Binding of Cytochalasin D to Platelet and Muscle Myosin*

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SUMMARY

Human blood platelets exposed to 2 μM tritiated cytochalasin D showed maximum incorporation within 5 min. Tritiated cytochalasin D was found to be bound to thrombosthenin M (platelet myosin), when subjected to gel exclusion chromatography in 0.6 M KCl. Using a Millipore filtration technique, binding at low ionic strength of tritiated cytochalasin D to purified preparations of contractile proteins, showed that platelet thrombosthenin M and muscle myosin, but not actin, was involved (0.9 mole of cytochalasin D per mole of protein). Prior treatment of these proteins with nonradioactive cytochalasin D, followed by exposure to the labeled alkaloid, showed little exchange under these conditions. Binding data gave a value of 0.99 ± 0.11 sites per 4.6 × 10^5 g of muscle myosin, an average intrinsic association constant of 6.2 × 10^7 M⁻¹ with an heterogeneity index of 1. Although the Ca²⁺- or Mg²⁺-stimulated ATPase activities of actomyosin from platelets (thrombosthenin) or muscle were not inhibited by 2 μM cytochalasin D, this concentration depressed thrombosthenin M and myosin ATPase. Recombination experiments indicated that actin and cytochalasin D compete for a binding region on myosin. The inhibitory effect of cytochalasin D on myosin ATPase was restored following dialysis against 0.6 M KCl; with concomitant removal of the drug. Furthermore, quantitative measurements of superprecipitation indicated that, although cytochalasin D did not interfere with dissociation of actomyosin by MgATP, increasing concentrations of cytochalasin D inhibited reassociation. Labeled cytochalasin D associated with thrombosthenin M could be removed by solvent extraction on thin layer chromatography and by sodium dodecyl sulfate gel electrophoresis, suggesting that the bond between cytochalasin D and thrombosthenin M or muscle myosin is not covalent.

Four major cytochalasins, designated A, B, C, and D, have been isolated and, although structurally similar, the C and D types exceed the A and B types in potency by at least 1 order of magnitude (3). Carter (3) first reported that exposure of fibroblasts in culture to low concentrations of cytochalasin B resulted in inhibition of cytokinesis, membrane ruffling, and locomotion. Higher concentrations of these drugs can actually mediate nuclear extrusion in a number of cultured mammalian cell lines (4). This led to several studies on the influence of cytochalasin B on those morphogenetic processes associated with cell motility (5–7). A diverse number of morphological observations have accumulated concerning the action of cytochalasin B, including inhibition of secretory processes (8, 9), phagocytosis (10–12), chemotaxis (13), pigment migration (14, 15), and many others (16–23). All of these functions, however, may be interrelated through the common denominator of the contractile mechanism of living cells.

Initial studies concerning the locus of action of cytochalasin B were morphological, and Schröder (24), studying sea urchin and HeLa cells, suggested that cytochalasin deranged the 40- to 50-A subunits of living cells. It has also been noted that cytochalasin B inhibited formation of arrowheads and contractile ring microfilaments. Bluemink (25) concluded, with drug-treated Xenopus eggs, that the site of cytochalasin B action was at the cytoplasmic membrane. Although a good deal of evidence was adduced in favor of inhibition of microfilament function by cytochalasin B, other data raised doubts concerning this conclusion (17, 18). Sanger and Holtzer (26) demonstrated mucopolysaccharide biosynthesis to be inhibited by cytochalasin B. Later experiments (27, 28) indicated that cytochalasin B inhibited transport of monosaccharides and amino sugars across cell membranes. It has also been noted that cytochalasin B formation of CO₂ from labeled glucose (12, 27, 29).

The foregoing studies employed cytochalasin B concentrations in the range of 1 to 10 μM, and more recent reports dealt with the mode of action of 1 μM on the biochemistry of contraction. Spudich and Lin (30) showed both a drop in viscosity when cytochalasin B was added to muscle actomyosin and inhibition of the ATPase of an actin-heavy meromyosin (A-HMM) complex. Myosin was unaffected by cytochalasin B. G-actin was able to polymerize in the presence of cytochalasin B, but reached only a low viscosity value comparable to F-actin plus drug. These data suggested that cytochalasin B reacted with actin rather than myosin, and actually competed with myosin for actin binding. On the other hand, Forer et al. (31) did not demonstrate cytochalasin B inhibition of A-HMM formation arrowheads and

The cytochalasins are a class of low molecular weight fungal metabolites that adversely affect a variety of cell functions (1, 2).

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1 The abbreviations used are: A-HMM, actin-heavy meromyosin; HMM, heavy meromyosin.
did not notice breakdown of F-actin, inhibition of ATP-induced release of HMM from the A-HMM, or any effect of cytochalasin B on the G-actin to F-actin transformation.

Thus the mode of action of cytochalasins is as yet unclear and published data have led to conflicting assumptions. Experiments now reported concern the effect of the relatively unstudied congeneric cytochalasin D on a target site within muscle and platelets, the contractile protein myosin and thrombosthenin M. The availability of high specific activity tritiated cytochalasin D (32) has allowed the interaction of this compound to be studied stoichiometrically.

**Materials and Methods**

Cytochalasin D was isolated from a strain of Zygosporium masonii, as described previously (32). The tritiated drug was prepared by the procedure of Hemberg et al. (33), which involves microwave discharge-catalyzed exchange with tritium gas. It was purified by repeated preparative thin layer chromatography, utilizing three solvent systems, to constant specific activity (0.610 mCi per mg). The labeled drug was 98% homogeneous, as judged by analytical combined thin layer chromatography-radiochemical assay. Details of the preparation, purification, and pattern of labeling will be published elsewhere. This sample of [3H]cytochalasin D was fully biologically active in a sensitive HeLa cell assay in which the dihydro derivative, prepared from unlabeled cytochalasin D, were shown to possess at the same molar concentration, 12 and 4%, respectively, as the controls.

Normal human blood was collected into 1/4 of its volume of acid-citrate-dextrose (34), and platelets were prepared by differential centrifugation (35). Thrombosthenin or thrombosthenin M was extracted from platelets pooled from three or four donors. Sephadex G-200 and Sephadex G-25 were obtained from Pharmacia Co. Bovine serum albumin was obtained from Pentex. Adenosine 5'-triphosphate-disodium salt (ATP) was purchased from Sigma. Celandra's reagent was obtained from Calbiochem.

**Protein Extraction**—Thrombosthenin was extracted from platelets by the method of Betten-Galland and Lüscher (36). Thrombosthenin M was prepared by the method of Puskin et al. (37). These proteins were also extracted after prior exposure of platelets to 2 µM [3H]cytochalasin D. Muscle actomyosin (38), myosin (39), and actin (40) were prepared from fresh rabbit back muscle. Protein concentrations were measured by the method of Lowry et al. (41) and standardized with bovine serum albumin dissolved in 0.1 or 0.6 M KCl 0.05 M Tris, pH 7.2.

**Determination of ATPase Activity**—ATPase activity of platelet proteins was determined by the release of inorganic phosphorus (P_i) from ATP according to Marsh (42) and adapted (43) to detect as little as 3 nmoles of Pi. Each milliliter of final mixture contained 0.04 M imidazole buffer, pH 6.8, 0.06 M KCl, 0.5 mM ATP, 1 mM MgCl₂ or CaCl₂, and 0.1 mM ouabain. The amounts of proteins added to the final mixture were 0.1 mg of thrombosthenin or thrombosthenin M, 0.01 mg of actomyosin or myosin, and 0.05 mg of actin. Assays were performed on each protein alone and after preincubation at room temperature for 2 min with 2 µM of cytochalasin D. The reaction was stopped by the addition of 0.4 ml of a 20% trichloroacetic acid per ml mixture. Blanks consisted of protein inactivated by trichloroacetic acid prior to addition of ATP. ATPase activity was estimated as the difference between P_i at 30 min and P_i at zero time for platelets and zero to 15 min for muscle proteins.

**Incorporation of [3H]Cytochalasin D into Platelets**—Platelet concentrates from individual donors were incubated with [3H]cytochalasin D at a final concentration of 2 µM. These platelet suspensions had a packed platelet volume of 7 to 10% and contained from 0.97 to 1.28 x 10¹⁰ platelet per ml. Two-milliliter aliquots were removed at intervals of 0 and 60 min and were added to 10 ml of Ringer's solution. After centrifugation at 1465 x g, supernatant fluid was removed and a platelet pellet was suspended in 5% trichloroacetic acid. The resulting precipitate was washed and dried, and glutamic acid, aspartic acid, glutamine, and asparagine were separated from the trichloroacetic acid supernatant fluid (35). Aliquots of plasma-Ringer's supernatant fluid, platelets of trichloroacetic acid-soluble fraction, trichloroacetic acid-precipitated proteins, and separated amino acids were transferred to vials containing 10% naphthaleine, 0.4% 2,5-diphenyloxazole, and 0.03% bis-methylstyryl benzene, 10 ml per vial. Radioactivity was determined by a Packard 1n-Carb liquid scintillation spectrometer where counting efficiency was 34% and quench corrections were made with internal standards.

**Protein Binding of Cytochalasin D**—Intact platelets preincubated with [3H]cytochalasin D were suspended in Weber-Fiedler (36) solution and disrupted in a Parr cell-disruption bomb for 30 min at 4° under N₂ pressure of 1,200 p.s.i. [3H]Cytochalasin D bound by extracted thrombosthenin or thrombosthenin M was determined after filtration on Sephadex G-200 by applying 2 to 8 mg of protein to a column (1.5 x 25 cm) previously equilibrated with 0.6 M KCl-Tris-HCl buffer, pH 7.2. Effluent was collected in 1-ml fractions and the degree of binding was calculated as radioactivity per mole of protein. Capacity to bind [3H]cytochalasin D of previously isolated thrombosthenin or thrombosthenin M from platelets, and of actomyosin and myosin from muscle, was determined in a similar manner.

**Binding of [3H]Cytochalasin D to Isoalted Thrombosthenin and Thrombosthenin M from Platelets and Actomyosin, Myosin, and Actin from Muscle**—Muscle actomyosin was also studied under low ionic concentrations using 0.45-µ Millipore filtration (Millipore Corporation). Each protein (0.1 mg) was incubated at room temperature with 0.04 M imidazole buffer (pH 6.8), 1 mM CaCl₂, and 0.32 µM [3H]cytochalasin D. After 5 min of incubation, the mixture was filtered through Millipore and both protein concentration and radioactivity were determined before and after filtration. Because of the lower molecular weight of G-actin, polymerization was induced after incubation with 0.1 M KCl plus 0.2 mM ATP. In addition, muscle G-actin binding of [3H]cytochalasin D was studied by gel filtration on Sephadex G-25 columns (1 x 20 cm) equilibrated and eluted with 0.2 mM ATP plus 0.2 mM ascorbate, pH 7.5.

Experiments were also carried out to determine the concentration dependence of binding of [3H]cytochalasin D to muscle myosin. Increasing concentrations of cytochalasin D were used to give a final concentration of 0.2 to 0.8 µM. From the [3H]cytochalasin D in the absence of proteins and the corresponding concentration with protein present, the amount bound could be calculated and plotted according to the equation 

\[ \frac{[\text{cytochalasin D}]}{[\text{cytochalasin D}]} = \left( \frac{n - \delta}{K_d} \right) \]

\[ \delta \] is the average number of moles of cytochalasin D per mole of protein; [cytochalasin D] is the free drug concentration at equilibrium; \( K_d \) is the apparent intrinsic association constant; and \( n \) is the apparent number of equivalent binding sites on each protein molecule (44). As \( \frac{[\text{cytochalasin D}]}{[\text{cytochalasin D}]} \rightarrow 0, \delta \) approaches \( n \). The molecular weight of myosin was...
taken as 460,000. The heterogeneity index α describes the dispersion of association constants about the average constant; $K_n$ was calculated from the generalized binding isotherm as defined by Sips in 1948 (45). For each titration, the log $\theta$ of $\theta$ was plotted versus log [cytochalasin D] by the method of least squares and was obtained as the slope, and $K_n$ from the intercept. When $α = 1$ all sites have the same association constant, and decreasing values of $α$ correspond either to increasing heterogeneity in respect to the association constant or to heterogeneity of the cytochalasin D used in the binding studies.

**Recrystallinity of Cytochalasin D Action**—One milligram of myosin was preincubated at room temperature with 0.1 M KCl and 20 μM $[^3H]$cytochalasin D. After 5 min of incubation, KCl concentration was increased to 0.6 M. The mixture was dialyzed for 24 hours against several changes of 0.6 M KCl-Tris buffer, pH 7.2, until no detectable radioactivity could be found in the external fluid. ATPase activity was determined on the myosin and reconstituted actomyosin (myosin + actin) prior to and after addition of cytochalasin D and on the 24-hour dialysate.

**Acrylamide Disc Electrophoresis**—Sodium dodecyl sulfate disc electrophoresis was performed on single 1% sodium dodecyl sulfate, 5% acrylamide gels. Quadruplicates of 125-μg protein samples were run on a Buchler electrophoresis apparatus. A current of 10 mA per gel was applied until the tracking dye reached the bottom of the gel. Duplicate gels were immersed for 1 hour in 1% Amido schwarz in 7% acetic acid and destained automatically with a diffusion destainer (Hoefer Scientific Instruments). The remaining pair of gels were cut into 1-mm slices and each slice was assayed for its radioactivity.

**Superprecipitation**—Superprecipitation was recorded by measuring absorbance of muscle actomyosin suspensions at 620 nm. Incubation time was 15 min at 25°.

<table>
<thead>
<tr>
<th>Cation</th>
<th>No. of preparation</th>
<th>Thrombochalin D-thrombosthenin</th>
<th>Myosin</th>
<th>Cytochalasin D-myosin</th>
<th>Cytochalasin D-thrombosthenin + actin</th>
<th>Thrombochalin D-thrombosthenin M + cytochalasin D</th>
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<tr>
<td></td>
<td>$\mu$moles P$_i$/mg protein/30 min</td>
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<td>1.92</td>
<td>2.10</td>
<td>0.79</td>
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**Effect of cytochalasin D on ATPase activity of muscle actomyosin, myosin, and reconstituted actomyosin**

The incubation medium contained 0.04 M imidazole buffer (pH 6.8), 0.06 M KCl, 0.05 μM ATP, 1 mM Ca$^{2+}$ or Mg$^{2+}$, 0.1 mM ouabain, 0.1 mg of thrombosthenin or thrombosthenin M, and 0.05 mg of actin. Incubation time was 15 min at 25°.

**RESULTS**

**ATPase Activity and Superprecipitation**—Changes in the ATPase activity of the actomyosin complex can reflect alterations in the ATPase activity of myosin alone, without influencing the interaction between actin and myosin. Also ATPase activity can be inhibited by agents which cause actin and myosin to be dissociated. The effect of cytochalasin D on both of these aspects was studied by measuring the influence of the drug on the ATPase activity of platelet and muscle contractile protein and the effect of cytochalasin D on muscle actomyosin dissociation and superprecipitation by ATP.

ATPase activity of actin, myosin, and actomyosin from muscle and thrombosthenin, thrombosthenin M from platelets, and various combinations with and without prior treatment with cytochalasin D is shown in Tables I and II. Cytochalasin D had no effect on either thrombosthenin or muscle actomyosin in regard to Ca$^{2+}$ and Mg$^{2+}$ ATPase activity. Cytochalasin D inhibited approximately 90 and 62% of the Ca$^{2+}$/ATPase activity of both myosin and platelet thrombosthenin M, respectively. When myosin and actin or thrombosthenin M and actin were combined first and cytochalasin D added later to the mixture, no inhibitory effect on the ATPase activity of either actomyosin or actin-thrombosthenin M complexes was seen. In contrast, when untreated actin was added either to cytochalasin D-treated myosin or cytochalasin D-treated thrombosthenin M, actin failed to restore either actomyosin or actin-thrombosthenin M ATPase activities. However, when untreated myosin and thrombosthenin M were combined with treated actin, ATPase activity of both actomyosin and actin-thrombosthenin M was restored.

Comparative behavior on superprecipitation of muscle actomyosin in relation to treatment with or without cytochalasin D
prior addition of Mg$^{2+}$ATP is described in the following experimental data. Results of a typical experiment are presented in Fig. 1. At an ionic strength of 0.15, the clearing phase of actomyosin was prolonged for 10 min, a condition found necessary to allow cytochalasin D to interact with the myosin moiety. Increasing concentrations of cytochalasin D progressively prolonged the clearing phase. Finally, at a concentration of 8 $\mu$m, protein suspension superprecipitation was completely inhibited. Interference by cytochalasin D with formation of actomyosin complex is consistent with results of drug inhibition of ATPase activity.

**Incorporation and Protein-binding of [3H]Cytochalasin D—**

Incorporation of platelets with [3H]cytochalasin D resulted in rapid incorporation of the label into the cell (Fig. 2). At 5 min, maximum platelet labeling was achieved with 63% cellular incorporation. The distribution of the 63% radioactivity within these platelets showed 78% in the trichloroacetic acid-soluble fraction and 22% in trichloroacetic acid-precipitated proteins. No radioactivity was found in the studied amino acids. Repeated washes of the precipitated proteins with 5% trichloroacetic acid showed release of radioactivity to the supernatant fluid.

The target site for cytochalasin D effect was investigated by studying its binding capacity to the major proteins of the contractile system. Purified thrombosthenin M fractions obtained from platelets preincubated with [3H]cytochalasin D exhibited minimal binding capacity. Fig. 3A shows the elution pattern of protein-bound cytochalasin D after filtration on Sephadex G-200 equilibrated with 0.6 M KCl. The protein emerged in the void volume and contained 0.15 mole of cytochalasin D per mole of thrombosthenin M fractions obtained from intact platelets preincubated with 2 $\mu$m [3H]cytochalasin D per ml of platelet suspension were filtered through Sephadex G-200 (1.5 X 35 cm) equilibrated with 0.6 M KCl-Tris buffer, pH 7.2. One-milliliter fractions were collected. Radioactivity associated with the protein peak represented 0.15 mole of cytochalasin D per mole of protein. B, thrombosthenin, obtained from intact platelets preincubated with 2 $\mu$m [3H]cytochalasin D and processed as described in A. C, previously isolated thrombosthenin M incubated with 2 $\mu$m [3H]cytochalasin D and filtered through Sephadex G-200 as described in A. The protein was eluted in the void volume devoided of radioactivity. Unbound [3H]cytochalasin D emerged after the proteins. D, binding of cytochalasin D to muscle G-actin was studied by gel filtration on Sephadex G-25. Two milligrams of protein incubated with 2 $\mu$m [3H]cytochalasin D were applied to a column (1 X 20 cm) equilibrated with 0.2 mM ATP-0.2 mM ascorbate, pH 7.5.
mole of protein. Unbound cytochalasin D emerged from the column after complete elution of the protein. Thrombosthenin (Fig. 3B) exhibited considerably less binding than thrombosthenin M (0.08 mole of cytochalasin D per mole of protein). When, on the other hand, thrombosthenin M was first extracted and purified and then incubated with cytochalasin D, a different pattern emerged. Fig. 3C shows the elution pattern on Sephadex G-200 of purified thrombosthenin M prepared in this way. No radioactivity was associated with the protein peak. Purified muscle actomyosin and myosin incubated with [3H]cytochalasin D also showed no protein binding when high ionic strength conditions were set. Sephadex G-25, equilibrated with ATP and ascorbate buffer, was used to study purified muscle G-actin incubated with [3H]cytochalasin D. However, no radioactivity was observed to be attached to the protein (Fig. 3D).

Binding of [3H]cytochalasin D to myosin and thrombosthenin M under conditions of low ionic strength was shown by Millipore filtration (Table III). Muscle myosin and thrombosthenin M bound 60% of added [3H]cytochalasin D, representing 0.88 and 0.86 mole, respectively, per mole of protein (mol wt 460,000). This binding, however, was lowered to only 3 to 4% when either protein was preincubated with nonradioactive cytochalasin D. Muscle actomyosin and platelet thrombosthenin M bound 16 and 11%, respectively, of added [3H]cytochalasin D, which was reduced to 1.6 and 3.8% when pretreated with cold cytochalasin D. Muscle actin bound only 5% and this could not be reduced by preincubation with nonradioactive cytochalasin D. Representative binding data for myosin under varying cytochalasin D concentrations are shown in Table IV. From Fig. 4a we found an average value of 0.99 ± 0.11 sites per 4.6 × 10^6 g mole^-1 of muscle myosin with an average intrinsic association constant of 6.2 × 10^4 M^-1.

**Reversibility of Cytochalasin D Action**—It was of interest to determine whether the effect of cytochalasin D on myosin ATPase activity was reversible or if cytochalasin D had produced alterations in the molecular conformation of myosin which were irreversible even after dissociation of cytochalasin D at high KCl concentration. After incubation with [3H]cytochalasin D, myosin Ca2+ATPase activity was inhibited, and actin could not restore the Mg2+ATPase activity. After dialysis, no measurable residual radioactivity was found to be associated with the protein. The Ca2+ATPase activity of the myosin and the ability of actin to activate Mg2+ATPase were restored (Table V).

**Acrylamide Disc Electrophoresis**—When thrombosthenin M obtained from platelets incubated in the presence of [3H]cytochalasin D was subjected to sodium dodecyl sulfate acrylamide disc electrophoresis, one major protein staining band of approximately 220,000 daltons and two very weak staining bands of

### Table III

**Binding of [3H]cytochalasin D to proteins at low ionic strength (Millipore filtration)**

<table>
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<tr>
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<th>[3H]CD bound to protein x 100</th>
<th>[3H]CD added</th>
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</thead>
<tbody>
<tr>
<td>Blank</td>
<td>2.2 ± 1.5a</td>
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</tr>
<tr>
<td>Myosin</td>
<td>60.8 ± 2.5</td>
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</tr>
<tr>
<td>CD pretreated myosinb</td>
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<tr>
<td>Actomyosin</td>
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<tr>
<td>CD pretreated actomyosinb</td>
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</tr>
<tr>
<td>Actin</td>
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<td>CD pretreated actinb</td>
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</tr>
<tr>
<td>Thrombosthenin M</td>
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</tr>
<tr>
<td>CD pretreated thrombosthenin Mb</td>
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</tr>
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<td>Thrombosthenin</td>
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<tr>
<td>CD pretreated thrombostheninb</td>
<td>3.8 ± 1.2</td>
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</table>

* Values given are mean ± S.D.

b The proteins were incubated with nonradioactive cytochalasin D prior to addition of [3H]cytochalasin D.

### Table IV

**Representative data on binding of cytochalasin D to myosin**

In incubation medium as in Table III, varying the cytochalasin D (CD) concentration between 0.2 to 0.8 μM.

<table>
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<td>μM</td>
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<tr>
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### Table V

**Reversibility of cytochalasin D action**

Myosin (1 mg per ml) was incubated with 20 μM [3H]cytochalasin D for 5 min at 25° with 0.1 M KCl buffer, pH 7.2. After incubation, KCl concentration was raised to 0.6 M and the protein solution was dialyzed for 24 hours against 0.6 M KCl. Conditions for ATPase determinations are described in the legend to Table II.

<table>
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<tr>
<th>Proteins</th>
<th>Ca2+</th>
<th>Mg2+</th>
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<tbody>
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<tr>
<td>Cytochalasin D myosin</td>
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<td>0.30</td>
</tr>
<tr>
<td>Dialyzed cytochalasin D myosin</td>
<td>9.5</td>
<td>0.32</td>
</tr>
<tr>
<td>Myosin + actin</td>
<td>11.9</td>
<td>10.5</td>
</tr>
<tr>
<td>Dialyzed cytochalasin D myosin + actin</td>
<td>10.1</td>
<td>8.7</td>
</tr>
</tbody>
</table>
smaller molecular weight were observed (Fig. 5). The [3H]-cytochalasin D was noted to migrate along with the tracking dye, to the bottom of the gels. Apparently, when sodium dodecyl sulfate depolymerized myosin into its subunits, [3H]-cytochalasin D was dissociated from the native protein sub-chain.

Thin Layer Chromatography—The entire radioactivity bound to thrombosthenin M could be removed from aqueous salt solution by organic solvent extraction. Furthermore, thin layer chromatographic analysis of the [3H]cytochalasin D-treated thrombosthenin M complex directly applied to Eastman 6060 silica sheets (solvent, toluene-methanol, 75:10) revealed that 90% of the radioactivity placed initially at the origin was located at one spot, RF 0.50, coincident with authentic cytochalasin D migrating on parallel guide strips. These results demonstrated that the alkaloid bond to the protein was not covalent in nature and also indicated that there had been no metabolism of the drug by the platelets during the uptake experiments.

DISCUSSION

Blood platelets have been reported to possess a considerable amount of an actomyosin-like protein, called thrombosthenin, which is believed to mediate clot retraction. Shepro et al. (46) and Wessells et al. (47), reported inhibition of clot retraction by cytochalasin B and recent work by White (48) extended these observations suggesting that cytochalasin B prevented changes in platelet shape as manifested by a prolongation of the primary aggregation phase that leads to inhibition of the platelet's clot-retracting capacity.

In the present report, an attempt was made to correlate platelet function with biochemical changes as manifested by the action of cytochalasins. Cytochalasin D was of interest because of its high potency and its availability as a tritiated compound, [3H]cytochalasin D.

The effect of cytochalasin D on the contractile mechanisms was studied first by measuring ATPase activity of thrombosthenin, thrombosthenin M, myosin, and actomyosin both before and after actin activation and alone or combined with cytochalasin D. It was apparent that cytochalasin D exerted considerable effect because cytochalasin D-treated myosin and cytochalasin D-thrombosthenin M had their Ca²⁺-ATPase activity inhibited. Furthermore, the addition of actin to either cytochalasin D-myosin or cytochalasin D-thrombosthenin M failed to restore Ca²⁺- or Mg²⁺-ATPase activity. Interestingly, the reverse combination, untreated myosin or thrombosthenin M with cytochalasin D-treated actin presented normal ATPase activity of the complex molecule. Extracted platelet thrombosthenin and muscle actomyosin, when incubated with cytochalasin D, showed no inhibition of ATPase activity. These data suggest that, when actin and myosin or thrombosthenin M and actin are combined, cytochalasin D does not affect ATPase activity of the complex.

The results obtained by mixing either untreated myosin or cytochalasin D-treated myosin with actin, and of mixing untreated thrombosthenin M or cytochalasin D-treated thrombosthenin M with actin suggest that actin and cytochalasin D bind to the same sites of both myosin and thrombosthenin M. Furthermore, it was apparent that when actin and cytochalasin D were added simultaneously to myosin or thrombosthenin M, actin was preferentially bound. However, when myosin or thrombosthenin M sites were previously blocked by cytochalasin D, actin was unable to react with either. These results differ from those of Spudich and Lin (30) who suggested that cytochalasin B appeared to interact with actin rather than with myosin. However, conditions of their assay were different; in particular, the high concentrations of cytochalasin B used could have been responsible for nonspecific effects. Spudich has further reported (49) that the activation of muscle HMM by platelet actin is not inhibited by cytochalasin B. Forer et al. (31), using the same drug concentration as Spudich and Lin (30) (1 mM cytochalasin B), concluded that F-actin was unaffected structurally or functionally, and that actin binding and release of HMM was normal. Their experiments were performed either by incubating actin with cytochalasin B to which HMM was added, or by mixing F-actin, cytochalasin B, and HMM. They found no blocking of HMM binding by actin nor inhibition of release of HMM from actin when ATP was added. This agrees with our results obtained when in the reaction mixture,
myosin, actin, and cytochalasin D were added simultaneously. Cytochalasin D did not interfere with the formation of the acto-myosin complex. Apparently under these conditions myosin binds preferentially to actin rather than to cytochalasin D. Restoration of the Ca$^{2+}$-ATPase activity and actin activation of the Mg$^{2+}$-ATPase on the dialyzed cytochalasin D-myosin indicated that the effect of cytochalasin D on myosin was reversible and that no irreversible alteration in the molecular conformation occurred.

From the results obtained by measuring the effect of cytochalasin D on superprecipitation, we found that cytochalasin D did not interfere with the dissociation of actomyosin into actin and myosin by MgATP. Dissociation of actomyosin by Mg ATP was evident by the decrease in optical density (clearing phase). When the clearing phase was prolonged at 0.15 M KCl, increasing amounts of cytochalasin D were found to inhibit proportionally reassociation of myosin to actin as manifested by complete inhibition of superprecipitation with 8 μM cytochalasin D. Wessels et al. (47), using cytochalasin B, failed to observe such an effect because superprecipitation was assessed only qualitatively and because conditions for clearing could not be established. Alternatively, differences in potential between cytochalasin B and cytochalasin D could account for the discrepancy of effects.

Our results are also consistent with those obtained by measuring the effect of cytochalasin D on the ATPase activity of thrombosthenin M and myosin (Tables I and II). On the other hand, dissociation of actomyosin into actin and myosin by ATP at 0.06 M KCl is nonmeasurable. Under these conditions cytochalasin D apparently could not bind to myosin; therefore, the ATPase activity of actomyosin was unaltered.

Intact platelets when incubated with [3H]cytochalasin D showed rapid incorporation of label, without detectable (thin layer chromatography) metabolite transformation of the drug. Platelet amino acids, including aspartic and glutamic acids and their amides, were not radioactive, which precluded degradation of the alkaloid through the tricarboxylic acid cycle.

Of the total amount of tritiated drug sequestered by the platelets, 22% was initially located in trichloroacetic acid-precipitated fractions. Serial trichloroacetic acid washings from such precipitates were found to contain labeled cytochalasin D; thus, it appears that the denaturing action of trichloroacetic acid affected the release of radioactivity from protein binding regions. The quantitative determination of cytochalasin D which could be bound in vitro by platelet- and muscle-derived proteins was difficult to assess by gel filtration because of the high ionic strength which was required (0.6) to maintain these proteins in solution; indeed, contractile proteins extracted from these sources and which were incubated with [3H]cytochalasin D failed to show coincident drug binding upon passage through Sephadex G-200. However, thrombosthenin and thrombosthenin M from platelets with prior exposure to the tritiated drug, when subjected to gel filtration, still contained measurable amounts of drug associated with these protein peaks. More radioactivity was associated with thrombosthenin M (0.15 mole of cytochalasin D per mole of protein) than with thrombosthenin (0.08 mole of cytochalasin D per mole of protein).

Consequently, other means were sought to determine binding at lower ionic strength. Initial attempts at equilibrium dialysis over a period of 24 hours proved unsuccessful, probably due to protein denaturation over this extended period of time. Binding of cytochalasin D by thrombosthenin, thrombosthenin M, actomyosin, myosin, and actin was measured by the technique of Millipore filtration. This evolved into the method of choice because it was rapid and, while it could be shown that free cytochalasin D minimally contaminated the filters, the proteins, as present in their aggregated states, were retained. To retain G-actin on the filter, it was necessary first to polymerize it to F-actin. Muscle myosin and thrombosthenin M were shown by this method to bind 0.88 and 0.86 mole of cytochalasin D per mole of protein, respectively. Proteins first exposed to nonradioactive cytochalasin D were completely blocked from further binding of [3H]cytochalasin D. These experiments indicated that cytochalasin D binding was nonexchangeable at low ionic strength and, more importantly, that binding of [3H]cytochalasin D was not due to nonspecific adsorption. Yet, a small amount of cytochalasin D was found bound to actomyosin from muscle and thrombosthenin from platelets. This result was interpreted as probably being due to the presence of dissociated myosin or thrombosthenin M in these preparations. Negligible amounts of radioactive alkaloid binding was associated with actin.

The interdependence of the two actin-binding sites has been reported by Barany (50). Young's (61) work has suggested that only one of the two subunits of the head portion of HMM may be able to bind to F-actin at any given time. The results obtained from kinetic studies of binding using different concentrations of cytochalasin D gave values of a maximal binding site of approximately 1. Therefore, it is possible that, if cytochalasin D binds to one of the actin-binding sites on myosin, the reactivity of the other actin binding site may be affected, resulting in the inability of actin to interact. Another possibility would be that cytochalasin D possesses two reactive groups capable of interacting with both sites on the myosin molecule. The fact that the heterogeneity index is 1 suggests that cytochalasin D binds to a homogeneous reactive site.

The effect of cytochalasin D on HMM was not studied. Efforts to prepare this protein by adapting methods used for muscle to human platelets failed to yield the like protein fragment; although a limited amount of platelet fragments can be obtained, efforts toward obtaining platelet HMM fragment are still in progress. Since subcellular localization of thrombosthenin in platelets is still unclear, it therefore cannot be concluded as to whether cytochalasin D penetrates the cell membrane to bind to the cytoplasmic thrombosthenin M or whether it binds to thrombosthenin M possibly located at the cell surface. It should be noted parenthetically, in work done with HeLa and MDBK cells, that radioautographic microscopy has provided evidence that [3H]cytochalasin D is present throughout the cytoplasma of these cultured cell lines.4

Thin layer chromatography of thrombosthenin M obtained after incubation of platelets with [3H]cytochalasin D showed that the label could be dissociated from the protein by organic solvents. In thin layer chromatography, the dissociated tritium label migrated identically to controls of free [3H]cytochalasin D on parallel lanes. The finding that cytochalasin D bound to thrombosthenin M was dissociated from protein species in sodium dodecyl sulfate acrylamide electrophoresis also reinforces the notion that cytochalasin D binds noncovalently to thrombosthenin M.

Some speculation on the nature of the binding of cytochalasin D to myosins is in order. Since the drug is lipophilic, and indeed contains neither functional nor potentially ionizable groups, hydrophobic bonding would appear to be one mechanism for an attachment which survives gel filtration and dialysis at low ionic strength which survives gel filtration and dialysis at low ionic strength.
strengths. It is hypothesized that a site on the multichain myosin which might involve such bonding could be formed by a cluster of amino acids at or near the ATPase catalytic centers, and one which would be rich in residues containing nonpolar side chains. Alternatively, the alkald might be bound to myosin by means of complex formation with a monovalent cation together with a negatively charged group on the protein, since it has been shown that cytochalasin B crystallizes as its silver fluoroborate complex (52).

The availability of high specific activity [3H]cytochalasin D has allowed the interaction of this compound to be studied stoichiometrically, thus explaining in part prior phenomena observed with congenic cytochalasins, such as cytochalasin B.

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