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# ON THE MOLECULAR BASIS OF ACTION OF CYTOCHALASIN B

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Tritium-labeled cytochalasin B binds rapidly and reversibly to mammalian cells, and a class of high-affinity sites ($K_D \cong 10^{-7}$ M) and a class of low-affinity sites ($K_D \geqslant 10^{-5}$ M) are detected. In red blood cells, the high-affinity binding sites (about $3 \times 10^5$ per cell) are associated with the plasma membrane, and at least 80% of these appear to be intimately related to the glucose transport system. Fractionation of cellular components of platelets by differential centrifugation and gel filtration chromatography reveals that the high-affinity binding sites in these cells are also associated with membranous materials. A substantial number of the low-affinity binding sites can be traced to platelet actin. The binding of cytochalasin B to actin is consistent with the alteration of intrinsic viscosity and morphology of actin filaments in vitro by the compound at concentrations of around $10^{-5}-10^{-4}$ M. The interaction of cytochalasin B with actin may account for its inhibitory effect on various forms of cell motility.

## INTRODUCTION

In recent years there has been much interest in the fungal metabolite cytochalasin B because of its rapid and reversible effects on a large number of biological processes (for reviews, see refs. 1–3). These effects can be classified into two groups. At low concentrations (around $10^{-7}$ M), cytochalasin B inhibits transport of sugars into various types of mammalian cells (4–9). At higher concentrations ($10^{-6}-10^{-4}$ M) it inhibits various forms of cell motility, such as cytokinesis, cell locomotion, phagocytosis, cytoplasmic streaming, and blood clot retraction (1). Evidence suggests that inhibition of motility is not merely a secondary effect of glucose deprivation (6, 7, 10, 11).

The molecular basis of action of cytochalasin B is unknown. Schroeder (12) and Wessells et al. (1) concluded from work involving electron microscopy that inhibition of cell motility by the drug may be related to the apparent disruption of microfilaments in the cell. The more recent observations that cytochalasin B inhibits sugar transport suggest that the plasma membrane may contain one or more receptors for the drug; indeed, it

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has been proposed that the effects of the drug in general may be caused by its interaction with the cell membrane (13–15).

We used biochemical and biophysical methods to study the molecular basis of action of cytochalasin B. This report summarizes the results of these studies, which are described in detail elsewhere (16–21).

## METHODS

Cytochalasin B was purchased from Imperial Chemical Industries, Ltd. $^3$H-cytochalasin B was prepared in our laboratory by reduction of cytochalasin A with $^3$H-$NaBH_4$ as previously described (19). This preparation had a specific activity of 6 Ci/mmole and a radiochemical purity of over 99%. The structure of the labeled compound (Fig. 1) was shown to be identical with that of authentic cytochalasin B by high-resolution mass spectrometry, thin-layer chromatography, and derivatization. The synthesized compound had the same potency as authentic cytochalasin B when tested in a number of biological systems.

We determined binding of cytochalasin B to intact cells, red cell ghosts, and large membrane fragments by a centrifugation method; the cells or membranes were separated from the assay media containing $^3$H-cytochalasin B, and the radioactivity of the two fractions was measured as described previously (19, 20). In experiments involving small membrane fragments or soluble materials, binding of the drug was determined by equilibrium dialysis (20, 21). Both methods gave the same results when used to measure binding of the drug to large platelet membrane particles.

## RESULTS

### Binding of Cytochalasin B to Intact Cells

The binding of cytochalasin B to HeLa cells, red blood cells, and platelets was found to be rapid and reversible at high ($10^{-5}$ M) and low ($10^{-7}$ M) concentrations of the drug (19, 20). The binding to various types of cells was studied using drug concentrations ranging from $10^{-9}$ to $10^{-4}$ M, and the data were analyzed by Scatchard plots (19). In these plots, the slope of the binding curve is equal to $-1/K_D$, and the intercept at the abscissa is equal to the number of binding sites present in the sample (22). Figure 2 shows that the binding curves for mammalian cells are biphasic, indicating the presence of a class of high-affinity binding sites ($K_D \cong 10^{-7}$ M; $10^6$ sites/HeLa cell, $3 \times 10^5$ sites/red cell, and $10^4$ sites/platelet) and a class of low-affinity binding sites ($K_D \geqslant 10^{-5}$ M). In contrast, the binding curves for nonmammalian cells show that slime mold amoebae bind cytochalasin B only with low affinity, while bacterial cells bind very little of the drug even at high drug concentrations.

### Binding of Cytochalasin B to Cellular Components of Bovine Platelets

To locate the different cytochalasin B binding sites, we studied the binding of the drug to major cellular components of platelets (21). Platelets were used because in these

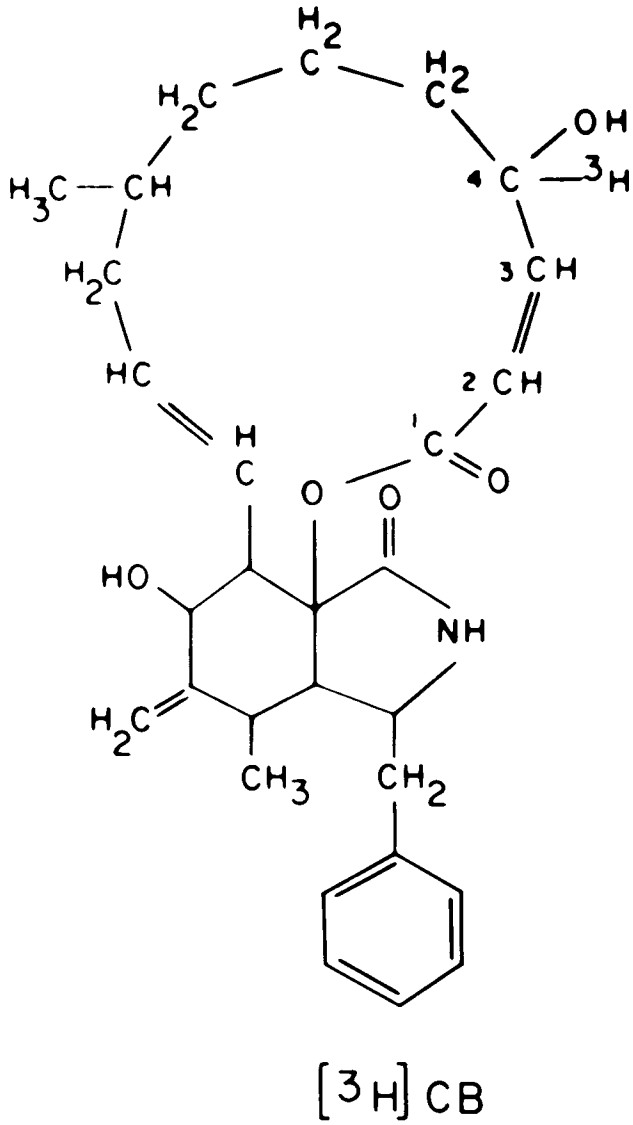


Fig. 1. The structure of $^{3}$H-cytochalasin B.

cells cytochalasin B inhibits utilization of exogenous glucose (23) and affects motility in the form of blood clot retraction (1, 17, 23, 24) and pseudopod formation (1).

Platelet lysates were separated into different fractions by low-speed and high-speed centrifugation followed by chromatography on a Sepharose 4B column. The fractions were then tested for cytochalasin B binding capacity. We found that the high-affinity binding sites of the cell are associated with membranous materials. Low-affinity binding sites are present in several fractions but are most concentrated in the fraction containing purified platelet actin (Fig. 3). Since actin, which represents over 10% of the total

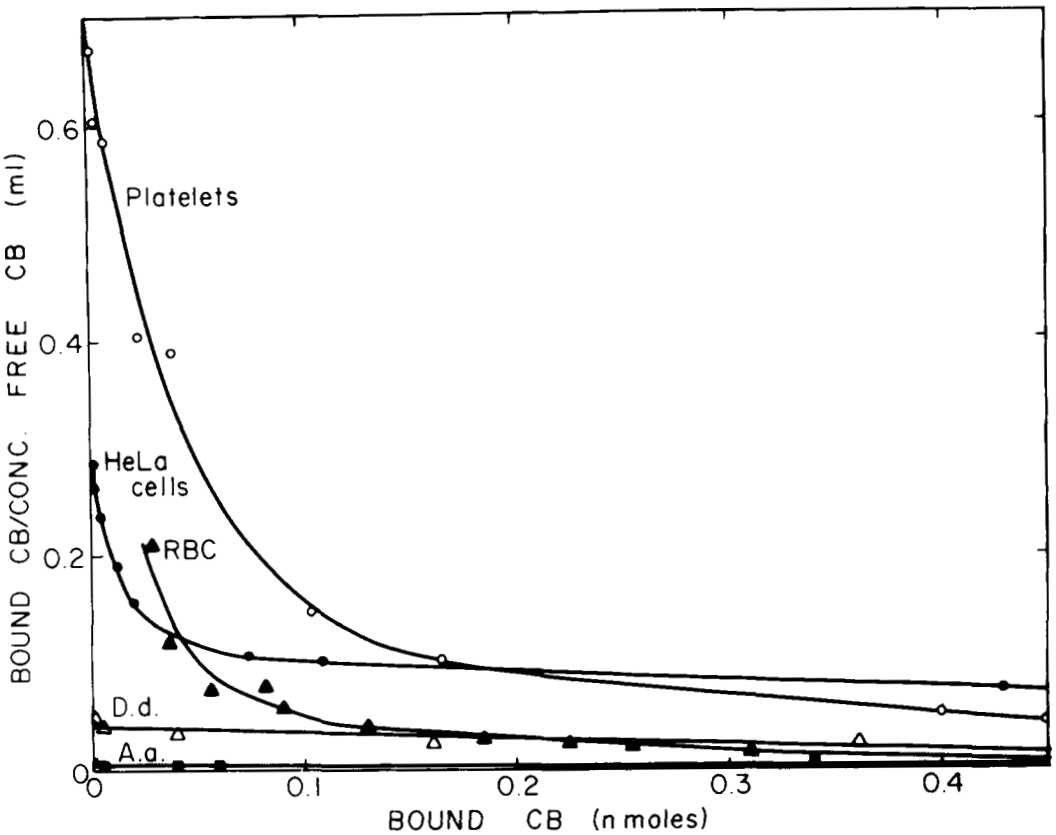


Fig. 2. Scatchard plots of binding of cytochalasin B to different types of cells. Samples contained $1.5 \times 10^9$ platelets (○), $6 \times 10^6$ HeLa cells (●), $10^8$ red blood cells (▲), $7 \times 10^6$ D. discoideum amoebae (△), or $7 \times 10^8$ A. aerogenes cells (■). Binding of the drug to the cells was determined as described previously (19).

cellular protein, is also present in significant amounts in several other fractions, much of the low-affinity cytochalasin B binding capacity found in these fractions might be accounted for by this protein. The fraction containing purified platelet myosin also binds cytochalasin B, but the affinity of this protein for the drug is even lower than that of actin (Fig. 3).

The binding of cytochalasin B to highly purified actin and myosin from rabbit skeletal muscle was studied in high and low ionic strength solutions by equilibrium dialysis and by the millipore method described by Puszkin et al. (25). As in the case of platelet actin and myosin, the muscle proteins bind the drug with low affinity.

### Effects of Cytochalasin B on the Structure of Actin Filaments

The binding of cytochalasin B to actin at drug concentrations around $10^{-5}$ to $10^{-4}$ M results in alteration of the structure of the actin filaments as judged by the following criteria. First, the intrinsic viscosity of filaments of highly purified muscle actin drops

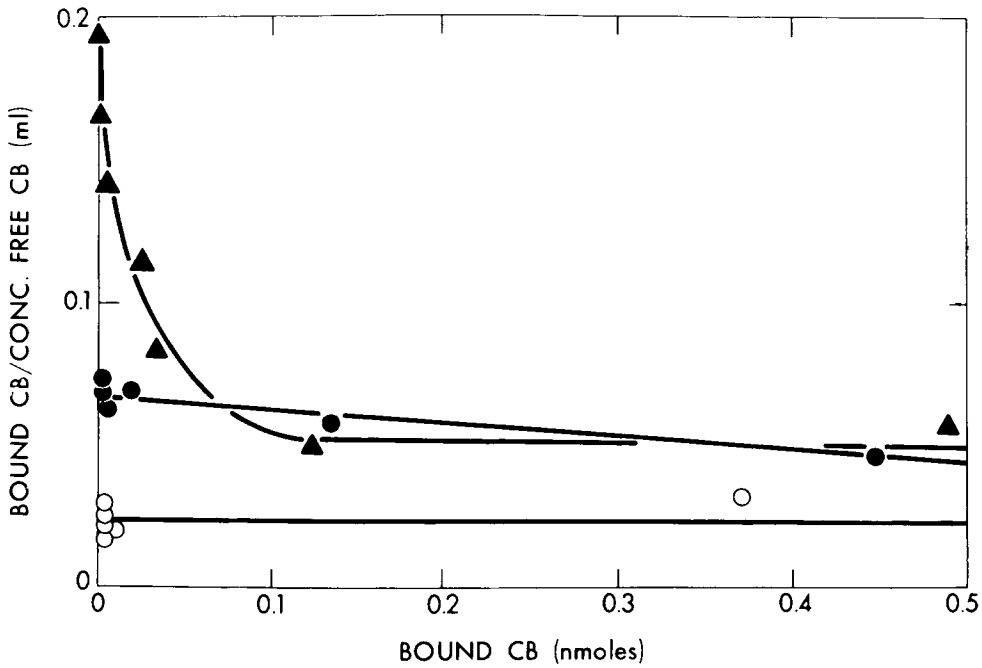


Fig. 3. Scatchard plots of binding of cytochalasin B to membranes, actin, and myosin purified from bovine platelets. Platelet lysate formed by disruption of the cells by freezing and thawing was fractionated as described elsewhere (21). Briefly, the lysate was centrifuged at 10,000 × g for 1 hr, and the resulting supernatant was then centrifuged at 100,000 × g for 2 hr. The pellet from the second centrifugation was resuspended in buffer and chromatographed on a Sepharose 4B column under conditions where the actin is in monomeric form and is dissociated from the myosin. The fractions eluting in the void volume (▲), which contained membranous materials, and the fractions containing purified actin (●) and myosin (○) were pooled, concentrated, and tested for cytochalasin B binding.

significantly in the presence of cytochalasin B (16). The concentration of the drug causing this effect is similar to that used to inhibit many forms of cell motility (e.g., blood clot retraction) (Fig. 4). Second, electron microscopy shows that filament networks of actin purified from muscle and from platelets become aggregated and disrupted when exposed to $7 \times 10^{-5}$ M cytochalasin B (17). This phenomenon resembles the reported disruption of microfilaments in cells incubated in similar concentrations of the drug (1).

Although myosin purified from muscle and from platelets binds cytochalasin B with low affinity, we failed to detect any alterations in its structural and enzymatic properties as a result of this interaction (21).

### Relationship Between High-Affinity Binding Sites and Sugar Transport

The binding studies using cellular components of platelets indicate that the high-affinity binding sites are associated with membranes. We used human red blood cells to study this type of binding sites in greater detail because their plasma membrane is easily isolated (20).

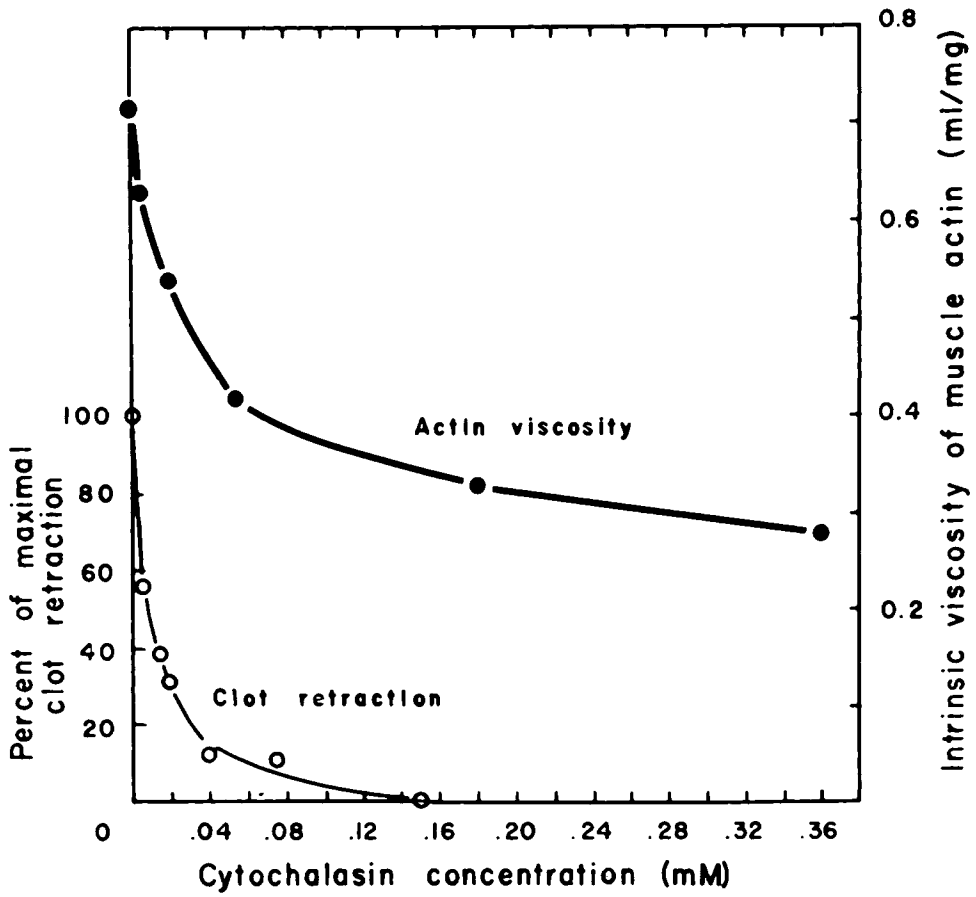


Fig. 4. Effects of cytochalasin B on the intrinsic viscosity of actin filaments and on blood clot retraction. The viscosities of solutions of highly purified muscle actin were determined with an Ostwald viscometer as described previously (16). The extent of clot retraction was determined by measuring the volume of serum expressed by the contraction of the clot (17). $CaCl_2$ was added to bovine platelet-rich plasma to initiate clot formation, and the clots were incubated at 37°C for 1 hr.

All of the high-affinity cytochalasin B binding sites of the red cell are in the plasma membrane (ghosts). These sites are not affected when cells are treated with pronase or with trypsin, but they are destroyed when ghosts are treated under conditions where the inner surface of the membrane is accessible to these enzymes. When intact red cells or ghosts are treated with p-chloromercuribenzoate, 80–90% of the high-affinity binding of cytochalasin B is eliminated, indicating that sulfhydryl groups are essential for binding. These results suggest that the high-affinity binding sites are protein components of the plasma membrane.

Since low concentrations of cytochalasin B ($10^{-7}$ M) inhibit glucose transport into red cells, we examined the effect of D-glucose on the high-affinity binding of the drug to ghosts. We found that increasing concentrations of the sugar result in increasing inhibition of the binding of the drug; 80–90% inhibition has been observed with 0.5 M D-glucose.

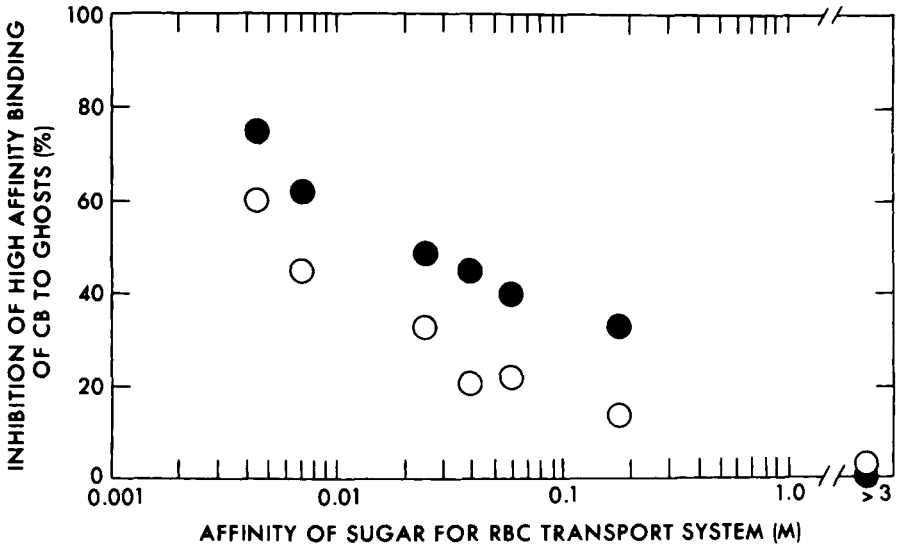


Fig. 5. Relationship between the effectiveness in inhibition of high-affinity cytochalasin B binding by various sugars and their affinity for the sugar transport system of the red cell. Samples containing ghosts were incubated in $10^{-7}$ M cytochalasin B in the presence of 0.1 M (○) and 0.5 M (●) of each sugar, as described previously (20). In order of decreasing effectiveness in inhibition of cytochalasin B binding, the points represent 2-deoxy-D-glucose, D-glucose, D-mannose, D-galactose, D-xylose, D-ribose, and L-glucose. The affinity of the sugars for the red blood cell transport system is expressed as the dissociation constant of the sugar-carrier complex in moles/liter, as given by LeFevre (26).

In contrast, L-glucose, which is not a substrate of the sugar transport system, has no significant effect on this binding. Similar experiments using seven different sugars showed that their effectiveness in inhibiting high-affinity binding of cytochalasin B to ghosts is related to their affinity for the sugar transport system of the red cell (Fig. 5).

### Initial Attempts to Identify the High-Affinity Cytochalasin B Binding Sites

Scatchard plot analysis of binding data indicates that there are $3 \times 10^5$ high-affinity cytochalasin B binding sites ($K_D = 10^{-7}$ M) per red cell. SDS-acrylamide gel electrophoresis of ghost proteins showed that there are about eight major protein components present at similar concentrations (27). Assuming that each binding site represents a single polypeptide chain, then one of these eight components must be the high-affinity cytochalasin B receptor. Selective elution of components 1 and 2 (the two subunits of spectrin), component 5, and component 6 from the membrane had no effect on the high-affinity binding, thus eliminating these as candidates for the high-affinity cytochalasin B receptor (20). Further experiments aimed at the identification and isolation of this receptor are in progress.

## DISCUSSION

Mammalian cells such as HeLa cells, fibroblasts, red blood cells, and platelets contain a class of high-affinity binding sites and a class of low-affinity binding sites for

cytochalasin B. The high-affinity binding sites ($K_D \cong 10^{-7}$ M) in red cells are apparently protein components of the plasma membrane. At least 80% of these sites appear to be intimately related to the sugar transport system of the cell, as judged by the following criteria. First, and most importantly, the high-affinity binding of cytochalasin B to ghosts is inhibited up to 80–90% by sugars which are substrates of the transport system. Second, the $K_D$ of the binding reaction is about $10^{-7}$ M, similar to the concentration of the drug required to produce half-maximal inhibition of glucose transport. Third, the number of high-affinity cytochalasin B binding sites per cell ($3 \times 10^5$) is close to the estimated number of transport-related D-glucose binding sites per cell ($2–5 \times 10^5$) reported by Kahlenberg et al. (28) and LeFevre (29). Fourth, the high-affinity binding of cytochalasin B is independent of pH in the range of 5.0 to 9.0, but is enhanced by high ionic strength (20). This is similar to the binding of D-glucose to the red cell membrane (28). Finally, as in glucose transport (30, 31) the high-affinity binding is not affected by treatment of intact cells with proteolytic enzymes but is sensitive to the sulfhydryl reagent p-chloromercuribenzoate.

In contrast to the situation with the high-affinity cytochalasin B binding sites, we are not as certain about the nature of the low-affinity binding sites. However, we have obtained evidence consistent with the notion that inhibition of cell motility at concentrations of cytochalasin B around $10^{-5}$ M is caused by the interaction of the drug with microfilaments in the cell. Actin purified from muscle and platelets has the capacity to bind cytochalasin B with low affinity. This interaction apparently leads to alterations in the structure of actin filaments which can be detected by viscometry and electron microscopy. Although myosin purified from muscle also binds cytochalasin B with low affinity, this interaction has no observable effect on the properties of this protein. These results are in contrast to the reported stoichiometric binding of cytochalasin D (a drug structurally related to cytochalasin B) with high affinity to myosin, leading to the inhibition of its ATPase (25).

The experiments involving actin do not rule out the possibility that binding of cytochalasin B to other low-affinity binding sites, or even to some high-affinity binding sites, causes inhibition of cell motility. For instance, we have demonstrated that high concentrations of the drug (around $10^{-4}$ M) affect mitochondrial functions in vitro (18), and it is conceivable that at these concentrations some forms of cell motility may be inhibited because of decreased levels of energy production. However, our data and evidence from other laboratories suggest that disruption of microfilaments is the most likely cause of the loss of many forms of cell motility reported in the literature. This is best illustrated by the studies on inhibition of cytokinesis by cytochalasin B. This is the first system where the effects of the drug were observed (32), and it has been the subject of detailed studies conducted by several investigators (12, 13, 33, 34). There is strong evidence unrelated to cytochalasin B that the contractile ring plays an important role in the cleavage of eukaryotic cells during cell division (12, 35). This organelle consists primarily of microfilaments which appear to be actin-like because of their ability to bind muscle heavy meromyosin in situ (36, 37). When cytochalasin B is present in the medium, cytokinesis is stopped and, in HeLa cells and Arbacia eggs, the contractile ring disappears (12, 35). In the case of Xenopus eggs, at times when cytochalasin B is apparently unable to enter the cell, injection of the drug into the cell in the immediate vicinity of the contractile ring results in local regression of the cleavage furrow (38). Thus, at least in this system, the

inhibition of cell motility is most likely the result of the action of the drug on the actin filaments of the cell.

There have been numerous instances recently where cytochalasin B was used as a diagnostic tool; the sensitivity of a biological process to the drug was taken as evidence for involvement of microfilaments in that process. The concentrations of cytochalasin B used in these studies were usually between $10^{-5}$ and $10^{-4}$ M. Since actin is not the only component of the cell which has the capacity to bind the drug with low affinity, it is dangerous to assume without other supporting evidence that the interaction of the drug with this protein is responsible for the inhibition of the biological process.

## ACKNOWLEDGMENTS

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