Nutrient Interactions and Toxicity

In Vivo Rates of Skeletal Muscle Protein Synthesis in Rats Are Decreased by Acute Ethanol Treatment but Are Not Ameliorated by Supplemental α-Tocopherol

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ABSTRACT Some studies have shown that reductions in tissue protein synthesis, under a variety of cytotoxic conditions, are ameliorated by α-tocopherol (ATC) supplementation. We have also shown evidence of increased oxidative stress and reduced protein synthesis rates in alcohol-exposed muscle. Serum levels of ATC fall and rates of muscle protein synthesis are reduced in patients with alcoholic myopathy. We therefore tested the hypothesis that treatment with ATC could ameliorate the ethanol-induced changes in muscle protein synthesis, a contributory event in the pathogenesis of alcoholic muscle disease. Studies were carried out on gastrocnemius (Type II fiber-predominant and usually considered representative of the musculature as a whole), soleus (Type I fiber-predominant) and plantaris (Type II fiber-predominant) muscles. For comparative purposes, we also investigated the liver. Young male Wistar rats (90 g body weight) were injected intraperitoneally (i.p.) daily with ATC (30 mg/kg body weight) in Intralipid fat emulsion (0.1 mL/100 g body, i.p.) for 5 d. Controls were similarly injected with the Intralipid vehicle alone. After ATC supplementation, rats were given ethanol (75 mmol/kg body weight, i.p., 2.5 h) or saline (0.15 mol/L NaCl, i.p.). Fractional rates of tissue protein synthesis (i.e., the percentage of the tissue protein pool renewed each day), k,s, and RNA activities [i.e., the amount of protein synthesis each day per unit RNA, kRNA, mg protein/d/mg RNA] were then measured. Supplementation increased ATC concentrations in plasma, gastrocnemius and liver. There was no effect of ATC supplementation alone on k,s in any of the tissues. ATC supplementation in the absence of alcohol increased kRNA in the plantaris muscle. In nonsupplemented groups, acute ethanol treatment reduced skeletal muscle (soleus, plantaris and gastrocnemius) k,s. Hepatic k,s was not altered by ethanol, although ATC concentrations in this tissue increased due to ethanol. However, none of the changes in muscle k,s or kRNA due to ethanol were significantly affected by ATC supplementation. In conclusion, ATC supplementation does not appear beneficial in ameliorating acute alcohol toxicity in skeletal muscle as defined by reductions in protein synthesis. J. Nutr. 130: 3045–3049, 2000.

KEY WORDS: α-tocopherol • muscle • liver • protein synthesis • rats • alcohol

Alcohol toxicity induces a variety of pathogenic reactions and affects a diverse range of tissues such as the salivary glands (Smith et al. 1996), gastrointestinal tract (Seitz and Poschl 1997), skeletal muscle (Xu et al. 1996), heart (Fernandez Sola et al. 1997) and brain (Zimakin and Deitrich 1997). A reduction in the rate of protein synthesis is a central feature of acute ethanol toxicity affecting all of the aforementioned tissues [see, for example Bonner et al. (2000), Marway et al. (1997), Preedy and Peters (1988), Proctor et al. (1993) and Siddiq et al. (1997)]. These reductions are reported to be related to changes in the activation of translation initiation factors involved in the binding of met-tRNA to the 40S ribosomal subunit, i.e., eukaryotic initiation factor 2B (eIF2B)3 and the initiation factors that are involved in the binding of mRNA to the 43S preinitiation complex elF4E (Lang et al. 1999). Muscle and liver respond differently with respect to these subcellular changes (Lang et al. 1999). In skeletal muscle, reductions in protein synthesis may be an initiating factor in the pathogenesis of the disease entity alcoholic myopathy (Preedy et al. 1990, Reilly et al. 1995). However, cellular factors for initiating the reductions in tissue protein synthesis in alcohol toxicity are unknown, although consideration must be given to the excessive generation of reactive oxygen species (ROS), leading to enhanced lipid peroxidation and/or membrane damage (Adachi et al. 2000b; Preedy et al. 1998a and 1998b). We found that two cholesterol-derived hydroperoxides, 7α-hydroperoxycholest-5-en-3β-ol (7α-OOH) and 7β-hydroperoxycholest-5-en-3β-ol (7β-OOH), were significantly elevated in both soleus and plantaris muscle of rats 24 h after acute ethanol treatment (75 mmol/kg body as used in this study). This reflects greater oxidative stress in the pathology of muscle of rats treated acutely with ethanol and, together with the observations of perturbations in membrane lipids in response to ethanol, has important implications for the pathogenesis of alcohol toxicity. We have also shown evidence of increased oxidative stress and reduced protein synthesis rates in alcohol-exposed muscle. Serum levels of ATC fall and rates of muscle protein synthesis are reduced in patients with alcoholic myopathy. We therefore tested the hypothesis that treatment with ATC could ameliorate the ethanol-induced changes in muscle protein synthesis, a contributory event in the pathogenesis of alcoholic muscle disease. Studies were carried out on gastrocnemius (Type II fiber-predominant and usually considered representative of the musculature as a whole), soleus (Type I fiber-predominant) and plantaris (Type II fiber-predominant) muscles. For comparative purposes, we also investigated the liver. Young male Wistar rats (90 g body weight) were injected intraperitoneally (i.p.) daily with ATC (30 mg/kg body weight) in Intralipid fat emulsion (0.1 mL/100 g body, i.p.) for 5 d. Controls were similarly injected with the Intralipid vehicle alone. After ATC supplementation, rats were given ethanol (75 mmol/kg body weight, i.p., 2.5 h) or saline (0.15 mol/L NaCl, i.p.). Fractional rates of tissue protein synthesis (i.e., the percentage of the tissue protein pool renewed each day), k,s, and RNA activities [i.e., the amount of protein synthesis each day per unit RNA, kRNA, mg protein/d/mg RNA] were then measured. Supplementation increased ATC concentrations in plasma, gastrocnemius and liver. There was no effect of ATC supplementation alone on k,s in any of the tissues. ATC supplementation in the absence of alcohol increased kRNA in the plantaris muscle. In nonsupplemented groups, acute ethanol treatment reduced skeletal muscle (soleus, plantaris and gastrocnemius) k,s. Hepatic k,s was not altered by ethanol, although ATC concentrations in this tissue increased due to ethanol. However, none of the changes in muscle k,s or kRNA due to ethanol were significantly affected by ATC supplementation. In conclusion, ATC supplementation does not appear beneficial in ameliorating acute alcohol toxicity in skeletal muscle as defined by reductions in protein synthesis. J. Nutr. 130: 3045–3049, 2000.

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3 Abbreviations used: 7α-OOH, 7α-hydroperoxycholest-5-en-3β-ol; ATC, α-tocopherol; elF2B, eukaryotic initiation factor 2B; elF4E, eukaryotic initiation factor 4E; i.p., intraperitoneal; k,s, fractional rate of protein synthesis; kRNA, RNA activity; ROS, reactive oxidative species; S,b, specific radioactivity of protein-bound amino acid; S,s, specific radioactivity of free amino acid in intracellular pools; S,p, specific radioactivity of free plasma amino acid.
alcohol-induced muscle disorders. Although increased hydroperoxides were shown to be elevated at 24 h (Adachi et al. 2000b), we have also shown significant increases in plantarls 2.5 h after ethanol treatment (Adachi et al. 2000a). A number of studies have implicated oxidant and antioxidant factors in the pathogenesis of other alcohol-related disorders such as alcoholic pancreatic (Van Gossen et al. 1996) and liver diseases (Chen et al. 1995).

Numerous studies have also implicated the generation of ROS and/or enhanced lipid peroxidation in the pathogenesis of reduced tissue protein synthesis. The shelfish toxin okadac acid reduces protein synthesis in cultures of vero cells, by a mechanism involving increased lipid peroxidation (Matias et al. 1999). Similarly, an increase in intestinal lipid peroxidation in Salmonella typhimurium infection perturburs amino acid transport, thereby leading to a reduction in enterocyte protein synthesis (Mehta et al. 1998). In metabolically degenerated neuronal tissue in vitro and liver tissue slices, there are also decreases in protein synthesis in response to enhanced lipid peroxidation (Fraga et al. 1989, Uto et al. 1995). These reductions in protein synthesis can be prevented by α-tocopherol (ATC) (Fraga et al. 1989, Matias et al. 1999, Uto et al. 1995).

From the above, we suggest that ATC supplementation may also have an ameliorative effect on ethanol-induced reductions in skeletal muscle protein synthesis. We tested this hypothesis by investigating changes in tissue ATC concentration and indices of protein metabolism in response to acute ethanol treatment in vivo, with or without ATC supplementation. Comparative reference was also made to the response of the liver because of its central role in both ethanol and ATC metabolism.

MATERIALS AND METHODS

Animals and reagents. Male Wistar rats were obtained from Charles River (Margate, Kent, UK) and consumed ad libitum a commercial pelleted diet containing crude protein, 17.9%; crude fiber 3.6%; carbohydrate, 57%; 13.3 MJ/kg (CRM diet, Special Diets Services, Essex, UK). They were housed in cages in an air-conditioned (20–25°C), humidified (40–60%) animal house with a 12 h light/dark cycle staring at 0800 h. Sterile α-(+–) -tocopherol (950 g/L) for injections was obtained from Sigma Chemical ( Lewes, Sussex, UK) and Intralipid intravenous fat emulsion from Kabi Pharma- cia (Tring, Buckinghamshire, UK). Pyrogallol, HPLC-grade ammonium acetate and HPLC-grade methanol were from Merck/BDH (Poole, Dorset, UK). All other materials and chemicals were from either Sigma Chemical or Merck/BDH.

ATC supplementation and ethanol dosage. Four groups of rats (60–70 g body weight) were weight-ranked into groups of equal mean body weights and allocated for treatment with either vehicle (Intralipid) or ATC (supplemented) and saline or ethanol as listed below. They were then fed and housed as described above for an initial 3-d period until they reached a body weight of ~90 g. Rats were then divided into four groups of equal mean body weight as a 2 × 2 factorial as follows: Group 1, vehicle-treated (5 d), saline-injected (2.5 h); Group 2, vehicle-treated (5 d), ethanol-injected (2.5 h); Group 3, ATC-supplemented (5 d), saline-injected (2.5 h); and Group 4, ATC-supplemented (5 d), ethanol-injected (2.5 h). The use of this ethanol treatment protocol has been reviewed previously (Preedy et al. 1996).

ATC suspensions were prepared by ultrasonic homogenization of α-(+–)-tocopherol in 20% (v/v) Intralipid fat emulsion (mass/L: fractionated soybean oil, 200 g; fractionated egg phospholipids, 12 g; glycercol, 225 g; energy content, 8400 kJ; and osmolality, 350 mOsm/kg) to a concentration of 300 g/L using a sterile Polytron (Phillip Harris, London, UK) homogenizer probe. Rats were injected intraperitoneally (i.p.) daily at 0800 h with either the ATC preparation or Intralipid vehicle as indicated above, for a 5-d period in the proportion of 0.1 mL/100 g body, after which time they weighed ~150 g. This regimen ensured a daily dose of 30 mg ATC/kg body weight, in addition to the small amount of mixed tocopherols present in the Intralipid vehicle (mass/L: Intralipid: 0.024 g RRR-α-tocopherol, 0.184 g RRR-γ-tocopherol and 0.085 g RRR-δ-tocopherol) which totaled 0.296 g. Although control (i.e., vehicle-injected) rats also received the small amount of mixed tocopherols in the Intralipid vehicle, the dose of ATC received represented ~1% that administered to the ATC-supplemented rats. In addition, the biological activities of γ-tocopherol and δ-tocopherol in protection against ROS are far less important than that of ATC (Chow 1985). After supplementation, rats were allowed a 1-d clearance period before i.p. ethanol or saline treatment. This was to ensure that uptake of i.p. injected saline or ethanol was not impaired by residual Intralipid or ATC.

Ethanol-dosed rats were injected with a single bolus of ethanol [75 mmol/kg body weight as 1.0 mL/kg body, i.p., (Preedy et al. 1996)]. Saline-injected controls were similarly injected with isovolumetric 0.15 mol/L NaCl (sterile). Treatment of saline-injected rats was identical to that of ethanol-injected rats.

At 140 min after ethanol or saline injection, rats were injected intravenously with a “floodling dose” of [4-H]-phenylalanine and killed by decapitation (Garlick et al. 1980). Tissue samples were taken for analysis of protein and RNA composition, ATC and fractional rates of protein synthesis (k(α)) as detailed below. Blood was collected in heparin- or fluoride-coated tubes for analysis of plasma ATC and ethanol, respectively. All tissue samples were stored at −70°C until processing; plasma was stored at −20°C (analytes) or −70°C (ethanol) before analysis.

Assays performed. Tissue ATC was extracted and assayed by reverse-phase HPLC (Heap 1994). Briefly, 1.5-mL aliquots of tissue homogenate (0.5 mL plasma) in water were mixed with pyrogallol (1.5 g/L in ethanol, 2.0 mL) and saponified at 70°C for 30 min with 0.45 mL of 10 mol/L KOH. Hexane (4 mL) was used as the extractant and was evaporated under nitrogen. The residues were then redissolved in 0.1 mL methanol. Aliquots of this extract were assayd on a MOS-2 glass-fiber column (Jones Chromatography, Hengoed, Wales) in a mobile phase of 5% of 1 mol/L ammonium acetate/95% (v/v) methanol by fluorescent spectrophotometry using excitation and emission wavelengths of 294 and 320 nm, respectively ([Hitachi F 1050 model fluorescent spectrophotometer; Hitachi, Tokyo, Japan (Heap 1994)]. Total RNA and protein were measured as previously described (Siddiqi et al. 1993).

“Floodling dose” determination of fractional synthesis rates in vivo. The fractional rate of protein synthesis (k(α)), defined as the percentage of tissue protein renewed each day, i.e., %/d was mea- sured as described previously (Garlick et al. 1980) for phenylalanine. Briefly, 1-[4-H]-phenylalanine (150 mmol/L, specific radioactivity −18.5 MBq/mmol, 1 mL/100 g body weight) was injected into rats via a lateral tail vein. Rats were killed by decapitation and tissues were quickly removed, blotted dry, and weighed and frozen in liquid nitrogen. All tissue samples were stored at −70°C until processing for specific radioactivities of phenylalanine in intracellular tissue free (Sf) and protein-bound (Sp) amino acid pools. The fractional rate k(α) was calculated as follows: k(α) = (Sf/Sp × 100)/(Sf × t), %/d. Where t (in units of days) was the period between injection of isotope and freezing of the tissue in liquid nitrogen, during which the label was incorporated. (Garlick et al. 1980). The RNA activity (kRNA), a dynamic measure of the activity of cellular RNA in protein synthesis, defined as the amount of protein synthesized per unit of RNA was derived by division of k(α) by protein “synthetic capacity” (C(α), mg RNA/g protein).

Statistics. All data are expressed as means ± SEM (n = 4–8). Differences between means were assessed by two-way ANOVA. Significant differences were indicated at P < 0.05.

RESULTS

Effects of α-ATC supplementation. ATC-supplementation significantly increased plasma concentrations of ATC (P < 0.001; Table 1). There were also large increases in ATC concentrations in gastrocnemius muscle (P < 0.025) and liver (P < 0.001). There were no significant differences in protein or RNA contents in any of the tissues due to ATC treatment
Liver 83.2 ± 2.8 81.8 ± 3.5 83.5 ± 2.6 86.2 ± 3.4 NS NS NS
Gastrocnemius 15.1 ± 0.5 10.9 ± 0.6 14.4 ± 0.3 11.9 ± 0.6 <0.001 NS NS
Soleus 23.9 ± 2.3 18.4 ± 1.1 22.2 ± 0.9 16.9 ± 0.4 <0.001 NS NS
Plantaris 14.2 ± 0.3 11.2 ± 0.8 15.6 ± 0.6 11.9 ± 0.4 <0.001 NS NS

1 Data are means ± SEM, n = 4–8. NS, not significant, P > 0.05.
2 At 140 min after acute intraperitoneal bolus injections of ethanol or saline, rats were injected with a “flooding dose” of L-[4-3H]Phenylalanine (150 mmol/L, 1 mL/100 g body) via a lateral tail vein. Exactly 10 min after injection of isotope, rats were killed and tissues removed and processed for determination of $S_p$, $S_i$, and $S_o$ specific radioactivities for calculation of $k_s$. 

**DISCUSSION**

The excessive generation of ROS leading to enhanced lipid peroxidation and/or membrane damage is an important feature of alcohol toxicity (Preedy et al. 1998a and 1998b). There are four areas of evidence to support the hypothesis that $\alpha$-tocopherol supplementation may be beneficial in ameliorating alcohol-induced biochemical lesions in muscle. The first pertains to studies (with the same treatment protocol as that used in this study, i.e., 75 mmol/kg body) that show increased oxidative stress as evidenced by elevations in skeletal muscle cholesterol-derivated hydroperoxides, 7α-OOH and 7β-OOH (Adachi et al. 2000a and 2000b). This is suggestive of pertur-
lations in membrane lipids in response to ethanol, which may have important implications for the pathogenesis of alcohol-induced muscle disorders. The second pertains to clinical studies showing reduced levels of serum tocopherol in UK alcoholics with skeletal muscle myopathy compared to patients without myopathy (Ward and Peters 1992). Third, alcoholic patients have reduced rates of muscle protein synthesis as measured by stable isotope incorporation studies (Pacy et al. 1991). Finally, a number of studies have implicated oxidant and antioxidant factors in the pathogenesis of reduced protein synthesis, which can be ameliorated by α-tocopherol (Fraga et al. 1989, Matias et al. 1999, Uto et al. 1995). From the above, we proposed that ATC supplementation may also have an ameliorative effect on ethanol-induced reductions in skeletal muscle protein synthesis especially because ATC protects against a variety of alcohol-induced pathologies (Mitchell et al. 1999, Nanji et al. 1996).

We tested this hypothesis by measuring tissue ATC concentrations and indices of protein metabolism in response to acute ethanol treatment in vivo, with or without prior ATC-supplementation. Comparative reference was also made to the response of the liver because of its central role in both ethanol and ATC metabolism.

Ethanol administration over extended periods causes marked anorexia. The acute ethanol treatment protocol used in this study circumvents this effect, allowing the appraisal of pathologic tissue changes due solely to high levels of circulating ethanol. Reductions in skeletal muscle (gastrocnemius, soleus and plantaris) protein synthesis due to acute ethanol administration were observed, confirming previous studies (Preedy et al. 1990, 1998a and 1998b). Acute dosing experiments reporting a relative lack of effect of ethanol on hepatic protein synthesis in vivo (Donohue et al. 1987) are also confirmed by the present results.

In this study, we focused on in vivo protein synthesis measurements using the “flooding dose” method with phenylalanine (Garlick et al. 1980). This has been reported to be the only feasible method for measuring protein synthesis in intact animals (Davis et al. 1999). None of the observed alterations in $k_{RNA}$ or $k_{RNA}$ in the present study were due to changes in the specific radioactivity of the precursor as reflected by measurement of $S_i$ ($P > 0.05$; data not presented). Recent studies have shown that with the “flooding dose” technique with phenylalanine, there is excellent agreement with actual measurement of aminoacyl tRNA specific radioactivities and free phenylalanine specific radioactivities in vivo (Davis et al. 1999). This does not appear to be influenced by hormonal or nutritional perturbations (Davis et al. 1999).

A number of processes have been proposed as causative mechanisms in alcohol-induced muscle damage, including excessive generation of ROS or impaired antioxidant systems (Reilly et al. 1995). Increased generation of ROS has been reported after acute ethanol administration [see, for example, Preedy et al. (1999) and Reinke et al. (1997)]. Increased skeletal muscle production of ROS has been suggested to arise from intramuscular xanthine oxidase activities causing raised lipid peroxidation of fatty acid moieties within the cellular and subcellular membranes (Reinke and McCay, 1996). The involvement of ROS in the pathogenesis of alcohol-related disorders has previously been assessed by attempts to reverse or ameliorate pathologic changes using antioxidant therapies. Beneficial effects of ATC supplementation on the extent of chronic ethanol-induced hepatic injury have been observed (Nanji et al. 1996). However, there are comparatively few studies on the protective effects of antioxidants on skeletal muscle. In skeletal muscle, the efficacy of ATC supplementation in ameliorating ROS-mediated protein oxidation has been shown after exercise (Meydani et al. 1993), glucocorticoid-induced myopathy (Ohtsuka et al. 1998) and immobilization atrophy (Appell et al. 1997). In ischemia or ischemia-reperfusion injury, α-tocopherol can ameliorate the reductions in muscle ATP, neutrophil infiltration and increased lipid peroxidation; some of the beneficial effects of α-tocopherol may be related to its ability to impede the expression of adhesion molecules such as ICAM-1 (Punz et al. 1998). However, in our studies, ATC supplementation did not significantly attenuate the ethanol-induced reductions in protein synthesis. The reason for this relative lack of effect is unknown but may pertain to the complex etiology of protein synthetic perturbations.

We used a 5-d protocol of ATC supplementation, which was sufficient to raise circulating, liver and muscle levels of ATC. Such acute regimens have proved efficacious in past studies on ROS-associated tissue or metabolic damage in animals (Bauersachs et al. 1993). For example, a single dose given before the onset of ischemia can be effective in ameliorating reductions in muscle ATP [Punz et al. 1998] or reduce malondialdehyde after hypoxia (Varskeviciene et al. 1984).

It is possible that the failure to observe a protective effect of ATC was due to a protocol that allowed 1-d clearance before ethanol administration. However, we do not think this is likely because muscle, liver and plasma ATC concentrations were still raised 1 d after the last dose. The presence of raised oxidative challenge (i.e., increased ROS) after ethanol administration is now well documented, as

### TABLE 3

<table>
<thead>
<tr>
<th>Protein</th>
<th>Vehicle +Saline</th>
<th>α-Tocopherol +Saline</th>
<th>Vehicle +Ethanol</th>
<th>α-Tocopherol +Ethanol</th>
<th>Two-way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>22.3 ± 0.7</td>
<td>21.8 ± 0.8</td>
<td>22.3 ± 0.9</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>17.4 ± 0.4</td>
<td>17.7 ± 0.4</td>
<td>14.6 ± 1.0</td>
<td>&lt;0.005</td>
<td>NS</td>
</tr>
<tr>
<td>Soleus</td>
<td>18.1 ± 1.6</td>
<td>16.9 ± 0.6</td>
<td>13.2 ± 0.5</td>
<td>&lt;0.001</td>
<td>NS</td>
</tr>
<tr>
<td>Plantaris</td>
<td>13.6 ± 0.3</td>
<td>15.6 ± 0.6</td>
<td>11.5 ± 0.5</td>
<td>&lt;0.001</td>
<td>NS</td>
</tr>
</tbody>
</table>

1 Data are means ± SEM, n = 4–8. NS, not significant, $P > 0.05$.
2 For details see legend to Tables 1 and 2.
is the role of ATC as an important antioxidant for membrane lipid moieties (Reinke and McCoy 1996). It is possible that the oxidative stress induced by ethanol may have been insufficient to cause changes in membrane oxidation or that muscle has a higher antioxidant capacity. This can be refuted because ethanol induces oxidative damage to muscle membrane lipids, an effect that is not seen in liver (Adachi et al. 2000a). However, it is important to realize that not all muscle pathologies or indices of muscle damage can be ameliorated by ATC supplementation. For example, raising muscle ATC concentrations by dietary supplementation produced observable improvements in muscle antioxidant capacity without improvement in indices of cellular injury induced by eccentric exercise (Siciliano et al. 1997) or myotonic dystrophy (Omdahl et al. 1994). It seems then that alcohol-induced muscle damage, as reflected by changes in tissue protein synthesis, may fall into this "nonresponsive" category. The inability of skeletal muscle to respond to ATC may reflect the fact that other processes may be involved in ethanol-induced reductions in protein synthesis. Candidate mechanisms include the formation of acetaldehyde-protein adducts, which will render inoperative cytoskeletal or other proteins involved in translation, by a process involving covalent binding (Niemela 1999).

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LITERATURE CITED

Fraga, C. G., Zamora, R. & Tappel, A. L. (1989) Damage to protein synthesis reflected by changes in tissue protein synthesis, may fall into the "nonresponsive" category. The inability of skeletal muscle to respond to ATC may reflect the fact that other processes may be involved in ethanol-induced reductions in protein synthesis. Candidate mechanisms include the formation of acetaldehyde-protein adducts, which will render inoperative cytoskeletal or other proteins involved in translation, by a process involving covalent binding (Niemela 1999).