

***In vivo* and *in vitro* effects of the mitochondrial uncoupler FCCP on microtubules**

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FCCP (carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone), a potent uncoupler of oxidative phosphorylation, induces the complete disruption of cellular microtubules. A further analysis of this effect on BHK21 cells has shown that a decrease in the number of microtubules can be observed 15 min after adding FCCP and there is complete disruption after 60 min. Regrowth of microtubules was initiated 30 min after removal of FCCP, in marked contrast with the rapid reversion observed when microtubules are disrupted by nocodazole. A similar delay was required for the recovery of mitochondrial function as assessed by rhodamine 123 labelling. The effect of FCCP on microtubules was partially inhibited by preincubation of the cells with NaN_3 , suggesting that FCCP acts on microtubules through mitochondria. FCCP did not depolymerize microtubules of cells permeabilized with Triton X-100. *In vitro* polymerisation of microtubule protein was only slightly diminished by concentrations of FCCP which provoke complete disassembly *in vivo*. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the microtubules polymerized *in vitro* in the presence of FCCP showed a reduced amount of high mol. wt. proteins, mainly MAP 2, associated with them. In an attempt to reproduce the mitochondrial effects of FCCP *in vitro*, we checked the effects of alkaline pH and calcium on microtubule protein polymerization in the presence of FCCP. FCCP did not influence the calcium inhibitory effect but did significantly increase the inhibitory effect of alkaline pH. We conclude that FCCP could depolymerise microtubules *in vivo* through a dual operation: increasing the intracellular pH by the disruption of the mitochondrial H^+ gradient and decreasing the stability of microtubules by impairing the binding of microtubule-associated proteins.

Key words: mitochondria/ionophores/microtubule-associated proteins/calcium/pH

Introduction

A physical and functional association between mitochondria and the cytoskeleton has been suggested from many studies (Smith *et al.*, 1977; Hegeness *et al.*, 1978; Johnson *et al.*, 1980; Couchman and Rees, 1982; Hirokawa, 1982). Mitochondrial distribution is dependent upon the microtubule network (Hegeness *et al.*, 1978; Johnson *et al.*, 1980; Couchman and Rees, 1982) but is independent of intermediate filaments (Lin and Feramisco, 1981; Ball and Singer, 1982; Couchman and Rees, 1982). Mitochondrial mobility depends on the actin system (Couchman and Rees, 1982) and mitochondrial membranes have recently been shown to have tubulin-binding activity (Bernier-Valentin and Rousset, 1982). We have previously reported that impairment of mito-

chondrial functions by metabolic inhibitors has an effect on the cytoskeleton (Maro and Bornens, 1982): FCCP induces the complete disruption of microtubules and the perinuclear aggregation of vimentin filaments in HeLa and KE 37 cells. We present further analyses of the effect of FCCP on microtubules performed on living cells (BHK 21 and HeLa cells), on cell ghosts obtained using non ionic detergent, or *in vitro* with rat brain microtubule protein purified by two cycles of polymerisation-depolymerisation.

Results

In vivo effects of FCCP

We used immunofluorescent staining of tubulin to study the kinetics of action of FCCP on microtubules of BHK 21 cells. These cells possess a primary cilium which allows the precise localisation of the centriole whatever the state of the cytoplasmic microtubular network (Figures 1–3). A decrease in the number of microtubules was observed after treatment for 15–30 min (Figure 1c,d). The residual microtubules began to disappear after 45 min (Figure 1e) and the disruption was complete after 60 min (Figure 1f,g). Upon the removal of the drug, a lag phase of 30 min was observed before regrowth of microtubules (Figure 1h–k). A complete microtubular network was restored within 60 min (Figure 1n). These effects of FCCP contrasted with those obtained with nocodazole, a microtubule disruptive drug which binds to tubulin and inhibits the binding of colchicine to tubulin dimers (Hoebeke *et al.*, 1976). In marked contrast with colchicine, nocodazole binding is reversible and, for this reason, was used in this study. Nocodazole-induced disruption was very rapid, being almost complete within 10 min (Figure 2b). Regrowth of a complete microtubular network was obtained within 10 min after removal of the drug (Figure 2d). The effect of FCCP on mitochondria was evaluated by following the accumulation of rhodamine 123 by mitochondria in living cells (Johnson *et al.*, 1980). FCCP abolished mitochondrial labeling in a very short time (~5 min), as already reported by others (Johnson *et al.*, 1981). Functional recovery of mitochondria was monitored after removal of FCCP. A weak staining of mitochondria was observed within 15 min and a strong staining at the end of 30 min. We must note, however, that the shape of the mitochondria was not yet restored to normal at that time (data not shown).

To investigate further the possibility that the FCCP effect was linked to mitochondrial events, we tested the effect of various inhibitors of mitochondrial functions (Table I). FCCP was the only drug tested able to disrupt completely microtubules in cells. Dinitrophenyl (DNP) and Nigericin had a weak effect leading only to a decrease in the number of microtubules.

To learn if a mitochondrial FCCP effect was responsible for disruption of the microtubules, we tried to protect the microtubular network from the action of FCCP by impairing some mitochondrial functions with other inhibitors (Table II). A preincubation of the cells with NaN_3 partially inhibited depolymerisation of microtubules induced by FCCP (Figure

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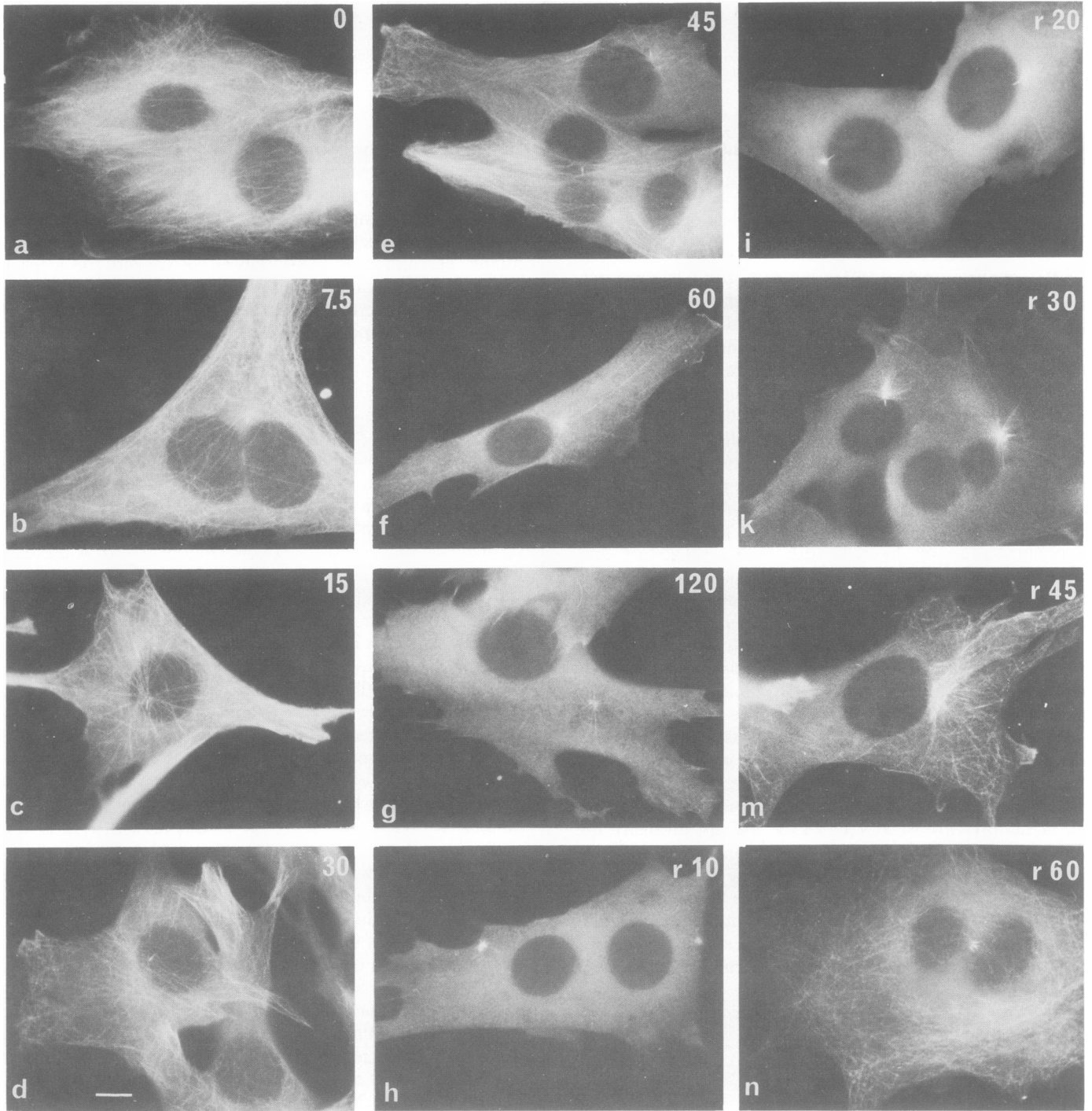


Fig. 1. Immunofluorescence staining of tubulin in FCCP-treated BHK 21 cells. **a**, control cells; **b–g**, cells treated with 6×10^{-5} M FCCP for various periods of time (**b**, 7.5 min; **c**, 15 min; **d**, 30 min; **e**, 45 min; **f**, 60 min; **g**, 120 min). **h–n**, cells treated with 6×10^{-5} M FCCP for 120 min and then in the absence of FCCP for various periods of time (**h**, 10 min; **i**, 20 min; **k**, 30 min; **m**, 45 min; **n**, 60 min). Bar in **d** represents 10 μ m.

3b). This was not observed with DNP (Figure 3a) or oligomycin.

In vitro effects of FCCP

We first tested the effect of FCCP on microtubules from cells extracted with Triton X-100 as described by Schliwa *et al.* (1981). FCCP at doses varying from 4×10^{-6} to 4×10^{-4} M had no effect on these *in vivo* polymerised microtubules.

The effect of FCCP on *in vitro* polymerisation of rat brain

microtubule protein was studied. We used FCCP concentrations between 4×10^{-7} and 4×10^{-4} M (Figure 4). At a microtubule protein concentration of 1 mg/ml, an inhibition of 20–25% was observed with 4×10^{-5} M FCCP (mol FCCP/mol tubulin dimer $\cong 5$). This concentration of FCCP leads to complete disruption of microtubules *in vivo* (Maro and Bornens, 1982). At 4×10^{-4} M FCCP (the highest concentration of the drug that could be obtained in aqueous solution), the inhibition was $\sim 45\%$ (mol FCCP/mol tubulin dimer $\cong 50$). The critical concentration of microtubule pro-

Table I. Effect of mitochondrial inhibitors on the pattern of microtubules in BHK 21 and HeLa cell lines

Treatment 2–4 h at 37°C	Maximum dose used	Action	Disruption of electro- chemical gradient ^a	Disruption of pH gradient	Calcium release	Disruption of microtubules network ^b
NaN ₃	2.0 x 10 ⁻² M	Inhibition of electron transport	+	+	+	0
Oligomycin	2.5 x 10 ⁻⁴ M	Inhibition of ATP synthetase	0	0	0	0
DNP	2.0 x 10 ⁻³ M	H ⁺ ionophore uncoupler	+++	+++	+	±
FCCP	6.0 x 10 ⁻⁵ M	H ⁺ ionophore uncoupler	+++	+++	+	+++
Nigericin	1.4 x 10 ⁻⁶ M	Exchange K ⁺ /H ⁺ ionophore	0	+++	0	±
Valinomycin	1.8 x 10 ⁻⁶ M	K ⁺ ionophore	+++	0	+	0

^aFollowed by rhodamine 123 staining of mitochondria in living cells (see also Johnson *et al.*, 1981).

^bFollowed by immunofluorescence staining of tubulin.

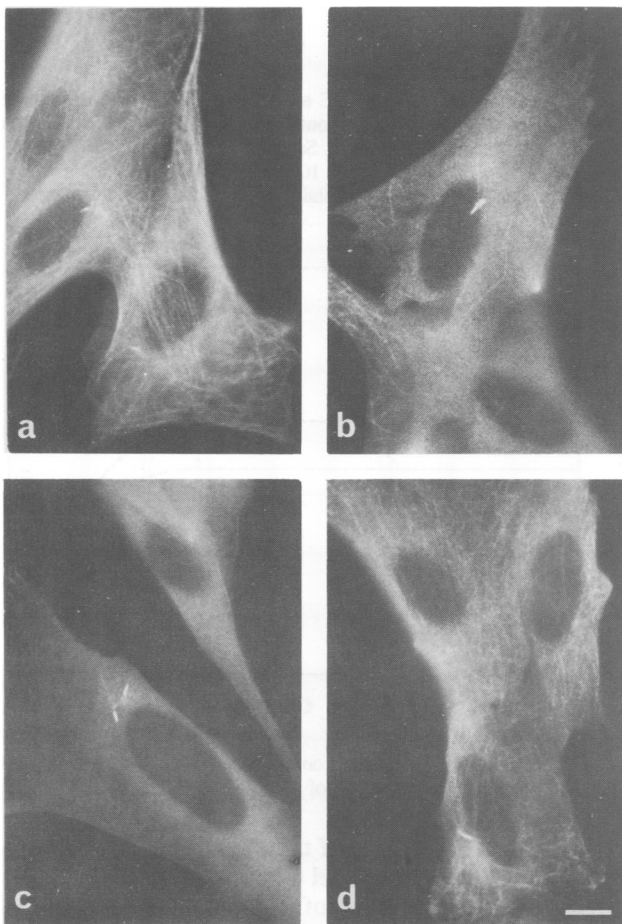


Fig. 2. Immunofluorescence staining of tubulin in nocodazole-treated BHK 21 cells. **a**, control cells; **b–c**, cells treated with 10⁻⁶ M nocodazole for 10 min (**b**) and 120 min (**c**). **d**, cells treated with 10⁻⁶ M nocodazole for 120 min then for 10 min in the absence of the drug. Bar in **d** represents 10 μm.

tein for polymerisation was not modified by FCCP. The inhibitory effect of FCCP on polymerisation was not enhanced by preincubating microtubule protein with FCCP for 30 min before adding GTP. The addition of FCCP after polymerisation of microtubules produced only a limited depolymerisation (10% inhibition at 4 x 10⁻⁵ M FCCP in 60 min).

Table II. Effect of various treatments on FCCP-induced disruption of microtubules in BHK 21 cells

Treatment at 37°C		Disruption of microtubule network ^a
0–4 h	2–4 h	
0	0	0
0	FCCP 6.0 x 10 ⁻⁵ M	+++
NaN ₃ 2.0 x 10 ⁻² M	0	0
NaN ₃ 2.0 x 10 ⁻² M	FCCP 6.0 x 10 ⁻⁵ M	+
DNP 2.0 x 10 ⁻³ M	0	±
DNP 2.0 x 10 ⁻³ M	FCCP 6.0 x 10 ⁻⁵ M	+++
Oligomycin 2.5 x 10 ⁻⁴ M	0	0
Oligomycin 2.5 x 10 ⁻⁴ M	FCCP 6.0 x 10 ⁻⁵ M	+++

^aFollowed by immunofluorescence staining of tubulin.

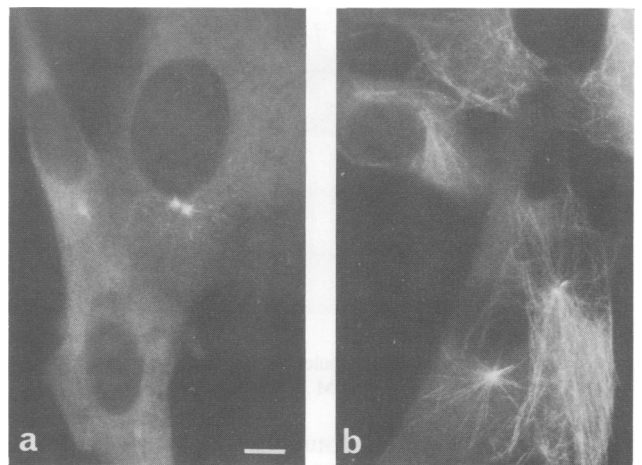


Fig. 3. Immunofluorescence staining of BHK 21 cells for tubulin. **a**, cells treated for 2 h with 10⁻³ M DNP followed by 6 x 10⁻⁵ M FCCP for 2 h; **b**, cells treated for 2 h with 2 x 10⁻² M sodium azide followed by 6 x 10⁻⁵ M FCCP for 2 h. Bar in **a** represents 10 μm.

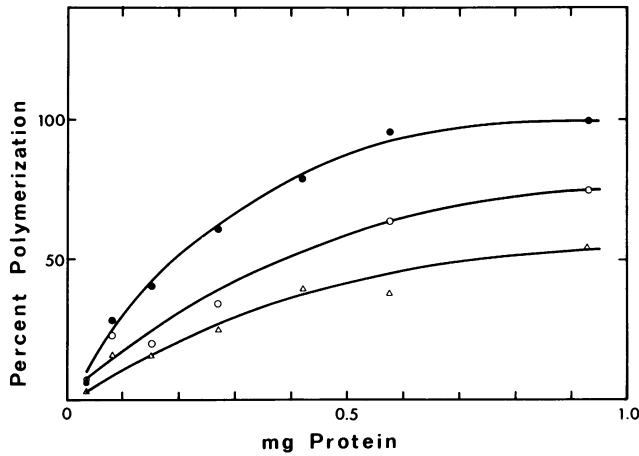


Fig. 4. Microtubule protein polymerisation in the absence (●) or in the presence of 4×10^{-5} M (○) or 4×10^{-4} M (△) FCCP. Polymerisation assays were performed as described in Material and methods.

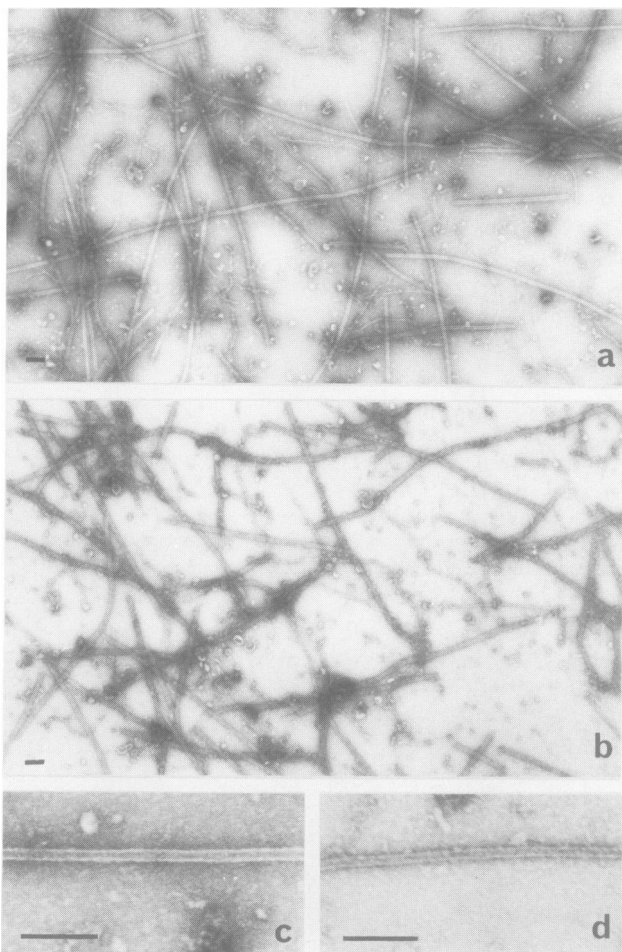


Fig. 5. Negative staining of microtubules polymerised in the absence (a,c) or in the presence (b,d) of 4×10^{-5} M FCCP. Bars represent 0.25 μm.

Negatively stained microtubules polymerised in the presence or absence of FCCP were examined by electron microscopy (Figure 5). The only difference between the samples was an insignificant shortening of the microtubules. The average length of microtubules treated with 4×10^{-5} M FCCP was 5.6 units \pm 3.4 compared with 6.7 units \pm 4.5 for the controls.

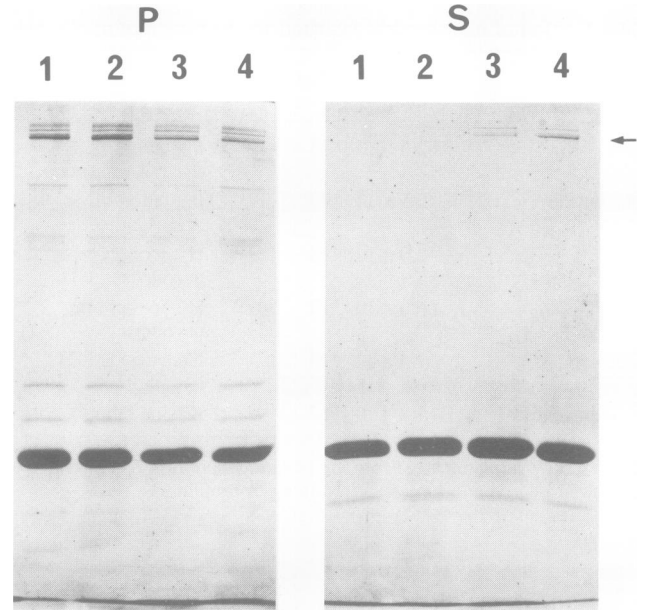


Fig. 6. 7.5% Acrylamide SDS-PAGE analysis of microtubules polymerised in the presence of FCCP. P, pellet containing microtubules; S, supernatant containing non-polymerised proteins. Samples 1, control; 2, 4×10^{-6} M FCCP; 3, 4×10^{-5} M FCCP; 4, 4×10^{-4} M FCCP. Each sample contained 25 μg of proteins. Proteins were stained with Coomassie blue. Arrows indicate MAP 2.

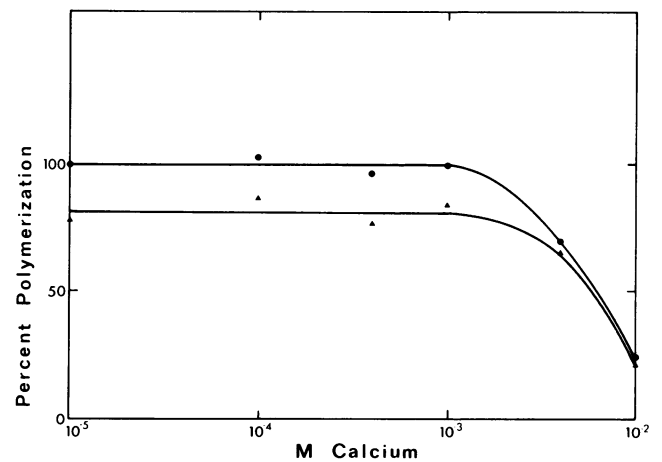


Fig. 7. Effect of calcium concentration on microtubules polymerised in the absence (●) or in the presence (▲) of 4×10^{-5} M FCCP.

The protein composition of these microtubules was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). A decrease in the amount of high mol. wt. microtubule-associated proteins (MAPs) linked with microtubules was observed, particularly for MAP 2 (Figure 6). Unbound MAPs were quantitatively recovered from the supernatant together with non-polymerized tubulin. This effect was observed with FCCP concentrations which completely disrupt microtubules *in vivo* ($\geq 4 \times 10^{-5}$ M).

Two mitochondrial effects of FCCP, the disruption of the mitochondrial pH gradient and the release of calcium from mitochondria, could be involved in FCCP-induced disruption of microtubules *in vivo*. The effect of calcium and H⁺ concentration on polymerisation of microtubule protein were studied *in vitro* in the presence of 4×10^{-5} M FCCP (a concentration which provokes total disruption of microtubules *in*

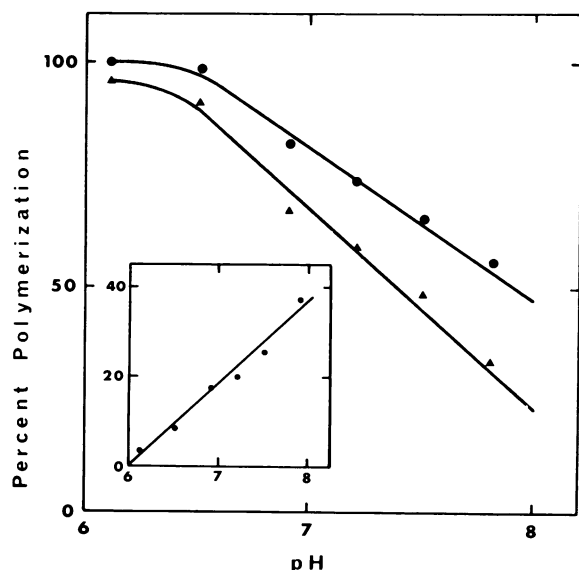


Fig. 8. Effect of pH on microtubules polymerised in the absence (●) or in the presence (▲) of 4×10^{-5} M FCCP. Insert, Percent inhibition of microtubules polymerisation by 4×10^{-5} M FCCP at various pH. 100% polymerisation was determined at pH 6.1.

Table III. Effect of FCCP on calcium-induced depolymerisation of microtubules in detergent-extracted HeLa cells

Calcium concentration 30 min at 37°C	Depolymerisation of microtubules ^a	
	No FCCP	FCCP 4×10^{-5} M
1.0×10^{-7} M	0	0
2.5×10^{-7} M	0	0
5.0×10^{-7} M	0	0
1.0×10^{-6} M	+	+
5.0×10^{-6} M	+++	+++

^aAfter the incubation with calcium the detergent-extracted cells were fixed and tubulin was stained by immunoperoxidase techniques as previously described (Maro and Bornens, 1982).

vivo). FCCP did not affect the inhibitory effect of calcium on polymerisation of microtubule protein (Figure 7) but enhanced the inhibitory effect of alkaline pH on its polymerisation (Figure 8).

We also examined the effect of FCCP on the calcium-induced depolymerisation of polymerised microtubules extracted from HeLa cells with Triton X-100. These microtubules are known to be sensitive to micromolar calcium (Schliwa *et al.*, 1981). 4×10^{-5} M FCCP did not modify this effect in a range of calcium concentrations varying from 10^{-7} M to 10^{-5} (Table III).

Discussion

The above experiments were designed to investigate the mechanism of action of FCCP on microtubules in living cells. FCCP has several effects on mitochondria: it disrupts the mitochondrial electro-chemical gradient and the pH gradient, inhibits mitochondrial ATP synthesis, and causes the release of mitochondrial calcium (Fiskum and Lehninger, 1982; Levenson *et al.*, 1982). It was of interest to know if the *in vivo* effects of FCCP on microtubules were related to these known effects on mitochondria. Two of our experiments suggested that this could be the case: (i) when cells were preincubated with NaN_3 , an inhibitor of electron transport, microtubules

were partially protected from the effect of FCCP (Figure 3); (ii) the recovery of mitochondrial function after removal of FCCP, as judged by rhodamine 123 staining, took place in the same period as the regrowth of microtubules.

The *in vivo* experiments indicated that the FCCP-induced disruption of the mitochondrial pH gradient rather than the calcium efflux was likely to be the most important effect leading to the disruption of microtubules. DNP and Nigericin, both of which disrupt the pH gradient, had a weak but significant effect on the microtubule network (Table I). Nigericin does not cause the release of mitochondrial calcium. Valinomycin, which releases calcium from mitochondria, and oligomycin, which blocks mitochondrial ATP synthesis, did not modify the microtubule network (Table I). Moreover, of three tested inhibitors (NaN_3 , DNP, and oligomycin) only NaN_3 protected microtubules from FCCP (Table II). NaN_3 blocks mitochondrial H^+ production but DNP and oligomycin do not. We did note, however, that the effects of FCCP on mitochondria were not sufficient to disrupt microtubules. The FCCP concentrations which caused microtubules to disrupt *in vivo* were higher than those needed to dissipate the mitochondrial electro-chemical gradient (Johnson *et al.*, 1981). Moreover, DNP which acts on mitochondrial metabolism in the same way as FCCP, is unable to disrupt totally microtubules *in vivo* (Table I).

A direct effect of FCCP on microtubule proteins was observed: FCCP decreases the amount of MAPs, particularly MAP 2, in *in vitro* polymerised microtubules. It decreases the amount of polymerised protein but does not prevent the polymerisation mechanism itself (Figures 4, 5, and 6). This effect is therefore quite distinct from the effects of nocodazole. The kinetics of *in vivo* action of FCCP and its reversal are also different from those obtained with nocodazole (Figure 1 and 2). MAPs are known to stimulate tubulin assembly (Sloboda *et al.*, 1976) and to stabilize microtubules in physiological conditions (Murphy *et al.*, 1977). It was also shown that MAP 2, when bound to microtubules, is not exchangeable and is incorporated at one end of the microtubule in a polymerisation-dependent manner (Manso-Martinez *et al.*, 1980). MAP 2 is not only present in brain but also in various other tissues (Valdivia *et al.*, 1982) and in cultured cell lines (Valdivia *et al.*, 1982; Weatherbee *et al.*, 1982). In the light of these data, the results presented here suggest that FCCP decreases the stability of microtubules by impairing the binding of MAPs. This effect on MAPs could explain why *in vivo* disruption of microtubules by FCCP was a slow process compared to the nocodazole effect (Figures 1 and 2). A longer time would be necessary to impair significantly the binding of MAPs to 'treadmilling' microtubules (Manso-Martinez *et al.*, 1980). From the many anti-microtubular agents known (for review, see Biswas *et al.*, 1981) only griseofulvin binds MAPs (Roobol *et al.*, 1977). It also induces aggregation of microtubule protein at 0°C (Weber *et al.*, 1976) and blocks the polymerisation of pure tubulin in the absence of MAPs (Wehland *et al.*, 1977). *In vitro* the effect of FCCP is different: it does not produce aggregates of microtubule protein, nor does it prevent polymerisation of microtubules.

FCCP enhances *in vitro* the disassembling effect of alkaline pH on microtubules (Regula *et al.*, 1981) while it does not modify the effect of calcium on microtubules. *In vitro* and *in vivo* experiments therefore give complementary results: the microtubules polymerised in the presence of FCCP are less stable and more sensitive to alkaline pH than controls, and

the most important *in vivo* effect of FCCP is the disruption of the mitochondrial pH gradient leading to an increase in the pH of the cytoplasm. These results support the conclusions of Regula *et al.* (1981) on the possible role of intracellular pH on the dynamics of microtubules in living cells. The FCCP-induced release of mitochondrial calcium in cells (Levenson *et al.*, 1982) could also affect microtubules, but according to the present study, this effect is only minor.

To our knowledge, FCCP is the first drug which totally disrupts microtubules *in vivo* but does not prevent polymerisation *in vitro*. More work is necessary to identify the molecular target of FCCP on microtubular proteins and especially to determine whether these proteins are part of the link between microtubules and mitochondria (Hirokawa, 1982; Bernier-Valentin and Rousset, 1982).

Materials and methods

Cell culture

BHK21 cells were cultivated in Glasgow modified Eagle's minimal essential medium supplemented with 10% foetal calf serum and 10% tryptose phosphate on glass coverslips for 24 h. HeLa cells were cultivated in Eagle's minimal essential medium (modified) supplemented with 10% foetal calf serum on glass coverslips for 48 h.

Immunofluorescence

Cells were fixed in phosphate buffered saline (150 mM NaCl, 10 mM Na₂HPO₄, phosphate pH 7.4) (PBS) containing 3% formaldehyde for 30 min at 37°C, then in methanol for 6 min at -20°C. The cells were finally extracted with 0.25% Triton X-100 in PBS for 2 min at 20°C. Immunofluorescence staining of the cells was accomplished using purified sheep anti-tubulin antibodies (Karsenti *et al.*, 1978) followed by fluorescein-labelled rabbit-anti-sheep-immunoglobulins. The antibodies were diluted in PBS containing 0.1% Tween-20 (PBS-Tween) and 3% bovine serum albumin. All the washing steps were performed in PBS-Tween.

Rhodamine 123 staining of mitochondria

Living cells were incubated for 10 min at 37°C in culture medium containing 5 µg/ml rhodamine 123 (Eastman Kodak) in the presence or absence of the various drugs tested. The cells were then washed twice in culture medium and observed in the fluorescence microscope.

Detergent extraction

Cells were extracted as described (Maro and Bornens, 1982) with an extraction buffer, pH 6.9, modified from Schliwa *et al.* (1981). We checked that the microtubules of these permeabilised cells were depolymerised by micromolar calcium (Schliwa *et al.*, 1981).

Tubulin polymerisation studies

Rat brain microtubule protein was purified by two cycles of temperature-dependent polymerisation-depolymerisation according to the method of Shelanski *et al.* (1973) using a 10⁻¹ M 2(N-morpholino)ethane sulfonic acid buffer, pH 6.9, containing 10⁻³ M ethyleneglycol-bis-(aminoethylether)NN'-tetraacetic acid, and 5 x 10⁻⁴ M MgCl₂ with 4 M glycerol during the polymerisation steps. Polymerisation was initiated in the same buffer by a temperature shift to 37°C and by the addition of 1 mM GTP. After 30-90 min incubation, microtubules were collected by centrifugation at 48 000 g for 30 min at 20°C. Determination of the percentage of polymerised protein was performed using the Coomassie blue protein assay (Sedmak and Grossberg, 1977). Light scattering could not be used for the quantitation of the effect of FCCP on tubulin polymerisation because FCCP absorbs strongly between 320 and 430 nm. The 100% polymerisation corresponded to the amount of pelletable proteins obtained with optimal control conditions of tubulin concentration. SDS-PAGE was performed according to Laemmli (1970).

Electron microscopy

Negative staining of polymerised microtubules was performed using 1% uranyl acetate in water.

Chemicals

FCCP (Pierce Chemical, USA) was made 4.0 x 10⁻² M in stock solution in 96% ethanol. Nocodazole (Aldrich Europe, Belgium) was prepared as a 10⁻² M stock solution in 100% dimethyl sulfoxide.

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