slender branches (Figs. 1B and 2). Two of the branches extend approximately 70 μm and terminate in rounded tips. The third branch is shorter, measuring 21 μm long, and exhibits an inflated tip. The tube measures 7 μm in diameter at its emergence from the grain, and the individual branches each measure approximately 3 to 4 μm in diameter.

One could possibly interpret this tube as a branched, filamentous fungal hypha of the type that occasionally occurs in petrified fossil material (*9*). This latter interpretation seems improbable, however, since the tube clearly extends from the distal sulcus of the grain (Fig. 1C). In addition, there is no evidence of fungal hypha within the ovule, or in the surrounding matrix. The tube is also unlike the other contents of the pollen chamber (Fig. 1, B and C) and therefore probably is not formed by the collapsed remnants of cell walls.

Branched pollen tubes are characteristic of living cycads, *Ginkgo*, and some conifers. The structure of the *Vesicaspora*-type grain compares most closely with that of some conifers in the Pinaceae (*8*), but the pollen in this family exhibits little or no branching of pollen tubes. On the other hand, the fossil pollen tube compares more favorably with the branched pollen tubes of some species in the Araucariaceae and Taxodiaceae (*10*). The available evidence does not indicate whether this type of fossil microgametophyte achieved siphonogamy (sperm-carrying pollen tubes), or whether the tube was merely haustorial in function. The discovery of this structure does, however, indicate that pollen tubes evolved as early as the Middle Pennsylvanian, and furthermore suggests that some pteridosperms produced relatively advanced microgametophytes comparable to those of many living gymnosperms.

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Cytochalasin B: Does It Affect Actin-Like Filaments?

Abstract. *An in vitro system was used to test the purported action of cytochalasin B. At concentrations 100 times those used for experiments in vivo, cytochalasin B did not cause the breakdown of F-actin, did not inhibit the transformation of G-actin to F-actin, did not inhibit the binding of heavy meromyosin to F-actin, and did not inhibit the adenosine triphosphate–induced release of heavy meromyosin from F-actin.*

Cytochalasin B (*1, 2*) "acts rather specifically in disrupting the function of contractile microfilament systems of cells" (*3*). The first evidence for such a specific effect was that cytochalasin B caused the disappearance of the 50- to 70-Å filaments of the "contractile ring" presumed to be responsible for cell cleavage (*4*). Other contractile systems which seem to function by the use of filaments have been affected by cytochalasin B, and "in every case so far examined microfilament morphology is altered by cytochalasin, and an identifiable biological process is concomitantly inhibited" (*3*).

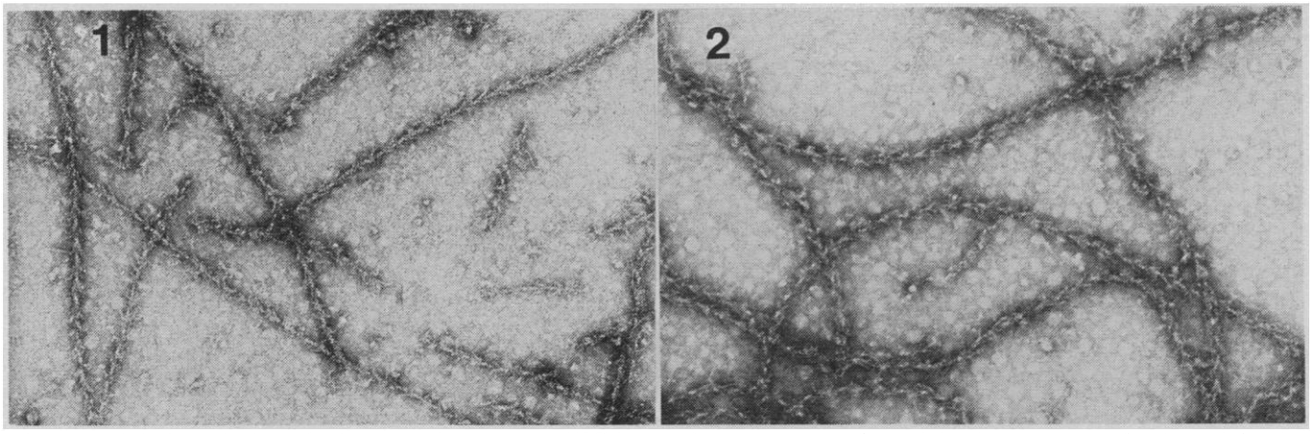
Cytochalasin B does not affect the contraction of all contractile filament systems, however; for example, it does not seem to affect the function of smooth muscle, skeletal muscle, or cardiac muscle (*5, 6*). Cytochalasin B does not block the formation of the cleavage furrow in all cells (*1, 7–9*), and, if we assume a common "contractile ring" mechanism for furrow formation, then cytochalasin B does not "disrupt the function" of the contractile ring filaments in all cells (*8*).

To clarify the action of this compound, we studied some effects of cytochalasin B on actin filaments in vitro. Since the contractile ring filaments include actin-like filaments that bind heavy meromyosin (*10*), and since blood platelets and other systems disrupted by cytochalasin B (*3, 6, 11, 12*) contain similar heavy meromyosin binding filaments (*12–14*), then one of the specific functions being disrupted may be the actin-myosin interaction. Thus, we tested whether cytochalasin B affects the structure or function (or both) of actin.

The G-actin was prepared from rabbit skeletal muscle (*15*). The F-actin was prepared by polymerization of G-actin by the addition of 2*M* KCl to the solution of G-actin to give a final concentration of 0.1*M* KCl; after 1 hour F-actin was collected by centrifugation (at 50,000*g* for 3.5 hours). Protein concentrations of G-actin and F-actin solutions were determined by the method of Lowry *et al.* (*16*) as described by Bailey (*17*). Heavy meromyosin (HMM) was prepared from rabbit skeletal muscle (*18*). Cytochalasin B (Imperial Chemical Industries, England) was dissolved in dimethyl sulfoxide as a stock solution, the concentration of cytochalasin B being 5 mg/ml, and when used was added to the aqueous medium to give the final concentration desired.

The structure of F-actin filaments was not altered by cytochalasin B. F-actin was mixed with cytochalasin B to yield final concentrations of 0.15 mg of F-actin and 0.5 mg of cytochalasin B per milliliter of solution, the final concentration of cytochalasin B being 100 times that generally used for in vivo experiments (*1, 3, 4, 6, 8, 12*). After 1 hour, portions were placed on a grid, fixed with 1 percent formalin, negatively stained with 1 percent uranyl acetate, and observed in a Philips EM 300 electron microscope (*13*). The F-actin filaments looked normal, and thus were not altered by cytochalasin B.

Since functional properties might be "disrupted" without a change in morphology, we then made tests to determine whether the functional properties of F-actin were affected by cytochalasin B. We used HMM-binding as a measure of function. The F-actin and cytochalasin B were incubated for 30 minutes, and then HMM was added to give final concentrations of 0.15 mg of F-actin per milliliter, 0.5 mg of cytochalasin B per milliliter, and 0.3 mg of HMM per milliliter. After 15 minutes, portions were taken for negative staining (by the procedure described above). In the presence of cytochalasin B, HMM formed arrowhead complexes with F-actin (Fig. 1). These complexes looked morphologically the same as those in the controls (Fig. 2). Thus cytochalasin B did not block HMM-binding to the



Figs. 1 and 2. Negatively stained preparation of actin-HMM arrowhead complexes formed in the presence (Fig. 1) and in the absence (Fig. 2) of cytochalasin B (× 76,000).

filaments. Since actin-myosin interactions in skeletal muscle involve cycles of binding and release (*19*), it was conceivable that cytochalasin altered functional properties by blocking the release of HMM after the HMM was bound to actin. We tested this possibility as follows: F-actin, cytochalasin B, and HMM were incubated, and then adenosine triphosphate (ATP) was added to cause the release of HMM from the actin (*20*). [The ATP was dissolved in standard salt solution (*14*) at a concentration of 0.01*M* and a final *p*H of 6.8. After addition of ATP to the mixture of F-actin, cytochalasin B, and HMM the final concentrations were 0.15 mg of F-actin per milliliter, 0.5 mg of cytochalasin B per milliliter, 0.3 mg of HMM per milliliter, and 0.004*M* of ATP.] Fifteen minutes after addition of ATP, portions were taken for negative staining. The actin had no arrowheads and looked normal. Thus cytochalasin B blocks neither the binding of HMM to actin nor the ATP-induced release of HMM from actin, and thus does not interfere with these functional properties of the actin-myosin system.

There is a further possibility that, although the cytochalasin B does not disrupt the function of filaments that are already present (*4*), it inhibits filament assembly. We therefore polymerized G-actin into F-actin in the presence of cytochalasin B. The G-actin, tris buffer (0.01*M*, *p*H 7.9 as measured at 4°C), mercaptoethanol, and cytochalasin B were mixed, and 60 minutes later 2*M* KCl was added in order to polymerize G-actin to F-actin. (The final concentrations were 0.35 mg of G-actin per milliliter, 0.008*M* tris, 0.04*M* mercaptoethanol, 0.5 mg of cytochalasin B per milliliter, and 0.1*M* KCl.) After 2 hours, portions were removed for negative staining (as described above). F-actin filaments were seen and appeared normal. In the continued presence of cytochalasin B the filaments reacted with HMM to form arrowhead complexes, and ATP caused release of the HMM from the F-actin. Thus, cytochalasin B did not prevent the assembly of functional F-actin filaments, nor the subsequent binding and release of HMM to these filaments.

We conclude that cytochalasin B does not "disrupt the function" of F-actin filaments in vitro and does not prevent the formation of functional F-actin from G-actin. Blood platelet filaments of actin-like material are also formed in the presence of cytochalasin B (*21*), and such filaments bind HMM (*22*). Functional actin in chick skeletal muscle is assembled in the presence of cytochalasin B (*6*). Thus we expect our results to apply to the in vivo experiments, and we expect no direct effect of cytochalasin B on cytoplasmic actin-like filaments.

It is possible that cytochalasin B affects the contractile filament systems in cells by acting on the myosin component; our experiments say nothing about this. Against this possibility, however, we should point out that in our experiments cytochalasin B had no apparent effect on HMM. In addition, other experiments have shown that (i) filamentous aggregates of myosin from blood platelets (*13*) are seen after treatment of the platelets with cytochalasin B (*22*); (ii) cytochalasin B has no effect on cardiac, smooth, or skeletal muscle function in Ascidian larvae (*5*); and (iii) cytochalasin B does not block the assembly of functional myosin in chick skeletal muscle in tissue culture (*6*). Furthermore, while cytochalasin B does not affect chromosome motion (*1, 4, 9*) and does not affect sperm tail motion (*3*), we have found actin-like filaments associated with chromosomes in spindles and in sperm tails, suggesting that actin-myosin interactions may indeed be responsible for chromosome and sperm motility (*23*). Similarly, actin-like filaments are found in the "contractile ring," and yet cytochalasin B does not block the furrowing in L cells (*1, 9*) or *Xenopus* eggs (*7, 8*). Finally, many effects of cytochalasin B are difficult to explain solely in terms of an effect on filament systems. (i) Nuclei are shunted out of cells on long cytoplasmic stalks and are sometimes even extruded from these cells (*1, 24*); (ii) mitotic rate is reduced (*1, 6*); (iii) nuclei are displaced toward the periphery of myotubes (*6*); and (iv) the adhesion of blood platelets is altered (*21*). Thus, since F-actin is unaffected structurally and functionally, since the HMM binding and release are unaffected, and since there is no evidence that cytochalasin B affects the myosin component in cellular cytoplasm, we must conclude that we do not know yet the sites of action of cytochalasin B.

We should also point out that even if cytochalasin B affected the myosin component of the contractile filament systems in cytoplasm, this does not eliminate the cell membrane or other sites of action which could be affected concomitantly (*25*).

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Wound-Induced Proteinase Inhibitor in Plant Leaves: A Possible Defense Mechanism against Insects

Abstract. *Wounding of the leaves of potato or tomato plants by adult Colorado potato beetles, or their larvae, induces a rapid accumulation of a proteinase inhibitor throughout the plants' tissues that are exposed to air. This effect of insect damage can be simulated by mechanically wounding the leaves. The transport of a factor out of damaged leaves takes place rapidly after the wound is inflicted and the levels of proteinase inhibitor, in both damaged and adjacent leaves, rises strikingly within a few hours. The rapid accumulation of a powerful inhibitor of major intestinal proteinases of animals in response to wounding of the leaves is probably a defense mechanism.*

The function of naturally occurring proteinase inhibitors in plant tissues has been the subject of speculation (*1*). These inhibitors are usually found in high concentrations in plant storage organs such as seeds or tubers. Some of these proteins have the capacity to inhibit proteolytic enzymes of insect and microbial origins, but rarely proteolytic enzymes of plant origin (*2*). Because of their specificities, they may be protective agents against invading microorganisms and insects (*3*, *4*). The arguments for proteinase inhibitors functioning in seeds as protective agents against insects (*4*) are based on the ability of several of them to inhibit insect digestive proteinases. Our recent discovery of the presence, in potato tubers, of a powerful inhibitor of the two major animal pancreatic exopeptidases (carboxypeptidase A and B) (*5*) further supports the argument for a protective function.

We have been studying the biochemistry, physiology, and function of a well-characterized protein from potato tubers called "inhibitor I," a potent inhibitor of the animal endopeptidases, chymotrypsin and trypsin (*6*). By immunological techniques, we found that this protein was present in potato and tomato leaves as well as in potato tubers. In leaves of both species, the inhibitor was a transient component and was present, in general, at periods preceding and during the breaking of apical dominance. In many instances, its presence in leaves could be correlated with a physiological event. Occasionally, however, differences in amount of inhibitor I in plants of the same age were extraordinary (five- to tenfold variations). Such results led us to suspect that some environmental factor might be responsible for this variability; microorganisms, insects, or physical injury were likely candidates.

Table 1. Colorado potato beetle-induced accumulation of chymotrypsin inhibitor I in tomato plants. Each value is an average obtained from 11 trifoliate leaves (from the second leaf down from apex). Ranges are given in parentheses. Adult beetles were allowed to feed randomly on plants for 24 hours. After an additional 24 hours the tissues were assayed immunologically for inhibitor I (*7*). Experiments were carried out in a greenhouse under natural light. Leaf damage varied from minor damage of a single leaflet to severe damage of all leaflets. The accumulation of inhibitor in leaves varied in proportion to the insect damage inflicted on the plants.

Leaf damage	Average inhibitor I concentration (μg/ml) in:		
	Leaves	Main stem	Roots
Beetle	202 (77–235)	52 (0–73)	<15
No damage	47 (0–120)	<15	<15

The effect of insect damage on inhibitor I concentrations was tested by allowing Colorado potato beetles, common pests of potatoes and tomatoes, to feed on the leaves of young tomato plants. Concentrations of inhibitor I in damaged and undamaged leaves of the plants were subsequently assayed immunologically (*7*). As shown in Table 1, we found that leaves of beetle-infested plants accumulated greater amounts of inhibitor I than did uninfested control plants. Data from individual assays showed that both damaged and apparently undamaged leaves from the beetle-infested plants had accumulated inhibitor I.

We confirmed the observation that damage by beetles to a single leaf could effect the concentration of inhibitor I in undamaged leaves. Adult beetles were then allowed to feed on a single leaf from each of three potato plants without access to the rest of the plants. The beetles fed for 48 hours on the individual leaves and nearly consumed them. At the end of the feeding period, the unwounded leaves of the three plants had an average of 336 μg of inhibitor I per milliliter of leaf juice whereas unwounded control plants had an average of 103 μg of inhibitor I per milliliter.

The accumulation of inhibitor I in leaf tissue far removed from the wounding site suggested that an inducing factor was introduced into the vascular system of the plant when it was wounded. The origin of this factor was undetermined, however, as it could have either entered the leaf cytoplasm from the beetle or originated within the leaf in response to the wound.

The wounding of the leaf appeared to be the primary cause of the induction of inhibitor I accumulation since nearly any type of crushing would cause the same induction. Reproducible results were obtained by using a paper punch