Plant Physiol. (1992) 98, 913–918
0032-0889/92/98/0913/06/$01.00/0

Received for publication June 3, 1991
Accepted August 20, 1991

Immunological Detection of Acetaldehyde-Protein Adducts in Ethanol-Treated Carrot Cells[1]

Pierdomenico Perata, Paolo Vernieri, Doretta Armellini, Massimo Bugnoli, Franco Tognoni, and Amedeo Alpi*

Dipartimento di Biologia delle Piante Agrarie, Universita' di Pisa, viale delle Piagge 23, I-56124 Pisa, Italy (P.P., P.V., F.T., A.A.); and Centro Ricerche Sclavo, via Fiorentina 1, I-53100 Siena, Italy (D.A., M.B.)

ABSTRACT

Polyclonal antibodies able to recognize protein-acetaldehyde conjugates were produced and characterized. The antibodies react with sodium cyanoborohydride-reduced Schiff's bases between acetaldehyde and a protein, independently of the nature of the macromolecule binding the acetaldehyde moiety. Only conjugates between acetaldehyde or propionaldehyde and a protein are recognized; conjugates obtained with other aldehydes are not reactive. Results concerning the formation of acetaldehyde adducts with carrot (*Daucus carota* L.) proteins are presented as well as the presence of such conjugates in ethanol-treated carrot cell cultures, a system highly sensitive to the presence of ethanol in the culture medium.

Acetaldehyde is the first oxidative product of ethanol metabolism in plants (4). The role of ethanol as a key factor in inducing anoxia injuries to plants has been contentious for a long time. In 1971, McManmon and Crawford (13) suggested that the diversification of the end products of fermentive metabolism could explain flooding tolerance in some species. Jackson *et al.* (9) questioned whether ethanol itself could be responsible for anoxic injuries, but we (17, 18) later demonstrated that ethanol can be toxic to specific plant systems even at concentrations comparable to those commonly found in plant tissues subjected to oxygen deficit.

Studies of the metabolism of ethanol in suspension-cultured carrot cells (15), a system remarkably sensitive to the presence of low concentrations of ethanol, revealed that, in addition to only small amounts of ethanol being metabolized, acetaldehyde production and accumulation could be observed in ethanol-fed carrot cell cultures. The role of acetaldehyde in the induction of injuries was tested, and the results indicated that ethanol toxicity to carrot cells could not be ascribed to ethanol *per se* but derived from its metabolization to acetaldehyde (16).

Monk *et al.* (14) suggested that the reaction of acetaldehyde with proteins to give cross-linkages, carbinolamines, and Schiff's bases may be responsible for cell death during the postanoxic phase. Acetaldehyde binds to proteins leading to the formation of both stable and unstable adducts (8); the latter, postulated to be Schiff's bases, can be stabilized *in vitro* by various reducing agents such as sodium borohydride, sodium cyanoborohydride, and ascorbate (7, 10, 22). In animal systems, it has been reported that acetaldehyde readily binds to blood proteins, including serum albumin (7) and hemoglobin (20), as well as to skeletal muscle actin (24). Likewise Mauch *et al.* (12) reported alterations in the catalytic activity of ribonuclease following binding to acetaldehyde. The authors named above hypothesized a role for acetaldehyde adducts to proteins in the pathogenesis of alcohol-induced liver injuries. Israel *et al.* (8) showed that antibodies can be obtained against epitopes containing the acetaldehyde residue in acetaldehyde-protein condensates and that the antibodies can recognize the adducts regardless of the nature of the protein binding the acetaldehyde moiety.

As part of our work concerning ethanol toxicity to plants (2, 15–18), we report the presence of reaction products of acetaldehyde with carrot cell proteins. We used an immunological approach, with the aim of verifying the presence of such conjugates in relation to the induction of injuries to ethanol-treated carrot cells.

MATERIALS AND METHODS

Plant Material and Culture Conditions

Carrot (*Daucus carota* L.) cells were grown in suspension as described by Perata and Alpi (16). Subculturing was performed every 20 d by inoculating 1 mL packed cell volume (after centrifugation for 5 min at 200*g*) into 25 mL fresh medium. Ethanol was added to the culture medium immediately after subculturing.

Tissue Homogenation and Extraction

Carrot cell proteins were extracted from carrot cells (7 d in culture) using potassium phosphate buffer (50 mM, pH 7), the cells were centrifuged (13000*g*, 10 min), and the supernatant was dialyzed against potassium phosphate buffer.

Preparation of Protein-Acetaldehyde Conjugates

Acetaldehyde was conjugated to KLH[2] as described by Israel *et al.* (8). This conjugate was used as immunogen. BSA conjugated to acetaldehyde, used to screen the sera, was

[1] Research supported by National Research Council of Italy, Special Project RAISA, Subproject 2, paper No. 264.

[2] Abbreviations: KLH, keyhole limphet hemocyanin; Ac, acetaldehyde; $NaCNBH_3$, sodium cyanoborohydride.

prepared as described for KLH. Control proteins were treated as described above with the omission of acetaldehyde.

Conjugates used to characterize the antibody were prepared by reaction of different proteins (100 μg/mL) with acetaldehyde (20 mM) for 3 h. Conjugates were stabilized by addition of sodium cyanoborohydride (6 M excess to acetaldehyde concentration for 3 h). Conjugates of BSA with different aldehydes (20 mM) were prepared as described above.

Carrot cell proteins were treated with acetaldehyde (2 mM) for 3 h and treated with sodium cyanoborohydride as described above.

All conjugates were dialyzed against sodium carbonate buffer (50 mM, pH 9.2) before use with the exception of the immunogen, which was dialyzed against (50 mM sodium phosphate and 75 mM NaCl, [pH 7.4]).

Protein-acetaldehyde conjugates obtained using low acetaldehyde concentrations were prepared as described by Israel *et al.* (8). Briefly, solutions of BSA at a concentration of 0.1 mg/ml in PBS were placed in dialysis bags (2.5 mL) and immersed for 7 d in 500 mL of PBS containing either 0, 0.01, 0.1, or 1 mM acetaldehyde in the presence or absence of 10 mM sodium cyanoborohydride. Subsequently, the BSA solutions were dialyzed against sodium carbonate (5 mM, pH 9).

Immunization Procedure

Immunogen (150 μg) in complete Freund's adjuvant was injected into rabbits (New Zealand whites) once a week for 3 weeks; thereafter, the animals received weekly injections (200 μg, intravenously) for 2 weeks. The rabbits were then bled and sera characterized by ELISA.

ELISA

ELISA plates were coated with 100 μL/well of a solution of acetaldehyde conjugate (10–100 ng/mL) in sodium carbonate buffer (50 mM, pH 9.6) and incubated overnight at 4°C. Plates were then washed with PBS-Tween (0.5% Tween 20), and 250 μL blocking solution (BSA 3% in PBS, pH 8) was added to each well. After washing, 100 μL antibody solution (in PBS [pH 7.4] and 1% BSA) was added to each well, and the plates were incubated at 37°C for 4 h. After washing, 100 μL secondary antibody (goat anti-rabbit immunoglobulin bound to alkaline phosphatase diluted in PBS [pH 7.4] and 1% BSA) was added to each well and incubated for 3 h at 37°C. Plates were then washed, and 200 μL alkaline phosphatase substrate (*p*-nitrophenyl phosphate, disodium, hexahydrate) was added to each well. The reaction was stopped by addition of 50 μL 5 N KOH. A_{405} was measured using a spectrophotometer.

Electrophoresis and Immunoblotting

Carrot cell extracts were subjected to SDS-PAGE in vertical slab gels. Acrylamide concentration in the separation gel was 8%.

Western blot and the dot blot assays were performed as described by Campbell (3), using goat anti-rabbit immunoglobulin bound to horseradish peroxidase (dot blot) or alkaline phosphatase (western blot).

Analytical Procedures

Samples of culture media were obtained and immediately assayed for acetaldehyde using the dimedone reagent as described by Donaldson *et al.* (6) with minor modifications (15).

Proteins were assayed using the Bio-Rad protein assay kit with BSA as the standard.

Chemicals

Enzyme-labeled antibodies, the alkaline phosphatase substrate, BSA, and the other proteins used were from Sigma except for KLH, which was from Calbiochem.

RESULTS

Antibody Characterization

The results of the immunization, reported in Figure 1 indicate that the antiserum obtained (DBPA2, serum 2B) is able to recognize acetaldehyde-modified BSA, whereas the control BSA is not reactive. The antiserum was further tested for reactivity against BSA treated with lower acetaldehyde concentrations. The results (Table I) indicate that even BSA treated with acetaldehyde at concentrations as low as 100 μM in the presence of the Schiff's base-reducing agent (sodium cyanoborohydride) is recognized by the antibodies. No reactivity was detected when acetaldehyde-treated BSA was tested in absence of sodium cyanoborohydride.

To verify whether the antibodies were able to recognize the products of conjugation of acetaldehyde, regardless of the kind of protein used, four different proteins were conjugated with acetaldehyde and the ability of the antibodies to recognize such conjugates was tested. The results (Fig. 2) show that the antiserum recognizes only the proteins treated with acet-

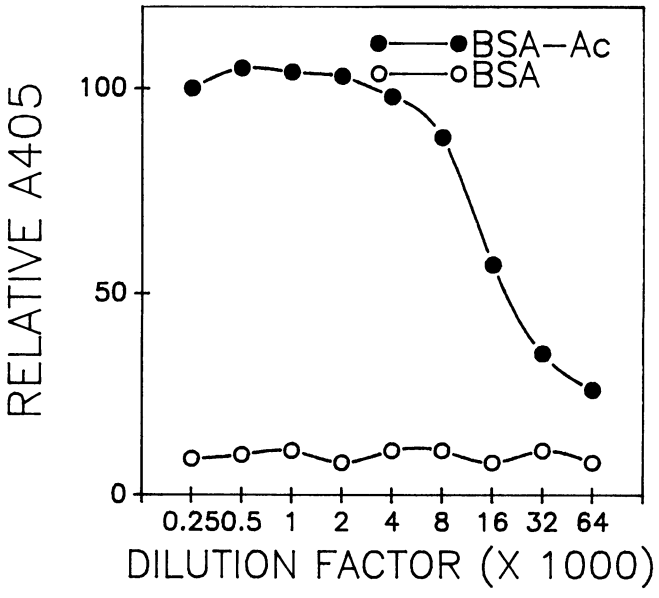

Figure 1. ELISA of serum from rabbit immunized with KLH protein-acetaldehyde conjugate and tested against BSA and BSA-acetaldehyde conjugate.

Table I. *ELISA of BSA-Acetaldehyde Conjugates Prepared at Low Acetaldehyde Concentrations in Presence or Absence of Sodium Cyanoborohydride*

BSA solutions were incubated with acetaldehyde as described in "Materials and Methods." $n = 5$.

Acetaldehyde Concentration	Sodium Cyanoborohydride Concentration	A_{405}
	mM	
0	10	0.25 ± 0.03[a]
0.01	10	0.34 ± 0.07
0.1	10	0.76 ± 0.09
1.0	10	2.70 ± 0.13
1.0	0	0.27 ± 0.02

[a] Mean ± SE.

Table II. *Specificity of the Antiserum Against Acetaldehyde-Protein Adducts*

BSA was used at a concentration of (100 μg/ml). Aldehydes were added to the reaction mixture at a concentration of 20 mM. Schiff's bases were reduced using sodium cyanoborohydride (6 M excess). Conjugates were tested for reactivity using the ELISA method.

Aldehyde	A_{405}	%
Formaldehyde	0.09	6.6
Acetaldehyde	1.35	100
Propionaldehyde	1.43	106
Butanal	0	0
2-Butanal	0	0
Isobutanal	0	0
Pentanal	0	0
Isopentanal	0	0
Esanal	0	0
Nonenal	0	0

aldehyde, whereas the control proteins are not reactive; an exception to this is represented by KLH, being the carrier protein used as immunogen. Nonetheless, affinity-purified serum recognizes acetaldehyde-conjugated KLH but not the unreacted protein (not shown).

The specificity of the antiserum against protein adducts with different aldehydes was also tested (Table II). The antiserum recognizes only the products of conjugation between the protein and acetaldehyde or propionaldehyde; the latter is absent in carrot cell cultures (15), allowing the use of the antibody without any risk of unspecific results.

Detection of Acetaldehyde-Protein Adducts in Carrot Cells

The ability of the antibody to recognize acetaldehyde adducts to carrot proteins was verified. Acetaldehyde was conjugated to proteins extracted from carrot cells, and the reactivity of the antibodies against this conjugate was tested. The results, shown in Figure 3, indicate the ability of the antibodies to recognize the product of stable conjugation of acetaldehyde with carrot proteins. No positive signal was obtained in testing control proteins from carrot cells (not shown).

Acetaldehyde supplied to carrot cells rapidly disappears, being reduced to ethanol by alcohol dehydrogenase, but ethanol-fed carrot cells produce acetaldehyde, which accumulates in the culture medium (15); the treatment with ethanol was, therefore, chosen to ensure a longer cell exposure time to the aldehyde. We first evaluated the presence of endogenously reduced stable adducts, detecting only a very faint signal of such a presence in long-term ethanol-treated cells (Fig. 4A) as well as in short-term acetaldehyde-treated cells (data not shown).

The presence of unstable, borohydride-reducible conjugates was verified by adding sodium cyanoborohydride (for 3 h) to the culture medium of 14-d-old ethanol-treated carrot cell cultures. The results, presented in Figure 4, indicate the presence of unstable (borohydride-reducible) conjugates in the cells treated with ethanol, whereas no response is evident for control cells if ethanol is absent from the culture medium.

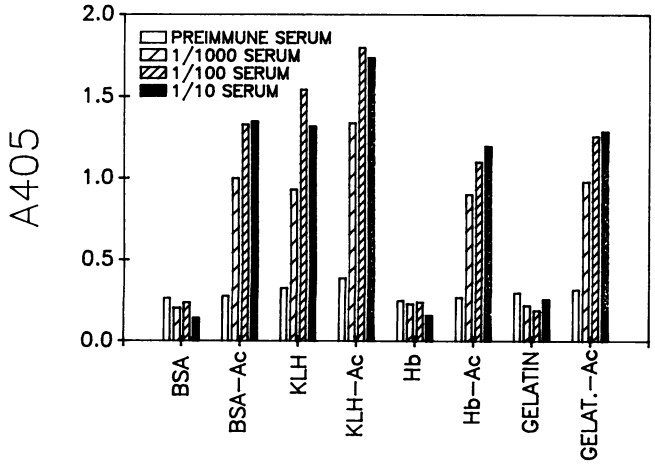

Figure 2. Serum against protein-acetaldehyde conjugates tested against different protein-acetaldehyde conjugates. Hb, hemoglobin; GELAT., gelatin.

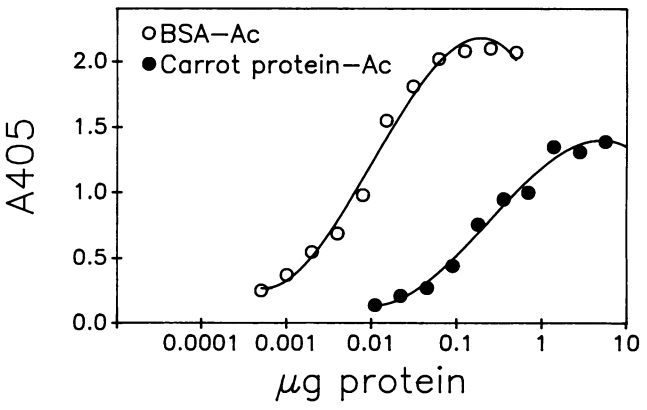

Figure 3. Immunological recognition of carrot proteins conjugated to acetaldehyde. The conjugates were prepared as described in "Materials and Methods" and tested by ELISA. The serum was diluted 1:2000.

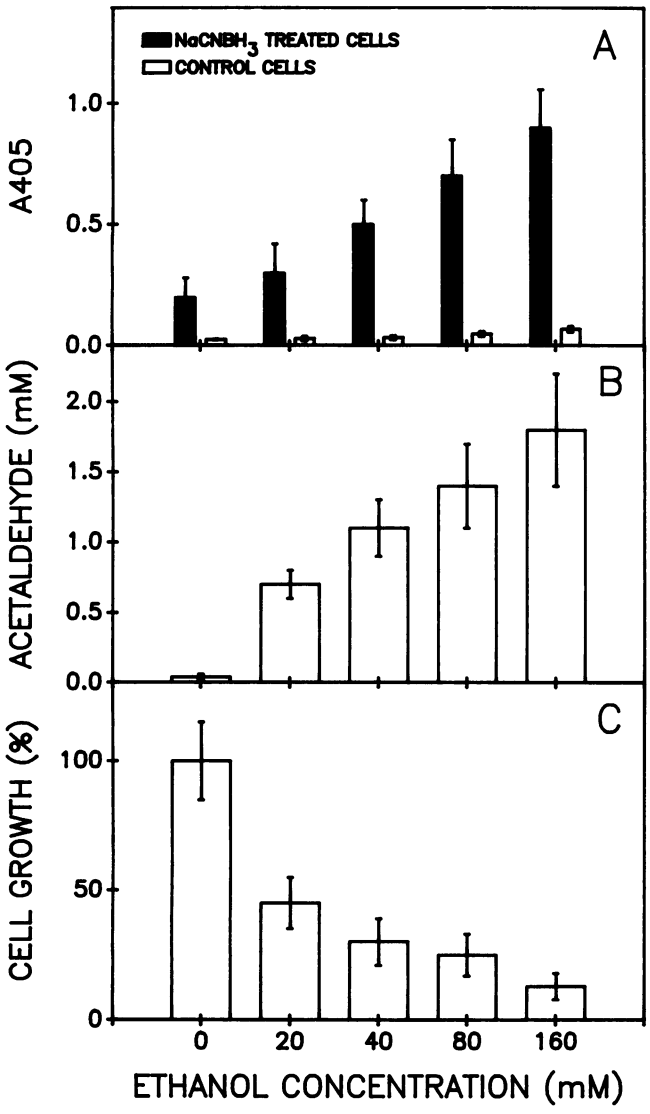

Figure 4. Carrot cells treated with different ethanol concentrations. A, ELISA of the proteins extracted from ethanol-treated carrot cells; B, acetaldehyde production in ethanol-treated carrot cell cultures (data are the mean concentrations of acetaldehyde in the culture medium for 1 to 14 d in culture); C, inhibition of cell growth due to the presence of ethanol in the culture medium. Bars, ± SD.

The amount of stabilized conjugates is positively correlated with the amount of acetaldehyde present in the culture medium and negatively with cell growth.

The results obtained were also confirmed by dot blot of the same extracts (Fig. 5), but the ELISA method proved to be more reliable and sensitive.

The results of the immunoblotting on electrophoretically separated carrot cell proteins (Fig. 6) indicated that the conjugation of acetaldehyde to carrot proteins is an nonspecific process, almost all the proteins being conjugated with the aldehyde even if a more intensely stained band at about 50 kD is also detectable.

DISCUSSION

Antibodies raised against acetaldehyde-modified protein can recognize the small acetaldehyde-containing epitope in macromolecules (8). The data reported by Israel *et al.* (8) indicate that the lysine-acetaldehyde adduct, comprising the *N*-ethyllysine residue, is immunologically recognized by the antibodies.

We report here that immunization with KLH-acetaldehyde conjugate results in the production of antibodies able to recognize the product of conjugation between acetaldehyde and other proteins, including those extracted from carrot cells. Moreover, the antibodies obtained show good specificity of the aldehyde used to produce the conjugate: only acetaldehyde and propionaldehyde conjugates are in fact reactive.

The antibodies only recognize sodium cyanoborohydride-reduced conjugates, indicating that the epitope recognized derives from the formation of a Schiff's base, between the carbonyl group of the aldehyde and an amino acid residue in the protein, reduced subsequently by sodium cyanoborohydride to a stable secondary amine. The reduction of a Schiff's base can occur *in vivo*, prrvided a physiological reducing agent is present in the cell (22).

Our results indicate that acetaldehyde readily forms unstable, borohydride-reducible, adducts with proteins in ethanol-treated carrot cells. Only a faint response was observed with endogenously stabilized conjugates.

Some additional observations should be noted when considering unstable conjugates. If a concentration of acetaldehyde comparable to that due to the presence of ethanol is added to the medium of control cells together with sodium cyanoborohydride, an ELISA signal comparable to that obtained from the extract of ethanol-treated cells (14 d of exposure to the aldehyde) can be detected (not shown). This fact indicates that the reaction of conjugation is immediate, whereas the reaction of reduction of the Schiff's base is strictly time dependent: no conjugates are detected if cells are washed before the addition of sodium cyanoborohydride, resulting in the fast hydrolysis of any unstable conjugates that may be present. The possibility of an amplification effect due to the contemporaneous presence of acetaldehyde and sodium cy-

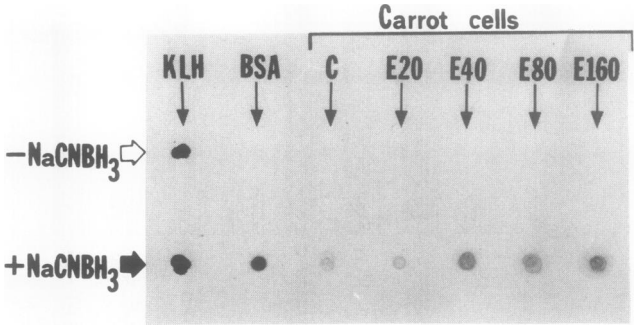

Figure 5. Dot blot of extracts from carrot cells fed with ethanol. Extracts, prepared as described in "Materials and Methods," were from the same cells as the experiment reported in Figure 4. C, Control; E20, E40, E80, E160, 20, 40, 80, 160 mM ethanol, respectively.

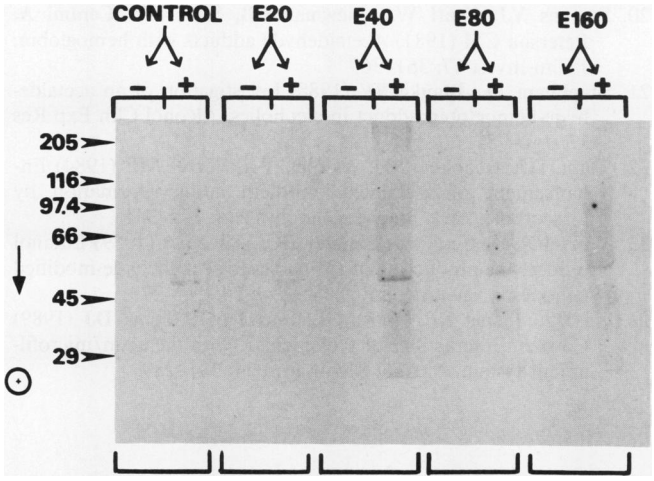

Figure 6. Immunoblotting of carrot cell proteins subjected to SDS-PAGE and Western blot. Extracts, prepared as described in "Materials and Methods," were from the same cells as the experiment reported in Figure 4. C, Control; E20, E40, E80, E160, 20, 40, 80, 160 mM ethanol, respectively. Numbers along left ordinate are molecular masses (in kD) of standard proteins; ⊕ indicates polarity.

anoborohydride should also be taken into account: although the reducing agent has no stimulating effect on the formation of Schiff's bases, the subtraction of the unstable conjugate (which is reduced) from the equilibrium between the free reagents and the Schiff's base could lead to the formation of new Schiff's bases, easily detected using the immunological method described here.

The formation of Schiff's bases between acetaldehyde and proteins may be harmful to carrot cells. This view is also supported by two lines of evidence: (a) acetaldehyde toxicity to carrot cells is stronger if cells are continuously exposed to the presence of the aldehyde (*e.g.* in ethanol-treated cells) and (b) the toxic effects of ethanol (due to its oxidation to acetaldehyde) are reversible: if cells are transferred to ethanol-free medium, normal growth is restored.

We failed to demonstrate clear evidence of the formation of endogenously reduced stable acetaldehyde adducts to proteins. This could indicate that stable conjugates are present in amounts not easily detectable by using the method described. Attempts to enhance the sensibility of the immunological method (different ELISA methods, new immunizations in rabbits and mice, different methods of treatment and extraction of the cells) were unsuccessful.

All of the data available concerning the presence of acetaldehyde adducts to proteins in animal systems indicate that chronic ethanol administration to laboratory animals leads to the generation of antibodies against protein-acetaldehyde conjugates, suggesting that these adducts are produced *in vivo* and can act as neoantigens (8, 23). Nevertheless, direct evidence concerning the *in vivo* formation of such aldehyde adducts is lacking (21).

Until now, no data were available concerning the presence of acetaldehyde conjugation products in plant systems, although acetaldehyde production in plants is well documented (1, 5, 11, 15, 19) and the toxic role of acetaldehyde has been proven in *in vitro* cultured carrot cells (16).

Mauch *et al.* (12) demonstrated that the covalent binding of acetaldehyde to catalytically essential lysyl residues can strongly inhibit the enzymatic activity of ribonuclease.

Our results indicate that the conjugation of acetaldehyde to carrot protein in ethanol-treated cells is a nonspecific process, as demonstrated by the immunoblotting of electrophoretically separated carrot proteins. As a consequence, conjugation of acetaldehyde can also occur with proteins having an essential function for carrot cell growth with adverse consequences if some of these macromolecules contain lysine residues in the active site or if the reaction of conjugation leads to conformational changes in the protein.

Nevertheless, evidence for a direct relationship between acetaldehyde conjugation to proteins and the induction of toxicity to carrot cells is still lacking and would probably require studies of the effects of acetaldehyde on a more defined target protein rather than on a whole living system such as carrot cell cultures.

LITERATURE CITED

1. **Adkins SW, Shiraishi T, McComb JA** (1990) Rice callus physiology: identification of volatile emissions and their effects on culture growth. Physiol Plant **78:** 526–531
2. **Alpi A, Perata P, Beevers H** (1985) Physiological responses of cereal seedlings to ethanol. J Plant Physiol **119:** 77–85
3. **Campbell AM** (1987) Monoclonal Antibody Technology. Elsevier, New York
4. **Cossins EA** (1978) Ethanol metabolism in plants. *In* DD Hook, RMM Crawford, eds, Plant Life in Anaerobic Environment. Ann Arbor Sciences Publishers, Ann Arbor, MI, pp 169–202
5. **Des S, Thomas D, Murashige T** (1979) Volatile emissions of plant tissue cultures. I. Identification of the major components. In Vitro **15:** 654–658
6. **Donaldson RP, Soochan P, Zaras A** (1985) Anaerobic stress in germinating castor bean, ethanol metabolism and effects on subcellular organelles. Plant Physiol **77:** 983–987
7. **Donohue TM, Tuma DJ, Sorrel MF** (1983) Acetaldehyde adducts with proteins: binding of [^{14}C]acetaldehyde to serum albumin. Arch Biochem Biophys **220:** 239–246
8. **Israel Y, Hurwitz E, Niemela O, Arnon R** (1986) Monoclonal and polyclonal antibodies against acetaldehyde-containing epitopes in acetaldehyde-protein adducts. Proc Natl Acad Sci USA **83:** 7923–7927
9. **Jackson MB, Herman B, Goodenough A** (1982) An examination of the importance of ethanol in causing injury to flooded plants. Plant Cell Environ **5:** 163–172
10. **Jentoft N, Dearborn DG** (1979) Labeling of proteins by reductive methylation using sodium cyanoborohydride. J Biol Chem **254:** 4359–4365
11. **Kimmerer TW, MacDonald RC** (1987) Acetaldehyde and ethanol biosynthesis in leaves of plants. Plant Physiol **84:** 1204–1209
12. **Mauch TJ, Tuma DJ, Sorrel MF** (1987) The binding of acetaldehyde to the active site of ribonuclease: alterations in catalytic activity and effects of phosphate. Alcohol Alcoholism **22:** 103–112
13. **McManmon M, Crawford RMM** (1971) A metabolic theory of flooding tolerance: the significance of enzyme distribution and behaviour. New Phytol **70:** 299–306
14. **Monk LS, Braendle R, Crawford RMM** (1987) Catalase activity

and post anoxic injury in monocotyledonous species. J Exp Bot **38:** 233–246

15. **Perata P, Alpi A** (1991) Ethanol metabolism in suspension cultured carrot cells. Physiol Plant **82:** 103–108
16. **Perata P, Alpi A** (1991) Ethanol-induced injuries to carrot cells. The role of acetaldehyde. Plant Physiol **95:** 748–752
17. **Perata P, Alpi A, LoSchiavo F** (1986) Influence of ethanol on plant cells and tissues. J Plant Physiol **126:** 181–188
18. **Perata P, LoSchiavo F, Alpi A** (1988) Ethanol production and toxicity in suspension-cultured carrot cells and embryos. Planta **173:** 322–329
19. **Righetti B, Magnanini E, Infante R, Predieri S** (1990) Ethylene, ethanol, acetaldehyde and carbon dioxide released by *Prunus avium* shoot cultures. Physiol Plant **78:** 507–510
20. **Stevens VJ, Fantl WJ, Newman CB, Sims RV, Cerami A, Peterson CM** (1981) Acetaldehyde adducts with hemoglobin. J Clin Invest **67:** 361–369
21. **Stockham TL, Blanke RV** (1988) Investigation of an acetaldehyde-hemoglobin adduct in alcoholics. Alcohol Clin Exp Res **12:** 748–754
22. **Tuma DJ, Donohue TM, Medina VA, Sorrel MF** (1984) Enhancement of acetaldehyde-protein adduct formation by L-ascorbate. Arch Biochem Biophys **234:** 377–381
23. **Worral S, De Jersey J, Shanley BC, Wilce PA** (1989) Ethanol induces the production of antibodies to acetaldehyde-modified epitopes in rats. Alcohol Alcoholism **24:** 217–223
24. **Xu DS, Jennet RB, Smith SL, Sorrel MF, Tuma DJ** (1989) Covalent interactions of acetaldehyde with the actin/microfilament system. Alcohol Alcoholism **24:** 281–289