# POLYMERIZATION AND DEPOLYMERIZATION OF MICROTUBULES IN VITRO AS STUDIED BY FLOW BIREFRINGENCE

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## 1. Introduction

Weisenberg [1] has recently reported the microtubule formation in vitro, and suggested a regulator role of $Ca^{2+}$ ions in polymerization of microtubules in vivo. In this and subsequent studies [2,3], microtubule formation is monitored by electron microscopy. We have attempted to follow the microtubule formation and breakdown in a quantitative way by measuring flow birefringence. Attention was paid to the requisite medium composition, and a kinetic analysis was made as to the process of microtubule polymerization.

## 2. Materials and methods

The procine brain was obtained within 2 hr of slaughter and chilled to 0°C. All subsequent procedures were carried out at 0–4°C unless otherwise indicated.

Cerebral tissue was freed of meninges and clotted blood, and 1.5 ml of homogenizing medium was added per gramme of tissue. The medium contained (final concentrations) PIPES*–KOH buffer, pH 6.5 (50 mM); EGTA (1 mM); GTP (0.1 mM); and $MgCl_2$ (0.5 mM) (cf. Borisy et al. [2]). Homogenization was performed in a Waring blendor operated at 7000 rev/min for three 15 sec periods with intervals of 45 sec. The homogenate was centrifuged at 5000 *g* for 10 min, and the supernatant was further at 70 000 *g* for 30 min. The final supernatant was stored frozen in small portions at –80°C. Immediately before use, the sample was thawed by inculating at 25°C for about 10 min, passed through a Sephadex G-50 column (3 cm × 12 cm) equilibrated with 50 mM PIPES–KOH buffer, pH 6.5, and appropriate portions of the void volume fraction were used for assay.

Flow birefringence was measured in a Rao–Edsall type apparatus (model No. A8, Rao Instrument Co.) at a velocity gradient of 2000 $sec^{-1}$. The standard assay medium (3.5 ml) contained (final concentrations) PIPES–KOH buffer, pH 6.5 (43 mM; 58 mM with respect to $K^+$); EGTA (0.1 mM); GTP (0.2 mM); $MgCl_2$ (0.43 mM); and 2–3 ml of the void volume fraction. Low $Ca^{2+}$ concentrations were prepared by equilibrating 5 mM EGTA or 5 mM EDTA with 1–4 mM $CaCl_2$ in the presence of free $Mg^{2+}$ added to 2 mM, and adjusting the pH to 6.5 with 2 M Tris. Free $Ca^{2+}$ concentrations were calculated on the basis of the binding constant of EGTA or EDTA with $Ca^{2+}$ or $Mg^{2+}$ ions [4].

For electron microscopy, a portion (generally 0.1–0.2 ml) of the assay medium was mixed with 1 ml of 1 M hexylene glycol–20 mM PIPES–KOH buffer (pH 6.5). A drop of this mixture was placed on a grid coated with 0.15% Formvar and carbon, negatively stained with 1% uranyl acetate, and examined with a model JEM-100B electron microscope (Nihon Denshi Co.).

* The abbreviations used are: PIPES, piperazine-*N,N'*-bis(2-ethanesulphonic acid); EGTA, ethanedioxybis(ethylamide)-tetra-acetate.

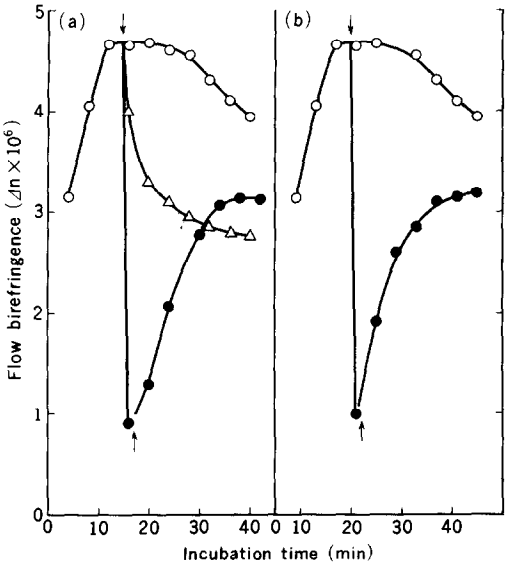


Fig. 1. Microtubule formation in vitro as measured by flow birefringence. Polymerization was allowed to proceed in the standard medium at 37°C (o–o–o). (a) $Ca^{2+}$ was added at ↓ to the final concentration of 2.9 mM, and EGTA at ↑ to 5.8 mM (•–•–•). In another assay, colchicine was added at ↓ to 2.9 mM (△–△–△). (b) Incubation medium was cooled to 0°C at ↓, and again warmed to 37°C at ↑ (•–•–•).

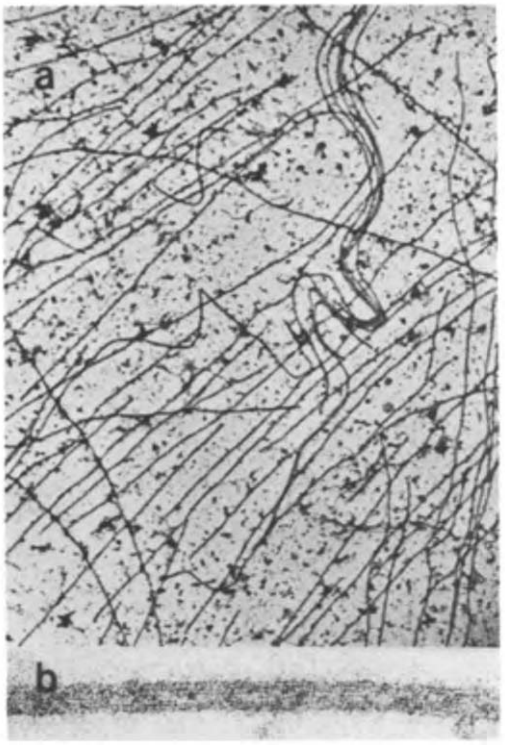


Fig. 2. Microtubules repolymerized during the incubation of brain extract at 37°C for 15 min. (a) A low-magnification view (× 7900); (b) A high-magnification view (× 149 000).

## 3. Results and discussion

When the brain extract was incubated at 37°C in the standard medium, the flow birefringence ($\Delta n$) of the solution was gradually increased until a plateau level was reached after 15–20 min (fig. 1). The increase of flow birefringence corresponded to microtubule polymerization, as was evident by parallel electron microscopic observations (fig. 2). $Ca^{2+}$ ions caused a steep fall of flow birefringence, indicating depolymerization of microtubules; subsequent addition of EGTA restored the flow birefringence, but to a limited extent (fig. 1a). Similar fail and rise of flow birefringence were observed on cooling the incubation medium to 0°C, and warming it again to 37°C (fig. 1b). Colchicine also caused a decrease of flow birefringence, but at a much slower rate as compared with $Ca^{2+}$ and cooling (fig. 1a). The process of colchicine binding to tubulin does not seem to limit the rate of depolymerization, because when added to the medium immediately before starting the incubation at 37°C, colchicine completely blocked the increase of $\Delta n$ at the final concentration of 0.1 mM or even lower. One possible explanation will be that $Ca^{2+}$ ions teract with tubulin molecules whether or not they are incorporated in microtubules, while colchicine shifts the polymerization–depolymerization equilibrium by binding only to tubulin in monomeric form (cf. [5]).

The degree of flow birefringence during the incubation at 37°C strongly depends on pH, ionic strength and ionic composition of the medium. Plateau value of $\Delta n$ became progressively lowered as the pH of the incubation medium was raised from 6.2 to 7.3, and at pH 7.3, the change in $\Delta n$ was reduced to nil.

In experiments shown in fig. 3, the concentration of PIPES–KOH buffer in the standard medium was reduced to 6 mM in order to decrease its contribution to $K^+$ concentration from 58 mM to 8 mM; the final $K^+$ concentration of the medium was then

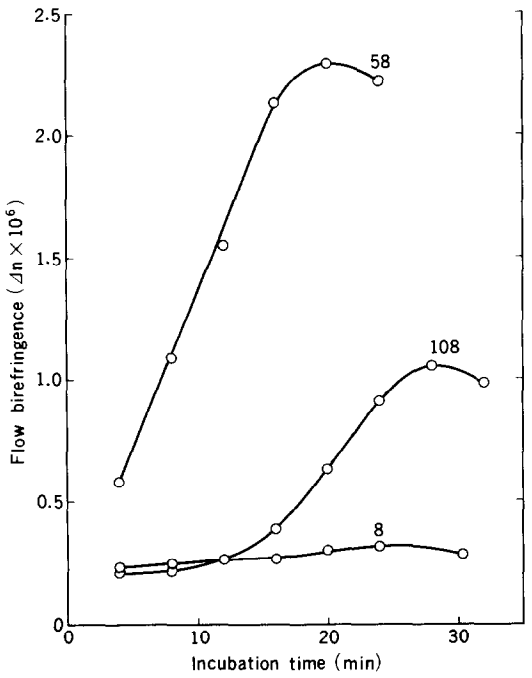


Fig. 3. Effects of $K^+$ on microtubule formation. In this experiment, concentration of PIPES–KOH buffer in the standard medium was reduced to 6 mM. Figures added to respective curves indicate the final $K^+$ concentrations in mM.

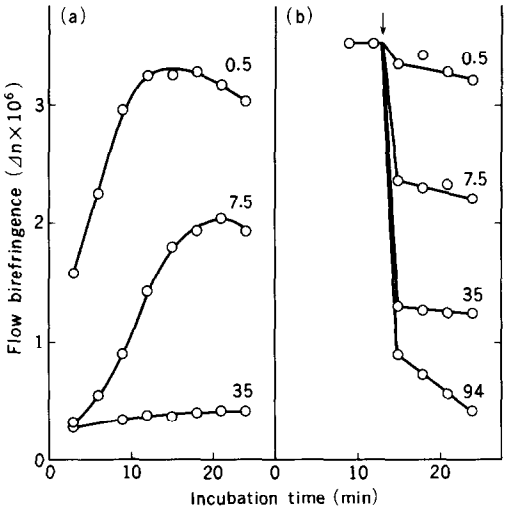


Fig. 4. Effects of $Ca^{2+}$ on microtubule formation. Figures added to respective curves indicate the final $Ca^{2+}$ concentrations in μM. (a) $Ca^{2+}$ was present from the beginning of incubation. (b) $Ca^{2+}$ was added at ↓ after flow birefringence levelled off.

modified by adding appropriate volumes of 1 M KCl. It is obvious from fig. 3 that an adequate concentration of $K^+$ (50–60 mM) is required to facilitate the microtubule formation; with higher concentration of $K^+$, $\Delta n$ was decreased. $K^+$ may be replaced by $Na^+$, suggesting that the factor involved is not the species of monovalent cations, but rather the ionic strength. The difference is that $Na^+$ begins to suppress the polymerization at lower concentration range as compared with $K^+$.

The presence of $Mg^{2+}$ is essential to the microtubule formation; removal of $Mg^{2+}$ by sufficient amount of EDTA leads to a complete blockade of polymerization. The optimal concentration range of $Mg^{2+}$ is 0.5–2 mM, which is comparable with the free $Mg^{2+}$ concentration in vivo [6]. $Mg^{2+}$ ions higher than 5 mM exert an inhibitory action; the $\Delta n$ value obtained in the presence of 10 mM $Mg^{2+}$ was one third of that with 2 mM $Mg^{2+}$.

$Ca^{2+}$ ions inhibit the polymerization either when they are present from the beginning of the incubation (fig. 4a), or when added after the polymerization comes to an equilibrium (fig. 4b). The effective concentration of $Ca^{2+}$ ranges from 1 to 30 μM in the standard medium containing 58 mM $K^+$. When $K^+$ concentration of the medium was raised to a physiological level, $Ca^{2+}$ concentration effective to suppress the microtubule formation became even lowered, although $\Delta n$ values were liable to somewhat wider variations, presumably because of an instability of microtubules under the higher ionic strength condition. The effective concentration range of $Ca^{2+}$ above-stated will support the view of Weisenberg [1] that $Ca^{2+}$ appears to be a logical candidate as a regulator of microtubule polymerization in vivo.

During the course of microtubule polymerization, the change in the monomer concentration may be given by

$$-\frac{dm}{dt} = k_1 m f_n - k_2 f_n \qquad \text{eq. (1)}$$

where $m$ is the monomer concentration, $f_n$ is the number of microtubules, and $k_1$ and $k_2$ are the rate constants of polymerization and depolymerization, re-

spectively (cf. [7,8]. It is assumed in equation (1) that polymerization occurs at the end of the growing microtubule upon collision with the monomer, and thus the overall rate of polymerization is proportional to $m \times f_n$, and that the rate of depolymerization is proportional to $f_n$. It has been shown that a kind of nucleation centre is required for initiating the microtubule polymerization in vitro [2]. This implies that $f_n$ equals to the number of nucleation centre. If we assume that the number of nucleation centres in a given medium does not change during the incubation period, i.e. $f_n$ remains constant independent of $t$, then the equation (1) can be integrated as

$$F_c = m_0 - m = m_0 - \frac{k_2}{k_1}[1 - \exp(-k_1 f_n t)] \quad \text{eq. (2)}$$

where $F_c$ is the concentration of monomer incorporated into microtubules, and $m_0$ is the monomer concentration prior to the start of polymerization. After the manner of actin polymerization [9], it may well be postulated that $\Delta n$ changes in proportion to $F_c$.

The relations between $\Delta n$ and $t$ at three different concentrations of the sample ($[S]$) in the standard medium are shown in fig. 5a. By performing appropriate plots, these results are found to fullfil the following equations.

$$\Delta n = \Delta n_{max}[1 - \exp(-kt)] \quad \text{eq. (3)}$$

$$k \propto [S] \quad \text{eq. (4)}$$

$$\Delta n_{max} \propto [S] - \text{constant} \quad \text{eq. (5)}$$

Since $m_0$ and $f_n$ are proportional to $[S]$, it is clear that equations (3), (4) and (5) conform well with the equation (2). Thus the assumption included in the equation (2) seems to substantiated.

Similar analysis was carried out on the results obtained at 38°C, 30°C and 23°C, and the apparent activation energy for binding of tubulin to microtubules was estimated to be 22 kcal/mole, which is comparable with that for actin polymerization [8].

The relations between $\Delta n$ and $t$ in media containing $Ca^{2+}$ or higher concentration of $K^+$ (figs. 5b and 5c) differ from those in the standard medium (fig. 5a), and do not conform with the equation (2).

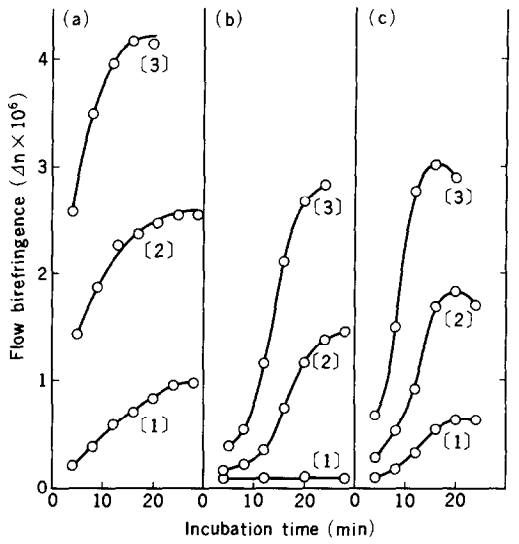


Fig. 5. Microtubule formation with three different concentrations of the brain extract. (a) Reaction was allowed to proceed in the standard medium. Volumes of the brain extract contained in the assay medium (3.5 ml) were 1, 2 and 3 ml in [1], [2] and [3], respectively. (b) Incubation media contained 143 mM $K^+$. Other conditions as in (a). (c) $Ca^{2+}$ was added to the final concentration of 7.5 μM. Other conditions as in (a).

This implies that the lowering of $\Delta n$ caused by $Ca^{2+}$ or high $K^+$ is not explained simply on the basis of decrease of polymerization rate and/or the increase of depolymerization rate of microtubules. In the presence of $Ca^{2+}$ or high $K^+$, $\Delta n$ begins to increase after a certain lag period, which is almost independent of the sample concentration. These characteristics suggest the occurrence of a step, such as an activation of monomer or of the nucleation centre, which becomes rate-limiting only when $Ca^{2+}$ or high $K^+$ is present. Such a step is expected to be closely associated with the regulation of microtubule polymerization in vivo.

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