INITIATION OF NEUROTUBULIN POLYMERISATION AND RAT BRAIN DEVELOPMENT

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1. Introduction

In previous publications [1,2] we have shown that the concentration of tubulin is probably not the rate limiting factor for the assembly of neurotubules during rat brain development. Various data suggest that most, if not all, neurotubulin which is formed during an early stage of development (15 days of gestation) do not polymerize efficiently. The assumption was made that some factor, different from neurotubulin, is responsible for the initiation of the polymerization process. It was also shown that this initiator is probably the limiting factor for microtubule formation at early stages of development. Recent publications by Kirschner et al. [3] have reported that adult 6 S neurotubulin is unable to polymerize by itself unless another protein, the τ factor, which acts as an initiator, is present. In order to determine whether this factor was responsible for the failure of the neurotubulin present in the brain supernatant of new born rats to polymerize normally, the effect of purified preparations of τ was studied in this work. From different experiments it may be concluded that in early stages of brain development τ, an 'initiator' of neurotubulin polymerization, is not present in sufficient amount to polymerize tubulin properly.

2. Materials and methods

2.1. *Preparation of supernatants*

3 and 30 day old Sprague-Dawley rats were killed by decapitation. The brain supernatants were prepared as described previously but EDTA 0.1 mM and MSH 1 mM were added to the reassembly buffer of Weisenberg [4].

2.2. *Colchicine binding assays*

Aliquots of supernatants were incubated for 2 h at 37°C (final volume 0.5 ml of modified Weisenberg's buffer) in the presence of 2.5×10^{-5} M colchicine labeled with 0.2 μCi of [^{3}H]colchicine per assay. Bound colchicine was measured according to a modification [1] of the procedure described by Weisenberg [5]. The units of tubulin concentration are those determined by the amount of colchicine that is bound.

2.3. *Preparation of the partially purified τ factor*

Tubulin was purified from rat brain by the assembly–disassembly procedure described by Shelanski et al. [6]. Immediately prior to use microtubule protein was repolymerized, pelleted at 105 000 g and resuspended in a MES–EDTA buffer containing 25 mM MES, 0.5 mM $MgCl_2$, 1 mM MSH, 0.1 mM EDTA, pH 6.4. After depolymerization at 4°C for 30 min undepolymerized material was eliminated by a 30 min centrifugation at 105 000 g. Microtubule protein was applied to a phosphocellulose column and eluted with MES–EDTA buffer. The protein factor τ was eluted with MES–EDTA buffer containing 0.3 M NaCl. The NaCl was removed by passage through a Sephadex G-25 column equilibrated with the modified reassembly buffer of Weisenberg. A further step in purification was performed by heating this fraction as described by Kirshner et al. [3]. Both the fraction partially purified by phosphocellulose chromatography and that further purified by heat

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treatment gave identical results. Most experiments were therefore performed with the partially purified fraction.

2.4. *Turbidimetry measurements*

Turbidimetry measurements during tubulin polymerization at 37°C were performed at 345 nm using Carl Zeiss PM 6 KS spectrophotometer with an automatic thermostated four sample changer.

3. Results and discussion

The 105 000 *g* supernatants from brain homogenates were prepared, as described by Shelanski [6], from new born (3 days) and adult (30 days) rats. In vitro polymerization of neurotubulin to neurotubules was studied by following at 37°C the rate of increase in turbidity at 345 nm. Fig.1 depicts the time-dependance of turbidimetry when identical concentrations of tubulin, based on colchicine binding assay, were used. Fig.1 shows that the initial rate of neurotubulin polymerization with the 3 days preparation was much lower than that obtained with the 30 days rat brain supernatant; in addition the lag

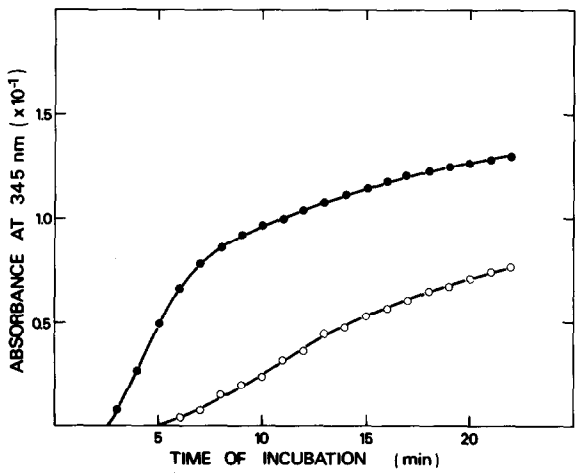

Fig.1. Turbidimetry time course at 37°C of 3 day and 30 day old rat brain supernatants. The supernatants were prepared and turbidimetry measurements were performed as described in Materials and methods. 3 day (○———○) and 30 day (●———●) old rat brain supernatant containing the same amount of colchicine binding tubulin (1920 pmol) were compared.

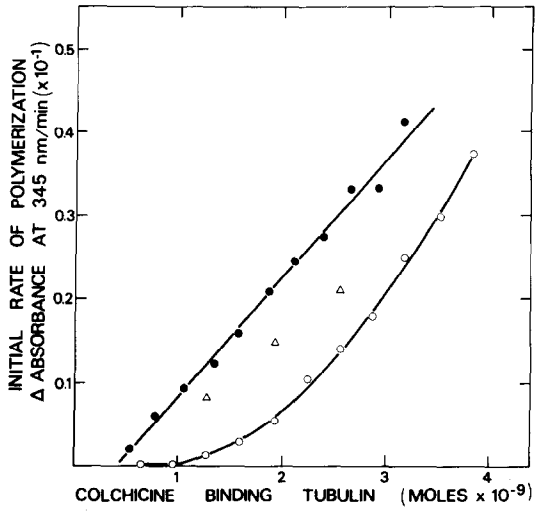

Fig.2. Relationship between initial rate of polymerization and colchicine binding tubulin concentrations of 30 day and 3 day old rat brain supernatants. Initial rates (Δ absorbance at 345 nm/min) were deduced from several kinetic experiments performed as described in Materials and methods at various dilutions of the 30 day (●———●) and 3 day (○———○) old rat brain supernatants. Initial rate of polymerization for 3 day supernatant to which τ factor was added is also represented (Δ).

period was much longer and the overall turbidity much lower. Gaskin et al. [7] have shown that both the initial rate of polymerization and the duration of the lag period probably depend on the concentration of 'initiator',

The polymerization was also studied with different dilutions of soluble fractions prepared from 3 and 30 day old rat brains. Fig.2 shows the relationship between the initial rates of polymerization and the concentration of neurotubulin (measured as colchicine binding protein) for the two ages. Clearly, for a given amount of tubulin, the initial rate was always much slower with the newborn preparation when compared to the adult. In addition the 'critical' concentration [C_c] was 2.6 times higher for the new born than for the adult (1300 pmol of colchicine binding tubulin for the new born instead of 500 pmol for the adult). Gaskin et al. have found that below a critical concentration [C_c] of neurotubulin the turbidity is the same as for the unpolymerized protein. These results, i.e. lower initial rates, and higher critical concentration,

may signify that an initiator is necessary to induce polymerization of the 6 S neurotubulin and that the initiator is the limiting factor at early stages of development. The addition to new born supernatant of very small volumes of adult supernatant containing an amount of tubulin lower than $[C_c]$ increased markedly the rate of polymerization, suggesting that the adult supernatant contains a non-limiting amount of 'initiator'. Similarly the addition of small amounts of sonicated fragments of adult microtubules to the new born supernatant resulted in a shorter lag period and in an increase in the rate of assembly.

While this work was in progress, Kirschner et al. [3] have isolated by anion exchange chromatography a protein factor, τ, which seemed to be necessary for the 'initiation' of neurotubulin polymerization. On the other hand 6 S neurotubulin, when freed from this τ factor, was unable to polymerize in vitro to neurotubules.

The assumption was made therefore that the factor which is limiting at early stages of development is precisely the τ protein. This factor has been added in increasing amounts to both new born and adult supernatants. Fig.3 shows that τ clearly and markedly increased the rate of polymerization of the new born

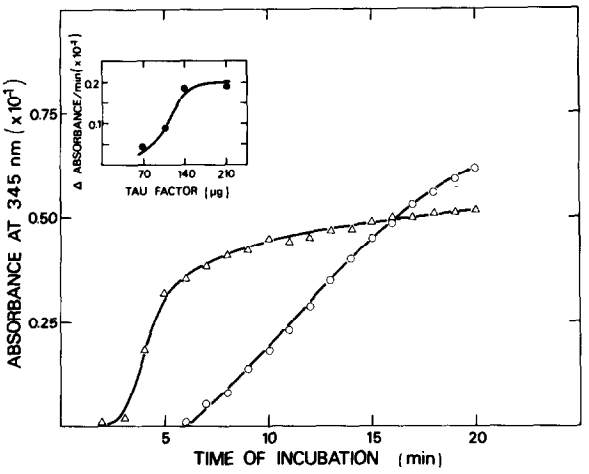

Fig.3. Effect of τ factor on turbidimetry time course of 3 day old rat brain supernatant. τ Factor was prepared as described in Materials and methods. 3 day old rat brain supernatant containing 1920 pmol of colchicine binding tubulin (○———○) to which 140 μg of τ factor were added (△———△). Insert: increase of the initial rate of polymerization with different concentrations of τ factor preparation.

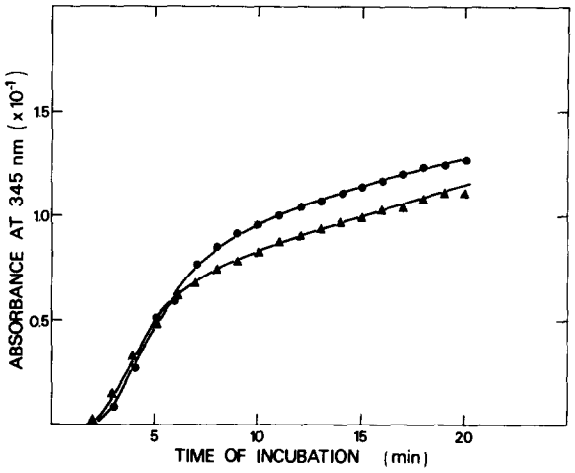

Fig.4. Effect of τ factor on turbidimetry time course of 30 day old rat brain supernatant (●———●) to which 140 μg of τ factor were added (▲———▲) (conditions and tubulin concentration as in fig.3).

supernatant. In addition the lag period was clearly reduced. Fig.3 (insert) shows that the rate of polymerization increases with the amount of τ fraction added to the new born supernatants. The increase in rate (due to τ protein) is also indicated for different dilutions of supernatant in fig.2. On the contrary, addition of τ protein to the adult supernatant was without effect (or even produced a slight inhibition) on the rate of polymerization (fig.4). The adult supernatant seems, therefore, to be saturated with the τ protein. Closer examination of figs.3 and 4 also shows that the final turbidity (at the end of the polymerization process, 30 min of incubation) decreased when the amount of τ increased. If τ is responsible for the formation of 'rings' [3], i.e. the first organized structure of a given number of 6 S tubulin subunits [8], it may be that increasing its concentration results in the formation of more rings and shorter microtubules. This possibility may be verified by electron microscopic studies by analyzing for the number of rings and the length of the microtubules.

In conclusion, the results reported in this work strongly suggest that an 'initiator' of neurotubulin polymerization is present in insufficient amounts at early stages of brain development. This initiator is very likely to be the τ factor isolated by Kirschner et al. [3]. Thus, although the amount of neurotubulin

(6 S) present at early stages of development is at least identical (if not higher) to that present in adult brain [1], the number of neurotubules should be low until the amount of initiator reaches a certain level.

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