PN ABS 442
5.143

Running Head: **Ethanol-induced chilling-resistance in cucumber seedlings**

To whom correspondence should be sent:

Chaim Frenkel

Department of Horticulture

Rutgers - The State University of New Jersey, Box 231

New Brunswick, New Jersey 08903

Telephone: (908) 932-9441

Bitnet: Frenkel @ Zodiac

SCI 936-5542

Title of Article: Arrest of chilling - stress in cucumber (*Cucumis sativus*) seedlings by endogenous and applied ethanol

Revised Accepted Date:

Authors: Chaim Frenkel and Amnon Erez

Department of Horticulture

Rutgers - The State University of New Jersey

New Brunswick, New Jersey 08903

Supported by State Funds, New Jersey Agricultural Experiment Station, Publication Number D -12140 - 12 - 9 and by the Agency for International Development Grant No. DDC-5542-G-5105-00

Present Address: Amnon Erez

ARO, The Volcani Center, Box 6

Institute of Horticulture

Bet Dagan 50250, Israel

Abbreviations: ADH, alcohol dehydrogenase

ABSTRACT

Five-day old etiolated cucumber seedlings that are held at 2 C for 72 h suffer chilling injury notably desiccation and collapse of the hypocotyl tissue and eventual plant death. Application of ethanol vapors to pre-chilled seedlings led to chilling-resistance, as evidence by freedom from injury and continued growth. Applied acetaldehyde by comparison led to marginal cold-tolerance. Seedlings that were held in hypoxia and accumulated ethanol and acetaldehyde also became chilling-resistant whereas arrest of volatile accumulation under these conditions, using bisulfite, negated the development of chilling-resistance in seedlings. Emergence of chilling-tolerance was time-dependent and developed in ethanol-enriched seedlings by additional holding up to 18 h at 25 C but decayed afterward.

Application of the volatile anesthetics chloroform and halothane reproduced the ethanol action and led to a substantial chilling-resistance suggesting that the anesthetic action of ethanol which maintain non-polar matrices in the disordered fluid state may oppose the cold-induced changes in macromolecular assembly. This view is supported further by showing that in ethanol enriched and chilling-resistant tissue the total lipid fraction did not undergo retailoring whereas acclimation by temperature cycling was accompanied by changes in fatty acid composition.

We conclude that ethanol is a native chilling-antagonist in plants. The activity of ethanol may arise from its anesthetic action.

INTRODUCTION

Ethanol and acetaldehyde accumulate in plants held in anoxia or hypoxia (James, 1953; Kennedy et al., 1992) and in tissues undergoing stress (Kimmerer and Kozlowski, 1992; Levitt, 1980) including chilling-stress (Murata, 1969). It is commonly believed that volatiles, particularly acetaldehyde are toxic (Perata and Alpi, 1991), apparently due to the formation of acetaldehyde-protein adducts (Israel, 1986) and volatiles were, accordingly, implicated as causative agents in cold injury (Murata, 1969). Other views hold that volatile production is coincidental to enhanced glycolytic activity that is recruited to offset the cold-induced disruption of respiration (Kimmerer and Kozlowski, 1992; Levitt, 1980; Lyons and Raison, 1970), or alternatively, a rescue mechanism for averting lactate accumulation and associated acidosis by redirecting glycolysis toward ethanol biosynthesis (Davies, 1980).

Other studies show that applied acetaldehyde or ethanol lead to respiratory upsurge in fruit (Janes et al., 1978b) or potato tubers (Rychter et al., 1979) and stimulate (cyanide-resistant) alternative respiration (Janes and Frenkel, 1978a) as well as fruit ripening processes (Janes et al., 1981) suggesting that volatiles may also modulate cellular metabolism. Volatile-induced accumulation of sugars (Paz et al., 1982), apparently by stimulating gluconeogenesis (Halinska and Frenkel, 1991) may contribute to cold-tolerance of tissues (King et al., 1988), in part, by stabilizing biopolymers against stress conditions (Clegg, 1985; Crowe et al., 1987). From this perspective, volatiles may function as protectants against stress rather than stress agents as previously suggested (Murata, 1969) or merely as glycolytic byproducts (Kimmerer and Kozlowski, 1992; Levitt, 1980; Lyons and Raison, 1970).

To test this conjecture, we examined if endogenous or applied volatiles

confer chilling-tolerance on chilling-sensitive cucumber seedlings. The obtained results indicate that while acetaldehyde may be toxic applied or endogenous ethanol led to chilling-tolerance. Furthermore, the activity of ethanol as cryoprotectant may arise from the anesthetic function of the compound.

MATERIALS AND METHODS

1. Plant Material

Cucumber (*Cucumis sativus*) plants are chilling sensitive (Saltveit and Morris, 1990; Wang and Baker, 1979) and, therefore, an appropriate test material for this study. Cucumber seeds, cv. Marketmore 76, (Harris Moran Seed Company, Rochester, NY) were germinated on wet vermiculite in 3.75x3.75 cm plastic containers. Each sample consisted of 6 containers containing 40 seedlings each. Seed germination and growth were at 25 C.

In pre-chilled plants light contributes to chilling-tolerance, apparently due to photosynthesis-driven sugar synthesis (King et al., 1988), whereas during chilling photooxidative damage aggravate the severity of stress (van Hasselt, 1990). To avoid the contrasting effects of light, seed germination, seedlings growth, cold acclimation and exposure to chilling conditions were in darkness. In the post-chilling period seedlings were kept under light-dark cycle to test their survival under standard physiological conditions.

2. Cold-acclimation of cucumber seedlings

Cucumber seedlings were acclimated to withstand chilling conditions by applied and endogenous volatiles. In one method, seedlings held in 9.5 L desiccators were ventilated with air containing different levels of volatiles. Air was purged through aqueous solutions of ethanol or acetaldehyde and the obtained gaseous mixtures were diluted further with air to final concentrations usually between 1,000 to 10,000 ppm of acetaldehyde or ethanol and applied to the seedlings at a rate of 400 mL/m. In an alternative application method measured

amounts of liquid ethanol or acetaldehyde, were introduced to sealed desiccators and allowed to evaporate yielding desired concentrations of volatiles. The concentration of gaseous ethanol or acetaldehyde obtained by this method was within 5% of the calculated level. This method was used also for the application of the anesthetics chloroform and halothane. Test compounds were applied by spraying aqueous solutions, usually 10 ml per sample using glass atomizer to deliver solutions in fine mist.

Production of volatiles in the seedlings was stimulated by anoxia (complete oxygen deficiency) and hypoxia (low oxygen conditions). Seedlings were held in desiccators and ventilated for various durations with nitrogen atmospheres containing 0, 1, 2, 4, 10 and 21% O_2 (air) at a rate of 100 mL/m. Gas mixtures were delivered from commercially obtained gas cylinders.

An additional acclimation protocol was by temperature cycling entailing holding pre-chilled plants at 12 C for 48 h followed by intermittent warming for 12 h at 25 C. This method was employed as reference for comparing the efficacy of volatile-induced cold-tolerance and for assessing the metabolic differences between the two acclimation protocols, as discussed below.

The various acclimation protocols were followed by a chilling duration of 72 h at 2 C in Fisher Isotemp refrigerator. On completion of the chilling period plants were transferred to room conditions (25 to 27 C) and placed under 12 h light-dark cycle using continuous fluorescent white light with an intensity of 14 μ mole $^{-2}$ Sec^{-1}. In chilling-sensitive seedlings cold-stress was manifested in the loss of turgor (Saltveit and Morris, 1990) and, subsequently, the collapse of the upper hypocotyl region followed by the death of the tissue. Seedlings that were injury-free, maintained their turgor and, furthermore, continued growth at the end of 5 days were considered chilling-tolerant. The percentage of

seedlings that survived cold conditions and defined as chilling-tolerant was used as estimate for chilling-resistance.

3. Extraction of volatiles from cucumber seedlings

The hypocotyl region from the entire seedling population in a sample (240 plants) were removed, washed thoroughly with ice-cold distilled water and homogenized in Waring blender for 1 min in 200 mL ice-cold water containing 1 mM NaN_3. The obtained homogenate was filtered once through Whatman filter paper No.1 followed by additional filtration through Millipore membrane with average pore size of 0.45 μm. The entire filtrate, containing around 220 ml, was transferred to 2 L round bottom retort-flask that was placed on a heating mantle. A Term-O-Watch L 7 SS regulator (Instruments for Research and Industry, Cheltenham, PA) was used to control heat output (80 volt setting). These conditions were maintained until 100 to 120 ml of the filtrate were distilled. The escaping vapors were fluxed through spiral glass condenser, cooled by circulating ice-cold water and collected in 200 ml round bottom flasks. The obtained samples were stored at 2 C and used for gas chromatography analysis as described below. The outlined protocol resulted in virtually complete recovery of volatiles from known test solutions (results not shown).

4. Gas chromatography estimation of volatiles and anesthetics

One to 2 μL in 5 to 6 replicates of the distillate (above) were injected onto 1/8 inch x 6 ft Poropaq Q column held at 140 C mounted in a Shimadzu 8 AM gas chromatogram equipped with flame ionization detector kept at 250 C. Signal output was recorded by a Shimadzu CR 501 Chromatopac Recorder that was used for estimating the peak area under each of the resolved compounds. Standard curves were made of aqueous solutions containing defined levels (v/v) of acetaldehyde

or ethanol. Figure 1 is an example of a chromatograph showing that acetaldehyde and ethanol were major components in the distillate mixture. The mixture contained also a fast migrating volatile that was identified as formaldehyde by gas chromatography and mass spectroscopy. Formaldehyde may have been formed by ADH-catalyzed oxidation of methanol (Blomstrand et al., 1979) but the reason for the abundance of the compound in the tissue is not clear. Enzymatically-catalyzed production of formaldehyde or other compounds during the extraction of tissues may be ruled out due to cold conditions and the presence of azide.

Head-space sampling was used for the estimation of applied volatiles including the volatile anesthetics chloroform and halothane. 0.5 mL gas samples in 5 to 6 replicates were removed from the head space using one mL syringe and the gas content used for the gas chromatographic estimation of acetaldehyde and ethanol using the conditions outlined above. A similar sampling protocol was used for estimating the concentrations of applied anesthetic compounds except that for the gas chromatograph estimation of anesthetics the Poropaq column was held at 180 C. Gas samples from 2 L flasks containing defined levels of the various volatiles in the gaseous sate were used for constructing standard curves.

5. Extraction and analysis of fatty acids in total lipid extract.

Total lipids from the hypocotyl region of 100 seedlings was extracted in water-methanol-chloroform mixture (Bligh and Dyer, 1959) and used for the estimation of the fatty acid composition essentially as outlined by Browse et al (1986).

RESULTS AND DISCUSSION

Cold Acclimation by Temperature Cycling

Cucumber plants that are kept in cold conditions develop abnormal metabolism including rapid water loss and consequently wilting and plant death (Saltveit and Morris, 1990; Wang and Baker, 1979). Figure 2 shows that cucumber seedlings tolerated chilling at 2 C for 24 h with little or no damage, but longer chilling periods for 48 h and 72 h, respectively, led to a progressive increase in wilting and death. Figure 2 shows also that temperature cycling, consisting of holding pre-chilled plants at 12 C for different time periods, with follow-up warming period for 12 h at 25 C, may be used for cold-acclimation. The results indicate that holding at 12 C for 12 h did not lead to chilling-resistance but chilling-tolerance developed progressively upon holding for 24 h and that 48 h at 12 C led to complete resistance up to 96 h at 2 C. We used temperature cycling as reference gauging for the efficacy of cold-acclimation by volatiles and for comparing changes in the fatty acids composition accompanying the two acclimation protocols (below).

Cold Acclimation by Applied Volatiles

We wanted to test the conjecture that ethanol and acetaldehyde, which accumulate in plants held under extreme conditions (Levitt, 1980) including cold-stress (Murata, 1969), may also lead to chilling-resistance. Application of ethanol vapors by continuous ventilation to room temperature-held seedlings resulted in chilling-tolerance (Fig. 3). The ethanol-induced cold-resistance intensified with time of application (Fig. 3) and approached the level in chilling-resistance achieved by temperatures cycling (Fig. 2). An important feature is the time-course development of the ethanol-induced chilling-tolerance

(Fig. 4). Seedlings showed some tolerance to cold when chilled immediately after ethanol application. However, ethanol application followed by additional holding of the seedlings at 25 C led to a time-course development of chilling-resistance, reaching a maximum after 12 to 18 h and decline afterward. Henceforth, chilling-acclimation was routinely by holding volatile-enriched plants at 25 C usually for 12 to 16 h. The reasons for the time-dependent emergence of the ethanol-induced resistance are not clear and reflect presumably the kinetics of ethanol migration to and activity at putative sites, as discussed below. The subsequent decay in the ethanol-induced cold-tolerance may reflect the metabolic turnover of the volatile.

In an alternative application method seedlings were held for 12 h in sealed desiccators supplemented with different levels of gaseous ethanol or acetaldehyde as described in the methodology section, followed by additional 16 h holding in volatile-free air (Fig. 5). The obtained chilling-resistance was in proportion to the levels of the ethanol and was comparable to the ethanol-induced tolerance obtained by ventilation (Fig. 3). Importantly, even high levels of applied ethanol (64,000 ppm) did not result in toxicity in further support of the view that plants can tolerate ethanol in high levels (Jackson et al., 1982).

Some cold-tolerance was obtained also by applied acetaldehyde (Fig. 5) but a dose response curve suggest that at supra-optimal concentrations the compound has became toxic (Perata and Alpi, 1991). Co-application of acetaldehyde and pyrazole, to inhibit the ADH-catalyzed conversion of acetaldehyde to ethanol (Blomstrand et al., Lelbach, 1969), resulted in the emergence of toxicity at progressively lower concentrations of the volatile (Fig. 6). Apparently, ADH-catalyzed conversion to ethanol may have minimized the toxicity of acetaldehyde whereas inhibition of ADH activity (by pyrazol) and, subsequently, retention of

acetaldehyde may have resulted in toxicity that intensified as the enzyme was progressively inhibited. Hence, the chilling-induced expression of ADH gene(s) and the attended increase in the enzyme activity (Christie, 1991) may have a survival value by detoxifying acetaldehyde to ethanol which can be tolerated by plants (Jackson et al., 1982). The present results suggest, furthermore, that the produced ethanol may function as chilling-antagonist and that ADH activity, leading to the production of ethanol, may have a role in stress amelioration.

Cold Acclimation by Endogenously Produced Volatiles

The view that ethanol biosynthesis leads to chilling-resistance may be tested further by examining if endogenously produced ethanol is a causative agent in the development of chilling-resistance. Production of volatiles is stimulated by cold (Murata, 1969) but in cucumber plants low temperatures lead also to the retailoring of membrane-lipid (Erez and Frenkel, 1991; Wang and Baker, 1979) and perhaps other changes. Under these conditions the effect of volatiles may not be sorted out from effects by other factors on cold-tolerance. We used instead anoxic and hypoxic conditions to depress aerobic respiration and to stimulate the production of ethanol and acetaldehyde (James, 1953; Kennedy et al., 1992).

Nitrogen atmosphere containing 1% oxygen appeared to support the development of a substantial chilling-resistance in the seedlings (Fig. 7). The time-course development, up to 12 h in 1% O_2, and a subsequent decay in chilling-tolerance corresponded to the time schedule in the emergence and disappearance of cold-tolerance obtained by applied ethanol (Fig.4). In nitrogen atmosphere containing 2% and progressively higher oxygen levels there was a precipitous decline in the ability of seedlings to develop chilling-resistance. Similarly, in complete anoxia (100% N_2) the development of chilling-tolerance was limited. To further illustrate the effect of gas regimes the information in Figure 7 was

restructured to show development of chilling-resistance as function of different oxygen concentrations (Fig. 8) and also for relating the emergence of chilling-resistance under the employed gas conditions to the accumulation of volatiles in seedlings (Fig. 9). Figure 9 indicates that the level of chilling resistance was well correlated with the level of ethanol and acetaldehyde that accumulated under these gas regimes; some chilling-resistance at 2% O_2 was associated with an incipient increase in volatiles level and an upsurge in resistance at 1% O_2 was clearly related to a sharp increase in acetaldehyde and ethanol levels. Although the highest accumulation of volatiles was in nitrogen atmosphere the development of chilling tolerance in complete anoxia was low. Apparently, the volatile-induced resistance may develop in hypoxic but not in anoxic conditions and accordingly, seedlings that were ventilated with ethanol and then held in 100% nitrogen failed to become chilling-tolerant (results not shown) further suggesting that the beneficial effect of volatiles may be nullified by extreme oxygen deficit.

Arrest of Volatile Production Negates Hypoxia-Induced Chilling-Tolerance

Early on Neuberg and Reinfurth (1918) used bisulfite in yeast culture to react with and form addition compounds with acetaldehyde thus blocking also the production of ethanol. Bisulfite may be used, by a similar rationale, to arrest the production of volatiles in seedlings and to further examine their role in the development of chilling-resistance under condition of hypoxia. Figure 10 shows that in seedlings held in 1% O_2, applied bisulfite led to a decline in chilling-resistance and, moreover, that the arrest in resistance was in proportion to the decrease in the content of ethanol and acetaldehyde. Applied bisulfite, up to 10 mM, did not interfere with chilling-acclimation by temperatures cycling (Fig. 11) indicating that the action of the compound may not be ascribed to

perturbations in cellular metabolism and instead may be attributed to the arrest of volatile accumulation. Taken together these results indicate that development of chilling-resistance at 1% O_2 is related to volatile accumulation rather than other near-anoxia conditions. Production of ethanol may serve to detoxify acetaldehyde that accumulate under these conditions and, moreover, protect tissues against chilling-stress.

Cold Acclimation by Volatile Anesthetics

Ethanol is believed to increase the fluidity of membranes (Littleton, 1983) and by this reason may restrain the cold-induced fluid-to-gel phase change in biomembrane (Lyons, 1973). The putative activity of ethanol resembles the mode of action of volatile anesthetics that migrate to and maintain non-polar cellular matrices in the disordered fluid state (Eyring et al., 1973) and anesthetics may be expected, therefore, to mimic the action of ethanol and lead to chilling-resistance. This conjecture was tested by applying chloroform and halothane, commonly used anesthetics (Eyring et al., 1973; Seeman, 1973), using the application protocol employed for volatiles. Figure 12 shows an incipient development of chilling-resistance in seedlings held for 12 h in air containing 1,000 ppm chloroform and a progressive increase in resistance as the levels of the anesthetic increased up to 8,000 ppm. At higher concentrations resistance declined, apparently due to the toxicity of the compound (Seeman, 1972). Applied halothane reproduced the chloroform dose-response pattern although becoming active, and subsequently toxic, at higher concentrations. At optimal concentrations (32,000 ppm) halothane led to practically complete chilling resistance. Ethanol at similar concentrations appeared to be less potent in cryoprotection than volatile anesthetics. Earlier work showed that lowering of freeze-killing point in plants by applied ethanol (Akerman, 1927) can be

reproduced by chloroform (Kessler, 1935) and that breakage of seed dormancy by ethanol is achieved also by applied anesthetics (Taylorson and Hendricks, 1979). These observations are consistent with the present results showing that the action of ethanol and anesthetics is interchangeable, apparently due to a common mechanism.

Changes in Fatty Acid Composition in Total Lipid Extract

It is believed that the hydrogen bond-breaking activity of ethanol and other volatile anesthetics result in the displacement of interfacial macromolecular waters and, thereby, disengagement of biopolymers assembly from the constraints of the aqueous microenvironment (Eyring et al., 1973). Cellular matrices that react with ethanol or other anesthetics assemble in a disordered relaxed conformation (Chiou et al., 1990) that may oppose the cold-induced macromolecular aggregation, for example, fluid-to-gel phase change in membrane-lipids (Lyons, 1973). Ethanol-induced chilling-resistance may arise then by changes in the reorganization rather than the composition of cellular matrices. This view is supported by the results (Fig. 13) showing no discernable differences in the lipid composition between ethanol-treated and chilling-tolerant seedlings and plants that were not exposed to ethanol and were chilling-intolerant. By comparison, acclimation by temperature cycling was accompanied in the seedlings by lipid retailoring, notably, enrichment in linolenic and depletion in other fatty acids. An emerging view suggest that cold conditions and the action of ethanol and other volatile anesthetics represent opposing forces on macro-molecular organization; anesthetics are known to antagonize pressure-induced fluid-to-gel phase change in membranes (Winter and Miller, 1985) and the present work indicates that similar changes that are induced by cold (Lyons, 1973) may also be opposed by volatile anesthetics, including ethanol.

Conversely, the metabolic effects of ethanol or other anesthetics are reversed by cold and pressure in animals (Alkana et al., 1985; Eyring et al., 1973) and, similarly, in plants (Hendricks and Taylorson, 1980; Taylorson and Hendricks, 1979).

Ethanol activity is apparently predicated on partitioning onto non-polar matrices where the anesthetic action of the compound leads to reassembly of biopolymers in the disordered expanded state (Chiou et al., 1990) suggesting that the metabolic action of ethanol may not consist of triggering cascade effect(s) and may entail instead alteration in the microenvironment of cellular matrices. This mode of action may account for the substrate levels of ethanol that are required for chill amelioration (Fig. 5 and 9) and resembles the action of polyols which act as stress protectants by occupying water residency sites in sufficient abundance and maintain macromolecules in a meaningful conformation (Clegg, 1985; Crowe et al., 1987). The proposed mode of action infers also that ethanol activity depends on populating biopolymers interfacial environment thus accounting for the time-course development of the volatile-induced chilling-resistance (Fig. 4).

A recent hypothesis (Frenkel, 1991) suggest that a fundamental effect of chilling is disruption of hydrophobic hydration and, subsequently, decay of hydration-dependent inter-molecular repulsive forces that maintain non-polar matrices in the disordered fluid state. Persistence of attractive (van der Waals) forces that go unmitigated may drive macromolecules to aggregate, for example, fluid-to-gel phase change in membrane lipids. From this perspective the presumed role of volatile anesthetic as water substituents in macromolecular assembly in a fluid state (Chiou et al., 1990; Eyring et al., 1973) may be a countermeasure to chilling-induced hydration changes and the attendant collapse in macromolecular organization.

We are led to the conclusion that chill amelioration by ethanol arises from the anesthetic action of the compound. The view that ethanol stabilize macromolecular organization against disruptive hydration changes raises the possibility of a similar role in other instances of hydration stress, including drought or salinity.

LITERATURE CITED

Akerman A (1927) Studien uber den kaltetod und die kalteresistenz der Pflanzen, In, Berlingska Boktuyck, Lund, Sweden, pp 1-232

Alkana RL, Finn DA, Soypin PJ, Malcolm RD (1985) Ethanol withdrawal in mice precipitated and exacerbated by hyperbaric exposure. Science 229:772-774

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911-917

Blomstrand R, Ostling-Wintzel H, Lofa, McMartin R, Tolf B-R, Hedstrom K-G (1979) Pyrazoles as inhibitors of alcohol oxidation and as important tools for alcohol research: An approach to therapy against methanol poisoning. Proc Natl Acad Sci USA 76:3499-3503

Browse JM, Court PJ, Somerville CR (1986) Fatty acid composition of leaf lipids determined after combined digestion and fatty acid methyl ester formation from fresh tissue. Anal Biochem 152:141-145

Chiou J-S, Ma S-M, Kamaya H, Ueda I (1990) Anesthesia cutoff phenomenon: interfacial hydrogen bonding. Science 248:583-585

Christie PJ, Hahn M, Walbot V (1991) Low-temperature accumulation of alcohol dehydrogenase-1mRNA and protein activity in maize and rice seedlings. Plant Physiol 95:699-706

Clegg JS (1985) The physical properties and metallic status of *Artemia* cysts at low water contents: The "water replacement hypothesis". *In*, AC Leopold, ed, *Membranes Metabolism and Dry Organisms*, Cornell Univ Press, Ithaca, pp 169-187

Crowe JH, Crowe LM, Carpenter, JF, Aurell, Wistrom C (1987) Stabilization of dry phospholipid bilayers and proteins by sugars. Biochem J 242:1-10

Davies DD (1980) Anaerobic metabolism and production of organic acids. *In* PK Stumpf, EE Conn, eds, *The Biochemistry of Plants: A Comprehensive Treatise*, Vol 2, Academic Press, New York, pp 581-611

Erez A, Frenkel C (1991) Role of temperature and oxygen regimes in chilling acclimation of cucumber seedlings. 88th Ann Meeting Am Soc Hort Sci, Univ Park, Penn (July 1991), No. 589

Eyring H, Woodsbury JW, D'Arrigo JS (1973) Molecular mechanism of general anesthesia. Anesthesiology 38:414-424

Frenkel C (1991) Disruption of macromolecular hydration--a possible origin of chilling destabilization of biopolymers. Trends Food Sci Tech 2 (2):39-42

Halinska A, Frenkel C (1991) Acetaldehyde stimulation of net gluconeogenic carbon movement from applied malic acid in tomato fruit pericarp tissue. Plant Physiol 95:954-960

Hendricks SB, Taylorson RB (1980) Reversal by pressure of seed germination promoted by anesthetics. Planta 149:108-111

Israel Y, Hurwitz E, Niemela O, Armon R (1986) Monoclonal and polyclonal antibodies against acetaldehyde-containing epitopes in acetaldehyde-protein adducts. Proc Natl Acad Sci USA 83:7923-7927

Jackson MB, Herman B, Goodenough A (1982) An examination of the importance of ethanol in causing injury to flooded plants. Plant Cell Environ 5:153-172

James WO (1953) Plant Respiration Oxford University Press, New York

Janes HW, Frenkel C (1978a) Promotion of softening processes in pear by acetaldehyde, independent of ethylene action. J Am Soc Hort Sci 103:397-400

Janes HW, Chin C-K, Frenkel C (1978b) Respiratory upsurge in blueberries and strawberries as influenced by ethylene and acetaldehyde. Bot Gaz 139:50-52

Janes HW, Rychter A, Frenkel C (1981) Development of cyanide-resistant respiration in mitochondria from ethanol, acetaldehyde, and acetic acid treated potato tubers. Planta 151:201-205

Kennedy RA, Rumpho ME, Fox TC (1992) Anaerobic metabolism in plants. Plant Physiol 100:1-6

Kessler W (1935) Uber der inneren ursachen der kalteresistenz der fplanzen. Planta 24:312-352

Kimmerer TW, Kozlowski TT (1982) Ethylene, ethane, acetaldehyde and ethanol production by plants under stress. Plant Physiol 69:840-847

King AI, Joyce DC, Reid MS (1988) Role of carbohydrates in diurnal chilling sensitivity of tomato seedlings. Plant Phsyiol 86:764-768

Lelbach WK (1969) Liver cell necrosis in rats after prolonged ethanol ingestion under the influence of an alcohol dehydrogenase inhibitor. Experientia 25:816-818

Levitt J (1980) Response of plants to environmental stresses: chilling, freezing, and high temperature stresses. *In*, TT Kozlowski, ed, *Physioloigical Ecology: A Series of Monographs, Texts and Treatises*, Vol. 1, Academic Press, New York, pp 23-64

Littleton JM (1983) Membrane reorganization and adaptation during alcoholic drug exposure. *In*, AR Cossins, P Sheterline, eds, *Cellular Acclimation to Environmental Changes*, Cambridge Univ Press, Cambridge, pp 145-159

Lyons JM (1973) Chilling injury in plants. Ann. Rev Plant Physiol 24:445-466

Lyons JM, Raison JK (1970) Oxidative activity of mitochondria isolated from

plant tissues sensitive and resistant to chilling injury. Plant Physiol 45:3876-389

Murata T (1969) Physiological and biochemical studies on chilling injury in bananas. Physiol Plant 22:401-411

Neuberg C, Reinfurth E (1918) Naturlich und erzwungene gleerinbildung bei der alkoholischen garung. Biochemische Zeitschrift 92:234-266

Paz O, Janes HW, Prevost BA, Frenkel C (1982) Enhancement of fruit sensory quality by post-harvest applications of acetaldehyde and ethanol. J Food Sci 24:315-317

Perata P, Alpi A (1991) Ethanol-induced injuries to carrot cells. Plant Physiol 95:748-752

Rychter A, Janes HW, Chin C-K, Frenkel C (1979) The effect of ethylene and end products of glycolysis on changes in respiration and respiratory metabolites in potato tubers. Plant Physiol 64:108-111

Saltveit ME, Morris LL (1990) Overview on chilling injury of horticultural crops. In, CY Wang, ed, *Chilling Injury of Horticultural Crops*, CRC Press, Boca Racon, pp 3-16

Seeman P (1972) The membrane action of anesthetics and tranquilizers. Pharmacol Rev 24:583-655

Taylorson RB, Hendricks SB (1979) Overcoming dormancy in seeds with ethanol and other anesthetics. Planta 145:507-510

van Hasselt PR (1990) Light-induced damage during chilling. *In* CY Wang, ed, *Chilling Injury of Horticultural Crops*, CRC Press, Boca Raton, pp 113-128

Wang CW, Baker, JE (1979) Effect of two free radical scavangers and intermittant warming on chilling injury and polar lipid composition of cucumber and sweet pepper fruit. Plant Cell Physiol 20:243-251

Winter PM, Miller JN (1985) Anesthesiology. Sci Am 252 (April):124-131

LEGEND FOR FIGURES

Figure 1. A chromatogram of a distillate obtained from cucumber seedlings tissue extracts as described in the Materials and Methods section. One to two μL of the solution were used for gas chromatography to resolve the compounds in the distillate. Peak 1 is the water solvent front. A fast migrating compound (peak 2) was found to be formaldehyde. Acetaldehyde and ethanol represents peaks 3 and 4, respectively. Peak 5 is an unknown.

Figure 2. Cold acclimation of cucumber seedlings by temperature cycling. Pre-chilled seedlings were held at 12 C for 0, 12, 24 and 48 h followed by intermittent warming at 25 C for additional 12 h. Seedlings were then kept at 2 C for different time periods and their percent survival used as an estimate of chilling resistance.

Figure 3. Time-course development of ethanol-induced chilling-resistance in cucumber seedlings. 3,000 ppm ethanol were applied by continuous ventilation to seedlings held at room temperature (25 to 27 C) in 9.5 L desiccators followed by additional holding for 16 h at 25 C in ethanol-free air. The seedlings were then chilled for 72 h at 2 C. The percent of the seedlings that survived the chilling regime was used as estimate of chilling-resistance.

Figure 4. Time-course development of chilling-resistance in ethanol-enriched cucumber seedlings as function of holding time at 25 C in ethanol-free air. Ethanol was applied as described in Figure 3 and the

seedlings were then held at 25 C in ethanol-free air for different time periods and then chilled. Control (air) consisted of seedlings held at room temperature in ethanol-free air during the time period of ethanol application and the follow-up warming period at 25 C. Chilling conditions and estimation of chilling-resistance were as described in figure 3.

Figure 5. Development of chilling-resistance in cucumber seedlings as function of different concentrations of applied ethanol and acetaldehyde. Liquid acetaldehyde and ethanol were introduced to sealed 9.5 L desiccators that held the seedlings. The resulting levels of the volatilized ethanol or acetaldehyde were within 5% of the calculated concentrations. Seedlings were held for 12 h at room temperature in the volatile-containing atmospheres and 16 additional h at 25 C in volatile-free atmosphere. Chilling conditions and estimation of chilling-resistance were as described in figure 3. Gas chromatography was used for estimating the levels of volatilized ethanol or acetaldehyde in the head space.

Figure 6. Effect of pyrazole on the development of chilling-resistance in acetaldehyde-treated cucumber seedlings. Ten mL aqueous solutions containing 0, 0.1 or 1.0 mM pyrazole (PRZL) were atomized and applied to the seedlings. Seedlings were then held for 12 h at room temperature in 9.5 L desiccators containing different acetaldehyde concentration and for additional 16 h at 25 C in volatile-free air. The subsequent chilling conditions and estimation of chilling-resistance were as outlined in figure 3.

Figure 7. Effect of different oxygen regimes on time-course development of chilling-resistance in cucumber seedlings. Seedlings were held at room temperature in 9.5 L desiccators and ventilated at a rate of 100 mL/m with nitrogen atmosphere containing 0, 1, 2, 4, 10 and 21% O_2 (air). Seedlings were removed periodically from the various gas regimes, held for additional 16 h at 25 C in air and then chilled. Chilling conditions and estimate of chilling-resistance were as described in figure 3.

Figure 8. Development of chilling-resistance as function of holding cucumber seedlings in different oxygen regimes. Values of percent survival of plants held for 12 h in different oxygen concentrations in figure 7 were rearranged to show emergence of chilling-resistance as related to different gas regimes.

Figure 9. Chilling-resistance in cucumber seedlings held at different oxygen regimes as related to the accumulation of acetaldehyde and ethanol. Volatile content was determined in seedlings as described in the Materials and Methods section. The experimental conditions were identical to those outlined in figures 7 and 8.

Figure 10. Effect of bisulfite on the development chilling-resistance and volatile accumulation in cucumber seedlings held in 1% O_2. 10 mL solutions containing different concentrations of sodium bisulfite were atomized and applied to the seedlings. The plants were held for additional 12 h at 25 C and were then ventilated for 12 h at room temperature with a nitrogen atmosphere containing 1% O_2. All

other conditions were as described in figure 8. Acetaldehyde and ethanol were extracted from the hypocotyl tissue and estimated as described in the Materials and Methods section.

Figure 11. Effect of bisulfite on chilling-resistance of cucumber seedlings that were cold-acclimated by temperature cycling. Bisulfite was applied as outlined in figure 10 and seedlings were then acclimated by temperature cycling as outlined in figure 2.

Figure 12. Development of chilling-resistance in cucumber seedlings as function of different concentrations of ethanol and the volatile anesthetics chloroform and halothane. The application of the compounds and all other experimental conditions are as outlined in figure 5.

Figure 13. Relative abundance of fatty acids in total lipid extracts from hypocotyl tissue of cucumber seedlings that were cold-acclimated by ethanol application or by temperature cycling. Acclimation by temperature cycling was described in figure 2. Ethanol was applied by continuous ventilation as outlined in Figure 3. Seedlings receiving 0 h ethanol were chilling-sensitive and became progressively chilling resistant as the ethanol application was prolonged to 1, 10 and 24 h (Fig. 3). The extraction and gas chromatographic assay of the lipids were described in the Materials and Methods section.

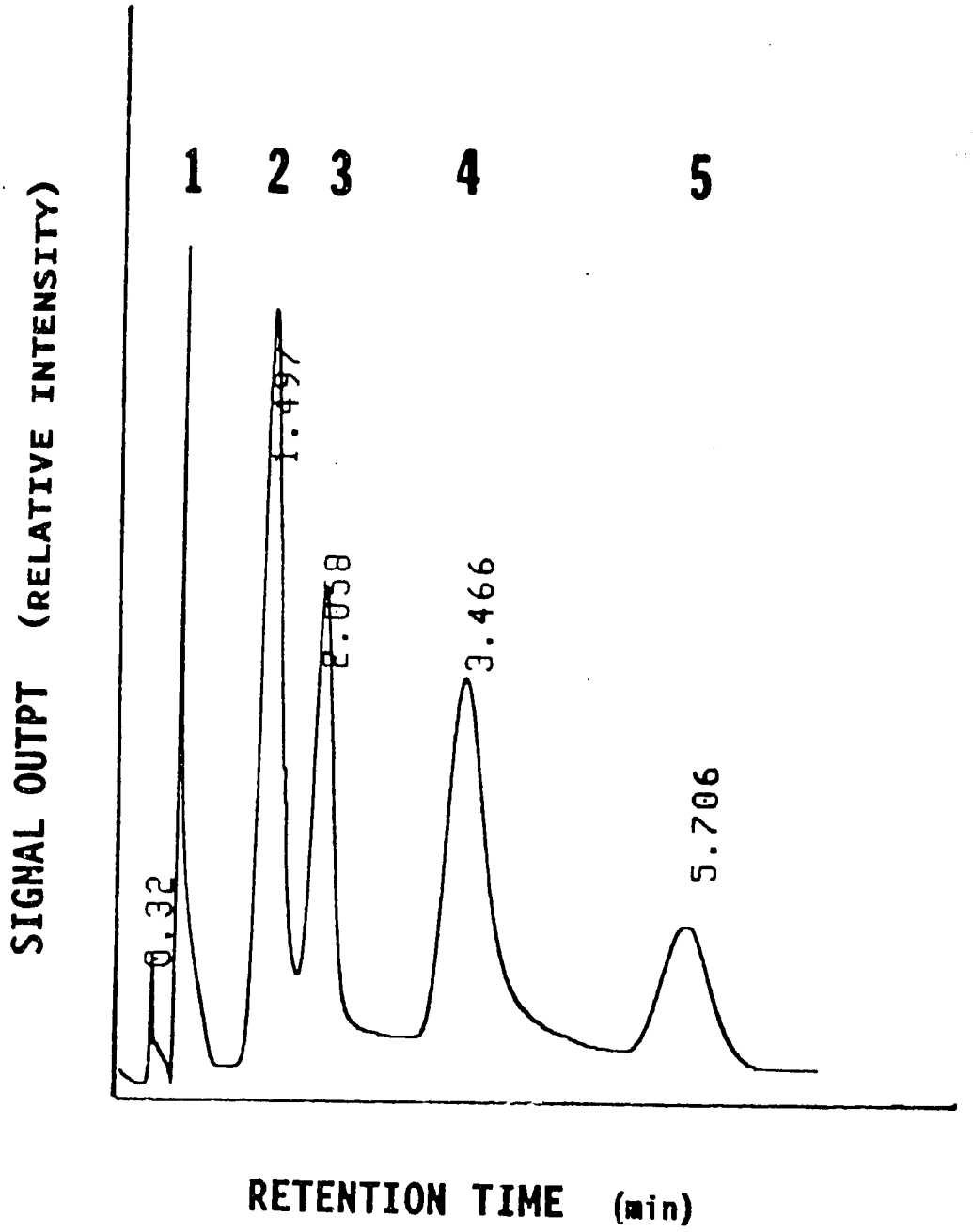
1
2
3
4
5
0.32
1.497
2.058
3.466
5.706
SIGNAL OUTPT (RELATIVE INTENSITY)
RETENTION TIME (min)

Figure 1

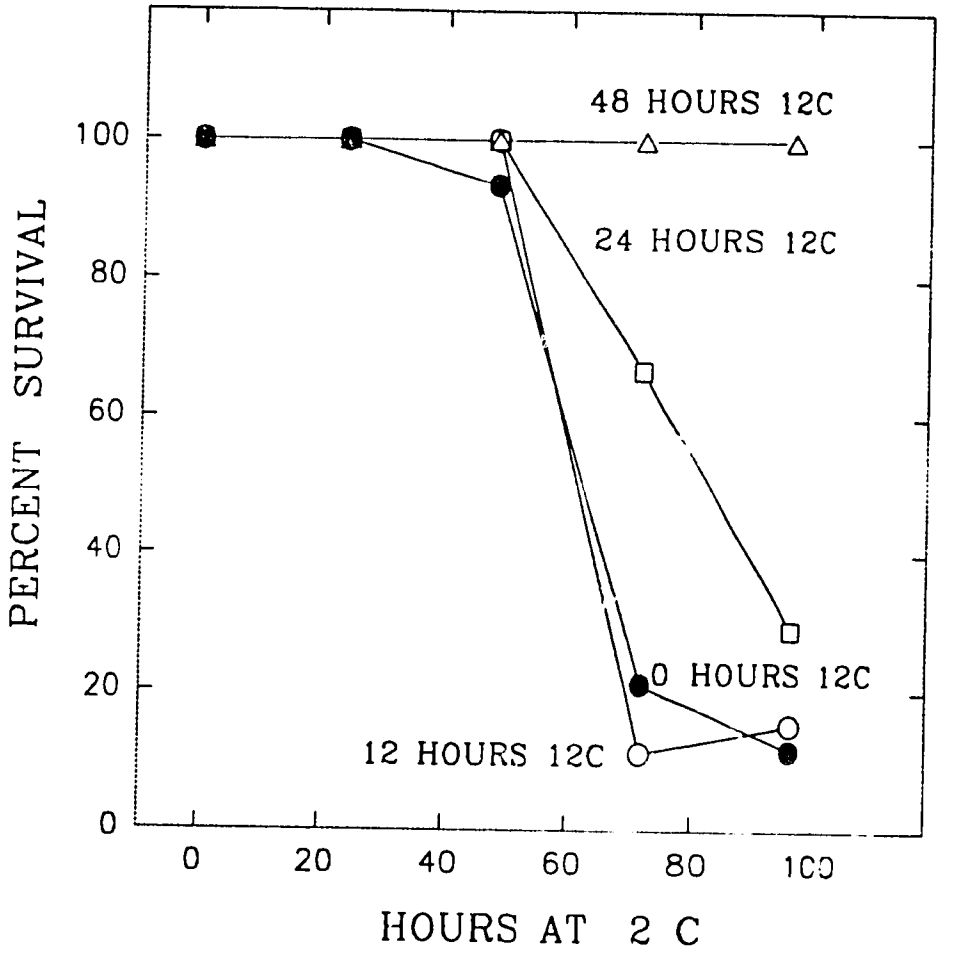
48 HOURS 12C
24 HOURS 12C
0 HOURS 12C
12 HOURS 12C
PERCENT SURVIVAL
HOURS AT 2 C
0
20
40
60
80
100

Figure 2

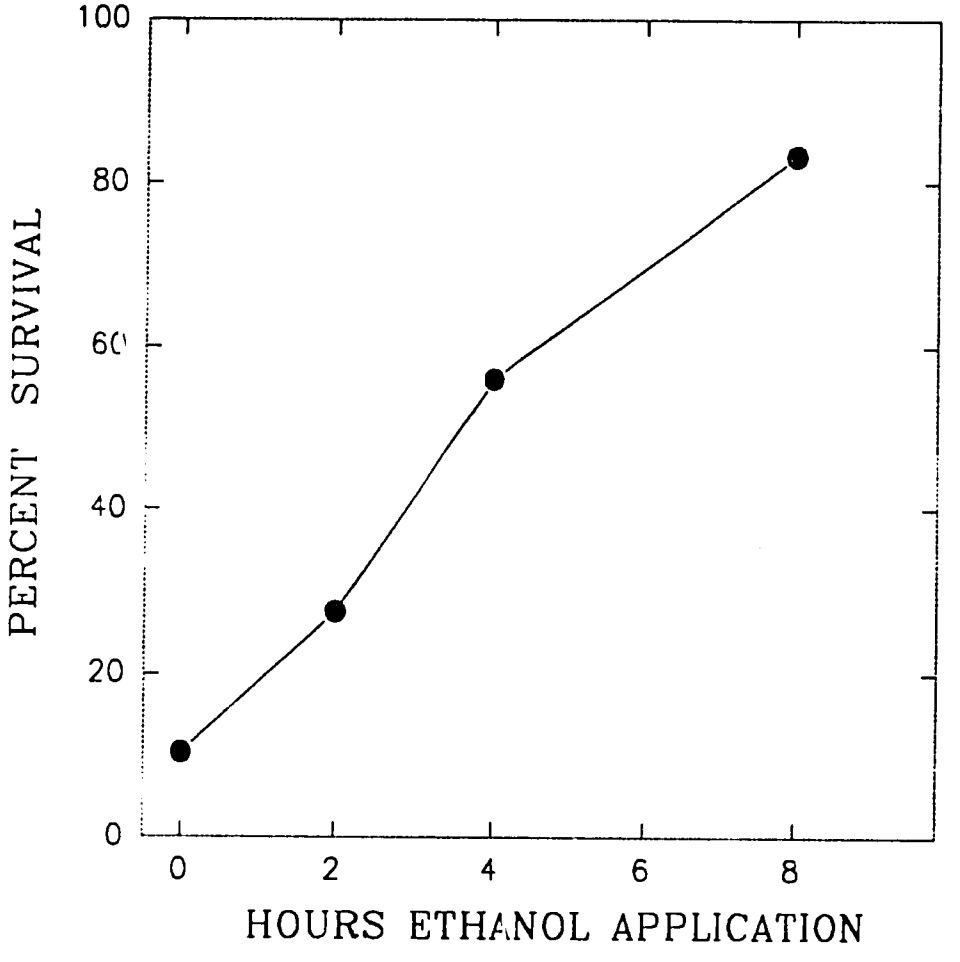
100
80
60
40
20
0
PERCENT SURVIVAL
0
2
4
6
8
HOURS ETHANOL APPLICATION

Figure 3

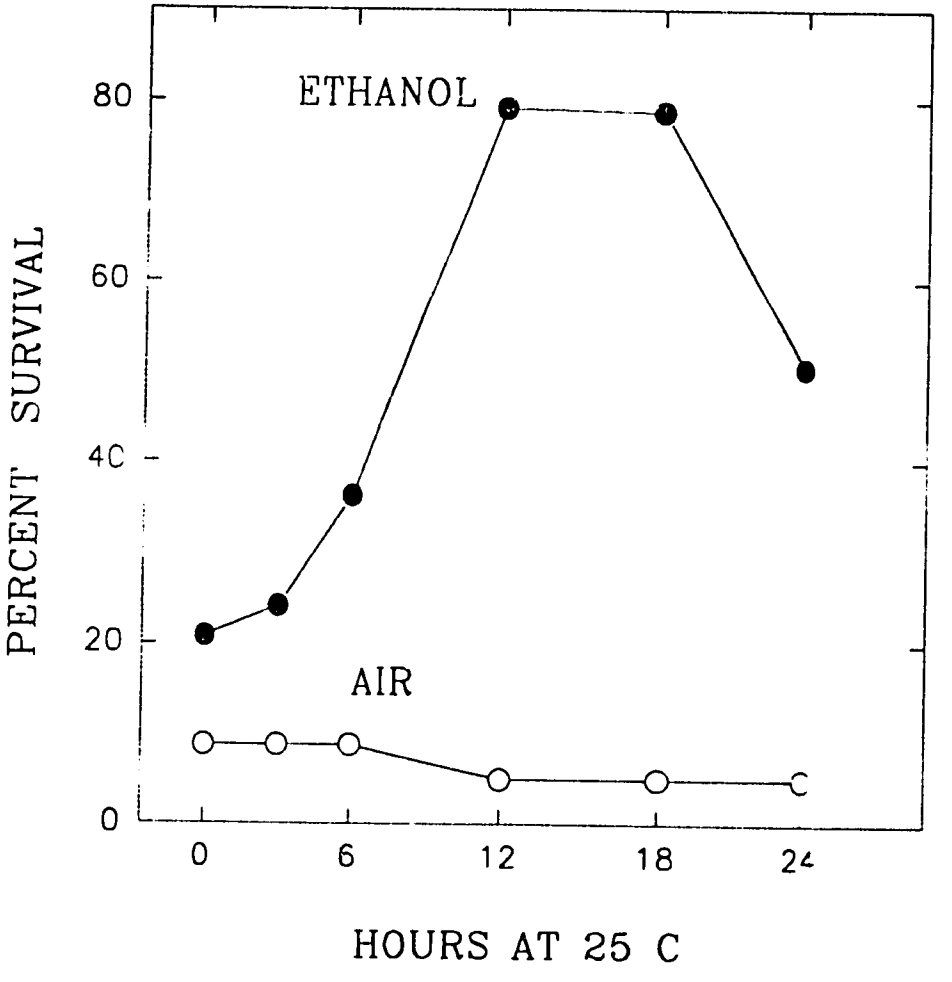
ETHANOL
AIR
PERCENT SURVIVAL
80
60
40
20
0
0
6
12
18
24
HOURS AT 25 C

Figure 4

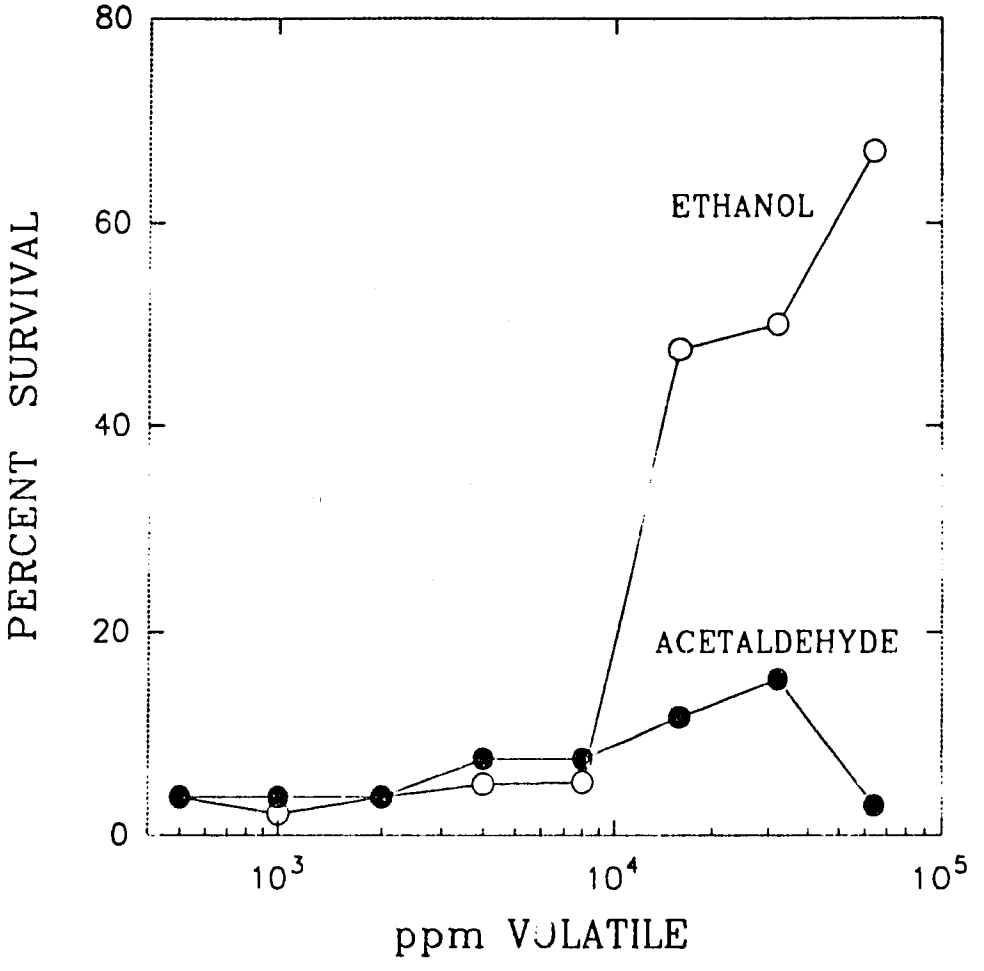
80
60
40
20
0
PERCENT SURVIVAL
ETHANOL
ACETALDEHYDE
10^3
10^4
10^5
ppm VOLATILE

Figure 5

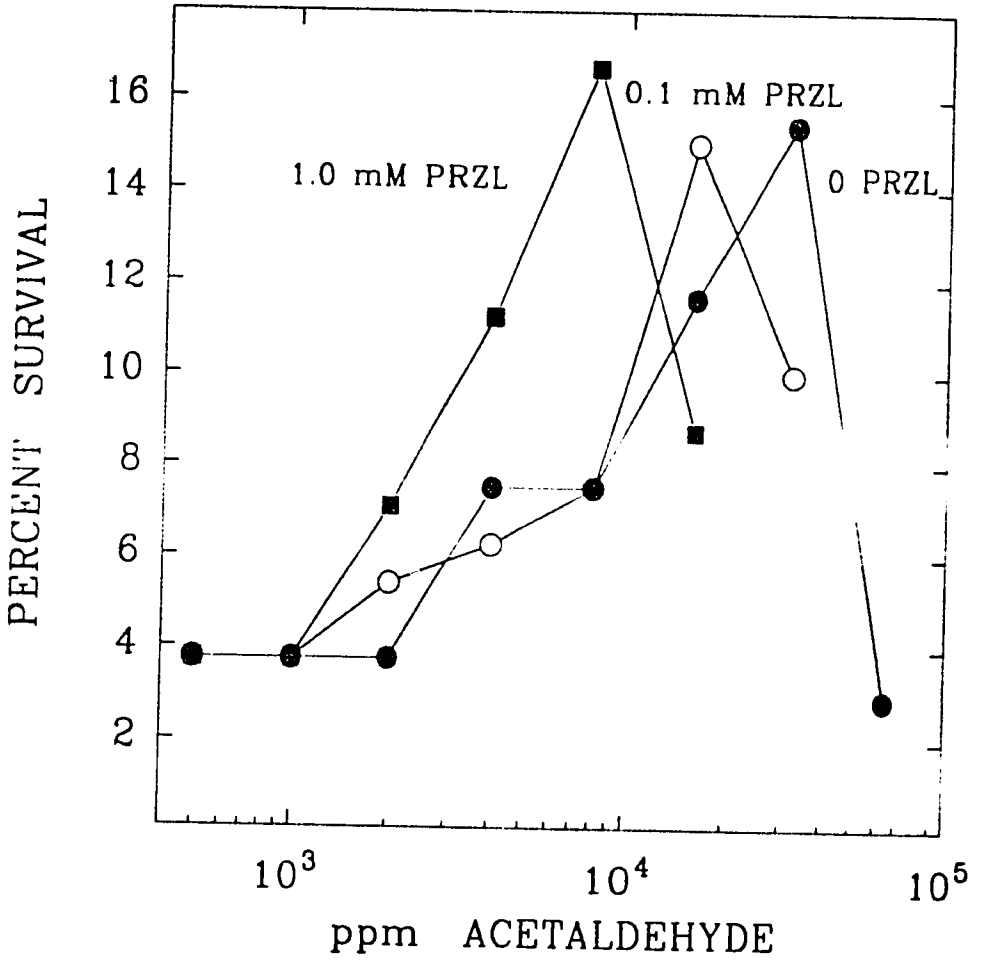
16
14
12
10
8
6
4
2
PERCENT SURVIVAL
1.0 mM PRZL
0.1 mM PRZL
0 PRZL
10^3
10^4
10^5
ppm ACETALDEHYDE

Figure 6

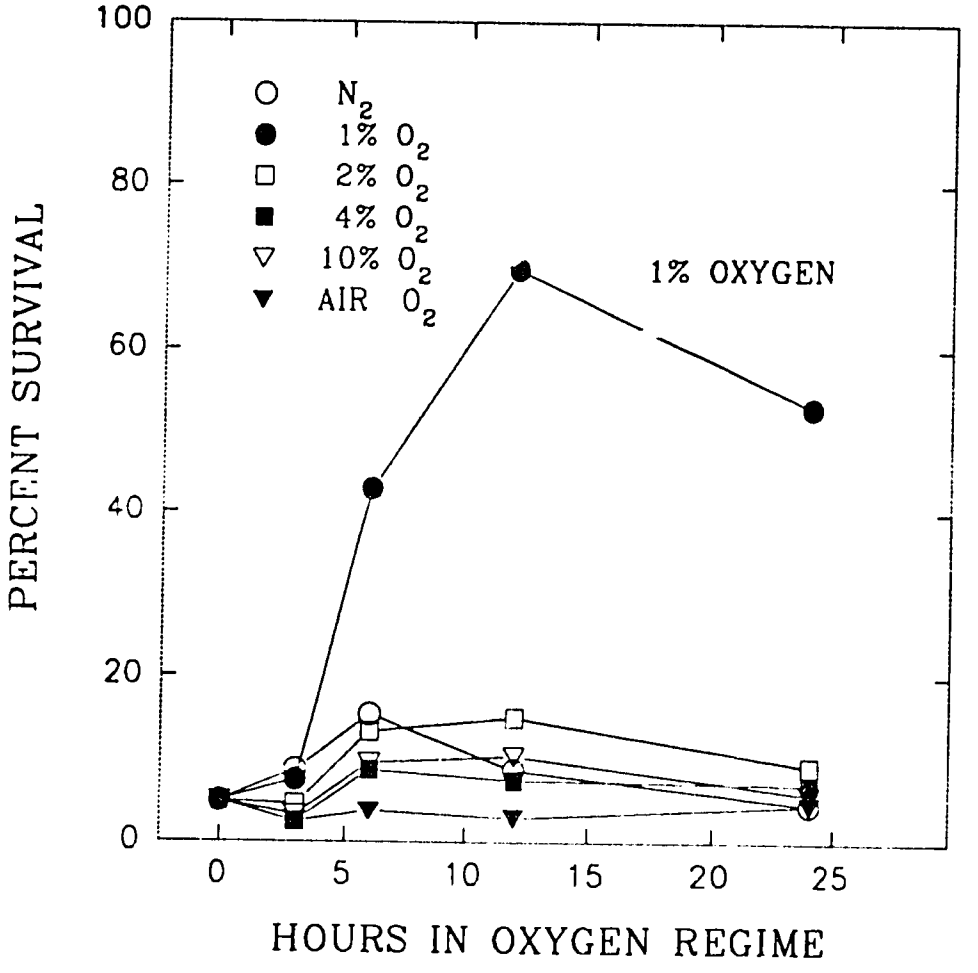
100
80
60
40
20
0
PERCENT SURVIVAL
N2
1% O2
2% O2
4% O2
10% O2
AIR O2
1% OXYGEN
0
5
10
15
20
25
HOURS IN OXYGEN REGIME

Figure 7

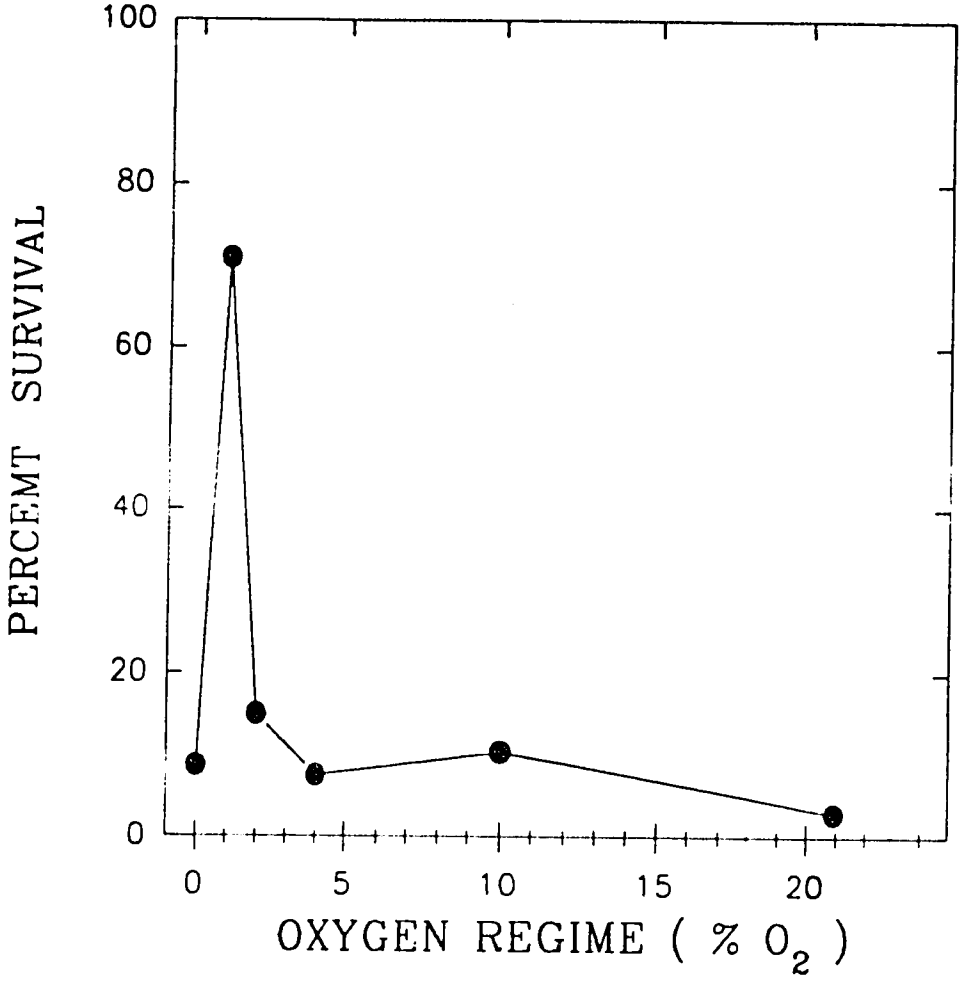

Figure 8

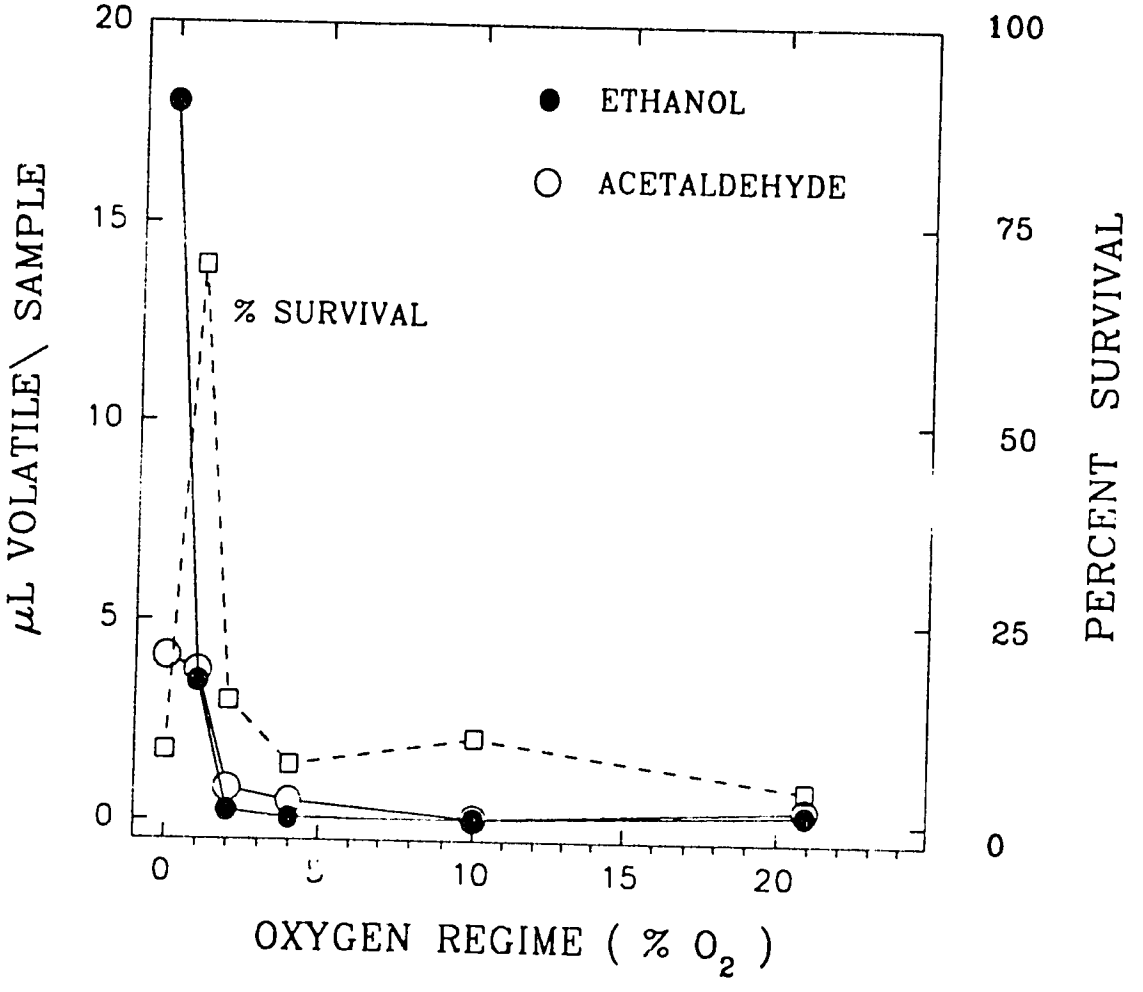
ETHANOL
ACETALDEHYDE
% SURVIVAL
μL VOLATILE\ SAMPLE
PERCENT SURVIVAL
OXYGEN REGIME (% O_2)
20
15
10
5
0
100
75
50
25
0
0
5
10
15
20

Figure 9

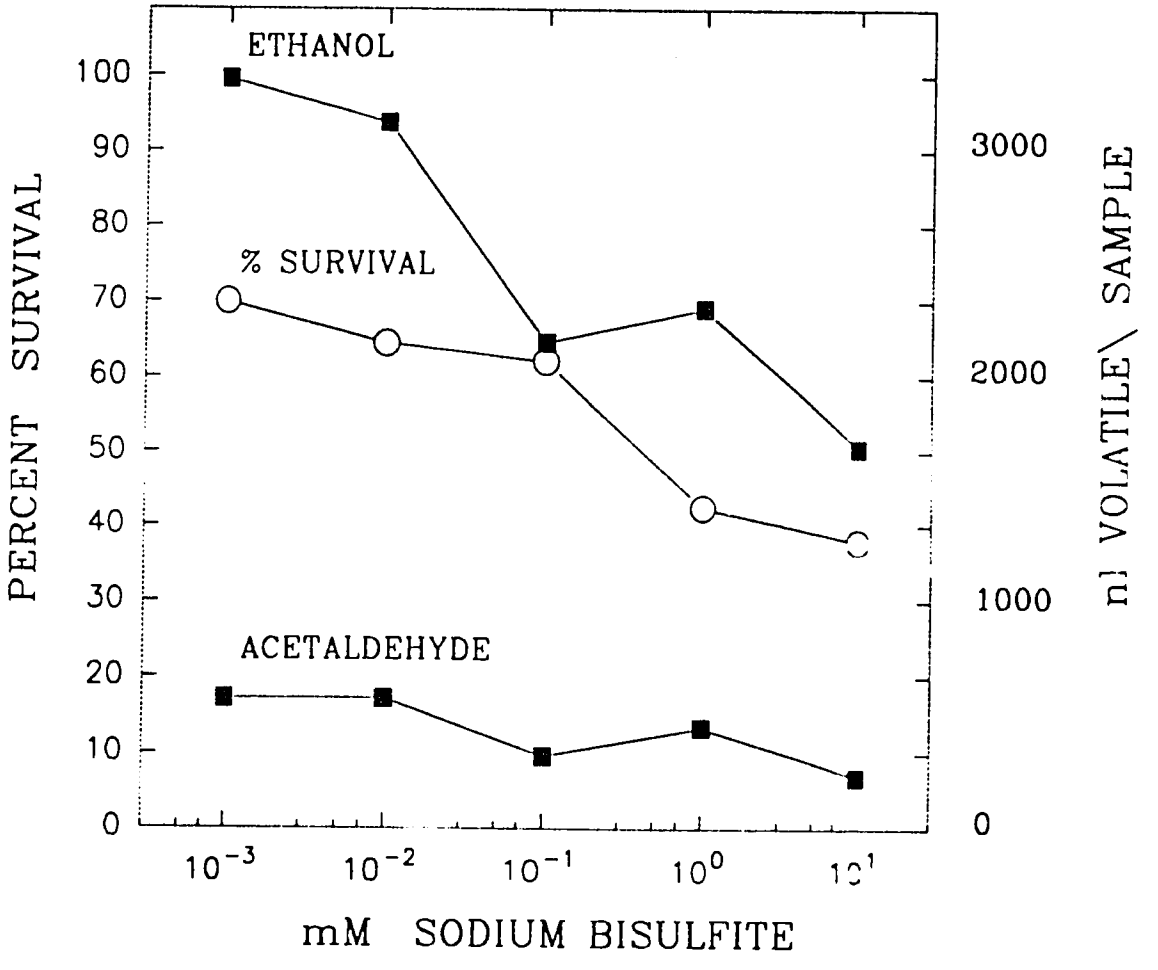
ETHANOL
% SURVIVAL
ACETALDEHYDE
PERCENT SURVIVAL
nl VOLATILE\ SAMPLE
mM SODIUM BISULFITE
100
90
80
70
60
50
40
30
20
10
0
3000
2000
1000
0
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}

Figure 10

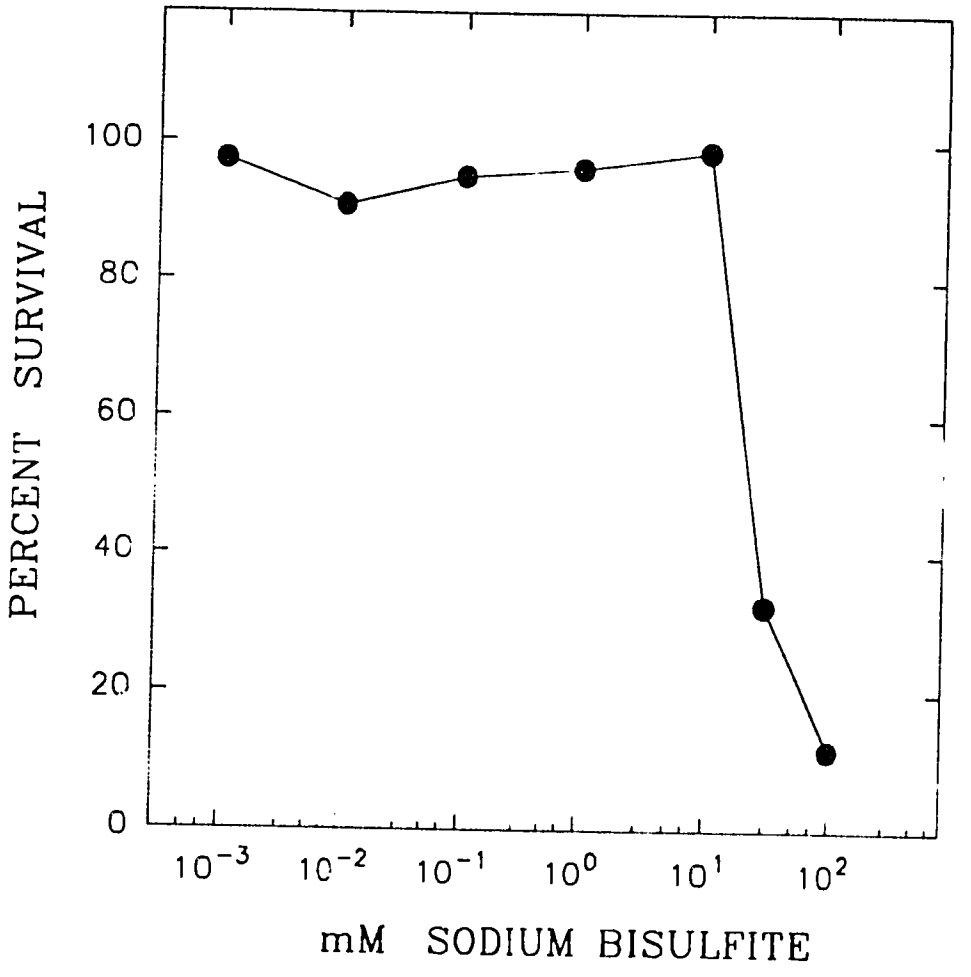
PERCENT SURVIVAL
100
80
60
40
20
0
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
mM SODIUM BISULFITE

Figure 11

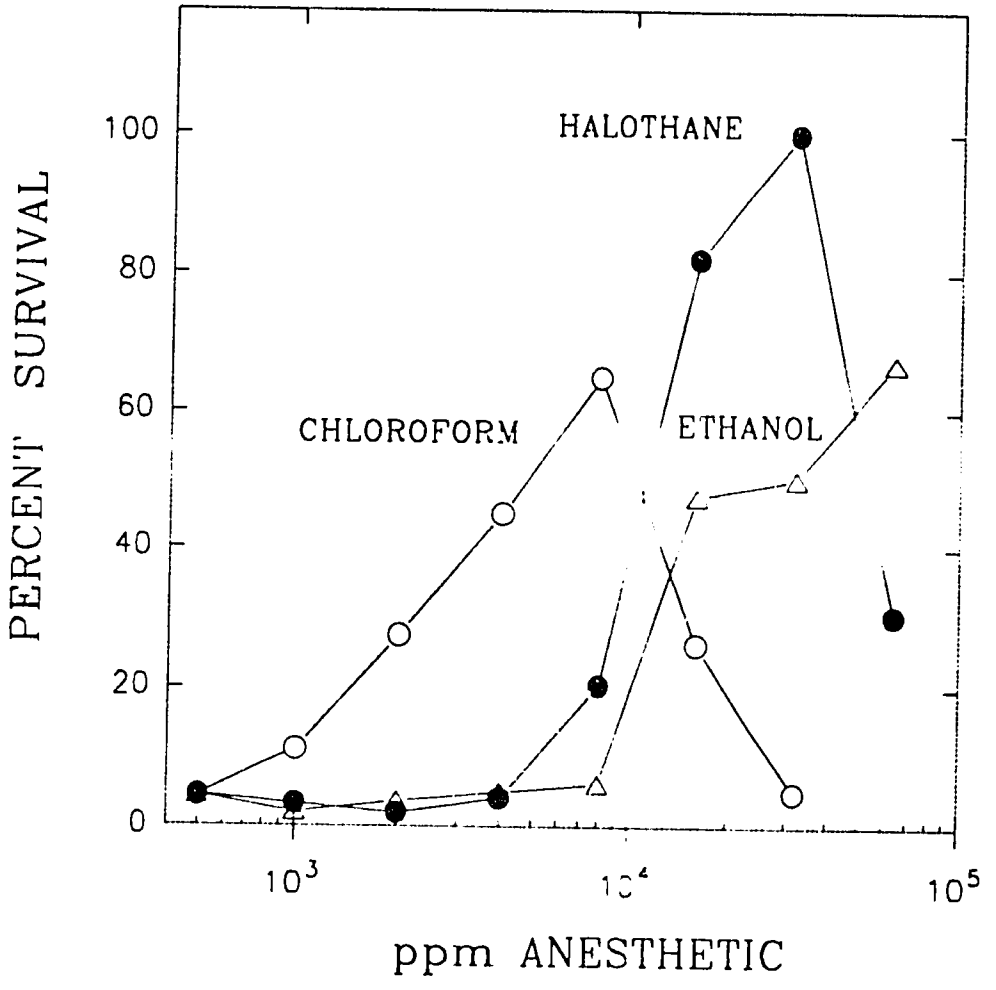
HALOTHANE
CHLOROFORM
ETHANOL
PERCENT SURVIVAL
100
80
60
40
20
0
10^3
10^4
10^5
ppm ANESTHETIC

Figure 12

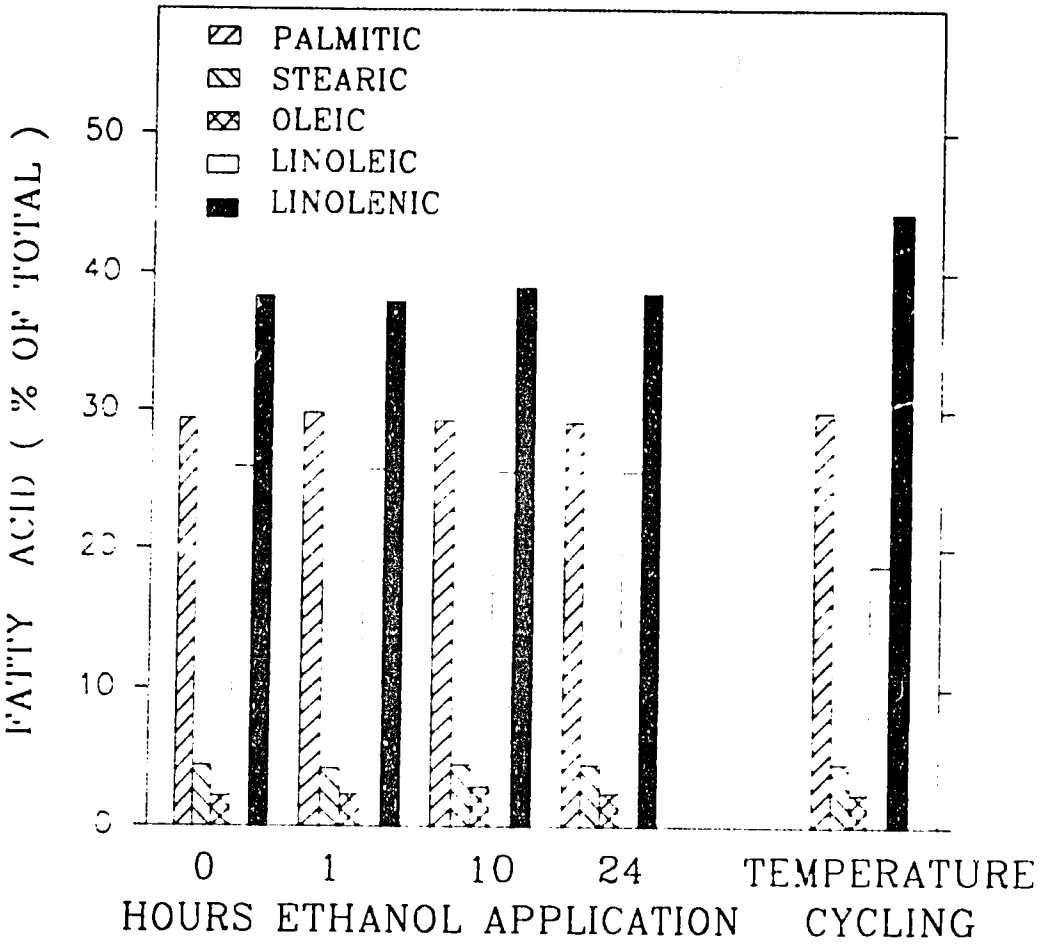
PALMITIC
STEARIC
OLEIC
LINOLEIC
LINOLENIC
FATTY ACID (% OF TOTAL)
50
40
30
20
10
0
0
1
10
24
TEMPERATURE
HOURS ETHANOL APPLICATION
CYCLING

Figure 13