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EVIDENCE FOR SELECTIVE SULFHYDRYL REACTIVITY IN CYTOCHALASIN A - MEDIATED BACTERIAL INHIBITIONS

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Summary: Cytochalasin A (CA) at 5 x 10^{-5} M strongly inhibits glucose transport in *Arthrobacter sialophilis*. This effect and other bacteriostatic and metabolic inhibitions of gram-positive bacteria are not caused by the closely related congeners cytochalasin B or D. Inhibitions by CA are nullified by prior drug incubation with sulfhydryl compounds. It was also found that the characterized adduct of CA with β-mercaptoethanol is devoid of biological activity. N-ethylmaleimide, p-chloromercuribenzoate and ethacrynic acid (a known, liposoluble, sulfhydryl reactant) were each shown at 5 x 10^{-5} M to be relatively ineffective in inhibiting D-glucose transport in *A. sialophilus*. These observations suggest that CA reacts at the molecular biological level in a site-specific manner.

INTRODUCTION

While the bulk of observations concerning the biological activities of cytochalasins relate to susceptible eukaryotic systems, and indeed, primarily involve use of the congener cytochalasin B (1), several reports of inhibitions produced by cytochalasin A (CA) have been published. Thus, apparently irreversible CA interference in immunological (2,3) and developmental (4) systems, as well as with the acellular slime mold *Physarum polycephalum* (5), have been described. The inhibition of extracellular cellulase secretion in *Achlya* (6), and of growth and ATPase activity in yeast (7), are also documented. We have assembled evidence (8) for the inhibition by CA of a number of physiological processes in gram-positive, as opposed to gram-negative, bacteria. We now report that such CA inhibitions are not observed with cytochalasins B or D nor with the CA-β-mercaptoethanol adduct (see below). Preincubation of CA with other compounds containing the sulfhydryl group also predictably nullify its

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action. Evidence for *in vitro* reactions of CA with the sulfhydryl groups of proteins has earlier been adduced (9,10). However, as measured by glucose transport in *Arthrobacter sialophilus* and by comparison with known sulfhydryl poisons, CA does not appear to be an indiscriminate group-specific reactant.

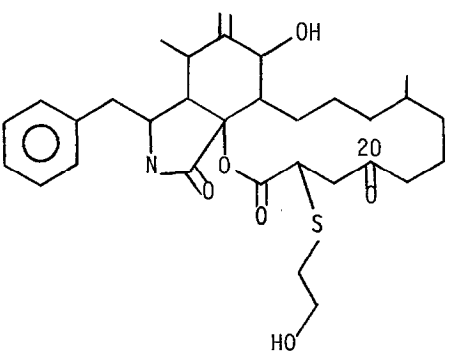
MATERIALS AND METHODS

Organism and Cultural Conditions - Growth of *A. sialophilus* was carried out as described elsewhere (8,11).

Transport of [^{14}C]-D-glucose - Substrate was added to stationary cultures kept on a rotary shaker at 30°C. Culture aliquots were removed at specified times and filtered through a membrane filter (pore size 0.45 μm, MF-Millipore, Millipore Corporation), immediately washed with 10 ml of 0.15 M LiCl, dried 1 hr at 60°C, and placed in vials containing 10 ml scintillation fluid (4 g 2,5-diphenyloxazole and 10 mg 1,4-bis[2(4-methyl-5-phenyl-oxazolyl)] benzene in 1 liter toluene). Radioactivity was measured with a Packard Model 200 liquid scintillation spectrometer.

Comparison of -SH Reagents - Each reagent was preincubated 10 min at 30°C with a stationary phase culture of *A. sialophilus* before the addition of [^{14}C]-D-glucose (0.2 μCi/ml) and 2 mM unlabeled D-glucose. Uptake was measured for 9 min as described above. Inhibition values in Table 1 reflect an average of two determinations.

Chemicals - Cytochalasins A, B (CB) and D (CD) were obtained from Aldrich. The CA-β-mercaptoethanol derivative, m.p. 169-170°, was prepared here by Dr. Carl A. Miller and was identified as the Michael adduct on the basis of appropriate physical and chemical characteristics. (NMR, mass spectrum, elemental analysis). Its preparation will be described in detail elsewhere. Ethacrynic acid, N-ethylmaleimide (NEM), and p-mercuribenzoic acid (PCMB) were commercial samples and were recrystallized before use.



CA-β-MERCAPTOETHANOL ADDUCT

RESULTS

Effects of CB and CD on Transport - Although CB and CD each have striking effects on eukaryotic cells, neither drug, in comparison to CA at 5.2 x 10^{-5} M,

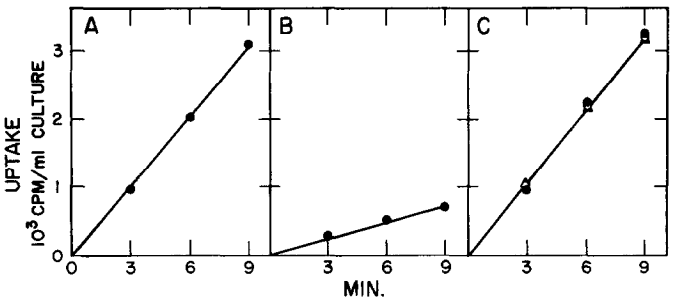


Figure 1. Effects of cytochalasins on glucose uptake. D-glucose (0.2 μCi/ml of [^{14}C]-D-glucose and 2 mM cold D-glucose) was added at time zero to stationary phase cultures of *A. sialophilus*, which had been preincubated 10 min with 5.2 x 10^{-5} M of the reagent tested. Panel A (control), panel B (CA), panel C (CB), ▲ (CD), O CA-β-mercaptoethanol adduct ● .

Table 1. Comparison of Effects of Sulfhydryl Reagents and CA on D-Glucose Transport in *A. sialophilus*

Reagent	Molarity	% Inhibition
CA	5.2 x 10^{-5}	76
NEM	1.0 x 10^{-3}	92
	1.0 x 10^{-4}	32
	5.2 x 10^{-5}	16
Ethacrynic acid	1.0 x 10^{-4}	39
	5.2 x 10^{-5}	8
PCMB	1.0 x 10^{-4}	30
	5.2 x 10^{-5}	6

had any activity as concerns growth, enzyme induction or respiration in six bacterial organisms previously studied (8). These findings are amplified by the data depicted in Fig. 1 (panels A-C), which show the unique and selective effect of CA on glucose transport in *A. sialophilus*. In the final frame (panel C), the ineffectiveness of the CA-β-mercaptoethanol adduct at the same molar concentration, is also shown.

Comparison of -SH Reagents on Glucose Transport - If CA acts as a group directed sulfhydryl reagent in producing its effects on susceptible bacteria, other sulfhydryl-reactive reagents might then be expected to reproduce the identical phenomenon. The results given in Table 1 show, at 5.2 x 10^{-5} M, that the sulfhydry

reagents, NEM, PCMB, and ethacrynic acid (12) had only a slight effect on glucose transport, whereas CA at this concentration inhibited transport 76%. However, 1.0×10^{-4} M NEM, PCMB and ethacrynic acid each resulted in moderate inhibitions, while 10^{-3} M NEM gave almost complete cessation of sugar transport.

Growth Reversal with Sulfhydryl Additions - We have reported the spontaneous recovery of *A. sialophilus* from CA inhibition, involving a time period of some 6-8 hours, and that this event did not involve selection of resistant clones (8). In Figure 2 is delineated the recovery of growth of *A. sialophilus* by administration of 0.5 mM L-cysteine. In contrast to the slow spontaneous release from CA inhibition (8), exponential growth of this microorganism commenced in less than two hours with such medium amendment.

DISCUSSION

The selectivity of CA as opposed to CB or CD in inhibiting a variety of physiological manifestations with susceptible gram-positive bacteria, is in keeping with an emerging although not understood pattern of discriminatory responses of microorganisms. Whereas cultured mammalian cells respond with quantitative differences to all cytochalasins tested, Thomas *et al.* (6) found that *Achlya* cellulase secretion is inhibited by CA but not by CB; Mante *et al.* described CA-specific morphological abberations with *P. polycephalum* (5); Betina *et al.* (13) indicated growth inhibition of yeast only by CA; and a number of fungi imperfecti are also markedly inhibited in hyphal growth or spore germination by this congener (14). Furthermore, evidence for specific binding of radioactive CB to *A. aerogenes* was also negative (15). The chemical difference between CB and CA resides in the fact that the former has a secondary alcohol rather than a carbonyl function at the C-20 position (see formula above). It is noteworthy that earlier studies by Tishler and coworkers (16) with a number of macrolide antibiotics demonstrated that antibacterial activities were similarly correlated with oxidation states at isologous carbons. It would appear that such subtle changes in regiospecific areas of antimicrobial agents of the macrolide class are discernable to bacterial acceptor macromolecules.

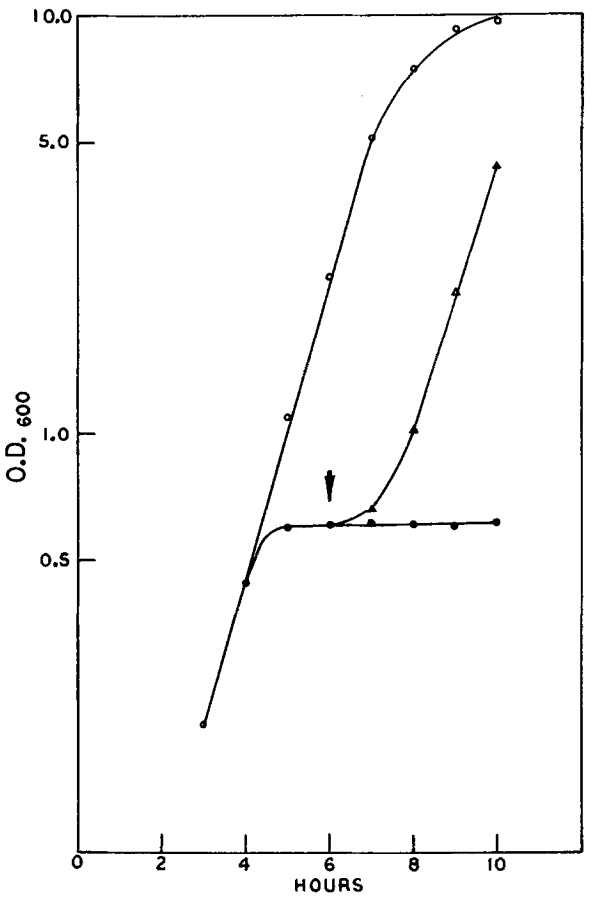


Figure 2. Reversal of CA growth inhibition by addition of cysteine. CA was added to logarithmic phase *A. sialophilus* at time 4 hrs. 0.5 mM cysteine was added to CA inhibited culture at the time indicated by the arrow. Control (O), 25 µg CA/ml (●), 25 µg CA/ml plus 0.55 mM cysteine (▲)

It has been suggested for *in vivo* CA-sensitive eukaryotic systems, that the drug reacts with macromolecular sulfhydryls because low molecular weight thiols reverse its actions (7,17). The reaction of CA with the thiol groups of proteins, especially following denaturation, has also been previously described *in vitro* (9,10). In keeping with this notion, are the results (Fig. 2) which demonstrated that growth recovery of *A. sialophilus* to CA was accelerated by addition of cysteine; a finding not unexpected in light of prior observations with CA inhibition of yeast (7). Our results further show that the characterized Michael-reaction adduct of CA and β-mercaptoethanol does not produce any of the anti-

bacterial effects observed with CA. This suggests that the reactive α,β-unsaturated keto moiety of CA is indeed essential for its biological activity. If CA produces its effects through Michael reaction with a critical sulfhydryl or other nucleophilic groups, other general sulfhydryl reactive reagents might be expected to mimick the action of this drug. However, NEM, PCMB and ethacrynic acid were each, at 5.2×10^{-5} M, 5-10 fold less effective than CA as inhibitors of *Arthrobacter* bacterial transport. It was necessary to increase NEM concentration to 1 mM in order to achieve a comparable degree of inhibition of hexose transport. The latter concentration of NEM has also been shown to inhibit D-lactate coupled portage of a number of low molecular weight solutes into *E. coli* membrane vesicles (18). The fact that CA is the most potent of the several group-directed inhibitors tested appears to rule out general sulfhydryl poisoning as the sole basis for its activity, and suggests that the mechanism of its action involves selective nucleophilic reaction with functional protein amino acid residues.

Some speculation on the nature of such presumptive acceptors of receptors for CA may be in order. Inhibition of permeation (Fig. 1) represents the gross mechanism whereby bacterial growth, enzyme induction, exogenous metabolite respiration and related phenomena become converged (8), in accord with known high-affinity sites for cytochalasin B blockage of transport in mammalian cells (19). Consideration of the multiciplicity of bacterial transport systems for even a single metabolite, as well as the several mechanistic classes into which these may fall (20), tends to make it unlikely that CA can inhibit all of these processes at the identical macromolecular level. A perhaps more tenable hypothesis is that the drug interferes with the orientation of membrane-associated or periplasmic proteins. This notion is compatible both with the presence of contractile-like elements in prokaryote membranes (21,22), and with patterns which are emerging for the locus of cytochalasin action in eukaryotic cells (23). It has furthermore been shown that the protein elongation factor EF-Tu from *E. coli* is seemingly actin-like in its biochemical properties (24). It is conceivable that the fine mechanism of CA action on gram-positive bacteria may

also include inhibition of the function of this factor in protein synthesis. These several modes of possible drug action are now amenable to experimental test.

ACKNOWLEDGMENTS

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REFERENCES

1. Tanenbaum, S. W. (1978) (Ed.), Cytochalasins, biochemical and cell biological aspects, pp. 1-564, North Holland Publ. Co., Amsterdam.
2. Bubbers, J. E. and Henney, C. S. (1975). J. Immunol. 115, 145-149.
3. Orange, R. P. (1975). J. Immunol. 114, 182-186.
4. Appleton, J. C. and Kemp, R. B. (1974). J. Cell. Sci. 14, 187-196.
5. Mante, S. D., Flashner, M. and Tanenbaum, S. W. (1978). Cytobiologie 17, 10-22.
6. Thomas, D. S., Lutzac, M. and Manavathas, E. (1974). Nature 249, 140-142.
7. Kuo, S. C. and Lampen, J. O. (1975). Biochim. Biophys. Acta 389, 145-153.
8. Cunningham, D., Schafer, D., Tanenbaum, S. W. and Flashner, M. J. Bacteriol., in press.
9. Himes, R. H., Kersey, R. N., Ruscha, M. and Houston, L. L. (1976). Biochem. Biophys. Res. Comms. 68, 1362-1370.
10. Lagunoff, D. (1976). Biochem. Biophys. Res. Comms. 73, 727-732.
11. Wang, P., Tanenbaum, S. W. and Flashner, M. (1978). Biochim. Biophys. Acta 523, 170-180.
12. Duggan, D. E. and Noll, R. M. (1965). Arch. Biochem. Biophys. 109, 388-396.
13. Betina, V., Micekova, D. and Nemec, P. (1972). J. Gen. Microbiol. 71, 343-349.
14. Thomas, D. S. (1978), *In* Tanenbaum, S. W. (Ed.), Cytochalasins, biochemical and cell biological aspects, pp. 258-272. North Holland Publ. Co., Amsterdam.
15. Lin, S., Santi, D. V. and Spudich, J. (1974). J. Biol. Chem. 249, 2268-2274.
16. Omura, S., Tishler, M., Nakagawa, A., Hironaka, Y. and Hata, T. (1972). J. Med. Chem. 15, 1011-1015.
17. Haslam, R. J. (1972). Biochem. J. 127, 34 p.
18. Kaback, H. R. and Patel, L. (1978). Biochemistry 17, 1640-1646.
19. Yung, C. Y. and Rampal, A. L. (1977). J. Biol. Chem. 252, 5456-5463.
20. Wilson, D. B. (1978). Ann. Rev. Biochem. 47, 933-965.
21. Ghosh, A., Maniloff, J. and Gerling, D. A. (1978). Cell 13, 57-64.
22. Nakamura, K. and Watanabe, S. (1978). J. Biochem. (Tokyo) 83, 1459-1470.
23. Tannenbaum, J., Tanenbaum, S. W. and Godman, G. C. (1977). J. Cell. Physiol. 91, 225-238.
24. Beck, B. D., Arscott, P. G. and Jacobson, A. (1978). Proc. Nat. Acad. Sci. 75, 1250-1254.