Nucleated Assembly of Microtubules in Porcine Brain Extracts

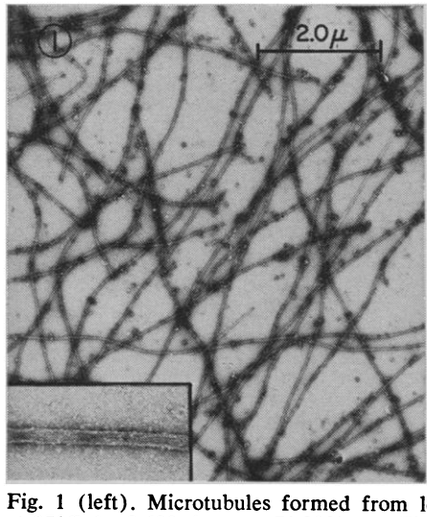
Abstract. *Disk-type structures found in extracts of porcine brain tissue appear to be required for microtubule assembly in vitro. From the morphology of the disks and the dependence of microtubule assembly on the presence of these structures, we propose that the disks are nucleation centers for the polymerization of microtubule protein.*

Until recently, efforts to reassemble microtubules from subunits in vitro have met with only limited success. Outer doublet microtubules from sea urchin spermatids have been dissociated with detergent into detergent-subunit complexes and then reconstituted upon dilution of the detergent solution (*1*). However, this assembly reaction proceeded at low temperatures and was not inhibited by colchicine, both of which are properties characteristic of microtubule assembly in vivo (*2*). A microtubule subunit aggregation system in which isolated subunits from porcine brain tissue are used has been defined and characterized as temperature dependent and colchicine inhibitable; however, the assembly products were mixtures of beaded and linear aggregates with microtubules being observed only rarely (*3*). Weisenberg (*4*) has recently discovered the importance of calcium ions in controlling microtubule formation in vitro from extracts of rat brain tissue. We have confirmed his observations, using porcine brain tissue and a slightly different method of preparation. However, in addition, we have found that microtubule assembly in the brain extracts requires the presence of disk-type structures which we suggest are nucleation centers.

Porcine brain tissue was obtained within 2 hours of slaughter and was chilled to 0°C; all further operations were carried out from 0° to 4°C unless otherwise indicated. In initial experiments, one part by weight of brain tissue was suspended in 1.5 volumes of 0.1*M* PIPES buffer [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], *p*H 6.5, and homogenized by at least ten passes in a hand homogenizer. The homogenate was centrifuged at 100,000*g* for 30 minutes and the supernatant was retained. Samples of the supernatant or of the supernatant diluted with buffer were applied directly to 400-mesh grids coated with Formvar and carbon, and were then negatively stained with 1 percent uranyl acetate for electron microscopy. As reported by Weisenberg (*4*), no microtubules were observed in supernatants held at 0°C, but microtubules formed within 30 minutes after warming a sample of the supernatant to 37°C. Microtubule formation was inhibited by the addition of 100 μM colchicine or 5 m*M* $CaCl_2$ to the supernatant. Addition of 1 m*M* guanosine triphosphate (GTP) to the homogenizing buffer resulted in microtubule formation within 10 minutes after the supernatant was raised to 37°C. Because of the apparent stimulatory effects of GTP and Mg^{2+} ions, and the inhibitory effects of Ca^{2+} ions on microtubule assembly (*4*), the homogenizing solution was subsequently modified to 0.1*M* PIPES buffer, *p*H 6.5, containing 1 m*M* EGTA [ethylenebis-(oxyethylenenitrilo)tetraacetate], 0.5 m*M* $MgSO_4$, and 1 m*M* GTP (PMEG solution).

Assembly of microtubules in the brain extracts also depended upon the extent of centrifugation used to prepare the extracts, and two sets of centrifugation conditions were used for the experiments described below. Homogenates were centrifuged at 25,000*g* for 1½ hours to produce an extract at low speed or at 230,000*g* for 1½ hours to produce one at high speed.

The evidence for nucleation of microtubule assembly can conveniently be discussed in terms of the following points. (i) Microtubules (260 ± 20 Å in diameter) appeared in the low speed extracts (50 to 100 per grid square) within 10 minutes after being warmed to 37°C (Fig. 1), but did not appear (1 to 2 per 10 grid squares) within 2 hours in the high speed extracts which had been warmed to the same temperature. This implied that a component, essential for microtubule assembly, was particulate. (ii) The calculated *S* values for particles which would sediment in the low speed and high speed extracts were, respectively, 300*S* and 35*S*, indicating that both supernatants would still contain the 6*S* microtubule subunits. (iii) Electron microscope observations of the low speed extracts kept at 0°C showed many disk-type structures 290 ± 40 Å in diameter with a 170 ± 20 Å hole (Fig. 2); however, no disk structures were observed in the high speed extracts. The disks had the dimensions and general appearance of microtubules in cross section, and so were plausible candidates for nucleation centers; similar structures have been described in the assembly of tobacco mosaic virus (*5*). (iv) The disk structures were not observed in low speed extracts that were raised to 37°C and allowed to polymerize. This observation is consistent with the disks being incorporated into the assembled microtubules. However, electron microscope observations of low speed extracts within 1 minute after initiation of polymerization have revealed short microtubules with an apparent curled structure at one end. (v) The disk structures reappeared when the tubules



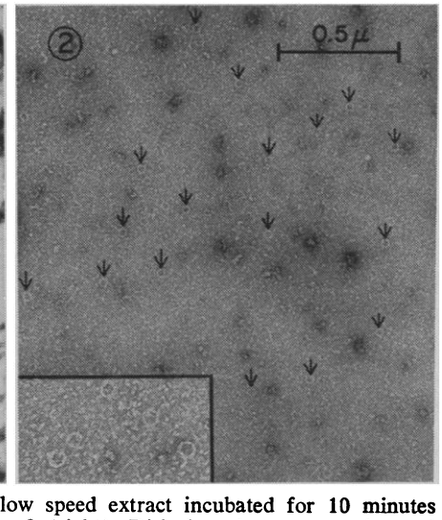


Fig. 1 (left). Microtubules formed from low speed extract incubated for 10 minutes at 37°C (× 9,600; inset, × 71,900). Fig. 2 (right). Disk-shaped structures observed in low speed extract held at 0°C (× 36,400; inset, × 71,900).

polymerized at 37°C were broken down by the addition of 5 m*M* Ca^{2+}, 100 μM colchicine, or by reducing the temperature to 0°C. This suggested that the disks were at least temporarily stable under conditions in which tubules were not, and that they probably originated from the dissolution of the microtubules. (vi) The microtubules formed in the low speed extracts appeared to be in equilibrium with subunits, since the microtubules slowly broke down (within 1 hour at 37°C) when diluted 1 : 10 with PMEG solution, but did not break down when diluted 1 : 10 with high speed extract.

From these observations, we conclude that the assembly of microtubules in porcine brain extracts proceeds by a nucleation mechanism and that the nucleation center is most probably a disk-type structure. Determination as to whether the disks are intermediates in the assembly of microtubules, or represent subunits modified to pack into a stable ring, must await the isolation of the disk structures. An interesting possibility is that the disk structures may be the microtubule organizing units postulated to exist in dividing and differentiating cells (*7*).

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Coding Properties of Reticulocyte Lysine Transfer RNA's in Hemoglobin Synthesis

Abstract. *Two isoacceptor transfer RNA's for lysine were found in rabbit reticulocytes. The codon recognition properties of these isoacceptors were studied in hemoglobin synthesis in a cell-free system. The two isoacceptors transferred lysine into different sites in hemoglobin, but showed no preference for one chain over the other. Codon cross recognition was less than 4 percent.*

Isoacceptor transfer RNA's (tRNA) for lysine have been found in rabbit reticulocytes, and their codon recognition properties have been investigated by measuring trinucleotide-induced binding of isoacceptors to *Escherichia coli* ribosomes (*1*, *2*). Results from our laboratory show the existence of two lysine isoacceptor tRNA's, one of which binds to ribosomes exclusively in the presence of AAG (A, adenosine; G, guanosine) while the other has a preference for AAA over AAG, in agreement with results of Rudloff and Hilse (*2*).

We were interested in determining whether or not these isoacceptors had the same codon specficity in protein synthesis as in the ribosome binding assay. Each isoacceptor was acylated with [^{14}C]lysine and then added to a highly active, reticulocyte, cell-free system (*3*) synthesizing many globin chains per ribosome during the course of an incubation. The tryptic peptides of the products synthesized in the presence of [^{14}C]lysine-labeled isoacceptor tRNA's were analyzed to determine the amount of lysine transferred into each site.

The results show that reticulocyte $tRNA^{Lys}_{I}$ (binds to ribosomes in the presence of AAG) transfers lysine to about the same extent into ten lysine sites in the α chain and ten lysine sites in the β chain. The reticulocyte $tRNA^{Lys}_{II}$ (binds preferentially to ribosomes in the presence of AAA) transfers lysine into the two remaining sites in the α chain and the two remaining lysine sites in the β chain. There appears to be very little overlap in the recognition of lysine sites by these two isoacceptors. Also, both RNA's recognize an equal number of lysine sites in the α and β chains, in contrast to results of Rudloff and Hilse (*2*), who report that the isoacceptor tRNA that binds to ribosomes in the presence of AAG transfers lysine mainly into the α chain and that the isoacceptor tRNA that preferentially binds to AAA transfers mainly into the β chain. We present our results and explore the reasons for the disagreements between the two findings.

The tRNA, prepared by phenol extraction of reticulocytes from anemic rabbits (*4*), was acylated with [^{14}C]-lysine with a reticulocyte synthetase preparation and fractionated into two isoacceptor species on benzoylated DEAE–(B-D) cellulose (*5*) and Freon reversed-phase (*6*) chromatography. The ratio of lysine I to lysine II tRNA as measured by acceptor activity was about 60 to 40 after B-D cellulose chromatography. The binding specificity of the two isoacceptors was similar to that reported by Rudloff and Hilse for their two major peaks of lysine isoacceptor activity (reticulocyte $tRNA^{Lys}_{I}$, which binds to AAG, corresponds to Rudloff and Hilse's $tRNA^{Lys}_{II}$; and our reticulocyte $tRNA^{Lys}_{II}$, which binds preferentially to AAA, corresponds to their $tRNA^{Lys}_{IV}$) (*7*).

Each of the acylated isoacceptor tRNA's was incubated in a reticulocyte cell-free system for 1 hour at 35°C (*3*). The cell-free systems sustained a linear rate of protein synthesis for approximately 15 minutes and synthesized more than eight globin chains per ribosome during an incubation. Although the amount of lysine tRNA added was about 100 times greater than that in the cell-free system, the amount of protein synthesized was depressed only slightly (about 10 percent), and the duration of synthesis was the same (about 30 minutes) as in the absence of added tRNA (*7*). Amino acid transfer from acylated tRNA's into hemoglobin was essentially complete within 5 minutes. Conditions were such as to minimize enzymatic deacylation of the added tRNA (*8*), and, furthermore, the specific activity of [^{14}C]lysine deacylated from the tRNA would have been so reduced by the pool of free [^{12}C]lysine in the mixture (50 μM) that its incorporation would have been below the level of detection.

Immediately after the incubation, the ribosomes were sedimented by centrifugation, uniformly labeled [^{3}H]lysine hemoglobin (prepared by whole cell incubation) was added to the supernatant, and globin was prepared by acid acetone precipitation (2 ml of concentrated HCl per liter of acetone).