

# Polymerization of Actin in the Absence and Presence of Cytochalasin B: Problems of Determining “Critical Concentration”

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Various concentrations of actin (0.3 or 1 mM MgCl<sub>2</sub>, 1 mM ATP, 1 mM EGTA) reached their final degree of polymerization (measured by a pyrene dye attached to actin) earlier in the presence of cytochalasin B than in its absence. The curves relating concentrations of polymeric F-actin to total actin concentration were under these conditions highly nonlinear making an unambiguous extrapolation to zero F-actin concentration (to deduce the “critical concentration” of actin polymerization) impossible. The concentration of actin, above which polymerization occurred, was unaltered by cytochalasin B (although for reasons not yet understood the specific fluorescence intensity of polymerized actin was lower in the presence of cytochalasin B than in its absence). The results show that a distinct “critical concentration” of actin polymerization must not always be well defined.

## Introduction

It is known for quite a long time that the fungal metabolite cytochalasin B disturbs actin structure and therefore interferes with the motile and cytoskeletal apparatus of the cell (*cf.* [1, 2]) since actin is a main constituent of this apparatus. These observations prompted many investigators to study the interaction between cytochalasins and actin during actin polymerization.

The main effects of cytochalasin B are retardation of actin filament elongation by binding to the fast-growing filament end [3–9] and acceleration of overall polymerization by facilitating the nucleation step which is a prerequisite for actin polymerization [10, 11]. The lower viscosity of filamentous F-actin in the presence of cytochalasin B (as compared to its absence) is generally interpreted as being due to generation of short actin filaments by cytochalasin B (either by fragmenting existing filaments or by generating an increased number of polymerization nuclei which give rise to an increased number of filaments with shorter length; see [12–14].

From the observation that cytochalasin B blocks the fast growing end of filaments it was often inferred that cytochalasin B should increase the “critical con-

centration” (that is the concentration of monomeric G-actin which, after completion of polymerization, coexists with filaments). This seems to be plausible since the critical concentration is the ratio of the ‘off rate’ and ‘on rate’ constant of filament elongation (see [15], for a classical review on actin polymerization). If cytochalasin B retards filament elongation without much influencing depolymerization one should indeed expect an increase in critical concentration but recent evidence [9] shows that both rate constants at the ‘barbed’ (fast growing) end are reduced so that only a minor alteration of critical concentration can be expected. Investigations determining the critical concentration after reaching polymerization equilibrium (which, in fact is mostly a steady state rather than a thermodynamic equilibrium) also could not demonstrate large alterations of critical concentration induced by cytochalasin B [13]. According to Löw and Dancker [16] the critical concentration might be even smaller in the presence of cytochalasin B than in its absence. Nonetheless many workers seem to favor the view that cytochalasins are “depolymerizing” rather than “polymerizing” agents with an overall tendency to increase critical concentration (see Tellam and Frieden [17] and Carlier *et al.* [18] for cytochalasin D). We therefore resumed the problem of critical concentration. In our experiments we exploited the experience of Kouyama and Mihashi [19] that a pyrene dye covalently attached to cysteine 374 of actin is a sensitive probe for the polymerization state of actin: in polymerized F-actin fluorescence intensity of the dye is much higher than

**Abbreviation:** EGTA, ethyleneglycol-bis-(2-aminoethyl-ether)-N,N'-tetraacetic acid.

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in unpolymerized G-actin. We conducted our experiments under conditions which favor actin polymerization only weakly (0.3 or 1 mM  $\text{MgCl}_2$ , 1 mM ATP) and show that there is no evidence for an increased critical concentration in the presence of cytochalasin B. We further show that a main problem in inferring critical concentration from steady state measurements can be posed by a nonlinear relationship between concentration of polymerized actin and of total actin.

## Methods

Actin (from mixed skeletal muscle of rabbit) was prepared as described [20]. After dialysis against G-buffer (2 mM Tris-HCl, pH 8.0, 0.1 mM ATP, 0.1 mM  $\text{CaCl}_2$ , 0.5 mM mercaptoethanol, 1.5 mM  $\text{NaN}_3$ ) and removal (by ultracentrifugation) of contaminations of polymerized actin, G-actin was stored in G-buffer.

Pyrene-actin was prepared essentially as described by Kouyama and Mihashi [19]. The buffer used for extraction, labelling, depolymerization and storage of actin was G-buffer without mercaptoethanol. As a pyrene dye we used N-(3-pyrene)maleimide (dissolved as  $10^{-2}$  M stock solution in acetone), which we attached to F-actin, the latter was then depolymerized to G-actin. Fluorescence was measured in a Shimadzu dual beam difference spectrofluorometer RF 520 with excitation wave length of 365 nm, emission wave length of 410 nm, the excitation slit was set at 5 nm in order to avoid photobleaching.

The reference cuvette contained the same concentration of pyrene-actin (as G-actin) as the sample cuvette. Actin in the sample cuvette was, depending on the conditions, G- and/or F-actin. Since F-actin fluoresces more intensely than G-actin (Kouyama and Mihashi [19]), the fluorescence signal of the instrument (*i.e.* the difference in fluorescence intensity of sample and reference cuvette) was proportional to the concentration of F-actin in the sample cuvette.

The experiments were conducted in the following way: various concentrations of G- or F-actin (as a mixture of unlabelled and pyrene-labelled actin, percentage of pyrene-labelled actin: between 1 and 20%) were stored in the dark and at room temperature in the desired media (0.3 or 1.0 mM  $\text{MgCl}_2$ , 1 mM ATP, 1 mM EGTA, 5 mM Tris-HCl, pH 8.0, 1.5 mM  $\text{NaN}_3$ , cytochalasin B, if desired, 30  $\mu\text{M}$ ). The references were without  $\text{MgCl}_2$ . (EGTA was included

because our earlier work has shown [16] that EGTA increases the effects of cytochalasin B.) At the times indicated in the figures the fluorescence intensity (as the intensity difference between sample and reference cuvette) of the samples was recorded and plotted against total actin concentration.

Protein concentration was determined by optical absorption assuming an extinction coefficient of 0.62 for 1 mg/ml actin at 290 nm [21].

N-(pyrene)maleimide and cytochalasin B were from Serva, Heidelberg, FRG. (Cytochalasin B was dissolved in dimethyl formamide as  $10^{-2}$  M stock solution.)

## Results

Actin polymerization proceeds until the critical concentration of monomeric G-actin, which remains in equilibrium with filamentous F-actin, has been reached. With this in mind one should infer from the experiments of Fig. 1 that in the presence of cytochalasin B actin polymerization is faster but leads to less polymerized actin than in the absence of cytochalasin B. But in both cases total actin concentration is above the critical concentration (otherwise no

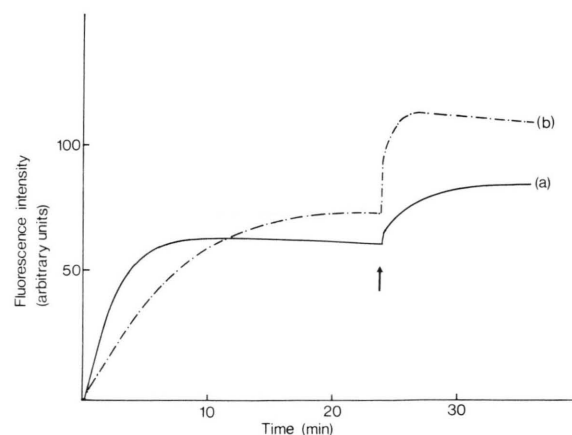


Fig. 1. Polymerization of actin in the presence and absence of cytochalasin B (CB). — The difference in fluorescence intensity between the sample and the reference cuvette (this difference is proportional to the concentration of polymerized actin in the sample cuvette) is plotted *versus* time. Actin concentration (1% pyrene-actin) was initially 0.75 mg/ml. At the time indicated by the arrow additional G-actin was added so that the final actin concentration was 1 mg/ml. Further concentrations: 5 mM Tris-HCl, pH 8.0; 1 mM ATP, 1 mM EGTA, 2 mM  $\text{MgCl}_2$ , CB (in (a)) 20  $\mu\text{M}$ . Polymerization was initiated by the addition of  $\text{MgCl}_2$ .

polymerization had occurred) so that addition of some more actin to both cytochalasin-free and cytochalasin-containing assays should give the same increment in polymerized actin since all actin above the critical concentration should polymerize. This kind of experiment is also presented in Fig. 1 showing that the same increment of polymerized actin generated a smaller increment in fluorescence intensity in the presence of cytochalasin B than in its absence. One has therefore to conclude that the lower final fluorescence in the presence of cytochalasin B is not due to a lower extent of polymerization (as has been inferred from similar results with cytochalasin D [17]) but to a lower fluorescence intensity of polymerized pyrene-actin in the presence of cytochalasin B than in its absence. Hence, experiments on critical

concentration as those described in the following should lead to curves with a smaller fluorescence increment per increment of F-actin in the presence of cytochalasin B than in the absence of cytochalasin B. Nonetheless it should be possible to extrapolate all curves to the X-axis provided they are sufficiently linear. In the following experiments it can be seen, that consistent with the result of Fig. 1, the curves relating total actin concentration to polymerized actin concentration are in fact less steep in the presence of cytochalasin B than in its absence.

The experiments of Fig. 2 and 3 were done by starting with various concentrations of unpolymerized G-actin. After initiating polymerization by addition of  $\text{MgCl}_2$ , the degree of polymerization was measured at various times. In the experiment of Fig. 2 actin was in a low-polymerizing medium (0.3 mM  $\text{MgCl}_2$ ). One main difference between the cytochalasin B-containing and cytochalasin B-free samples is that in the presence of cytochalasin B no

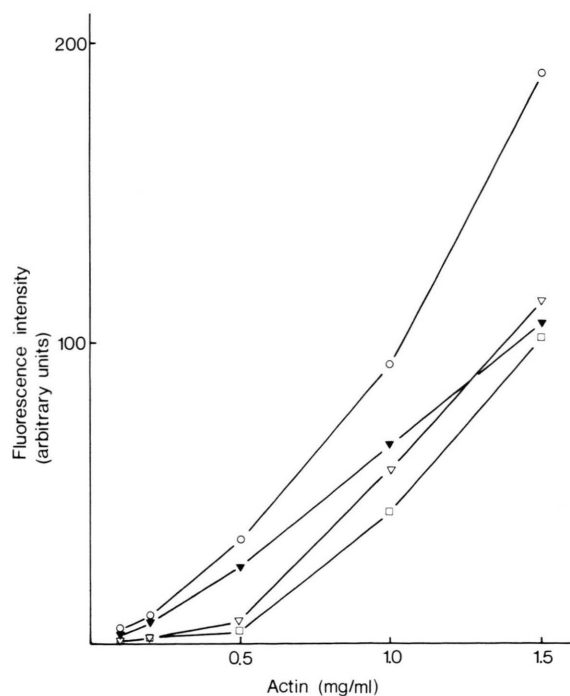


Fig. 2. Extent of polymerization of actin, measured after various times, in low  $\text{MgCl}_2$  concentration. — 3 ml assays of G-actin at the concentrations indicated at the abscissa were prepared and polymerization was initiated by adding  $\text{MgCl}_2$  to 0.3 mM. At various times after start of polymerization 2 ml aliquots were transferred from each assay to the fluorometer. After measuring fluorescence the aliquots were given back to the polymerization assays. Further concentrations: 5 mM Tris-HCl, pH 8.0; 1 mM ATP; 1 mM EGTA; 30  $\mu\text{M}$  cytochalasin B (CB) (if present).  $\square$ — $\square$ : without CB, after 24 h;  $\nabla$ — $\nabla$ : without CB, after 48 h;  $\circ$ — $\circ$ : without CB, after 1 week;  $\blacktriangledown$ — $\blacktriangledown$  with CB, after 24 and 48 h.

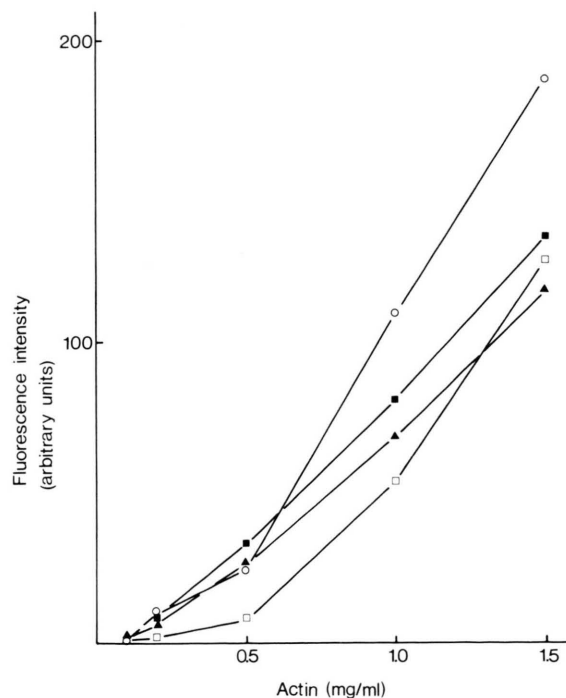


Fig. 3. Extent of polymerization after various polymerization times in high  $\text{MgCl}_2$  concentration. — In this experiment polymerization was initiated by adding  $\text{MgCl}_2$  to a final concentration of 1 mM. For further details see Fig. 2  $\square$ — $\square$ : without cytochalasin B (CB), after 2 h;  $\circ$ — $\circ$ : without CB, after 24 h;  $\blacktriangle$ — $\blacktriangle$ : with CB, after 2 h,  $\blacksquare$ — $\blacksquare$ : with CB, after 24 h.

further increase in polymerization occurred after 24 h, whereas in the absence of cytochalasin B polymerization continued to increase even after 48 h. After 48 h of polymerization the fluorescence signal was higher in the presence of cytochalasin B whereas after one week the actin samples had a much higher fluorescence in the absence of cytochalasin B than in its presence. After the period of one week the curve in the absence of cytochalasin B was highly nonlinear so that determination of the critical concentration by extrapolating it back to the X-axis becomes rather difficult. When one does this extrapolation with the other curves one would clearly deduce a lower critical concentration of actin in the presence of cytochalasin B. However, if one looks at that actin concentration where the first fluorescence signal appears (that is at the origin of the curves), it is obvious that all curves start from the same concentration varying only in degree of increase and curvature.

The experiment of Fig. 3 differs from that of Fig. 1 in that  $\text{MgCl}_2$  concentration was higher (1 mM  $\text{MgCl}_2$  rather than 0.3 mM) providing more favourable polymerizing conditions. Again only after longer time periods there is a higher fluorescence signal in the absence of cytochalasin B as compared to its presence. Again the final degree of polymerization was reached earlier in the presence of cytochalasin B than in its absence. As in the lower Mg concentration extrapolation of the linear part of the curves to the X-axis would indicate a lower critical concentration in the presence of cytochalasin B than in the absence.

The other strategy to measure the critical concentration, namely to dilute F-actin to different concentrations, thus approaching the steady-state of coexisting monomers and polymers from the F-actin side (rather than from the G-actin side) was applied in the experiment of Fig. 4. This experiment looks different from the preceding ones in the following respects: there is no significant difference in the fluorescence signals after 24 and 48 h and the curves are much more linear than those from the experiments starting with G-actin. But once again the slope is slighter in the presence of cytochalasin B than in its absence. In this case extrapolation of the steeper part of the curve referring to the presence of cytochalasin B could suggest a higher critical concentration in the presence of cytochalasin B, but the true origin suggests that the concentration above which actin remains polymerized is the same both in the absence and presence of cytochalasin B.

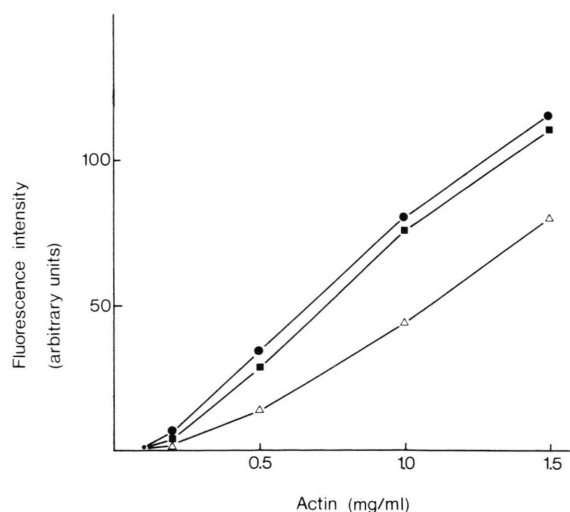


Fig. 4. Extent of depolymerization of F-actin after various depolymerization times. — 3 ml assays of F-actin were prepared by diluting a F-actin stock solution (polymerized in 2 mM  $\text{MgCl}_2$ , 1 mM ATP, 1 mM EGTA, 5 mM Tris-HCl, pH 8.0 in the absence or presence of  $30 \mu\text{M}$  cytochalasin B) to the concentrations (in 3 ml) indicated at the abscissa. The solvent conditions remained unaltered except that  $\text{MgCl}_2$  was lowered to 0.3 mM. After the times indicated 2 ml aliquots were measured as described in Fig. 2. ■—■ : without cytochalasin B (CB), after 24 h; ●—● : without CB, after 48 h; △—△ : with CB, after 24 and 48 h.

## Discussion

The following features emerge from the experiments presented:

(1) The polymerization of various concentrations of G-actin continued to proceed over the whole range of time measured. Particularly after longer periods of polymerization the curves relating concentration of polymerized actin to total actin concentration were remarkably non-linear making a linear extrapolation to zero F-actin concentration impossible.

(2) In the presence of cytochalasin B polymerizing actin reached its final degree of polymerization earlier than in the absence of cytochalasin B, so that initially the curves referring to the presence of cytochalasin B lie above those referring to the absence of cytochalasin B. This reflects the fact that cytochalasin B accelerates overall actin polymerization (Löw and Dancker [16]).

(3) When F-actin was under conditions where depolymerization should occur, the final fluorescence intensity was reached rather early.



(4) The fluorescence increment per increment of actin concentration was smaller in the presence of cytochalasin B than in its absence. This was true for both sets of experiments (starting with G- or with F-actin). The reason for this is not clear.

The non-linearity of the curves in Fig. 2 and 3 is difficult to explain particularly with respect to the more or less linear appearance of the curves in Fig. 4. Since polymerization velocity depends on actin concentration one should expect that higher actin concentrations reach their final degree of polymerization earlier than lower ones so that the curves obtained at earlier stages of polymerization should be more non-linear than those obtained later, but just the opposite was the case. Hence the non-linearity is probably not due to different polymerization velocities. Then, in the experiment of Fig. 4, which started with F-actin, depolymerization would not have reached — for some kinetic or other reason — the same ratio between G- and F-actin as had been reached when G-actin was induced to polymerize.

Extrapolating the steeper part of the curves in Figs. 2 and 3 back to zero F-actin concentration would suggest a lower critical concentration in the presence of cytochalasin B (consistent with [16]). This however, might result from the fact that in the presence of cytochalasin B the final polymerization values are reached earlier than in the absence of cytochalasin B. The following argument appears to be more realistic: According to the operational definition of critical concentration as that particular concentration of total actin where the curve relating F-actin concentration to total actin concentration originates at the abscissa, one would infer the same critical concentration both in the absence and presence of cytochalasin B. It is noteworthy that this origin does not depend on the time, at which the curves were obtained. One has to assume that under the conditions of the present work actin begins to polymerize both in the presence and absence of cytochalasin B already at very low concentrations. The non-linearity at the curves can be explained on the assumption that with increasing total actin concen-

tration both unpolymerized G-actin and polymerized F-actin increase in concentration, so that the concentration of G-actin that coexists with F-actin depends on total actin concentrations.

Newman *et al.* [22] deduced from the formalism of Oosawa's theory on actin polymerization (*cf.* [15] for a summary) that below the critical concentration there should be actin "oligomers" and that the concentration of these oligomers should be the higher the lower the free energy of nucleus formation. In a recent paper which appeared during the preparation of our manuscript Carlier *et al.* [18] observed that in the presence of cytochalasin D the critical concentration curves deviated from linearity at low actin concentrations and interpreted these observations according to [22]. Newman *et al.* [22] distinguish between "oligomers" (occurring below critical concentration) and filamentous actin (occurring above critical concentration), but it is doubtful whether this distinction can be really made. We deduce from our observations (and the similar observations recently published by Carlier *et al.* [18]) that a distinct critical concentration can only then be defined well enough if the cooperativity during polymerization is high, that is, when elongation is much more favored than nucleation (*cf.* also Wegner and Engel [23]). Our experiments which started from G-actin suggest that under the conditions of the present work (0.3 mM or 1 mM  $MgCl_2$ , 1 mM ATP) this cooperativity might not be high enough to define an unambiguous critical concentration.

In conclusion: the present work shows that a critical concentration of actin polymerization is not always well defined. Moreover, the present work does not support the view that cytochalasin B increases the critical concentration of actin, but it rather demonstrates that cytochalasin B stimulated overall actin polymerization.

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