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Toxic Effects of Some Alcohol and Ethylene Glycol Derivatives on *Cladosporium resinae*

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Eleven commercially available alcohol and ethylene glycol derivatives were tested for their toxicity toward a problem organism in jet fuel, *Cladosporium resinae*. In the presence of glucose, 20% (vol/vol) ethylene glycol monomethyl ether prevented spore germination and mycelial growth, and 10% (vol/vol) 2-ethoxybutanol, 10% 2-isopropoxyethanol, 10% 3-methoxybutanol, 5% 2-butyloxyethanol, 5% ethylene glycol dibutyl ether, and 5% diethylene glycol monobutyl ether were found to have similar effects. In a biphasic kerosene–water system, 3-methoxybutanol, 2-butyloxyethanol, and diethylene glycol monobutyl ether were again found to be more toxic than ethylene glycol monomethyl ether. Considerable potassium efflux, protein leakage, and inhibition of endogenous respiration were observed in the presence of the more toxic compounds. 2-Butyloxyethanol also caused loss of sterols from cells.

Microbiological contamination of jet aircraft fuel systems has been a major concern of airline operators, particularly in the tropics. The microorganisms usually form a thick slimy sludge which is firmly attached to the floor and sides of the integral fuel tanks whenever moisture is present to produce a fuel–water interface. The filamentous fungus *Cladosporium resinae* has been shown to be the predominent species present in the biological sludge (3, 4, 10) and is mainly responsible for the extensive corrosion frequently found in fuel tanks of both commercial and military jet aircraft (9).

Previous work from this laboratory has shown that *C. resinae* can utilize C_9 to C_{18} *n*-alkanes for growth, whereas the shorter-chain *n*-alkanes, particularly *n*-hexane and *n*-heptane, do not support growth of this organism and inhibit its growth on glucose (14). Similar effects were also observed when shorter-chain fatty acids and alcohols (C_6 to C_{12}) were incubated with *C. resinae* (13). Ethylene glycol monomethyl ether (EGME), a short-chain glycol used primarily as an anti-icing additive in military jet aircraft, has been found to be toxic to *C. resinae* (5–7). The present paper reports work done on the action of some derivatives of ethylene glycol and other alcohols on *C. resinae*.

MATERIALS AND METHODS

Test organisms and conditions. Isolate 35A, isolated from Australian soil and identified as *C. resinae* forma *avellaneum* by D. G. Parbery, School of Agriculture, University of Melbourne, was used in all experiments unless otherwise stated. The cultures were maintained on Bushnell-Haas glucose agar slants (2). Strains ATCC 22711 and ATCC 22712 were obtained from J. J. Cooney, Department of Biology, University of Dayton, Dayton, Ohio.

All liquid culture experiments were carried out in triplicate in standard 250-ml Erlenmeyer flasks. For the preparation of liquid inoculum, the organism was grown on Bushnell-Haas glucose agar slants (2) for 1 to 2 weeks at 30°C. A spore suspension practically free of mycelium as observed under a microscope was obtained by gently shaking the spores off an agar slant with 10 to 15 ml of sterile distilled water or basal salts medium. For each experiment, samples of the spore suspension were counted with a hemacytometer and diluted to give a concentration of 6×10^7 spores per ml. A known volume of the continuously agitated spore suspension was then pipetted into each flask.

Chemicals. The ethylene glycol derivatives and other alcohols were obtained from Fluka, Basel, Switzerland, and E. Merck AG, Darmstadt, West Germany. Commercial jet fuel (Jet A-1) was kindly supplied by Shell Co., Singapore. D-[*U*-^{14}C]glucose was purchased from Radiochemical Centre, Amersham, England. All other compounds were analytical grade or the best grade available and were from Merck or Sigma Chemical Co., St. Louis, Mo.

Abbreviations. The names of the various compounds are abbreviated as follows: ethylene glycol monomethyl ether or 2-methoxyethanol, EGME; 2-ethoxyethanol, 2-EE; 2-isopropoxyethanol, 2-IE; 3-methoxy-1-butanol, 3-MB; 1-methoxy-2-propanol, 1-MP; 2-butyloxyethanol, 2-BE; diethylene glycol monomethyl ether, DEGMME; diethylene glycol dimethyl ether, DEGDME; diethylene glycol monoethyl ether, DEGMEE; diethylene glycol diethyl ether, DEGDEE; diethylene glycol monobutyl ether, DEGMBE; and ethylene glycol dibutyl ether, EGDBE.

Effect of compounds on spore germination and mycelial growth of *C. resinae* in glucose medium.

A 0.5-ml amount of the spore suspension in water was added to 40 ml of a mixture of Bushnell-Haas mineral salts medium containing 1% glucose and appropriate volumes of the substances being tested. The final concentrations of each of the substances tested ranged from 1 to 20% (vol/vol). The flasks were incubated statically at 30°C for 21 days. To test for effects on spore germination, flasks containing 40 ml of mineral salts medium with 1% glucose and a test compound were inoculated with 0.5 ml of spore suspension. Germination was considered inhibited if white mycelial colonies were not observed after 21 days of incubation at 30°C. To test for inhibitory effect on mycelial growth, 1-ml samples of the filter-sterilized test compounds were added aseptically to flasks containing mineral salts medium, 1% glucose, and germinating spores in the form of tiny white mycelial colonies. Incubation was carried out at 30°C, and evidence for increase in mycelial mass was observed for 20 days. Flasks were incubated for an additional 21 days if no growth was observed.

Effects of compounds on *C. resinae* in a biphasic system containing Jet A-1 fuel and mineral salts solution. The ratio of fuel to water found in the fuel tanks of airplanes is usually between 1,000:5 and 1,000:1. The experiments were therefore carried out using one of these ratios. A 0.1-ml amount of a spore suspension in Bushnell-Haas salts medium was added to 100 ml of Jet A-1 fuel in a 250-ml Erlenmeyer flask. The test substance was added so that its final concentration ranged from 0.05 to 0.2% (vol/vol) with respect to the fuel.

Effect of antifungal compounds on potassium efflux and protein leakage. The procedure for these experiments has been reported previously (15). Potassium was analyzed by flame photometry, and protein was analyzed by the method of Lowry et al. (8).

Extraction of sterols by the test compounds. A cell suspension in mineral salts medium (4 ml, 1 to 2 mg of cells per ml) was gently shaken with 1 ml of test compound at 30°C for 5 h in a 25-ml flask. Also, cells were incubated without the addition of any test compound. At the end of the incubation period, the cells were harvested by vacuum filtration over preweighed Whatman glass fiber filter paper. The filtrate was shaken vigorously with 5 ml of chloroform–methanol (2:1, vol/vol) in a 50-ml screw-capped tube for 15 min at room temperature. The organic phase was separated, dried over anhydrous Na_2SO_4, and analyzed for sterols by the colorimetric method of Searcy and Bergquist (12). The reagent volumes were reduced proportionately to enable the assay of 10 to 50 μg of sterols. Ergosterol was used as the standard.

Glucose uptake. Glucose uptake in the presence and absence of the test compounds was measured by a [^{14}C]glucose uptake method described previously (15). The final concentration of the antifungal compounds tested was from 1 to 5% (vol/vol).

Endogenous respiration. Cells were initially grown in Bushnell-Haas medium containing 1% glucose in 1-liter Fernbach flasks for 3 to 7 days. Harvested cells were washed free of glucose and resuspended in the salts medium to give a cell concentration of 5 to 10 mg (dry weight) per ml. A 50-ml sample of the cell suspension was incubated with 1 ml of D-[^{14}C]glucose solution (5 μCi/ml, 0.125 μCi/μmol) at 30°C for 2 h. The labeled cells were harvested, washed thoroughly with distilled water (three times, 50 ml each) by vacuum filtration on Whatman glass fiber filter paper, and finally resuspended in the mineral salts medium to give a cell concentration of 2 to 3 mg (dry weight) per ml. Production of $^{14}CO_2$ from the washed labeled cells was measured in the presence and absence of the various compounds. A 3-ml amount of the cell suspension was placed in a screw-capped bottle (30-ml capacity) equipped with a rubber seal through which materials could be injected into the bottle. A scintillation vial, which contained 1 ml of $^{14}CO_2$-trapping agent (16) and was tightly capped with a rubber stopper, was connected to the cell chamber by a length of polythene tubing. At zero time, 1 ml of 20% (vol/vol) test compound was injected into the cell chamber. After a 2-h incubation at 30°C in a shaker bath, 1 ml of 40% trichloroacetic acid was injected into the cell suspension to stop the reaction. About 100 ml of nitrogen gas was pumped into the cell chamber to drive the gas produced by the cells into the vial. After standing for 4 h or longer in the shaker bath, the vial was removed, and 10 ml of scintillation fluid [4 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis-2-(5-phenyloxazolyl)benzene in 1 liter of toluene] was added to it. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS AND DISCUSSION

The two most common biocides currently being used in jet aircraft are Biobor JF (a product of U.S. Borax and Chemical Corp.) and EGME. Both are commercially available. The former is a mixture of two boron compounds, 2,2-oxybis-(4,4,6-trimethyl-1,3,2-dioxaborinane) and 2,2-(1-methyltrimethylenedioxyl)-bis-(4-methyl-1,3,2-dioxaborinane). It has been observed that Biobor JF at the recommended concentration of 270 μl/liter of fuel gives rise to a deposit of boric acid after standing for some length of time, both in the laboratory and in fuel tanks (unpublished data). The effectiveness of EGME, an anti-icing agent, as a biocide is still doubtful, as shown by the occasional isolation of *C. resinae* from storage tanks containing fuel treated with 0.05 to 0.15% (vol/vol) EGME (7, 11).

Eleven commercially available aliphatic alcohols, mostly related to EGME, were tested for antifungal activity in the present study. The effects of these compounds on the growth of *C. resinae* 35A in 1% glucose–mineral salts medium are shown in Table 1. The toxicities of all the compounds tested were at least comparable to that of EGME. 2-EE, 2-IE, 3-MB, 2-BE, DEGDEE, and DEGMBE were more toxic than EGME; 20% EGME prevented spore germination and mycelial growth, whereas 10% 2-EE, 10% 2-IE, 10% 3-MB, 5% 2-BE, 5% DEGMBE, and 5% EGDBE were found to have a similar

TABLE 1. *Growth of C. resinae 35A in 1% glucose–mineral salts medium in the presence of various compounds*[a]

Compound	Spore germination and mycelial growth at the following concn:				Mycelial growth at the following concn:			
	1%	5%	10%	20%	1%	5%	10%	20%
EGME	++++	+++	+	−	++++	+++	+	−
2-EE	+++	+	−	−	+++	+	−	−
2-IE	+++	+	−	−	+++	+	−	−
3-MB	+++	+	−	−	+++	+	−	−
1-MP	++++	+++	+	−	++++	+++	+	−
2-BE	+++	−	−	−	+++	−	−	−
DEGDME	++++	+++	+	−	++++	+++	+	−
DEGDEE	+++	+++	+	−	+++	++	+	−
DEGMME	++++	+++	+	−	++++	+++	+	−
DEGMEE	++++	+++	+	−	++++	+++	+	−
DEGMBE	+++	−	−	−	+++	−	−	−
EGDBE	+++	−	−	−	+++	−	−	−

[a] ++++, Growth of *C. resinae* in controls without inhibitors; +++, ++, and + refer to the degree of growth relative to that of the controls. −, No visible growth after 42 days of incubation at 30°C.

TABLE 2. *Growth of C. resinae 35A in a static two-phase fuel–water (1,000:1) system containing various compounds*[a]

Compound	Spore germination and mycelial growth after 30 days with the following concn (% of total vol):		
	0.05	0.1	0.2
EGME	+++	+	−
2-EE	+++	+	−
2-IE	+++	+	−
3-MB	++	−	−
2-BE	++	−	−
DEGDEE	++	+	−
DEGMBE	++	−	−
EGDBE	++	−	−

[a] ++++, Growth of *C. resinae* in controls without inhibitors; +++, ++, and + refer to the degree of growth relative to that in the controls.

effect. Similarly, the potencies of these six compounds, when evaluated under simulated field conditions by observing the growth of *C. resinae* 35A in static two-phase fuel–water systems (1,000:1), were greater than that of EGME (Table 2). No growth was observed when EGME was used at 0.2%, whereas 3-MB, 2-BE, and DEGMBE were effective in controlling the growth of the fungus at 0.1%.

Figures 1 and 2 show the uptake of D-[*U*-^{14}C]glucose in the presence of various concentrations of the compounds tested. At a final concentration of 1% 2-BE, glucose uptake was only 25% that of the controls. For DEGMBE, the inhibition was about 25% at a 1% concentration and 40% at a 3% concentration. With 3% (final concentration) 2-BE and EGDBE glucose uptake by the cells was almost completely inhibited. EGME at a 5% concentration, on the other hand, did not inhibit glucose uptake at all.

The question as to the cause of the antifungal activity of these alcohol derivatives led to an investigation of their effects on the fungal cell membrane by studying potassium loss from the cell. It was found that 2-BE at a 20% concentration caused almost complete loss of cellular potassium, whereas at the same concentration EGME resulted in the efflux of only 27% of the potassium. In fact, EGME, when compared with the other alcohols tested, caused the least amount of potassium efflux (Table 3). A greater loss of cellular soluble protein was also observed in the presence of alcohols other than EGME (Table 4). Thus, it appears that one of the effects of these alcohols is on the membrane of *C. resinae* since the amount of potassium efflux and loss of cellular soluble proteins would reflect in some measure the degree of damage to membrane function. EGME is therefore the least toxic of all of the compounds tested.

The probable mechanism of action of these alcohols on cell membranes was then investigated by testing the incubating medium for the presence of membrane components, such as sterols. Except for 2-BE, no sterols were found in the medium after the cells were incubated with the alcohol derivatives at 20% (vol/vol) concentrations for 5 h. About 6.0 μg of sterol (as ergosterol) per mg of cell dry weight was released by 20% 2-BE. Thus, 2-BE appears to cause damage to the cell membrane by removing some of the integral components of the membrane. Thus, the effect of the other antifungal ethylene glycol derivatives on the membrane was probably to cause a disarrangement of membrane structure

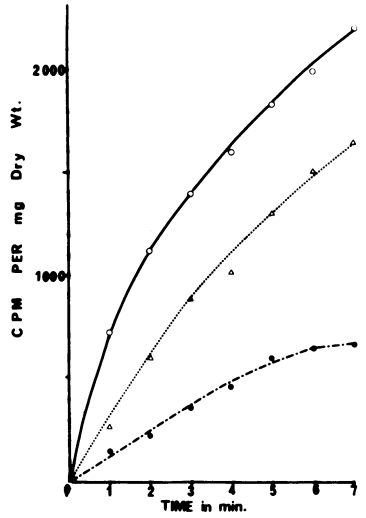

FIG. 1. *Effect of test compounds on the uptake of D-[^{14}C]glucose. Values are expressed as counts per minute per milligram (dry weight) of cells. A 1% final concentration of each was used. Symbols: ○, control; ●, 2-BE; △, DEGMBE.*

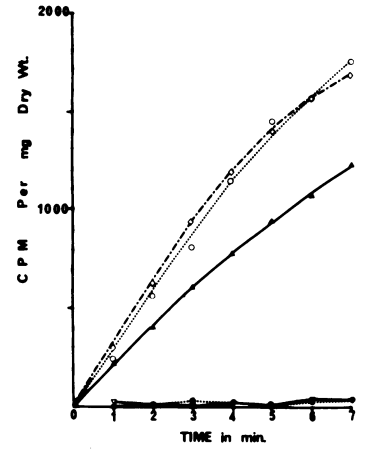

FIG. 2. *Effect of test compounds on the uptake of D-[^{14}C]glucose. Values are expressed as counts per minute per milligram (dry weight) of cells. A 5% final concentration was used for EGME, and a 3% final concentration was used for the other compounds. Symbols: ○, control; ◇, EGME; ▲, DEGMBE; ●, 2-BE; ▽, EGDBE.*

without causing the disintegration of the components of the membrane. This disorganization could therefore have resulted in the inhibition of the uptake of substrates, such as glucose.

Furthermore, the endogenous respiration of *C. resinae* was inhibited by some of these compounds (Table 5). In the three strains studied, the degree of inhibition ranged from 51% in the presence of DEGMBE for ATCC 22711 to 70% in the presence of 2-BE for isolate 35A. No inhibition was observed with the same concentration of EGME. The reason for the differences in the sensitivities to these alcohols observed among the different strains of *C. resinae* is not known. The action of these compounds in inhibiting endogenous respiration might be attributed to their effect on the intracellular membrane or their nonspecific blocking of enzymic sites or both (1).

TABLE 3. *Effect of antifungal substances on potassium efflux of C. resinae 35A at 30°C*

Substance (20% final concn)	K^+ loss after a 5-h incubation (% of total cellular K^+)
None (control)	0.95
EGME	27.33
2-BE	91.00
DEGDEE	58.20
DEGMBE	58.20
EGDBE	45.79
3-MB	56.68

TABLE 4. *Effect of antifungal substances on protein leakage of C. resinae 35A at 30°C*

Substance (20% final concn)	Protein leakage after a 5-h incubation (% of total soluble protein)
None (control)	2.26
EGME	8.51
2-BE	25.27
DEGDEE	25.75
DEGMBE	34.80
EGDBE	28.60
3-MB	25.22

TABLE 5. *Effect of compounds on the endogenous respiration of three C. resinae strains*[a]

Compound (5% final concn)	Endogenous respiration (% of controls) in:		
	35A	ATCC 22711	ATCC 22712
EGME	98	99	97
2-BE	30	50	53
DEGMBE	40	59	55
DEGDEE	50	54	52

[a] Rate of $^{14}CO_2$ expiration was measured as counts per minute per milligram of cell dry weight after a 2-h incubation at 30°C.

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