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The effects of cytochalasins on onion seedling growth and development and their possible mode of action.

Robert W. Seagull
University of Windsor

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THE EFFECTS OF CYTOCHALASINS ON ONION SEEDLING GROWTH AND DEVELOPMENT AND THEIR POSSIBLE MODE OF ACTION

BY

ROBERT W. SEAGULL

A Thesis
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APPROVED

Wm. des O. Thomas

Donald L. Gallen

Norman F. Taylor

ABSTRACT

Changes in morphology of 4 day old seedlings of onion, Allium cepa, elicited by cytochalasin B (CB) at 20 ug/ml, are due to decreased water uptake. Decreased seedling and cell extension is accompanied by the development of curvatures in the cotyledon. All cytochalasins tested (CA, CB, CE, and CD) act similarly but vary in the concentration needed and the magnitude of the response. Inhibition of water uptake by CB is accompanied by 30% and 60% inhibition, respectively, of glucose uptake and incorporation into walls.

The effectiveness of cytochalasins as sulfhydryl reagents was predicted on the basis of the presence of an unsaturated ketone structure in the region of carbons 1 through 4, thus resembling the sulfhydryl active agent N-ethyl maleimide (NEM), and tested using simultaneous addition of a SH-protecting agent, Cysteine (CYS). The most potent cytochalasin, CD, 10 times more effective than CB, predicted to a weak sulfhydryl reagent, was only partially inactivated by adding CYS, while the weaker cytochalasins B, and E, were completely inactivated by CYS. This decreased susceptibility of interference by sulfhydryl groups may account for the high relative potency of CD. Addition of NEM (1.0 mM) to a low, normally ineffective concentration of CB (5.0 ug/ml), elicits a significant cytochalasin response. This cytochalasin-sparing effect of a SH-reagent indicates again that cytochalasins tend to be inactivated by binding to non-specific sulfhydryl groups. In general, the effectiveness of a cytochalasin, in this system, is inversely related to its potency as a sulfhydryl reagent.

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INTRODUCTION

Initial growth and development of the onion seedling is dependent on cell expansion. Plant cells expand using water uptake as the driving force (22). During the first nine days of growth, the seedling increases in fresh weight 6-7 fold. There is very little cell division in the rapidly elongating cotyledon (6). The cells of the developing cotyledon, after 9 days of growth, have elongated to 7-8 times their original length (6).

As elongation occurs, pushing the embryo out of the seed coat, an acute angled bend develops at an interior point along the length of the cotyledon. This bend forms the soil penetrating organ or "knee", which is carried upward by elongation of the parts of the cotyledon on either side of it (6).

Cytochalasins, fungal metabolites of Helminthosporium dematioideum, are found to produce a wide variety of effects in eukaryotic cells. Classical cytochalasin responses, as described by Carter (4), are rapid, reversible, and occure at low concentrations (1-2 ug/ml). Cytochalasin effects can be divided in two categories. The first includes phenomena involving cellular movement, both taxis and intracellular movements, presumably related to mechanochemical transductions (17). The second involves trans-membrane activities such as uptake and secretion (17).

Cytochalasins are being studied in higher plant systems. The most commonly used cytochalasin (CB) does reversibly inhibit phenomena such as streaming (11), mitosis (16), and tip growth (5), but at relatively high concentrations (20-50 ug/ml). Cytochalasin D is effective at much lower concentrations (2.0 ug/ml). The results most commonly obtained with plant materials are not "classical" cytochalasin responses in that they are slow, poorly reversible, and require high concentrations. Plant morphogenesis has been altered in several systems by cytochalasin B. Root growth in soy beans has been inhibited by CB (18). Cytochalasin B counteracts kinetin stimulation of root hair development in lettuce seedlings (13).

Cytochalasins have been studied as to their affect on onion growth and morphogenesis. Root elongation in the onion is reversibly suppressed by CB treatment of 30 ug/ml (16). Mitotic division is reversible inhibited

by CB in root tips of onions but not in other plant systems (16). The cell is altered from a typical cylindrical shape to a spheroid form (16). Cytochalasin B, when applied to the growth medium of developing onion seedlings, alters cotyledon morphology. After 48 hours treatment, a 5 day old onion seedling develops curvatures in the cotyledon on both sides of the "knee" (20). All cytochalasins tested (CA, CB, CE, and CD) produce curvatures in the onion. This ability seems to be restricted to the cytochalasins since common plant growth regulators, such as IAA, GA_3, kinetin, and ethrel (2-chloroethyl phosponic acid) all produce little or no curvatures (13).

This study was designed to examine the possible causes of the curvature response in the onion seedling. An ability to produce curvature in the onion, if similiar to curvatures in other plant systems, may be due to differential cell expansion.

The tendency for water to flow in to a cell during expansion depends on the interaction of forces related as shown below:

$$\Psi_{cell} = \Psi_{\pi} + \Psi_{p}$$

where, Ψ_{cell} = water potential of the cell. The ability of water to flow either in or out of the cell.

Ψ_{π} = osmotic potential. The contribution made by dissolved solutes to force water from an area of high concentration to one of low. (eg. from the outside to the inside of the cell)

Ψ_{p} = turgor pressure. The pressure exerted by the cell wall as water enters the cells which tends to prevent excessive water entry.

For water potential to be positive, and water to enter, osmotic potential must exceed turgor pressure. This implies that either the cell must concentrate more solutes in the cytoplasm, or the the turgor pressure must be lowered by wall expansion. Normal cell growth is associated with simultaneous changes in osmotic pressure and turgor pressure. Hence, the effects of cytochalasins on cell wall synthesis as well as on factors affecting osmotic potential were examined.

METHODS AND MATERIALS

Plant Materials

Onion seeds (var. Sweet Spanish) were surface sterilized, for 5 minutes, using 0.1% $HgCl_2$, and rinsed with distilled water. Seeds were sown on damp paper towels, and incubated 4 days, in the dark, at 22°C.

After 4 days, seedlings weighing 8-10 mg were transfered to petri dishes, each containing a Whatman #2 filter disk, soaked with 2 ml of treatment solution. For initial curvature and cell length studies, the seedlings were covered and incubated in the dark at 22°C. For water uptake, glucose uptake, and incorporation into walls, another layer of filter paper soaked with an additional 2 ml of treatment solution, was placed on the seedlings before they were covered and incubated.

Treatment Solutions

Cytochalasin stock solutions were made to contain 1 ug/ul of 100% absolute ethanol. Cytochalasin treatment solutions were made to contain, in addition to the appropriate amount of cytochalasin, a total ethanol concentration of 2% (v/v). The controls received the same ethanol concentration.

To study glucose uptake and metabolism, it was necessary to use two types of glucose solutions. Total glucose uptake into cells was determined using tritium labelled 2-deoxy-D-(1-H^3) glucose (H^3-DOG). Using this non-metabolizable form of glucose minimized respiratory loss of incorporated glucose. Treatment solutions contained 2.5 x 10^{-4} uCi/ml, in 10^{-5}M glucose. Carbon-14 labelled glucose was diluted with 10^{-5}M glucose, giving a working solution containing 1.25 uCi/ml, in order to follow the metabolized glucose into the cell walls. A much higher specific activity of C-14 glucose was needed for the metabolism study since most of the glucose taken into the cells was metabolized into CO_2, thus leaving a low percentage of glucose going into the wall. Cysteine (CYS) and N-ethyl-maleimide (NEM) solutions were made to 1.0 mM and contained 2% ethanol.

Water Uptake; Cotyledon Curvature

For water weight increase studies, seedlings were weighed prior to treatment and then weighed for 5 consecutive days. At the termination of the experiment, seedlings were dried, using a heat lamp, for 2 hours, and reweighed. Cotyledons were scored as curved when one or both arms of a cotyledon had a curvature greater than 90 degrees.

Glucose Uptake and Metabolism

Seedlings of different ages (3 and 5 days) were incubated with and without CB (20 ug/ml) for 24 hours. The seedlings were then placed in 5 cm petri dishes, each containing a filter disk soaked with 1 ml of H^3-DOG solution. After a 3 hour uptake period, the seedlings were washed in unlabelled 10^{-5}M glucose, and individually placed into scintillation vials containing 5 ml of Warner's Solubilizing Cocktail (21) and counted.

Glucose Incorporation Into Walls

Five day old seedlings, pretreated 24 hours with CB (20 ug/ml), were placed onto filter disks soaked with labelled glucose (C^{14}). After 3 hours, the seedlings were plasmolysed for 1 hour in 1.0 M sucrose, to promote separation of the plasma membrane from the wall, and homogenized, using a glass homogenizer, in the plasmolysing medium. The homogenate was centrifuged (3000 x g, 15 min). The pellet was suspended in distilled water, rehomogenized, and centrifuged (12,000 x g, 10 min). Following three distilled water washes and centrifugations, the pellet was suspended in 70% ethanol and centrifuged (12,000 x g, 10 min). Using light microscopic observation, it was seen that the pellet contained only cell wall fragments, with no whole cells observed. The cell wall fragments were collected on glass fiber filters and the dry weights of the wall fractions obtained. The wall material, on the filter disk, was counted in 5 ml of Warner's Cocktail.

Mode of Action of Cytochalasins

In comparative studies on the affects of cysteine (a sulfhydryl protecting agent) and N-ethyl-maleimide (a sulfhydryl binding agent) on the ability of cytochalasins to alter plant morphogenesis, the parameters of water uptake and curvature were examined. Experiments were set up

identical to those for water uptake, except that only initial and final weights after 2 days of treatment were measured, and the layer of filter paper covering the seedlings was omitted.

RESULTS

Cell Extension

Cytochalasin treated seedlings, in addition to having curvatures above and below the knee, show decreased seedling length (Table 1), accompanied by decreased individual cell lengths. In the curved cotyledons, CB treatment decreases the ratio of inside (concave) to outside (convex) cell lengths, thus providing an anatomical explanation for the observed curvatures. Bilateral CB application caused 54% of the seedlings to produce curvatures as compared to unilateral application, producing 73.3% of the seedlings curved (Table 2).

Water Uptake

Neither age (Table 3) nor cytochalasin treatment (Table 4) affects the dry weight of the onion seedlings. Using gravimetric analysis of seedling growth, CB at 20 ug/ml was found to decrease water uptake during a 5 day treatment (Fig 1a). A similar decrease was found using a CB concentration of 10 ug/ml (Fig 1b). Concentrations of 2.5 and 5.0 ug/ml showed no treatment affect (Fig 1c). Cytochalasin D decreased water uptake to the same extent as CB, but at one-tenth (2.0 ug/ml) the concentration (Fig 1d).

Glucose Uptake and Incorporation into Walls

A 24 hour pretreatment with CB at 20 ug/ml decreased the amount of H^3-DOG taken into the cells of 5 day old onion seedlings by 30% during a three hour incubation period in labelled medium (Table 5). By using C^{14}-glucose, CB was found to decrease the amount of label found in the washed wall fraction of 5 day old seedlings by 60% (Table 6), after a three hour incubation period. Repeating the same experiment using three day old seedlings, CB was found not to alter glucose uptake (Table 5) or incorporation into walls (Table 6).

Table 1: The Effect of 48 hour CB treatment (20 ug/ml) on seedling length, cell length, and length ratio, using 4 day-old onion seedlings.

TABLE 1

Parameter	Treatment Control 2.0% Etoh	CB 20 ug/ml	Statistical analysis t-test Comparison	Significance
Seedling length (cm)	3.74 ± 67[a]	2.26 ± .54	control x CB	0.01
Cotyledon length (cm)	2.25 ± .32	1.34 ± .35	"	0.01
Cell length convex side * (u)	10.54 ± .32	6.65 ± .48	"	0.01
Cell length concave side ** (u)	9.35 ± .50	3.77 ± .39	"	0.01
Cell length ratio concave/convex	0.89	0.57		

a = mean ±standard deviation
* = convex side; side of the curved cotyledon on the outside of the curve.
** = concave side; side of the curved cotyledon on the inside of the curve.

Table 2: The effect of unilateral and bilateral cytochalasin application on curvature of 6 day old onion seedlings pretreated for 48 hours.

TABLE 2

Treatment	Curvature # of seedlings curved as a % of total seedlings observed. Unilateral	Bilateral	Statistical analysis t-test Comparison	Significance level
Control (C)	9.2 ± .98a	8.3 ± 1.23	$C_{1*} \times C_{2*}$	NS_b
CB (CB) 20 ug/ml	73.3 ± 8.6	54.0 ± 7.6	$C_1 \times CB_1$	0.005
			$CB_1 \times CB_2$	0.01
CD (CD)	76.3 ± 5.3	50.9 ± 11.3	$CD_1 \times CD_2$	0.01
			$CB_1 \times CD_1$	NS

a = mean ± standard deviation
* = subscripts 1 and 2 denote unilateral and bilateral application, respectively
b = not significantly different

Table 3: The Effect of seedling age on wet weight and dry weight of the onion seedling.

TABLE 3

Parameters	Seedling age (days)			Statistical analysis t-test Comparison	Significance	
	0	5	9			
Dry weight (mg)	3.63 ± .57[a]	3.56 ± .46	3.29 ± .60	0 x 5	NS*	0.005**
				0 x 9	NS*	0.005**
Wet weight	———	7.21 ± 2.15	19.2 ± 3.76	5 x 9	NS*	0.005**

a = mean ± standard deviation
* = comparison of dry weight data
** = comparison of wet weight data

Table 4: The effect of CB concentration on wet and dry weights in 9 day old onion seedlings with 4 day pretreatment.

TABLE 4

Parameters	CB concentration (ug/ml) 0	10	20	Statistical analysis t-test Comparison	Significance	
Wet weight (mg)	18.2 ± 2.46[a]	13.3 ± 2.13	12.8 ± 2.13	0 x 10	NS*	0.005**
				0 x 20	NS*	0.005**
Dry weight (mg)	3.4 ± 0.39	3.4 ± 0.30	3.5 ± 0.38	10 x 20	NS*	0.005**

a = mean ± standard deviation
* = comparison of dry weight data
** = comparison of wet weight data

Figure 1a: The effect of CB (20 ug/ml) on wet weight increase of 4 day old onion seedlings over a subsequent 5 day treatment period.

Figure 1a

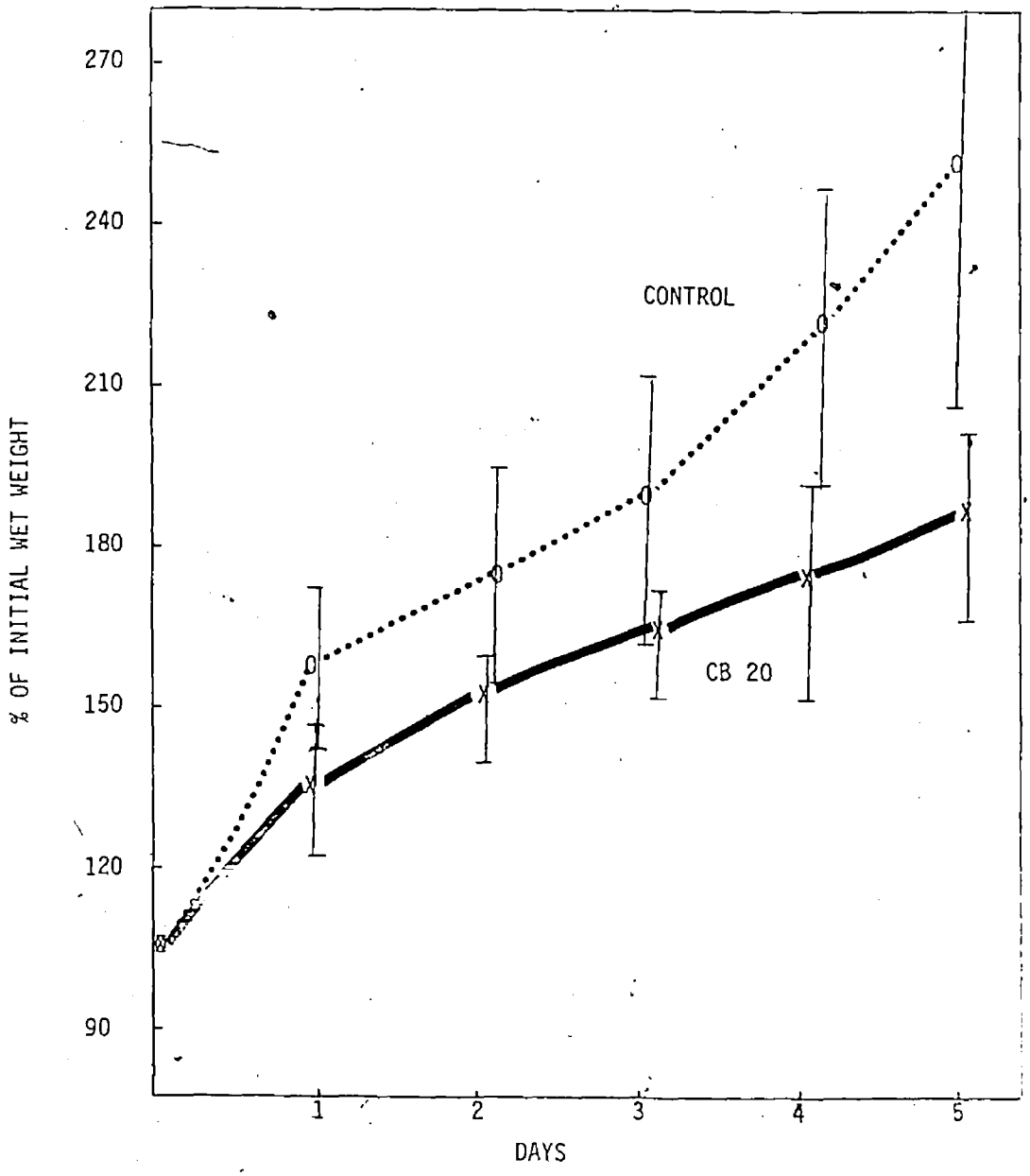


Figure 1b: The effect of CB (10 ug/ml) on wet weight increase of 4 day old onion seedlings over a subsequent 5 day treatment period.

Figure 1b

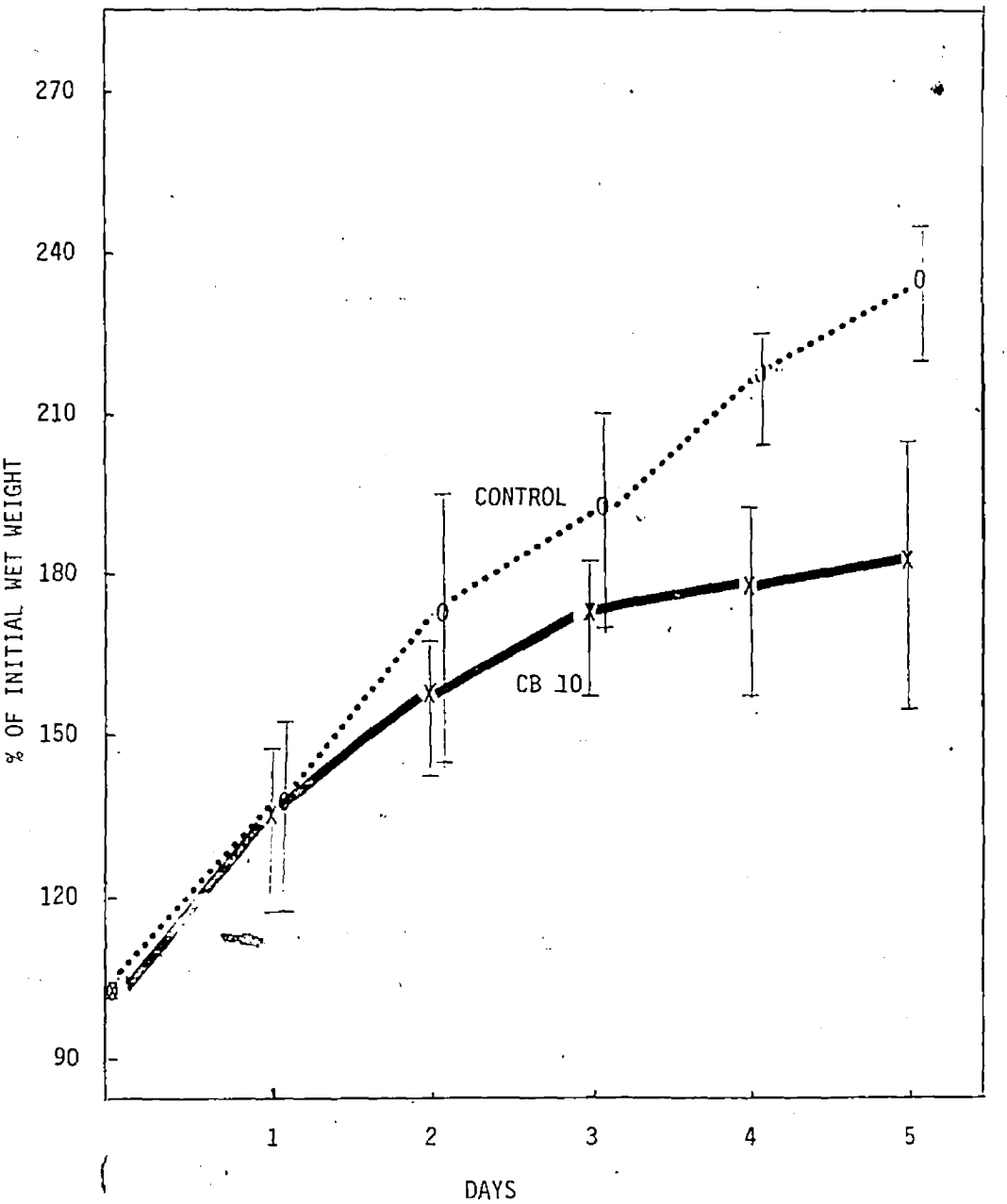


Figure 1c: The effect of low CB concentration (2.5 and 5.0 ug/ml) on wet weight increase of 4 day old onion seedlings over a 5 day treatment period.

Figure 1c

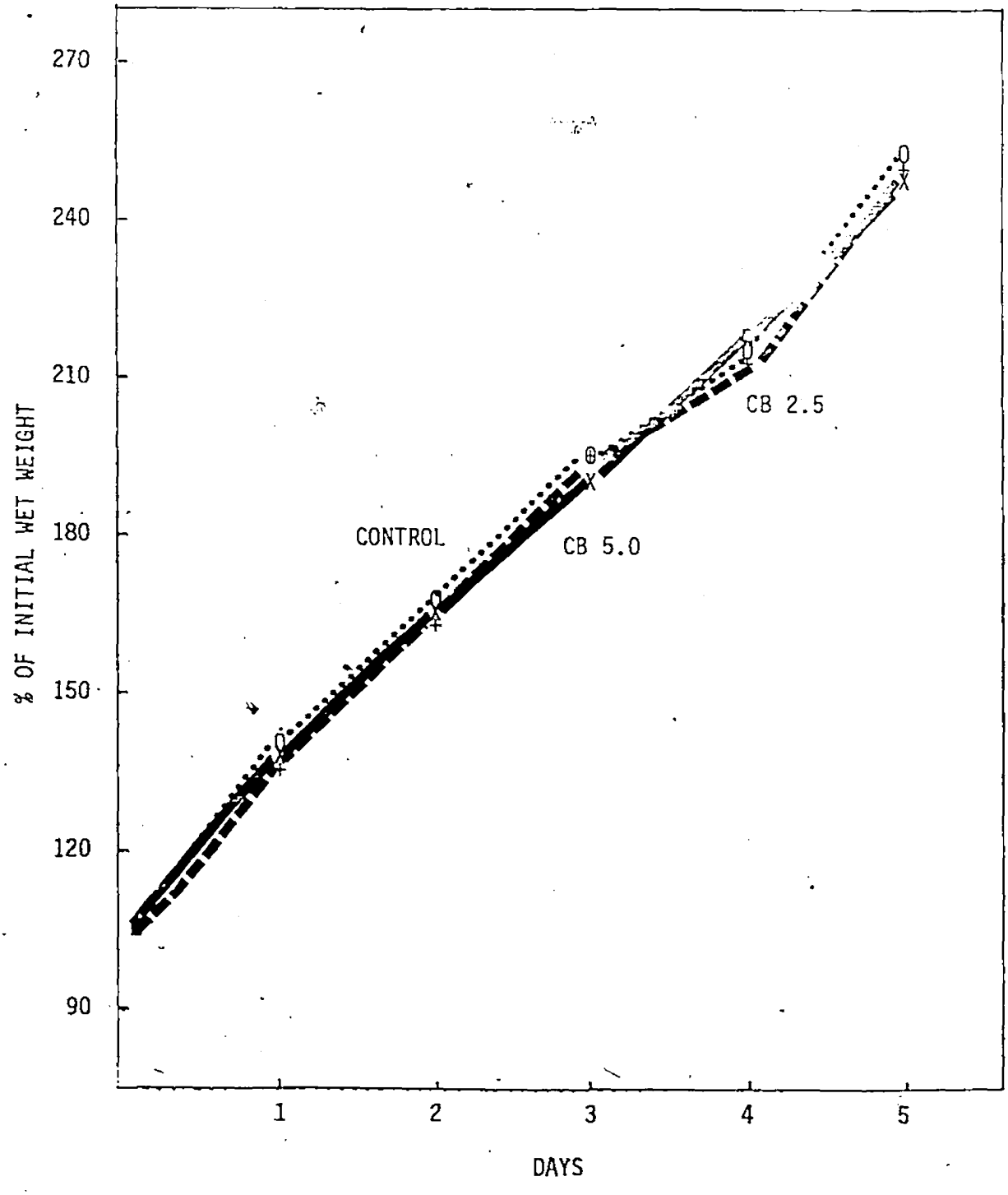


Figure 1d: The effect of CD (0.1, 1.0, and 2.0 ug/ml) on wet weight increase of 4 day old onion seedlings over a 5 day treatment period.

Figure 1d

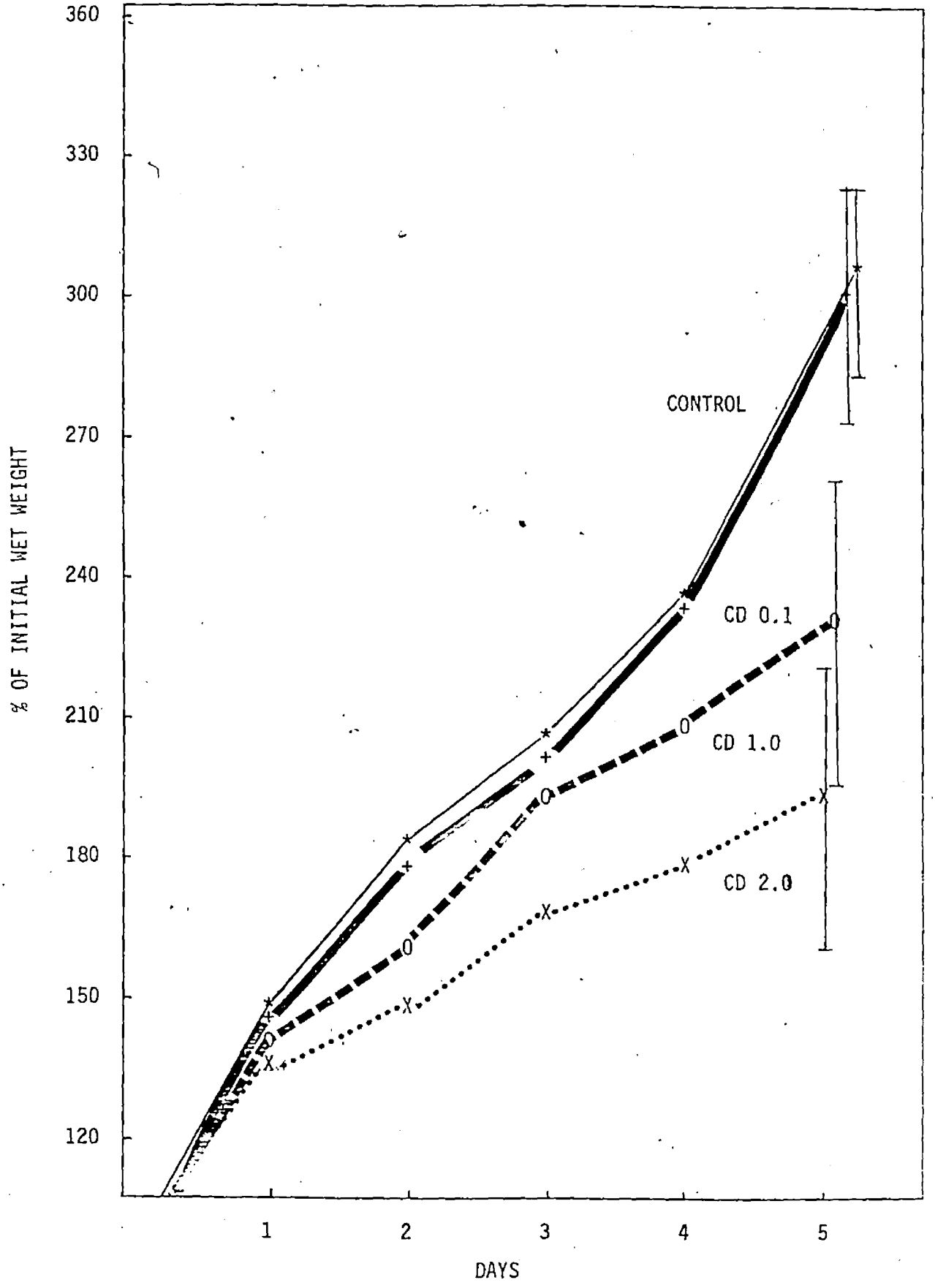


Table 5: The effect of a 24 hour pretreatment with CB (20 ug/ml) on H^3-deoxy-glucose uptake, over a 3 hour period, in 3 and 5 day old onion seedlings.

TABLE 5

Parameters	Seedling age (days)	Treatment Control 2% Etoh	CB 20 ug/ml	Statistical analysis t-test Comparison	Significance
H^3-DOG uptake CPM/mg fresh weight	3	215 ± 64[a]	214 ± 84	C x CB*	NS
	5	326 ± 84	196 ± 38	C x CB**	0.005

a = mean ± standard deviation
* = comparison of 3 day old seedling data
** = comparison of 5 day old seedling data

Table 6: The effect of a 24 hour pretreatment with CB(20 ug/ml) on C^{14}-glucose incorporation into washed wall fractions of 3 and 5 day old onion seedlings.

TABLE 6

Parameters	Seedling age (days)	Treatment Control (C) 2% Etoh	CB (CB) 20 ug/ml	Statistical analysis t-test Comparison	Significance
C^{14}-glucose incorporated into washed wall fraction CPM/mg washed wall	3	1225 ± 14[a]	1236 ± 26	C x CB*	NS
	5	1730 ± 176	523 ± 21	C x CB**	0.005

a = mean ± standard deviation
* = comparison of 3 day old seedling data
** = comparison of 5 day old seedling data

Cytochalasins: Possible Action as Sulfhydryl Agents

Cytochalasin B, when applied in the presence of 1.0 mM cysteine, has no affect on curvature or water uptake (Table 7) in 4 day old seedlings. Cysteine alone showed no effect on the parameters measured. Cytochalasin E showed similar results to CB (Table 8). The addition of cysteine (1 mM) tends to diminish the effects of CD (2.0 ug/ml) on curvature and water uptake in 4 day old seedlings (Table 9). The addition of the sulfhydryl binding agent, NEM, to low, normally ineffective CB concentrations (5 ug/ml) produces significant inhibition of water uptake, accompanied by an increase in seedling curvatures (Table 10). Applied alone, NEM produces no detectable response in the onion seedling.

Table 7: The effect of a 48 hour treatment with CB (20 ug/ml), with and without the addition of cysteine (1.0 mM), on curvature and water uptake in the 4 day old onion seedling.

TABLE 7

Treatment	Seedlings with curvatures (%)	Wet weight after 48 hour treatment (mg)	Statistical analysis t-test Comparison	Significance
Control (C) 2% Etoh	0	15.6 ± 1.6[a]	C x CB*	0.005
			C x CYS*	NS
CB (CB) 20 ug/ml	60	12.8 ± 2.0		
			C x CYS+CB*	NS
Cysteine (CYS) 1.0 mM	0	16.3 ± 2.8	CB+CYS x CB*	0.01
			CB+CYS x CYS	NS
CB + CYS	15	15.5 ± 1.7		

a = mean ± standard deviation
* = comparison of wet weight data

Table 8: The effect of a 48 hour treatment with CE (20 ug/ml), with and without the addition of cysteine (1.0 mM), on water uptake and curvature in 4 day old onion seedlings.

TABLE 8

Treatment	Seedlings with curvatures (%)	Wet weight after 48 hour treatment (mg)	Statistical analysis t-test Comparison	Significance
Control (C) 2% Etoh	0	16.3 ± 2.3[a]	C x CE*	0.005
CE (CE) 20 ug/ml	70	13.5 ± 1.8	C x CYS*	NS
Cysteine (CYS) 1.0 mM	0	15.6 ± 1.9	C x CE+CYS*	NS
CE + CYS	45	15.1 ± 1.5	CYS x CE+CYS	NS
			CYS x CE*	0.01

a = mean ± standard deviation
* = comparison of wet weight data

Table 9: The effect of a 48-hour treatment with CD (2.0 ug/ml), with and without the addition of cysteine (1.0 mM), on curvature and water uptake in the 4 day old onion seedling.

TABLE 9

Treatment	Seedlings with curvatures (%)	Wet weight after 48 hour treatment (mg)	Statistical analysis t-test Comparison	Significance
Control (C) 2% Etoh	0	16.3 ± 2.3[a]	C x CD*	0.005
			C x CYS*	NS
CD (CD) 2.0 ug/ml	80	11.5 ± 1.5	C x CD+CYS*	0.001
Cysteine (CYS) 1.0 mM	0	15.1 ± 2.0	CYS x CD*	0.005
			CYS x CD+CYS*	0.005
CD + CYS	70	12.8 ± 2.1		

a = mean ± standard deviation
* = comparison of wet weight data

Table 10: The effect of a 48 hour treatment of a low CB concentration (5.0 ug/ml), with and without the addition of NEM (1.0 mM), on curvature and water uptake in the 4 day old onion seedling.

TABLE 10

Treatment	Seedlings with curvatures (%)	Wet weight after 48 hour treatment (mg)	Statistical analysis t-test Comparison	Significance
Control (C) 2% Etoh	0	16.1 ± 3.7[a]	C x CB*	NS
			C x NEM*	NS
CB (CB) 5 ug/ml	30	16.6 ± 2.7	C x CB*	NS
NEM 1.0 mM	30	14.1 ± 2.8	C x CB+NEM*	0.001
CB + NEM	70	11.4 ± 1.8	NEM x CB+NEM*	0.005

a = mean ± standard deviation
* = comparison of wet weight data

DISCUSSION AND CONCLUSION

Response to Cytochalasins

The initiation of cotyledon curvature, in the onion, is associated with a decrease in cell extension. Increased curvature produced by unilateral, as opposed to bilateral application of CB indicate that CB is not readily transported in the seedling. Tissues in close contact with CB are inhibited to a greater extent than those not in direct contact.

Growth, in plant cells, is dependent on an influx of water. Water movements are regulated by two opposing forces. Osmotic potential determines water entry into the cells and is dependent on the ionic and molecular concentration inside the cell. The cell membrane tends to concentrate certain molecules and ions inside the cell (1), resulting in water influx to maintain osmotic balance. The observed inhibition of glucose uptake (30%) by CB, if it results in reduced cellular osmotic potential, would account to some extent for the CB inhibition of water uptake. The plasmalemma, if unrestrained by the cellulose-reinforced jacket secreted by the cell, would burst (1). Thus the cell wall acts as a physical barrier to uncontrolled cell expansion. The resulting turgor pressure acts in opposition to the osmotic potential and prevents excessive swelling. For growth, cell wall synthesis must take place. Cellulose, the major component of the wall (1) is composed entirely of glucose units linked together by B-1, 4 linkages (1). The observed decrease in wall incorporation of glucose (60%) indicates that synthesis and, or, secretion of wall material may be inhibited.

Inhibition of glucose uptake is a characteristic mode of action of cytochalasins (14), as is ~~inhib~~ition of secretion in animals (3, 15) and in the fungus Achlya (19), where CA inhibits synthesis and secretion of cellulase, an enzyme important in wall morphogenesis. Both of the two possible mechanisms for the CB inhibition of water uptake appear applicable. Both parameters regulating the water potential (osmotic potential and turgor pressure) are altered by CB treatment in a manner as to decrease water uptake. The lack of CB responsiveness in young onion seedlings

(3 day) is consistent with the results of Thomas, Lager, and Manavathu (16), which show that CB applied from the onset of germination does not affect mitosis or cell length until the fifth day of treatment. This may be related to the high level of the thiol compound, glutathione, which has been reported during the early days of seedling germination (7).

Mode of Action

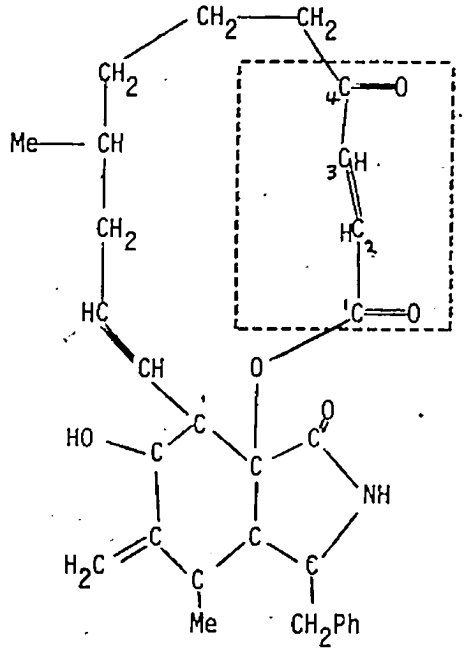
The onion seedling is well suited for comparative studies of the mode of action of cytochalasins since all the cytochalasins tested (CA, CB, CE, and CD) produce curvatures and decrease water uptake in this system. Depending on the cytochalasin used, one can elicit the "classical", low concentration response (CD at 1.0 ug/ml), and the high dose response (CA, CB, and CE at 20 ug/ml). Most higher plant responses to cytochalasins are poorly reversible, slow, and require high concentrations (20-50 ug/ml). In order to elucidate the mode of action of cytochalasins, it is useful to account for the two major types of responses (ie. classical and high dose).

Work by Kuo and Lampen (8) has indicated that CA, but not CB, acts via sulfhydryl interference. Comparing CA in the region of carbons 1-4, with the sulfhydryl-binding agent N-ethyl maleimide (NEM), it is clear that both contain two ketone groups separated by an alkene (Fig 2a). Although CA is trans in this region, and NEM is cis, it has been pointed out that CA undergoes a slow, light catalysed, isomerization to the cis form (2). Comparing the structures of CB, CE, and CD, (Fig 2b) it is evident that all contain the alkene function (carbons 2-3), but differ in the substituents on the adjacent carbon groups (carbons 1 and 4).

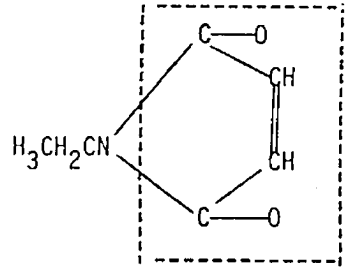
Cysteine, a compound having reactive SH-groups, protects endogenous sulfhydryl functions in a system by providing alternative binding sites for sulfhydryl reagents. Cytochalasins B and E, effective only in high concentrations, were found to be inactive when applied in the presence of cysteine (Table 7 & 8). Cytochalasin D, producing the low concentration, "classical" response was not affected by cysteine (Table 9). It appears then, that the effectiveness of a cytochalasin may be inversely related to its activity as a general sulfhydryl reagent.

Figure 2a: Comparison of structural similarity between cytochalasin A and N-ethyl maleimide (NEM).

Figure 2a



CYTOCHALASIN A (CA)



N-ETHYL MALEIMIDE (NEM)

Figure 2b: Comparison of cytochalasins A, B, E, and D in the region of carbons 1-4.

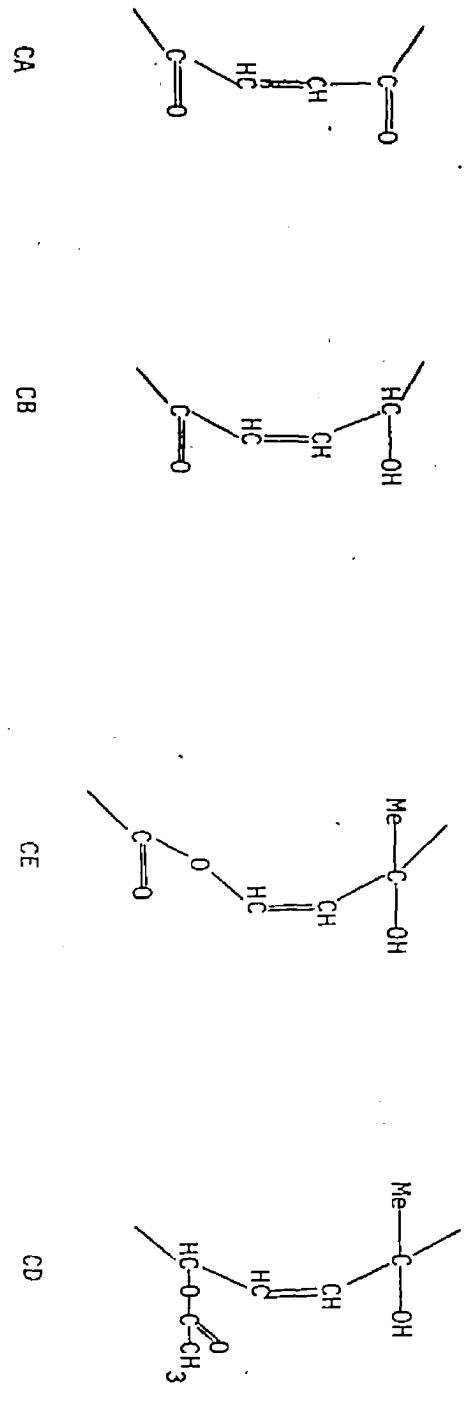


Figure 2b

The high potency of CD, a poor SH-reagent, and the fact that a strong sulfhydryl reagent, such as NEM, fails to mimic the cytochalasin response, indicated that non-specific SH-binding is not the major mode of action of cytochalasins, in the plant system. Although NEM, on its own elicits no response, it significantly enhances the potency of CB applied in low, normally ineffective concentrations (5.0 ug/ml). The effectiveness of the usual high dose of CB (20 ug/ml) presumably requires initial saturation of SH-groups, with the residual free CB acting on mechanisms such as glucose uptake. Inactivation of sulfhydryl groups with NEM lowers the threshold concentration of CB required to elicit onion seedling responses. The CB-sparing affect of NEM may the the result of NEM competing for cellular sulfhydryl groups which would otherwise bind and inactivate CB. The presence of sulfhydryls in plant wall material has been documented (9).

Cytochalasin B has at least two binding sites in cells. Non-specific sulfhydryl binding occurs preferentially since only when these sites are occupied, either by excess CB or by NEM, will CB bind to the sites responsible for regulating cell growth. Cytochalasin D, as evidenced by incomplete cysteine inhibition has minimal non-specific sulfhydryl binding ability, which may account for the fact that its potency exceeds that of the other cytochalasins by a factor of ten in onions and in mammalian systems (4).

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VITA AUCTORIS

Born: July 21, 1950. Windsor Ontario.
Son of Mr. and Mrs. Michael Seagull Sr.

Elementary Education:
Windsor, Ontario.

Secondary Education:
W.F. Herman C.I., Windsor, Ontario, 1964-1965.
Massey Secondary School, Windsor, Ontario, 1965-1969.

University Education:
University of Windsor, Windsor Ontario, 1969-1974.
B.Sc. in Biology.