C for 20 h. The rate of axonal transport was studied by incubations at 20°C for 1–8 h. The distribution of [^{3}H]leucine material in the nerves was determined and the front of radioactive material plotted against time, for calculations of the transport rate.

2.3. *Preparation of microtubule proteins*

Bovine brain microtubule proteins were isolated by two cycles (2 × MT) of assembly-disassembly as described in [6]. Microtubule proteins from cod brains were isolated as follows. Brains (weighing approx. 0.3–0.5 mg each) were removed, washed in ice-cold buffer (100 mM Pipes and 0.5 mM $MgSO_4$, pH 6.8), prepared free of blood and blood vessels, blotted and weighed. The brains were homogenized in cold assembly buffer (100 mM Pipes, 0.5 mM $MgSO_4$, 1 mM GTP and 1 mM EGTA, pH 6.8) with a knife homogenizer and then twice in a glass-Teflon homogenizer. The homogenate was centrifuged at 30000 × g for 20 min at 4°C and the supernatant at 180000 × g for another 90 min. The supernatant was filtered through a Millex-HA 0.45 μm filter (Millipore) and GTP added to 1 mM (crude extract). Microtubules were assembled at 30°C for 45 min, and centrifuged at 30°C at 150000 × g for 35 min. The pellet was resuspend by gentle treatment in a Dounce homogenizer in assembly buffer (1/5 of the volume of the crude extract), kept on ice for 30 min and centrifuged at 100000 × g for 20 min at 4°C (1 × MT). Most of the experiments were done with this supernatant. For some experiments an additional assembly-disassembly cycle was performed. Microtubules were assembled for 45 min at 30°C and centrifuged at 100000 × g for 30 min at 30°C. The pellet was resuspended in 20–25% of the volume of the 1 × MT in assembly buffer, kept on ice for 30 min and again centrifuged at 100000 × g for 20 min at 4°C (2 × MT).

Assembly of microtubules was measured in a temperature-controlled Perkin-Elmer Lambda 3 spectrophotometer. GTP (1 mM) was added to the protein solution and the assembly monitored continuously at 30°C by the change in absorbance at 350 nm.

Protein concentrations were determined according to [7] using bovine serum albumin as a standard.

The microtubule proteins were analyzed by SDS-polyacrylamide gel electrophoresis on a 1.5 mm thick vertical slab gel (LKB, Sweden) using a linear gradient of 5–12% polyacrylamide according to [8].

2.4. *Electron transport*

The splanchnic nerves with the ganglion coeliacum from 9 cods were dissected out and incubated under slight stretching (comparable to the in vivo situation) for 23 h at 0, 4 and 11°C in cod Ringer's solution. The nerves were then fixed in Karnovsky's solution [9] for 1 h at the same temperature, followed by 1% osmium tetroxide in cacodylate buffer, dehydrated and embedded in Epon. Thin sections were made on an LKB ultramicrotome and viewed in a Zeiss 109 transmission electron microscope.

Negatively stained specimens were prepared from 5 μl of the sample which was applied on a formvar-coated copper grid, dried after 20 s with filter paper and stained with 5 drops of 1% uranyl acetate.

3. RESULTS

3.1. *Axonal transport and nerve microtubule content*

Analysis by linear regression indicates a transport of 85 mm/24 h at 20°C (fig.1). The transport of radiolabelled material was shown to be dependent on temperature and an optimum was found at 8°C, when expressed as per cent cpm per total nerve (fig.2A). The amount of radiolabelled proteins was lower at 2°C, indicating a low synthesis of ^{3}H-proteins (fig.2B). No obvious disassembly of microtubules was observed in nerves incubated at 0, 4 and 11°C, as judged from electron micrographs of embedded nerves (fig.3).

3.2. *Microtubule proteins*

Cod brain microtubules could only be isolated

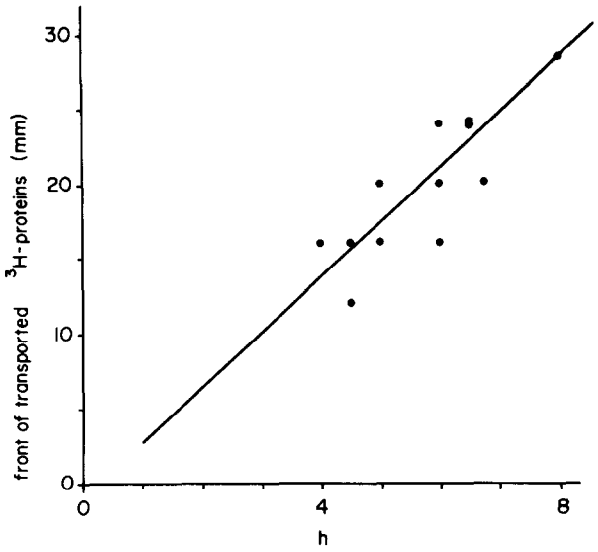

Fig.1. The transport of radiolabelled proteins in nervus splanchnicus. The linear regression analysis was performed by the method of least squares, giving a correlation coefficient of 0.78; $0.001 < P < 0.01$.

from fresh brains. The critical concentration for microtubule assembly in 1 × MT was determined from three experiments which all showed a critical concentration of approx. 0.8 mg/ml. The results from one experiment are shown in fig.4.

SDS-polyacrylamide gel electrophoresis performed on 1 × MT and 2 × MT and as comparison bovine brain microtubules (2 × MT) is shown in fig.5. No or very low amounts of high-M_r proteins were found in 1 × MT, while several low-M_r proteins in addition to tubulin were present. 2 × MT was shown to consist primarily of tubulin.

The assembled microtubules were found to be cold-labile. Incubation of assembled microtubules at 0–4°C for 20 min was enough to disassemble them. When the temperature was increased again to 30°C, the microtubules reassembled to approximately the same level. This procedure could be repeated several times as shown in fig.6.

4. DISCUSSION

Axonal transport is known to be a microtubule-based function which is temperature-dependent. Studies of axonal transport often include determination of accumulated labelled proteins in front

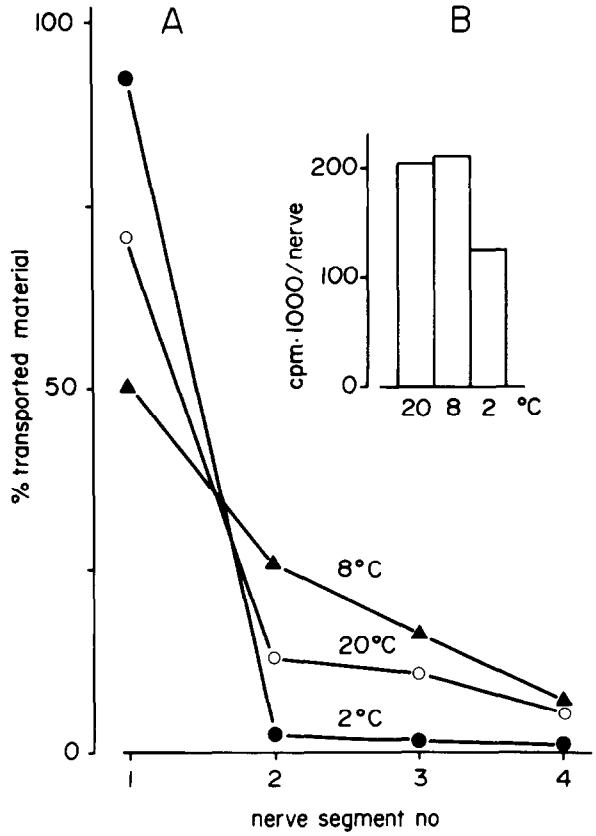

Fig.2. (A) Distribution of ^{3}H-labelled material in nervus splanchnicus incubated at different temperatures expressed as per cent per nerve. (B) Total amount of radiolabelled proteins in nervus splanchnicus at different temperatures.

of a ligature or an area of the nerve which is cold-blocked. Here, the transport was not completely inhibited by a cold-block (not shown), indicating the presence of cold-stable microtubules. This was confirmed by the presence of microtubules in the nerves even at 0°C. Although a transport optimum was found at 8°C, factors such as energy for synthesis of ^{3}H-proteins and for transport influence the final transport picture. These factors might be of minor importance within certain temperature intervals, differing with species, in this study from 20 to 8°C, while they become important at higher or lower temperatures, for example at 2°C. In favour of this hypothesis is the finding that the calculated transport rate was not markedly affected by lowering the temperature from 20°C in vitro in this study to 10°C in vivo [10].

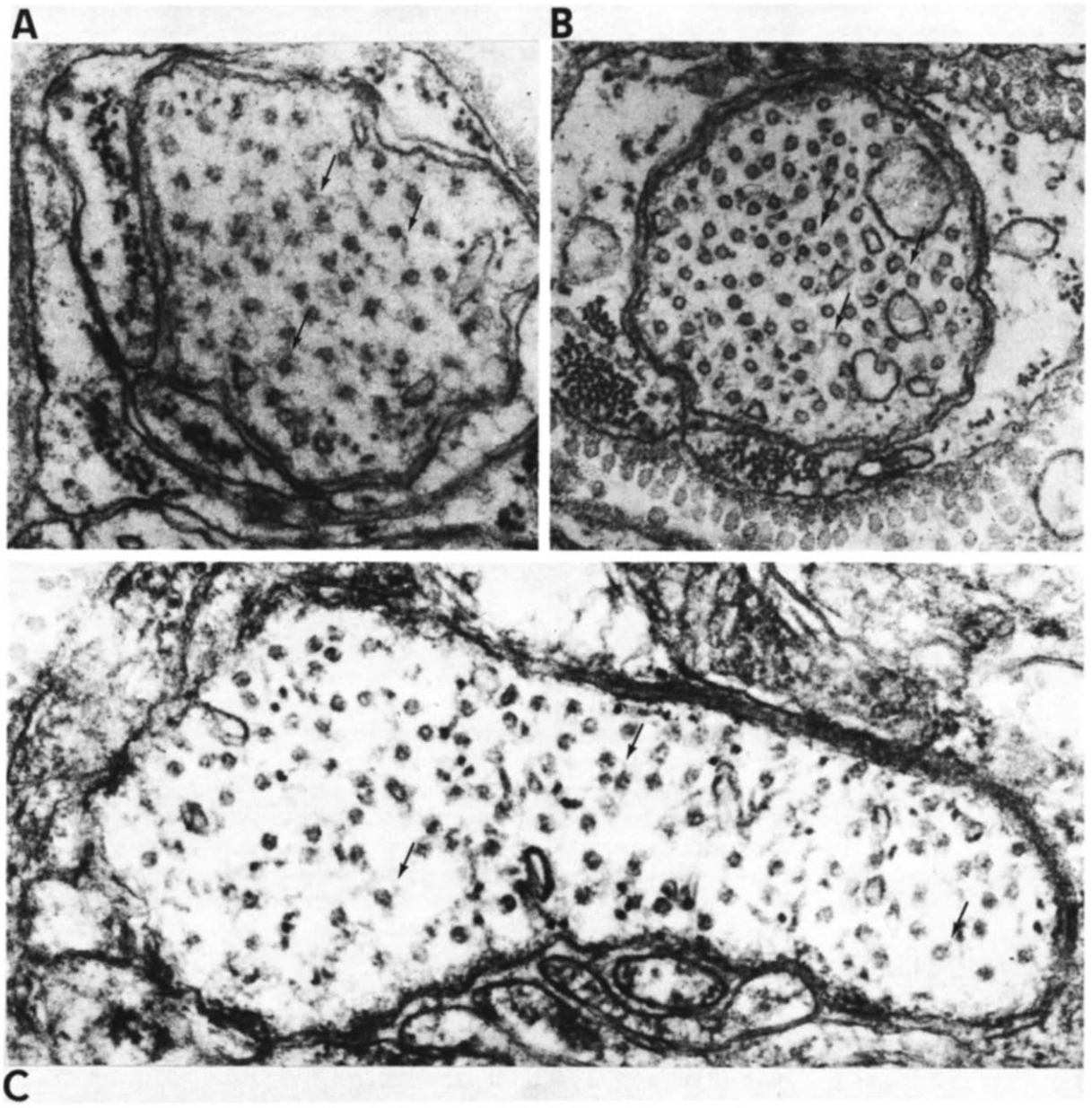

Fig.3. Electron microscopy of nervus splanchnicus incubated in cod Ringer's solution for 23 h at 0°C (a), 4°C (b) and 11°C (c). Magnification 90000 ×. Projections on the microtubule surface are indicated by arrows.

However, when microtubules were isolated from the brain, only a cold-labile fraction of microtubules was purified, the assembly of which was fully reversible after cold treatment. The yield was low (approx. 0.07 mg/g brain tissue), which might be caused by the very high critical concentration found for the assembly (0.8 mg/ml) compared to 0.1–0.2 mg/ml for the critical concentration for bovine brain microtubule assembly [11].

Microtubule-associated proteins such as microtubule-associated protein 2 and tau proteins are known to facilitate assembly and lower the critical concentration for assembly of mammalian brain microtubules [1]. The cod brain microtubule

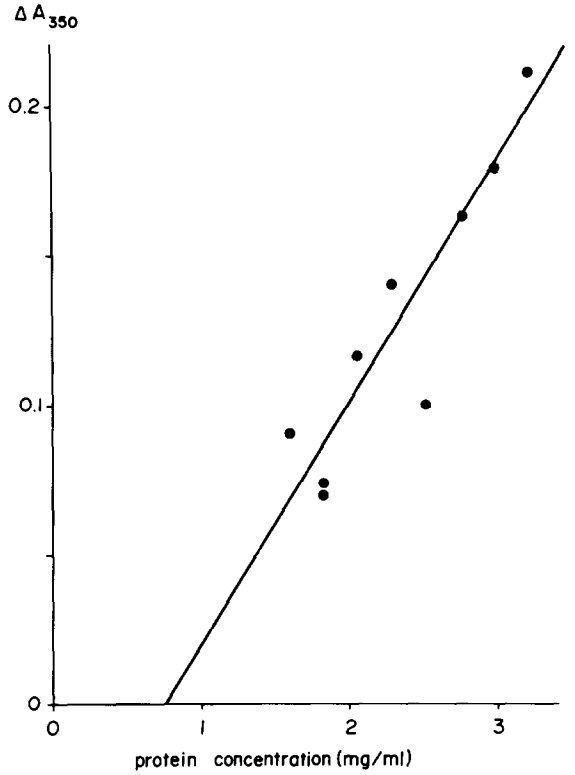

Fig.4. Microtubule assembly at different protein concentrations. The steady-state level of assembly is plotted vs the change in turbidity at 350 nm.

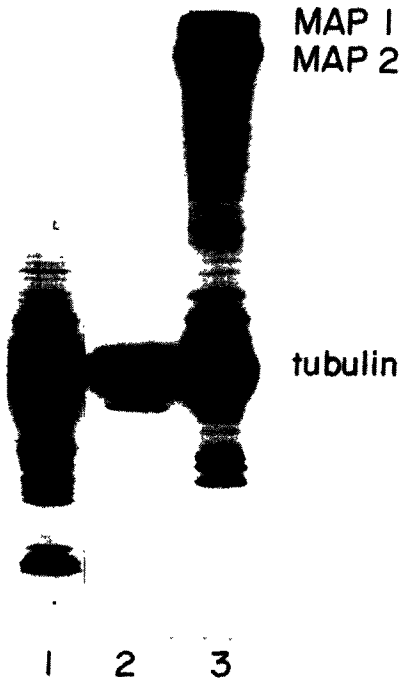

Fig.5. SDS-polyacrylamide gel electrophoresis of microtubule proteins from cod brain 1 × MT (lane 1), cod brain 2 × MT (lane 2) and bovine brain 2 × MT (lane 3).

protein preparation was depleted of several microtubule-associated proteins, which might be another reason for the low yield. It is uncertain whether microtubule-associated proteins, and in particular the high-M_r proteins, are present in the cod brain and nerves and are lost during the purification process. However, the presence of lateral projections from microtubules in sections of cod nerves indicates the presence of high-M_r microtubule-associated proteins in vivo.

Microtubules which were found to assemble even at low temperatures have recently been isolated from Antarctic fish [12]. The cold stability was proposed to be an intrinsic property of tubulin, since the microtubules were composed of almost pure tubulin. In contrast, cold stability of mammalian brain microtubules has been conferred to a cold-stabilizing factor [2]. These microtubules were disassembled by treatment with Ca^{2+}. However, cod brain microtubules did not

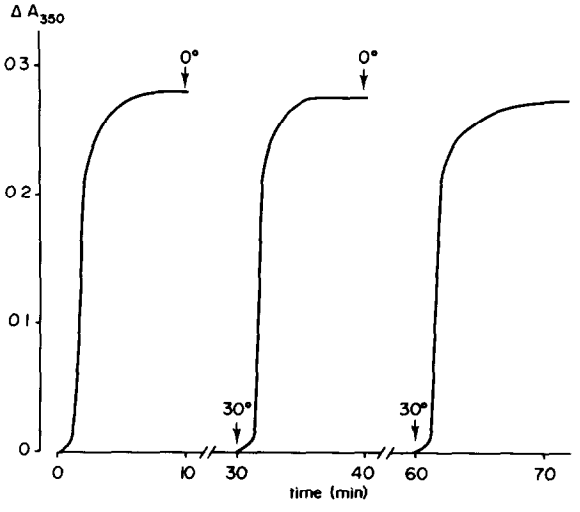

Fig.6. Microtubule assembly measured at 30°C. Cold disassembly was induced by transferring the cuvette to 0°C for 20 min.

disassemble upon addition of Ca^{2+} (not shown), making it impossible to determine whether calcium-labile, cold-stable microtubules exist in cod brain.

Our results indicate that cod nerve microtubules are cold-stable, but that the brain microtubules which can be isolated for in vitro studies are cold-labile. It is not yet possible to discriminate between the possibilities whether a cold-stabilizing factor or cold-stable microtubules are lost during the preparation, or if cold-stable microtubules are components of peripheral axons only.

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