calpain I which has a specificity for intermediate filament proteins[12,15]. For calpain II, the effectiveness of inhibition by the polyamines is less than that reported for calmodulin. Concentrations of spermine required to give a 50% inhibition of calmodulin effects on target enzymes were about 1 mM[6], whereas our studies indicate that for a 50% inhibition of the calpain II enzymes, the spermine concentration needed is about 25 mM. Spermidine is a less effective inhibitor of calpain II than spermine as concentrations of about 50 mM are required for 50% inhibition. The inhibitory effects of the polyamines on calpain II were synergistically increased in the presence of the endogenous calpain inhibitor calpastatin, with the concentration of spermine required for 50% inhibition being lowered to about 6 mM in the presence of calpastatin. It is therefore possible that the in situ inhibitory effectiveness of the polyamines is of physiological importance, particularly in those tissues which have high calpastatin: calpain II ratios[7]. Further work will be needed to identify the regions of the calpain molecule which are involved in polyamine binding, and to explain the reasons for the differences in inhibitory effectiveness of the polyamines on calpains I and II.

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In vivo inactivation of transglutaminase during the acute acrylamide toxic syndrome in the rat

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Summary. The activity of liver and brain transglutaminase is rapidly lost following i.p. injection of acrylamide (50–200 mg/kg). Other enzymes investigated were not modified by the treatment, with the exception of brain enolase.
Key words. Transglutaminase; in vivo inactivation; acrylamide; neurotoxicity.

Professional and experimental exposure to acrylamide leads to severe poisoning, with morphological and functional changes in the central nervous system, the liver and the gonads[1–4]. The nervous alterations have been thoroughly investigated because of their prominence in the clinical picture[5], but their pathogenesis is still unclear. Interference of the chemical with the energy metabolism of the neuron and with axoplasmic transport has been reported[6–8].

Quite recently, we discovered that acrylamide inactivates purified erythrocyte transglutaminase in a calcium-dependent way[9]. Independent data suggested that this enzyme is of critical importance for the release of granules of secretion in the central nervous system and in the endocrine pancreas[10–12]. These considerations led us to investigate the effects of acrylamide on transglutaminase in vivo to evaluate the possibility that the enzyme is involved in the pathogenesis of the toxic syndrome. The data we report here document that the enzyme is effectively inactivated in vivo by treatments which induce acute toxicity, and that its sensitivity to the chemical compares favorably with that of other proteins whose sensitivity to acrylamide was previously described.

Materials and methods

Analytical grade chemicals and enzyme reagents were obtained from Sigma or Boehringer. Acrylamide was purchased from Merck and recrystallized from ethyl acetate before use. Stock solutions were prepared in phosphate buffered saline and the concentration measured spectrophotometrically[13]. Male Wistar rats (b.wt 200–250 g), maintained on standard rat diet with free access to water, were injected i.p. with acrylamide at the specified dosages; control rats received an equivalent amount of vehicle. At the required time, rats were killed by decapitation and the liver and brain were removed and homogenized with a teflon-glass Potter-Elvehjem apparatus in 6

and 2 volumes respectively of 50 mM Tris, 250 mM sucrose, 1 mM EDTA and 5 mM mercaptoethanol pH 7.5. The homogenates were centrifuged for 10 min at 12,000 rpm in a refrigerated Eppendorf centrifuge and the supernatants were saved for enzyme assays. Transglutaminase activity was determined by a filter paper assay as previously detailed[9]; for the brain extract, the incubation time was extended to 1 h and the specific activity of putrescine was increased to 3500 cpm/nmole. The activities of enolase, lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and glycogen synthetase (total activity) were determined according to published procedures[14-18]. Results are presented as units per milligram of soluble protein[19]. The statistical significance of the acrylamide induced changes was determined by the Wilcoxson test for unpaired data.

Results and discussion

Acrylamide is considered to be a substance which has only relatively mild effects on proteins[20]. The contrast between the impressive effects of the reagent in vivo and the chemical inertness was explained by the observation that only a few proteins are selectively inactivated by the drug. This was observed in vitro[21-23] and, less frequently, in vivo[24,25]. In the majority of cases, the reagent modifies cysteine residues by addition on the double bond, as in substituted maleimides[26]; a notable exception is erythrocyte transglutaminase which is inactivated by a reaction involving the amide moiety of acrylamide[9].

To evaluate the potential of acrylamide for affecting enzymes in vivo, we measured the activity of 6 enzymes in tissue extracts of control and treated animals. Three of these enzymes had previously been reported to be sensitive to acrylamide[9,14,16]. The results, summarized in the table, document a marked decrease of the activity of transglutaminase in liver and brain; under our conditions the total activity of enolase is only marginally decreased in the brain, although it is known that the neuronal $\gamma\gamma$ isoenzyme is selectively altered by the treatment[27]. At variance with previous reports[28], glyceraldehyde phosphate dehydrogenase was not altered under our experimental conditions, nor were other enzymes (glycogen synthetase, lactate and 6 phosphogluconate dehydrogenase) which were chosen as potential controls for the treatment.

The dose-effect plots for the in vivo inactivation of liver transglutaminase (fig. 1), assayed at a constant time from injection (4 h), allow the calculation of a dosage of 110 mg/kg as that required for a 50% decrease of enzyme activity; this value, approximately 2 mM, is of the same

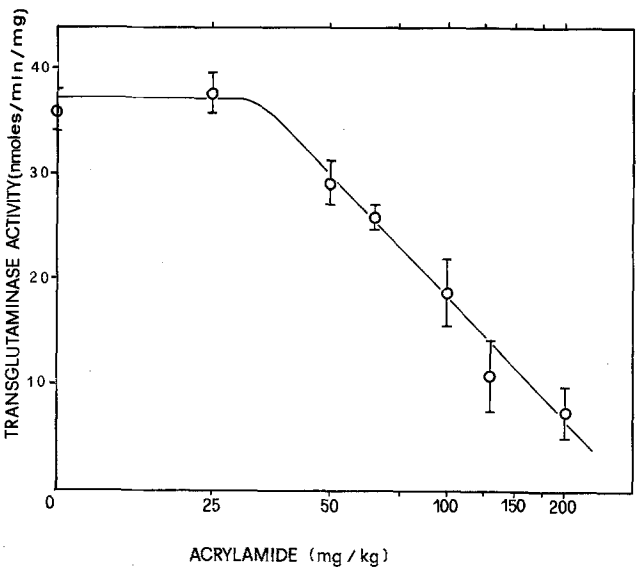

Figure 1. Dose-dependent inactivation of liver transglutaminase by in vivo treatment with acrylamide. Animals (6 per experimental point) were treated with acrylamide as described in Methods and sacrificed 4 h later. The enzyme activity was determined in the supernatant obtained after removal of cell debris from the homogenate by centrifugation.

Influence of in vivo treatment with acrylamide on the activity of liver and brain enzymes

Treatment	Glycogen synthetase		Enolase		Lactate dehydrogenase		6P Gluconate dehydrogenase		Glyceraldehyde-3P-dehydrogenase		Transglutaminase	
	Liver	Brain	Liver	Brain	Liver	Brain	Liver	Brain	Liver	Brain	Liver	Brain
Control	1.57 ± 0.16 (8)	0.49 ± 0.14 (8)	0.148 ± 0.015 (8)	0.318 ± 0.019 (8)	0.070 ± 0.008 (7)	0.023 ± 0.011 (7)	0.040 ± 0.012 (5)	0.0096 ± 0.0015 (5)	0.85 ± 0.13 (6)	0.68 ± 0.13 (6)	0.60 ± 0.05 (14)	0.060 ± 0.014 (13)
Acrylamide 100 mg/kg	1.93 ± 0.16 (6)	0.47 ± 0.18 (6)	–	–	0.065 ± 0.012 (5)	0.021 ± 0.009 (5)	–	–	–	–	0.29 ± 0.06 (8) **	0.025 ± 0.008 (8) **
130 mg/kg	1.61 ± 0.41 (4)	0.38 ± 0.11 (4)	0.144 ± 0.026 (6)	0.281 ± 0.042 (6)	–	–	0.043 ± 0.012 (5)	0.0084 ± 0.0025 (5)	0.91 ± 0.12 (6)	0.73 ± 0.12 (6)	0.18 ± 0.02 (6) **	0.022 ± 0.006 (6) **
200 mg/kg	–	0.46 ± 0.12 (4)	0.135 ± 0.025 (6)	0.265 ± * 0.022 (6)	0.065 ± 0.016 (6)	0.023 ± 0.011 (6)	–	–	0.71 ± 0.14 (6)	0.61 ± 0.16 (6)	0.13 ± 0.03 (8) ***	0.017 ± 0.007 (8) **

Activities (mean ± 2 SEM) are presented as units/mg of soluble proteins (μmoles/min/mg) except for glycogen synthetase and transglutaminase where nmoles/min/mg are presented. When present, asterisks denote statistical significance *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$.

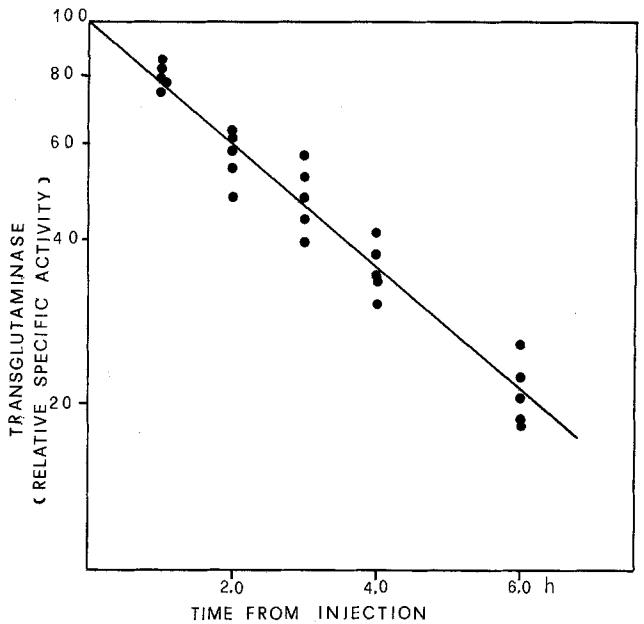

Figure 2. Time-dependent inactivation of rat liver transglutaminase by in vivo treatment with acrylamide (100 mg/kg). Rats were treated with acrylamide and activity assayed as described. Each experimental point is the average of a duplicate determination on a single animal. Activity was normalized to that of control homogenates prepared from two animals at each time point.

order of magnitude as the acrylamide concentration required for the inactivation of the enzyme in vitro [9]. Similar results were obtained when the activity of transglutaminase was determined in extracts from brain (data not presented). The kinetics of inactivation are those of an apparent first-order reaction, with a half-life of approximately 3.5 h at a dosage of 110 mg/kg (fig. 2). In these experiments the animals died between 6 and 8 h after injection, when the activity of the enzyme had decreased to 10–15% of control values. Attempts to reactivate the enzyme by dialysis against mercaptoethanol and EDTA-containing buffers – conditions which promote the reactivation of erythrocyte transglutaminase treated in vitro with acrylamide [9] – failed to restore the activity. It is not yet clear whether these differences depend on intrinsic properties of the two enzymes or rather on differences in the conditions of treatment.

In conclusion it appears that rat tissue transglutaminase is effectively inactivated in vivo by acrylamide, at concentrations which compare favorably with those required to inactivate the enzyme in vitro, or are even better if the in vivo catabolism of the chemical is considered [29]. Any attempt to correlate these findings with a pathogenetic mechanism for the acute acrylamide toxic syndrome would, however, be premature, because of uncertainties about the physiological role of the enzyme itself.

Transglutaminases catalyses calcium-dependent protein crosslinking reaction via ε (γ-glutamyl) lysine isopeptide bonds [30]. In resting cells the enzyme is probably in a latent state, and it is activated by a rise in intracellular free calcium [31], to modify predominantly contractile proteins [32]. Several pieces of evidence suggest a correlation between the activity of cell transglutaminase and the growth potential of the tissue [33, 34]. More interestingly, experiments with perfused pancreatic islets demonstrated the suppression of secretogogue-stimulated insulin secretion in the presence of competitive transglutaminase inhibitors [11] and, conversely, the appearance of aggregated intracellular proteins [12] crosslinked via isopeptide bonds during glucose-induced insulin secretion. It is thus likely that crosslinking of cytoskeletal proteins by transglutaminase is an important event during the release of secretory granules. It is tempting to speculate that the acrylamide-directed in vivo inactivation of transglutaminase interferes with neuromediator release and triggers thereby the appearance of the neurologic symptoms. Further studies are required to substantiate the involvement of transglutaminase during chronic acrylamide poisoning and in the pathogenesis of other toxic neuropathies which share the central-peripheral pattern with acrylamide neuropathy [35].

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Role of active oxygen species in diethyldithiocarbamate-induced gastric ulcer in the rat

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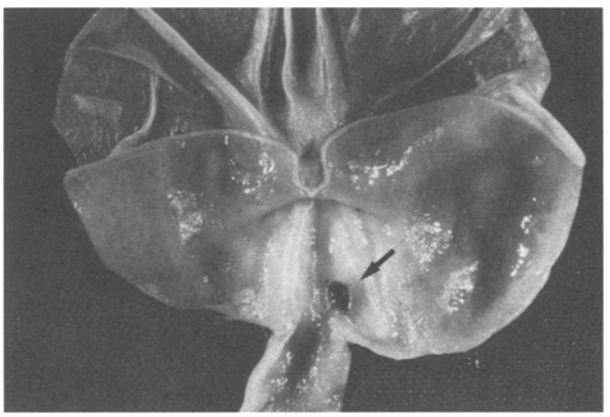
Summary. Diethyldithiocarbamate, an inhibitor of Cu,Zn-superoxide dismutase, was recently found to be ulcerogenic in the rat stomach, and active oxygen species were found to be responsible for its ulcerogenicity. To clarify which active oxygen species play a role in ulcerogenesis, the effects of various scavengers and iron-chelators were studied. As superoxide dismutase and catalase reduced the ulcerogenesis induced by diethyldithiocarbamate, the superoxide radical and hydrogen peroxide were considered to play a pathogenic role in this ulcer model.
Key words. Diethyldithiocarbamate; gastric ulcer; superoxide dismutase; catalase.

Recent studies have indicated that active oxygen species contribute to mucosal injury in the alimentary tract, and as superoxide dismutase (SOD) prevents this mucosal injury, its role in the alimentary tract has received much attention[1,2]. We previously reported that diethyldithiocarbamate (DDC), an inhibitor of Cu,Zn-SOD, showed ulcerogenicity in rat stomach, and that the effect was due to active oxygen species[3–6]. In this study, we investigated which active oxygen species played a role in the induction of antral ulcers by DDC in the rat.

Materials and methods

The experimental ulceration method used in this study has already been described in detail[6]. Briefly, rats, each weighing 200–230 g, previously fasted for 24 h, were anesthetized with ether. After pylorus ligation, DDC 800 mg/kg was injected subcutaneously and 1 ml of 0.1 N HCl was administered orally. Seven hours later, a large ulcer was observed in the antrum along the lesser curvature, penetrating into the muscular layer (fig.). The rats were killed and the stomach was filled with 10% formalin. The ulcer index was measured under a dissecting microscope with a square-grid eyepiece and expressed as the area of the antral ulcer (mm^2).

Study 1: In a preliminary study, the effect of the concomitant administration of HCl was investigated. Two groups, one treated with 0.1 N HCl and another without HCl, were studied. Both groups were subjected to the same procedures except for HCl administration.



Photograph of a stomach with antral ulcer (arrow) induced by diethyldithiocarbamate, pylorus ligation and 0.1 N HCl.

Study 2: Eleven different pretreatment regimens were assigned to groups of animals as follows:
1) superoxide dismutase (SOD) (Sigma Chemical Co.) 60,000 U/kg; 2) catalase (Cat) (Sigma Chemical Co.) 500,000 U/kg; 3) SOD plus Cat; 4) mannitol (Wako Pure Chemical Industry) 400 mg/kg, three times; 5) dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industry) 20 mg/kg; 6) DMSO 40 mg/kg; 7) 5% DMSO 1 ml; 8) diethylenetriaminepentacetic acid (DETAPAC) (Wako Pure Chemical Industry) 250 mg/kg; 9) deferoxamine (DFO) (Ciba-Geigy) 30 mg/kg; 10) DFO 60 mg/kg; 11) DFO 100 mg/kg.