THE ROLE OF COENZYME-A IN ACRYLAMIDE NEUROTOXICITY IN THE RAT

by

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STATEMENT OF AUTHOR

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To my loving husband, Matt, whose emotional and intellectual support made this endeavor possible. Also, to "Baby", whose impending delivery helped me finish this project on time.

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ABSTRACT

Acrylamide produces central-peripheral distal axonopathy. The mechanism of acrylamide-induced neuropathy is currently unknown, but has been postulated to occur through the inhibition of energy producing pathways within the nerve. Since coenzyme-A (CoA) is actively involved in neuronal energy production, the role of CoA in acrylamide neurotoxicity was examined. The enzymatic function of CoA was not inhibited by acrylamide *in vitro*. CoA content of neural and non-neural tissues was elevated after a cumulative acrylamide dose of 250 mg/kg i.p. Animals demonstrated signs of neurotoxicity following a cumulative dose of acrylamide of 100 mg/kg i.p. Diet supplements which were designed to bypass putative energy blocks produced by acrylamide had no effect on either the onset or magnitude of acrylamide neurotoxicity. Acrylamide produced anticholinergic effects which were assessed by the ocular zingerone test. This test was found to be a simple, sensitive measure of neuronal alterations induced by acrylamide.

INTRODUCTION

Physical and Chemical Data

Acrylamide (CH_2:$CHCONH_2$) is a vinyl monomer which is widely used in industry. Over 70 million tons of acrylamide are manufactured each year. The world's largest producer is the American Cyanamid Co. which synthesizes in excess of 35 million tons per year. Acrylamide is prepared from acrylonitrile by catalytic reaction with hydrochloric or sulfuric acid. The resulting white flakey monomeric compound has a molecular weight of 71.08, a melting point of 84.5 ^{o}C and is highly soluble in water (21.5 g/l at 30^{o}C). The solid readily polymerizes under ultraviolet light and at temperatures near the melting point.

Uses

The major commercial uses of acrylamide are the synthesis of polyacrylamides which are used as waste water flocculants by the mining industry to separate solids from aqueous solutions (40% of production) and in the paper industry as strengtheners for cardboard and other paper products (20% of production). Acrylamide is also used for the synthesis of polyacrylamide gels used by the scientific community and as industrial adhesives and flooding agents for petroleum recovery. Only 5% of produced acrylamide is used commercially in the monomeric form. Monomeric acrylamide is pumped into the soil and polymerized *in situ*. This process stabilizes and waterproofs the soil, making acrylamide an excellent grouting agent for construction of tunnels, dams and

foundations (U.S. Dept. Commerce, 1976). In situ polymerization is no longer used due to the extreme hazard associated with this procedure (Mast, personal communication). More recently, monomeric acrylamide has been used by the scientific community as a research tool in the study of peripheral neuropathy in animals.

Sources of Exposure

Soon after acrylamide was first manufactured, the neurotoxic properties of acrylamide were recognized (Fullerton and Barnes, 1966). It was noted that monomeric acrylamide, but not polymeric acrylamide, was neurotoxic. Most reported cases of intoxication involved workers in the synthesis and polymerization processes of acrylamide manufacture (Garland and Patterson, 1967). For this reason, prophylactic measures, such as wearing protective clothing and use of face masks, are recommended to minimize industrial intoxication. The threshold limit value (TLV) for acrylamide in the work place is 0.3 mg/m^3 and it is recommended that daily exposure of workers to acrylamide should not exceed 0.05 mg/kg (Spencer and Schaumburg, 1974).

The potential environmental hazard of acrylamide is minimal due to the high biodegradability of the monomer via microbial degradation (Croll, Arkell and Hodge, 1974). However, concern that excessive acrylamide concentrations may occasionally occur in drinking supplies has led to placing ceiling limits of 0.05% residual monomer in polymers used as paper strengtheners or as flocculants for the purification of drinking water and 2% residual monomer in polymers used as flocculants in other processes (Spencer and Schaumburg, 1974). Small concentrations

of acrylamide monomer have been found in sewage effluent in Great Britain (50 ppb)(Croll et al., 1974) and at least one case of acrylamide intoxication from drinking well water has been reported (Igisu et al., 1975). This case involved a family in Japan which became severely intoxicated after their drinking water became contaminated with 400 ppm of acrylamide from a nearby sewage construction site.

Symptomatology of Intoxication

Human

Clinical features of acrylamide toxicity in humans varies depending on the concentration and duration of exposure. Early symptomology is predominantly of central nervous system origin. These effects include drowsiness, lack of concentration, slurred speech, dizziness, retention of urine and truncal ataxia without ataxia of the limbs (Garland and Patterson, 1967; Kesson, Baird and Lawson, 1977; Igisu et al., 1975). The truncal ataxia is thought to be a cerebellar rather than sensory disorder since sensory function appears normal even during severe ataxia.

After several months of exposure to acrylamide, peripheral neuropathy develops. Symptoms include sensory loss, such as numbness of the hands and feet, and loss of vibrational and occasionally positional sense. Diminished motor function is evidenced by clumsiness of the hands, weakness in the legs and feet, and difficulty in walking. Other symtoms of intoxication include excessive sweating and redness and peeling of the skin, particularly on the hands (Spencer and Schaumburg,

1974). The time of onset and severity of symptoms is largely dependent upon the length of exposure and concentration of acrylamide (Garland and Patterson, 1967). In all cases, symptoms of peripheral neuropathy do not develop until weeks or months after the initial central nervous systems aberrations are evident.

The only known treatment for acrylamide intoxication is removal from the source of exposure. Recovery from acrylamide toxicity is usually complete and occurs within weeks or months depending on the severity of the initial exposure (Auld and Bedwell, 1967). However, in severely disabled patients, neuronal function may be permanently impaired.

Laboratory Animals

The neurotoxic symptoms of acrylamide are manifest following parenteral, oral or dermal exposure (Hamblin, 1956). Early work done by Kuperman (1958) in cats demonstrated that the neurotoxic effects of acrylamide were not manifest until a cumulative threshold dose of approximately 100 mg/kg had been reached. Neither the route of administration nor the magnitude of the daily dose employed affected this threshold level required to induce toxicity. Fullerton and Barnes (1966) confirmed the cumulative nature of acrylamide toxicity in rats. More recent studies in rats by Tilson, Cabe and Spencer (1979) indicated that when acrylamide was given in spaced doses, rather than daily doses, a higher cumulative dose of acrylamide was needed to produce toxicity.

The acute effects of acrylamide intoxication are quite different than symptoms seen after low level chronic dosing. At high acute doses

of acrylamide in cats, marked behavioral changes such as viciousness and apprehension, tonic and clonic seizures, severe ataxia, tremors and death due to respiratory failure were observed (Kuperman, 1958). McCollister, Oyen and Rowe (1964) reported similiar findings following high acute doses of acrylamide in rats, guinea pigs, rabbits, cats and monkeys. The LD_{50} measured for acrylamide in rats, guinea pigs and rabbits was 150-180 mg/kg. Cats and monkeys were found to be more susceptible to acrylamide than are other species ($LD_{50} \simeq 100$ mg/kg) (McCollister et al., 1964).

After a subacute dosing regimen, animals predominently exhibited symptoms of peripheral neuropathy. Symtomology included incoordination, trembling, ataxia, and hindlimb weakness progressing to paralysis. Other symptoms included weight loss, grossly enlarged and distended bladders, and testicular atrophy. A loss of tendon reflexes has also been reported in baboons (Hopkins, 1970).

Quantitative Assessment of Acrylamide Toxicity

A variety of behavioral and motor dysfunction testing methods have been employed to assess acrylamide-induced toxicity. Early behavioral changes may be measured by quantifying ambulation and rearing of rats in an open field during a three minute period (Gipon et al., 1976). These workers found significant open field behavioral changes in rats following a cumulative dose of 150 mg/kg.

Kaplan and Murphy (1972) described a rotorod test which was designed to assess motor incoordination in acrylamide-treated animals. Rats were force trained to maintain their balance on a rotating rod for

two minutes and were tested daily after receiving acrylamide. The onset of acrylamide toxicity was assessed by determining the number of daily treatments required before rats could no longer maintain their balance on the rotorod. Animals usually failed this test after a cumulative dose of 350 mg/kg was reached.

Edwards (1977) described a simple technique for early detection of acrylamide-induced neuropathy known as the "dropping foot-splay test". Rats are dropped from a standard height and the spread of their hindlimbs are quantified upon landing. Changes in foot-splay were noted after a cumulative dose of 150 mg/kg had been reached. This method was not suitable for quantifying severe dysfunction, since maximum foot-splay is rapidly reached as neuropathy progresses.

Cabe and Tilson (1978) assessed neuropathy by determining the force with which the hindlimbs were capable of extending. The hindlimbs of rats were placed on a pressure bar and their forelimbs on a platform which was 3 cm horizontal to and 1.5 cm above their hindlimbs. A blast of air was then directed at the anus of each animal eliciting an escape response and hindlimb extension pressure was recorded. Significant changes in hindlimb stength were detected after cumulative doses of 100 mg/kg of acrylamide.

Cabe et al. (1978) also reported a forelimb grip strength test. The force required to pull a rat free from a grasping ring held by the forepaws was measured. This method proved to be less sensitive for assessment of acrylamide toxicity than methods described above.

Morphological Studies

Acrylamide caused a specific type of axonal degeneration which is known as "dying back". This term is used to describe neuropathies in which damage is first noted at the distal ends of peripheral neurons and then progresses proximally up the axon. More recently, Spencer and Schaumburg (1976) classified acrylamide-induced neuronal degeneration as a "central-peripheral distal axonopathy" since degeneration of axons in both the peripheral and central nervous systems was observed with acrylamide.

Acrylamide attacked sensory neurons before motor neurons. The earliest detectable morphological change observed in the nerves of acrylamide-intoxicated animals was loss of the filopod axon processes at the Pacinian corpuscles in the toepads of cats (Schaumburg, Wisniewski and Spencer, 1974). In the rat, initial changes in morphology were seen at the plantar sensory nerves (Spencer and Schaumburg, 1977). Following the initial involvement of sensory nerves, motor nerves (predominently in the sciatic) began to degenerate. Concurrently, degenerative changes were observed in the long ascending tract of the spinal cord. Initial changes were seen in the gracile nucleus and dorsal spinal cerebellar tracts and in the caudal region of long descending fibers in the cerebrospinal tract (Spencer and Schaumburg, 1977). Progressive degeneration in both central and peripheral nerve tracts proceeded proximally up the axon as intoxication progressed.

It has also been observed that long fibers and large diameter myelinated neurons in rats (Fullerton and Barnes, 1966) and cats (Spencer and Schaumburg, 1974) were affected before smaller neurons.

This effect was also noted in baboons (Hopkins, 1970) and in man (Fullerton, 1969). Unmyelinated neurons were relatively resistent to acrylamide except during severe intoxication. As with myelinated nerves, the long and large diameter unmyelinated fibers were first affected. Also, unmyelinated fibers innervating the gastrocnemius were affected to a greater extent then were somatic nerves (Post, 1978).

Morphologically, degeneration was characterized by swellings of the axon at, or near, the nodes of Ranvier in the distal portions of the nerve. Prineas (1969) and later Schaumburg et al. (1974) found that the nodal swelling resulted from an accumulation of neurofilaments, vesicles, neurotubules, smooth endoplasmic reticulum and mitochondria. Although these cellular organelles were normal in appearance and number, their spatial arrangement within the swelling were in marked disarray. For example, swirling patterns of neurofilaments were observed rather than the normal linear alignment. These swelling were usually seen proximal to the nodes of Ranvier but occasionally occurred distal to the nodes. The distal swelling predominently contained numerous enlarged or degenerating mitochondria and very few neurofilaments. As these nodal swellings enlarge, the myelin sheath surrounding the axon retracted away from the nodal region. The myelin and axon degenerated and resulting material accumulated in dense membranous bodies which collect around the axon further constricting the nodal region.

Neurophysiology

Neurophysiologic studies in humans and laboratory animals correlate well with pathological alterations observed during acrylamide

intoxication. Early studies by Fullerton and Barnes (1966) demonstrated that acrylamide decreased peripheral motor nerve conduction velocity and action potential amplitude in rats. Fullerton (1969) confirmed this finding in humans and further demonstrated that the distal portions of peripheral nerves were more severely affected by acrylamide than were proximal portions. Sensory neurons were found to be more susceptible than motor neurons and unmyelinated neurons appeared resistant to the actions of acrylamide. Using single fiber muscle stretch afferent (muscle spindles) techniques, Sumner and Asbury (1975) showed that impulse conduction in the nerve fibers from acrylamide intoxicated rats failed initially at distal terminals while the remainder of the fiber was capable of normal impulse conduction. As the severity of neuropathy increased, the proportion of nerve fibers showing failure also increased.

Lowndes et al. (1978) suggested that the initial symptoms of acrylamide-induced neuropathy, such as loss of position sensitivity, were a result of muscle spindle dysfunction. Both primary and secondary muscle spindle endings showed increased threshold and decreased discharge frequencies at times which correlated with the appearance of acrylamide-induced ataxia in cats. Dysfunction occurred after a cumulative acrylamide dose of 105 mg/kg in cats regardless of the magnitude of the daily dose administered. Also, cats which were asymptomatic at a cumulative acrylamide dose of 75 mg/kg demonstrated normal spindle function. Since primary muscle spindle endings provide information on dynamic changes in muscle length, position and movement,

spindle dysfunction would account for the observed clinical features of acrylamide poisoning, such as muscle incoordination and ataxia.

Electrophysiologic studies on the autonomic nervous system revealed decreases in conduction velocities and action potential amplitudes in myelinated sympathetic fibers (Post and McLeod, 1977). Initially it was reported that unmyelinated sympathetic fibers were unaffected by acrylamide (Hopkins and Lambert, 1972), however, recent electrophysiologic and histologic studies have demonstrated that post-ganglionic unmyelinated fibers were affected in severely intoxicated cats (Post and McLeod, 1977).

Distribution and Metabolism

Acrylamide was found to be rapidly distributed throughout all tissues, metabolized and readily excreted independent of dose (Miller, Carter and Sipes, 1982). The distribution and elimination of acrylamide in rats was identical following several different routes of administration (i.v., p.o., dermal) and at widely separated acute and chronic dose levels (Young et al., 1979).

Utilizing a single intravenous 10 mg/kg dose of 2,3-^{14}C-acrylamide, Miller, Carter et al. (1982) demonstrated that the concentration of radiolabel in all tissues except erythrocytes was equivalent and decayed in a biexponential fashion with time ($t_{\frac{1}{2}} \simeq 5$ hours). Only erythrocytes demonstrated a significant retention of radiolabel with time accounting for approximately 12% of the total dose administered (Pastoor and Richardson, 1981; Miller, Carter et al., 1982). A small percentage (<5%) of radiolabel persists in tissues for

several weeks ($t_{\frac{1}{2}} \simeq 8$ days). Hashimoto and Aldridge (1970) previously reported that the tissue associated radiolabel was protein bound since it could be precipitated with trichloroacetic acid. However, no consideration was given to the possibility that radiolabel resulting from the metabolism of acrylamide to one or two carbon species may be incorporated into newly synthesized proteins.

The elimination of parent acrylamide was faster than total radioactivity ($t_{\frac{1}{2}} \simeq 2$ hours) in all tissues. Thus, acrylamide was not selectively retained by neural tissues. Also, no selective accumulation of radiolabel was seen in neural tissues following chronic dosing with acrylamide (Young et al., 1979; Rylander-Yueh and Carter, 1982).

Within 24 hours, approximately 60% of an acrylamide dose was excreted in the urine with an additional 5% excreted in the feces (Miller, Carter et al., 1982). Approximately 15% of an acrylamide dose was excreted in the bile within 6 hours indicating that acrylamide may undergo enterohepatic circulation. An additional 6% of an acrylamide dose was exhaled as $^{14}CO_2$ when 1-^{14}C-acrylamide was administered (Hashimoto and Aldridge, 1970).

The predominent route by which acrylamide is metabolized involves glutathione conjugation (Pastoor, Heydens and Richardson, 1980; Miller, Carter et al., 1982). This route is presumably detoxifying since Dixit, Husain et al., (1980) demonstrated an earlier onset of acrylamide-induced toxicity after depleting hepatic glutathione stores with diethylmaleate. Acrylamide rapidly reacted with hepatic glutathione to form S-3-propionamideglutathione (Edwards, 1975a) which was eventually excreted as mercapturic acids in the urine. The major

urinary metabolite of acrylamide was identified as N-acetyl-S-(3-amino-3-oxypropyl)-cysteine which accounted for 48% of the excreted dose (Pastoor et al., 1980; Miller, Carter et al., 1982). Acrylamide inhibits glutathione-S-transferase activity in vitro (Dixit, Mukhtar et al., 1980) and in vivo (Dixit, Husain et al., 1981). Thus, acrylamide would appear to inhibit its own biotransformation along this pathway, although this has not been demonstrated directly.

Acrylamide is apparently partially metabolized by microsomal oxidation in the liver although conflicting reports appear in the literature concerning the significance of cytochrome P-450 mediated metabolism of acrylamide. Kaplan, Murphy and Gilles (1973) reported that acrylamide disappeared faster in S-9 fractions of livers obtained from phenobarbital pretreated rats when compared to controls. Kaplan et al. (1973) reported that pretreatment of rats with microsomal inducers (phenobarbital or DDT) delayed the onset of acrylamide-induced toxicity as assessed by rotorod. However, Edwards (1975b) could not reproduce the findings of Kaplan et al. (1973) and reported that the pretreatment of animals with phenobarbital or DDT had no affect on the development of acrylamide-induced neuropathy in rats. Recently, Hashimoto, Sakamoto and Tanii (1981) reported a delayed development of acrylamide-induced neurotoxicity in mice which were pretreated with phenobarbital.

The effects of acrylamide on liver include decreased cytochrome P-450 content and increased liver porphorin levels (Edwards, Francis and DeMatteis, 1978). No effect of acrylamide on hepatic UDP-glucuronyl transferase activity, total hepatic protein or microsomal protein content was observed (Howland and Lowndes, 1978).

Acrylamide Analogs

Several structural analogs of acrylamide have been tested for toxicity in order to determine the active or toxic portion of the acrylamide molecule (Hashimoto and Aldridge, 1970; Edwards, 1975a,b; Hashimoto et al., 1981). Analogs which produced neurotoxicity include N-isopropylacrylamide, N-methylacrylamide, methacrylamide, N-hydroxymethylacrylamide and N,N-diethylacrylamide (Hashimoto et al., 1981). None of these compounds were as toxic as acrylamide. The analogs tested which produced toxicity (except methacrylamide) were different from acrylamide in substitution on the amide group. Interestingly, other amide substituted analogs, such as methylene(bis)acrylamide and N,N-pentamethylene acrylamide had no detectable neurotoxic actions. Reduction of the double bond of acrylamide or deletion of the nitrogen atom always eliminated the neurotoxic potential of the compound.

Edwards (1975b) demonstrated that N-hydroxymethylacrylamide reacted with glutathione to the same degree as did acrylamide. However, reactivity towards glutathione was not suggestive of neurotoxicity since other toxic analogs were minimally reactive with glutathione and the non-toxic analog methylene(bis)acrylamide reacted to a large extent with glutathione.

Using various analogs of acrylamide, Hashimoto et al. (1981) demonstrated that the toxic effects of acrylamide on testes involved a different biochemical mechanism than was involved in the neurotoxic effects. The non-neurotoxic analog methylene(bis)acrylamide produced

testicular atrophy in mice whereas neurotoxic analogs, such as methacrylamide had no testicular effects. All other neurotoxic analogs of acrylamide produced testicular atrophy. Pretreatment with phenobarbital prevented testicular damage caused by acrylamide and N-isopropylacrylamide, and reduced the extent of testicular damage resulting from N-hydroxymethylacrylamide. These data indicated that acrylamide-induced testicular atrophy involved an action of acrylamide itself and not a cytochrome P-450 mediated metabolite. Alternatively, a non-microsomal metabolite could have caused the testicular changes induced by acrylamide.

Neurochemical Data

Acrylamide is a highly reactive compound which has been shown to actively bind to non-protein sulphydryl groups and central nervous system proteins (Hashimoto and Aldridge, 1970). Therefore, it was suspected that the actions of acrylamide may involve alterations in critical sulphydryl containing enzymes or cofactors. Acrylamide has been shown to bind to the sulhydryl portion of glutathione (Edwards, 1975a; Hashimoto and Aldridge, 1970; Dixit, Husain et al., 1980), glutathione-S-transferase (Dixit, Husain et al., 1981) and alcohol dehydrogenase (Dixit, Mukhtar and Seth, 1981). Also, several glycolytic enzymes were inhibited by acrylamide. Acrylamide inhibited neuronal specific enolase and glyceraldehyde dehydrogenase in neuronal tissues of acrylamide intoxicated rats (Howland, Vyas and Lowndes, 1980) and cats (Howland, 1981). *In vivo* inhibition of neuronal specific enolase was more pronounced in the distal versus proximal segments of sciatic nerve

(Howland, 1981). Several authors (Howland, Vyas, Lowndes and Argentieri, 1980) have also reported *in vitro* inhibition of phosphofructokinase activity by acrylamide, but this inhibition could not be demonstrated *in vivo*.

Although certain glycolytic enzymes could be inhibited by acrylamide, no changes in mitochondrial oxidative phosphorylation have been demonstrated (Hashimoto and Aldridge, 1970). In addition, levels of high energy phosphate sources such as ATP, phosphocreatine, glucose and glycogen were not altered by acrylamide *in vivo* (Johnson and Murphy, 1977). Further, no change in neuronal levels of glucose-6-phosphate, glyceraldehyde-3-phosphate, glutamate, dihydroxyacetonephosphate, 6-phosphogluconate or pyruvate were observed (Hashimoto and Aldridge, 1970; Johnson and Murphy, 1977). At high doses of acrylamide (>500 mg/kg) there were an increases in lactate, NAD, and the NAD dependent enzyme α-glycerophosphate. No change in the normal ratio of oxidized to reduced forms of NAD were found.

Kaplan et al. (1973) suggested that since nicotinamide structurally resembles two molecules of acrylamide, acrylamide could be competing with nicotinamide for NAD and NADP production sites. Sharma and Obersteiner (1977) showed that addition of NAD, NADP, nicotinamide or glutathione prevented the cytotoxic action of acrylamide in chick dorsal root ganglia cultures. Although Johnson and Murphy (1977) reported increased NAD levels after high doses of acrylamide, they concluded that acrylamide does not interfere with pyridine nucleotide formation *in vivo*. This was further supported by the fact that neither

pyridoxine nor thiamine deficiency in rats had any influence on the development of acrylamide-induced neuropathy (Kaplan et al., 1973).

Protein synthesis rates and amino acid incorporation were altered by acrylamide. The incorporation of ^{3}H-leucine (Schotman et al., 1977) and ^{14}C-valine (Schotman et al., 1978) into neuronal protein was decreased after cumulative acrylamide doses in excess of 200 mg/kg. However, these data could not be reproduced in vitro. The incorporation of amino acids increased during the recovery stages of acrylamide intoxication. The role of inhibition of protein synthesis in acrylamide-induced neuropathy is questionable since the non-neurotoxic analog of acrylamide, methylene(bis)acrylamide, also inhibited the rate at which amino acids were incorporated into neuronal protein (Schotman et al., 1978).

Hashimoto and Ando (1973) studied the incorporation of radiolabelled lysine and methionine into protein of neuronal tissues obtained from acrylamide intoxicated rats. No alteration in the incorporation of these amino acids was seen in liver or cerebral cortex. However, an increased rate of incorporation was seen in the spinal cords of severely intoxicated animals. In contrast, sciatic nerve initially demonstrated decreased lysine, but not methionine, incorporation into protein. At higher acrylamide doses, the incorporation of both lysine and methionine into protein was increased. Rates at which amino acids were incorporated into protein reached maximum values after acrylamide exposure was terminated. Therefore, Hashimoto and Ando (1973) concluded that alterations in the incorporation of these amino acids were

associated with regenerative processes and not caused by a direct action of acrylamide.

Recently, several researchers have examined changes in central nervous system neurotransmitters and receptor content following acrylamide treatment. Dixit, Husain et al., (1980) demonstrated that dopamine, norepinephrine and serotonin concentrations in the brains of acrylamide intoxicated rats were reduced. Pretreatment with diethylmaleate, a compound which depletes glutathione, resulted in further reduction in the brain levels of these biogenic amines. Agrawal et al. (1981) found that a single oral dose of acrylamide (25 or 50 mg/kg) increased the specific binding of ^{3}H-spiroperidol. No change in affinity of ^{3}H-spiroperidol for dopamine receptors was induced by acrylamide. These data suggest that acrylamide alters dopaminergic pathways in the brain. This hypothesis was supported by studies in which acrylamide administration resulted in decreased behavioral responses to the dopamine receptor agonist apomorphine. Agrawal et al. (1981) concluded that acrylamide alters dopaminergic pathways probably by uncoupling dopamine receptors which would then induce up-regulation of dopamine receptors. No changes in glycine, serotonin or muscarinic cholinergic binding was observed. In addition to increased radioligand binding to dopamine receptors, Bondy, Tilson and Agrawal (1981) reported that repeated administration of acrylamide also increased radioligand binding to glycine, serotonin, muscarinic cholinergic and GABA receptors.

Other neurochemical alterations induced by acrylamide include increased plasma acetylcholinesterase activity during the early stages

of intoxication (Bass and Goldberg, 1982) and increased β-glucuronidase activity in the sciatic nerves of animals recovering from acrylamide neuropathy (Kaplan and Murphy, 1972). These alterations may be potentially useful as markers for early detection of peripheral neurotoxicity and as a way of quantifying the degree of nerve damage incurred during acrylamide-induced neurotoxicity, respectively.

Mechanisms of Toxic Damage

Acrylamide produces a characteristic "dying-back" type of axonal degeneration in both the central and peripheral nervous system. However, the mechanism by which acrylamide induces this lesion remains to be established. Several hypothesis have been suggested to explain the underlying mechanism of typical "dying-back" neuropathies (Cavanagh, 1964; Schoental and Cavanagh, 1977; Ochs and Worth, 1978; Spencer et al., 1979).

Ochs and Worth (1978) suggested that neurotoxins, such as acrylamide, which produce distal axonal degeneration, interfered with axoplasmic transport mechanisms. This would result in a decreased delivery of essential nutrients to the distal portions of nerve fibers. Acrylamide administration resulted in a slight decrease in fast anterograde axoplasmic transport but no detectable change in slow anterograde axoplasmic transport in the peripheral nerves of cats (Bradley and Williams, 1973). These results were confirmed in rats by Griffin, Price and Drachman (1977). The slight change in fast anterograde axonal transport produced by acrylamide was not considered to be of a large enough magnitude, in and of itself, to account for the

actions of acrylamide. Therefore, the changes in anterograde axonal transport induced by acrylamide were probably a result, not a cause, of acrylamide-induced neuropathy.

Recently, Miller, Burks and Sipes (1982) demonstrated that retrograde axoplasmic transport was inhibited in a dose-dependent manner by acrylamide ($ED_{50} \simeq 25$ mg/kg). Further, maximal inhibition of retrograde axonal transport (90%) occurred prior to the onset of detectable peripheral neuropathy. These data suggest that alterations in retrograde axoplasmic transport may be involved in the etiology of acrylamide-induced neuropathy. The initial biochemical event responsible for altered retrograde transport was not described. Since transport mechanisms are entirely dependent upon energy derived from glycolysis or tricarboxylic acid cycle (TCA) dependent oxidative phosphorylation (Ochs, 1974; Ochs and Worth, 1978), a decrease in energy production could account for the defect in retrograde transport.

Biochemical and pathological evidence suggested that acrylamide may cause a defect in neuronal energy producing pathways. The first sign of acrylamide toxicity was the appearance of swellings which contain enlarged, or degenerating, mitochondria and increased numbers of neurofilaments at, or near, the nodes of Ranvier in the distal portions of peripheral nerves (Prineas, 1969; Suzuki and Plaff, 1973; Schaumburg et al., 1974). Nodal regions normally consume large amounts of energy (approximately 14% of the total energy demand of the adult animals) (Sumner and Asbury, 1974) and participate in the maintenance of axoplasmic transport mechanisms (Pleasure, Mishner and Engel, 1969; Griffin and Price, 1976). Thus, a block in energy

producing pathways could account for alterations in axonal transport mechanisms, decreased protein synthesis rates and degeneration at nodal regions.

Recent studies by Howland (1981) demonstrated that acrylamide inhibited several glycolytic enzymes both *in vitro* and *in vivo* further supporting the hypothesis that neuronal energy production is impaired by acrylamide. Presently, it is unknown whether acrylamide compromises the somal metabolism machinery required for normal synthesis of essential glycolytic enzymes and cofactors (Cavanagh, 1964) or if acrylamide exerts a direct action on these factors as they are transported within the axon (Spencer et al., 1979). In either case, the distal portion of nerves would be most susceptible to diminished energy supplies since it is furthest from the cell body. Acrylamide may be damaging the axon directly since early morphological changes were not at the actual nerve terminus but were preterminal and multifocal (Spencer et al., 1979).

Relationship Between Coenzyme-A and Acrylamide Toxicity

Suggestions that coenzyme-A (CoA) may be involved in certain "dying back" types of neurological disorders have appeared in the literature (Schoental and Cavanagh, 1977). Animals maintained on diets deficient in pantothenic acid, the precursor of CoA, demonstrate peripheral neuropathy which has similiar pathology to acrylamide-induced neuropathy (Novelli, 1953). A genetic disease known as acute porphyria causes neurological disorders (Cavanagh, 1964). In animals with experimentally induced porphyria, the neural lesions were not observed unless the animals were maintained on pantothenic acid

deficient diets (DeMatteis and Remington, 1962). Acrylamide has also been shown to slightly increase liver porphyrin production (Edwards et al., 1978) which is the initial symptom of porphyria.

It has been postulated that acrylamide interferes with energy producing pathways in nerves (Spencer et al., 1979). The cofactor, CoA, is intimately involved in energy production since it is responsible for incorporating carbon atoms which are derived directly from pyruvate into the TCA cycle. Schoental and Cavanagh (1977) suggested that acrylamide may bind directly to CoA thus decreasing the available CoA content necessary for neuronal metabolism and maintenance of axonal integrity. Acrylamide actively binds to sulphydryl groups (Hashimoto and Aldridge, 1970), and CoA contains an essential terminal sulhydryl group which is responsible for its enzymatic function. Decreases in CoA would lead to decreased pyruvate utilization in the TCA cycle, thus inhibiting the production of high energy phosphates required by nerves (Novelli, 1953; Lehninger, 1977). Recently, Dairman et al. (1981) showed that rats whose diets were supplemented with pyruvate demonstrated a delay in the onset of acrylamide-induced neurotoxicity. Since pyruvate levels in intoxicated rats are normal (Johnson and Murphy, 1977), increases in pyruvate could competitively inhibit potential interactions between acrylamide and CoA.

STATEMENT OF THE PROBLEM

Monomeric acrylamide, a widely used industrial compound, produces central-peripheral distal axonopathy in humans. The mechaism by which acrylamide produces toxicity is unknown. One hypothesis is that acrylamide interferes with either glycolysis or the tricarboxylic acid cycle (TCA) to produce a defect in energy production required for normal neuronal function and integrity. The effects of acrylamide on coenzyme-A were examined in this study. Coenzyme-A is responsible for transfering the acetyl group derived from pyruvate (the end product of glycolysis) into the TCA cycle. This process is essential for adequate energy production. Alterations in CoA levels would suggest that acrylamide produces a biochemical aberration in energy producing pathways. The specific aims of this study were:

1. To determine if acrylamide alters the normal function of CoA by inhibiting the ability of CoA to act as a carrier for acetyl groups.
2. To determine the fate of CoA, an essential cofactor in neuronal energy production, in nervous tissue of acrylamide intoxicated animals.
3. To identify and characterize an acrylamide-CoA adduct, if formed, *in vivo* and *in vitro*.
4. To demonstrate a causal relationship between alterations of CoA in neuronal tissues and acrylamide-induced neuronal toxicity.
5. To see if acrylamide toxicity could be inhibited or delayed by supplementing the diet of rats with alternate energy sources which would bypass putative glycolytic-TCA lesions produced by acrylamide.

METHODS

Materials and Animals

Acrylamide (99+ %, electrophoresis grade) and pyruvic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). Coenzyme-A (CoA), acetyl-CoA, propionyl-CoA, S-acetyl-CoA synthetase, tryptamine, succinate, oxaloacetic acid, citric acid, dl-β-hydroxybutyric acid, atropine and neostigmine were obtained from Sigma Chemical Co. (St. Louis, MO). Pilocarpine was purchased from Regis Chemical Co. (Chicago, IL). Tetrabutylammonium phosphate was supplied by Eastman Kodak Co. (Rochester, NY) and zingerone was obtained from Pfaltz and Bauer, Inc. (Stamford, CN). The 2,3-^{14}C-acrylamide (0.512 mCi/mmol) was purchased from New England Nuclear (Boston, MA) and was purified in our laboratory by dissolving the monomeric form in 100% ethanol and removing insoluble polymer by filtration. The 1-^{3}H-acetic acid (4.4 Ci/mmol) and 1-^{3}H-acetyl-CoA (15 Ci/mmol) were obtained from Amersham Corp (Arlington Heights, IL) and ICN Pharaceuticals Inc. (Irvine, CA), respectively. All other solvents and chemicals used were of the purest grade available. Male Sprague-Dawley rats (200-300g) were used in all experiments.

Coenzyme-A Assay

Coenzyme-A was assayed using the method of Chan and Ebadi (1981). Tritium labelled acetic acid is added to tissue homogenates containing CoA. The enzyme, S-acetyl-CoA synthetase, is used to

transfer the tritium label to available CoA to form tritiated acetyl-CoA. Tryptamine is then added to the reaction mixture and the tritium label in transfered to tryptamine via the enzyme N-acetyltransferase to form ^{3}H-N-acetyltryptamine. This final product is extracted from the reaction mixture and quantified. The general scheme of this assay is shown below:

$$^{3}\text{H-acetic acid} + \text{CoA} \xrightarrow{\text{S-acetyl-CoA synthetase}} {}^{3}\text{H-acetyl-CoA}$$

$$^{3}\text{H-acetyl-CoA} + \text{tryptamine} \xrightarrow{\text{N-acetyltransferase}} {}^{3}\text{H-N-acetyltryptamine}$$

Isolation of N-acetyltransferase

N-acetyltransferase (NAT) was isolated from rat liver as described by Weber (1971). Rat livers were homogenized in four volumes of ice-cold 0.1 M potassium phosphate buffer (pH 7.4). All steps that follow were carried out at 0-5^{o}C. The homogenate was centrifuged at 10,000 x g for 15 min in a Sorvall Superspeed RC2-B refrigerated centrifuge (Sorvall Inc., Newtown, CN) and the precipitate formed was discarded. The resulting supernatant was then centrifuged at 100,000 x g for 50 min in a Beckman L8-55 Ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA) and the clear supernatant was subjected to further fractionation. Saturated ammonium sulfate solution (pH 7.2) was added slowly to the supernatant layer until 0.45 saturation was reached. After 1 hr, the precipitate formed was discarded and the supernatant was further saturated to 0.60 with ammonium sulfate. After 15 min the sample was centrifuged and the supernatant was discarded. The remaining precipitate was resuspended in 10 ml of 0.1 M potassium phosphate buffer

(pH 7.2) and dialyzed 3 times against 100 volumes of 0.005 M potassium phosphate buffer (pH 7.2) for 3 hr each. The dialysate was centrifuged at 10,000 x g for 15 min and the supernatant was assayed for protein content and NAT activity. The purified enzyme preparation was stored at -80°C in 10% (v/v) ethylene glycol, 0.1 mM EDTA and 1 mM mercaptoethanol.

Assay of Enzyme Protein Content

Protein content was determined by the Biuret method (Gornall, Bardawill and David, 1949). Figure 1 shows the standard curve and protein concentration of the purified NAT preparation (18.17 mg protein/ml) using this procedure.

Assay of NAT Activity

N-acetyltransferase activity was determined using the method of Parfitt et al. (1975). Twenty ul of 0.1 M sodium phosphate buffer (pH 7.5) containing 200 pmole 1-^{3}H-acetyl-CoA (15 Ci/mmol) was mixed with 10 ul of 40 mM tryptamine. Varying amounts of NAT (2.5-50 ul) were added to the mixture which was then incubated at 37°C for 15, 30 or 60 min. Reactions were stopped by the addition of 1 ml water-saturated chloroform. Samples were vortexed for 10 sec, centrifuged and the aqueous layer was discarded. The organic layer was washed with 200 ul 0.1 M sodium phosphate buffer (pH 7.5). The aqueous layer was discarded. Aliquots (100 ul) of the organic phase were placed in scintillation vials, evaporated to dryness, and 7 ml BetaPhase^{R} scintillation fluid (WestChem Products, San Diego, CA) was added. Samples were quantified for ^{3}H-N-acetyltryptamine by liquid

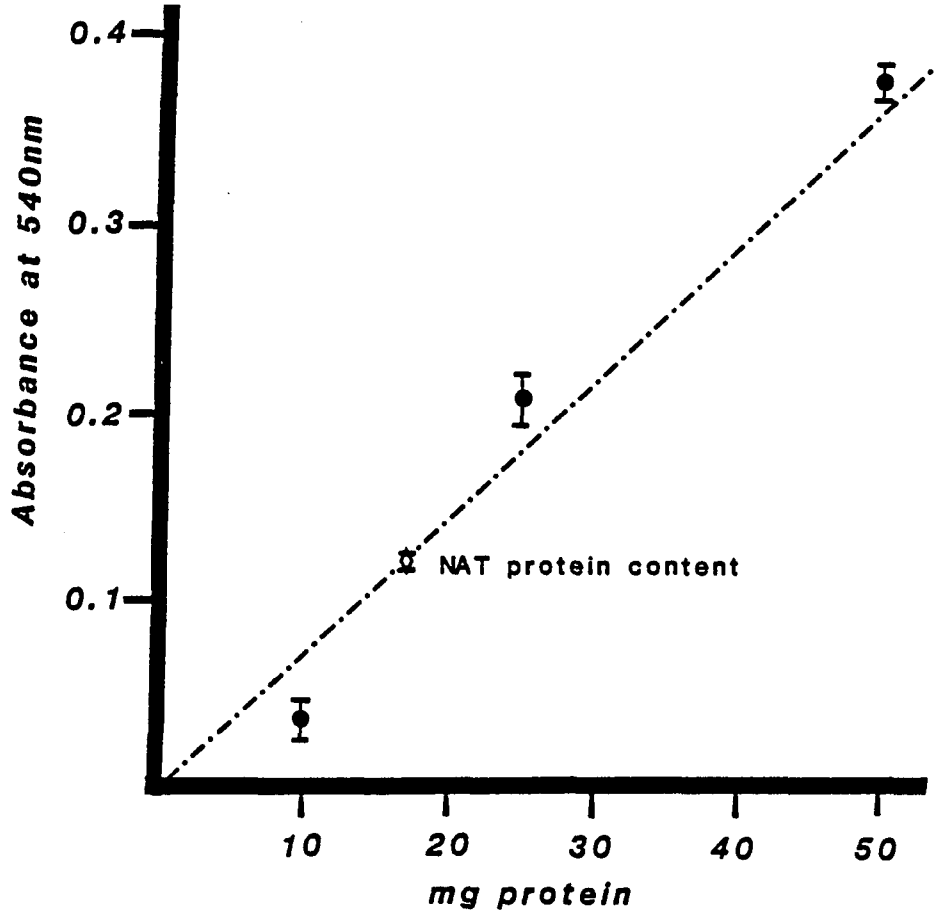

Figure 1. Protein standard curve.

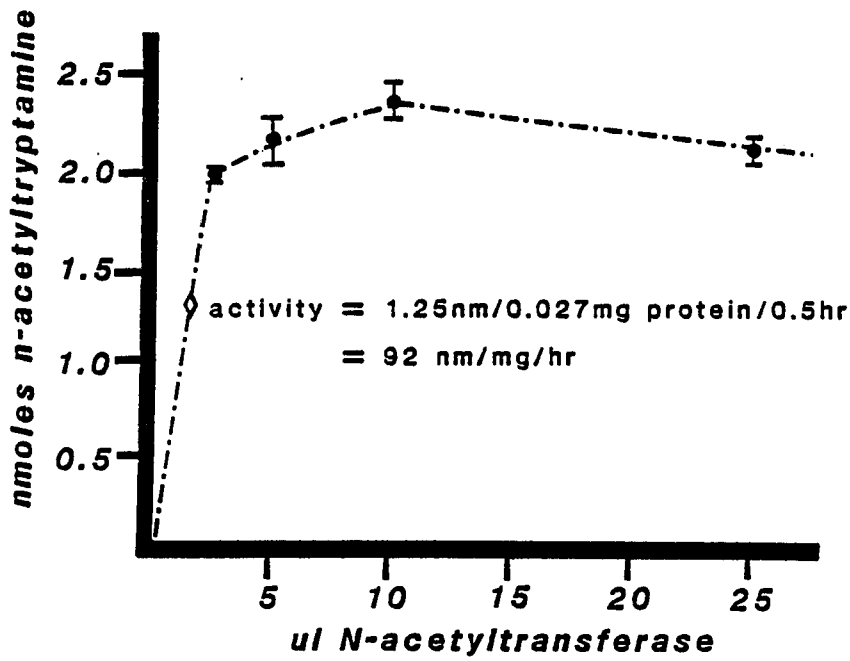

Figure 2. Determination of NAT activity and optimal amount (ul) of NAT needed to transacetylate 200 pmoles of acetyl-CoA. -- Incubations were carried out for 30 min.

scintillation counting (LSC). As shown in Fig. 2, the specific activity of purified NAT was 92 nmoles/mg of protein/hr and 10 ul NAT was the optimal amount of enzyme needed to maximally acetylate tryptamine in this assay. The optimal incubation time needed for this reaction was 30 min (Fig. 3).

Tissue Preparation

Rats were decapitated and their internal organs were immediately removed, frozen on dry ice, and stored at -80°C until CoA assays could be performed. In order to inactivate endogenous CoA metabolizing enzymes, frozen tissues were sonicated in boiling 0.1 M sodium phosphate buffer, pH 7.5 (1 mg wet weight/15 ul). The homogenates were cooled and centrifuged at 50,000 x g. The clear supernatant solutions were used to assay for CoA.

CoA Assay Procedure

Twenty ul aliquots of the tissue homogenate supernatant were added to 10 ul of a solution containing 0.1 M sodium phosphate buffer, (pH 7.5), 15 mM $MgCl_2$, 30 mM ATP, 0.15 M KF, 0.36 mM 1-^{3}H-acetic acid (4.4 Ci/mmol), 30 mM GSH and 0.025 units (umoles/min) of S-acetyl-CoA synthetase. The mixtures were incubated at 37°C for 30 min in a water bath. Samples were then boiled for 2 min and cooled in ice. To these samples, 10 ul of 40 mM tryptamine, dissolved in 0.1 M sodium phosphate buffer, (pH 7.5), and 10 ul of rat liver NAT were added. The reactions were allowed to proceed for 30 min at 37°C and then stopped by the addition of 1 ml water-saturated chloroform. Samples were quantified for ^{3}H-N-acetyltryptamine as previously described. The standard curve

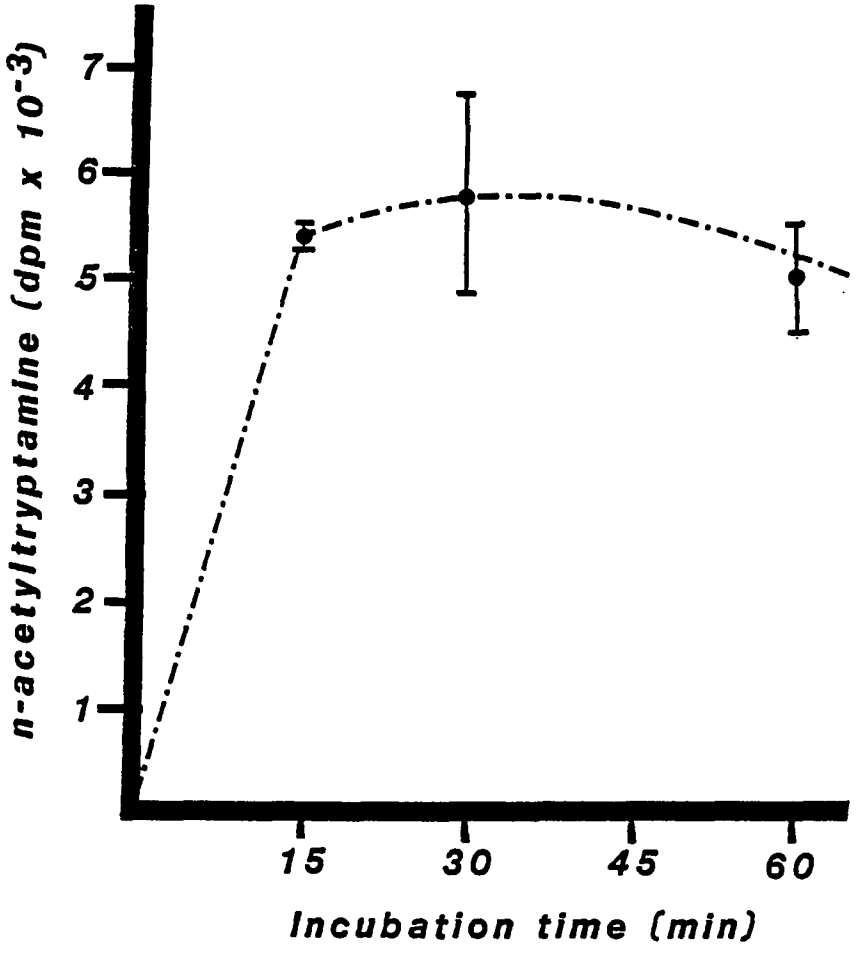

Figure 3. Determination of optimal incubation time needed to transacetylate 200 pmoles of acetyl-CoA using 10 ul NAT.

for CoA was obtained by adding known amounts of CoA (0-200 pmole) to the assay system. A typical standard curve is shown in Fig. 4.

Heat Stability of CoA and Acetyl-CoA

The heat stability of CoA and acetyl-CoA was tested by boiling 20 ul of 200 pmole CoA or 1-^{3}H-acetyl-CoA for 2 min. The samples containing boiled CoA were carried through the procedure for CoA assay as described above. The samples containing boiled 1-^{3}H-acetyl-CoA were carried through the procedure for N-acetylation of tryptamine as previously described. The concentration of ^{3}H-N-acetyltryptamine formed in these reactions was identical to control reactions. Therefore, CoA and acetyl-CoA concentrations were unaffected by boiling for 2 min.

Behavioral Testing Methods

Rats (n=6/group) were administered 10 daily doses of acrylamide (50 mg/kg/day i.p.) or saline. A variety of behavioral tests were then performed on both treated and control animals. These tests included:

a) determining if rats felt pain by the presence or absence of a withdrawal response to a pin prick on their hindpaw.

b) determining the response to heat by timing how long it took for animals to withdraw their tails from a 58^{o}C water bath. This test is referred to as the "tail-flick" test.

c) determining the response to pressure by use of a Von Frey's anesthesiometer (Stoelting Co, Chicago, IL). This device consists of 9 plastic threads varying in tensile strength from a very light thread (tensile number=1) to a very sturdy thread (tensile number=9).

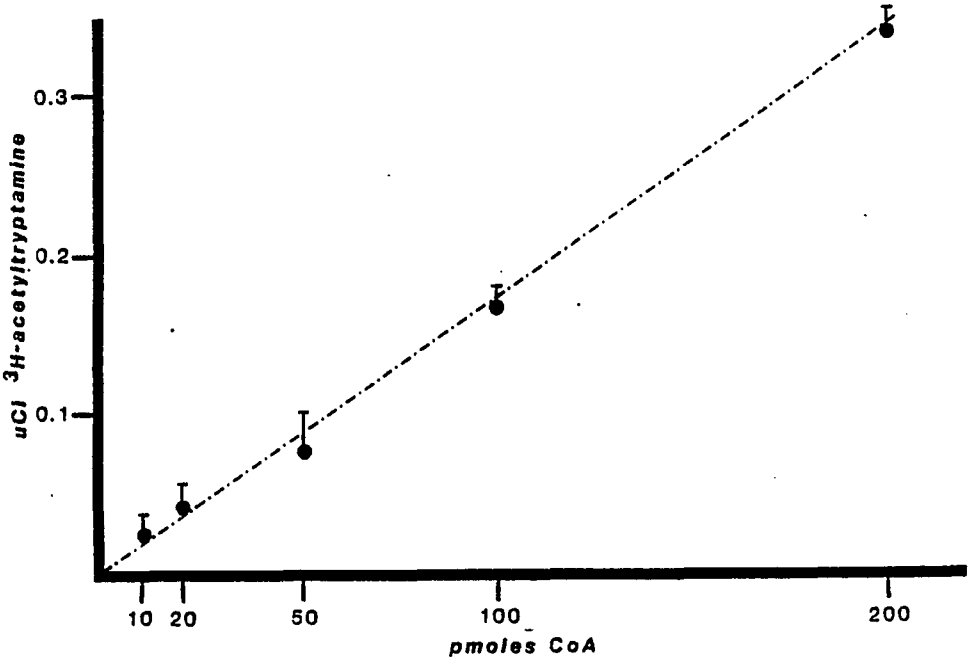

Figure 4. CoA standard curve.

Each thread was pressed onto a denuded portion of the animal's back until a skin flinch response was seen. The tensile number of the lightest thread which elicited a response was recorded.

d) measuring the duration of response to a chemical irritant applied to the eye. A 1% solution of zingerone, which is the irritating component of ginger, was used as the chemical irritant. The zingerone response consisted of repeated blinking and wiping of the eye. This test is referred to as the "ocular zingerone" test.

e) measuring forepaw grip strength using the method of Cabe et al. (1978) described in the introduction.

f) measuring alterations in foot-splay using the "dropping foot-splay test" (Edwards,1977) also previously described in the introduction.

The sensitivity of the ocular zingerone test and the dropping foot-splay test were assessed. One group of rats (n=4/group) received daily doses of acrylamide (50 mg/kg/day i.p.) or saline. Animals were tested for changes in response to ocular zingerone and alterations in foot-splay following cumulative acrylamide doses of 50, 150, 250, 400 and 500 mg/kg. A second group of rats (n=6/group) were administered 15 mg/kg/day i.p., of acrylamide or saline and were similarly tested after a cumulative acrylamide dose of 75, 150, 225, 300 or 375 mg/kg was reached. The rats in this latter group were also rated for their observable degree of toxicity in a single-blind observation study. In this study, individual rats from each group were selected randomly and rated as follows for neurotoxic symptoms: 1=none, 2=slight (slightly wide gait; shaking), 3=moderate (wide gait; incoordination) and 4=severe

(incoordination with foot drag or paralysis). The viewer did not know what dose of acrylamide, if any, each rat received. The control rats in this experiment received daily doses of saline for 25 days. All rats were rated for neurotoxic symptoms on the same day.

Mechanism of Acrylamide-induced Effects on the Ocular Zingerone Test

A variety of experiments were performed in order to determine if the response associated with the ocular zingerone test was due to an anticholinergic effect caused by acrylamide. Rats (n=5/group) were administered varying doses of atropine (0.025-0.8 mg/kg i.p.), an antimuscarinic agent, or saline. Thirty min later, a 1% solution of zingerone was applied to the eye and the duration of response was recorded. To determine if the alteration in response to zingerone induced with atropine could be reversed, a second set of rats were administered 0.8 mg/kg (i.p.) of atropine and thirty min later were administered varying concentrations of pilocarpine (25 ul, 0.05-0.8%), a direct-acting muscarinic agonist, or saline in the eye (n=5/group). After 1 min, these animals were tested for their duration of response time to the ocular zingerone test. A similar experiment was performed on atropine treated animals (0.8 mg/kg i.p.) using varying doses of neostigmine (6.25-100 ug/kg i.p.), an indirect-acting muscarinic agonist. The response to the ocular zingerone test was determined 15 min following administration of neostigmine.

In a second set of experiments, rats were administered 50 mg/kg/day (i.p.) of acrylamide until a cumulative neurotoxic dose of 400 mg/kg was reached. One day after receiving the final dose of

acrylamide, rats (n=5/group) were administered varying doses of pilocarpine (25 ul, 0.05-0.8%, applied to the eye), neostigmine (6.25-100 ug/kg i.p.) or saline. The duration of response to the ocular zingerone test was determined in these animals.

The interactions between acrylamide and neostigmine on the response time to the ocular zingerone test was also determined. Acrylamide treated rats (n=6/group) (50 mg/kg/day i.p., for 8 days) were administered 100 ug/kg (i.p.) of neostigmine or saline. Fifteen min later, animals were tested for their duration of response to a 1% solution of zingerone applied to the eye. A control group of rats (n=6/group) were similarly treated with neostigmine or saline and their response time in the ocular zingerone test was determined.

In Vivo Studies

Effects of Acrylamide on CoA Content

The dose-response relationship between alterations in CoA levels and the dose of acrylamide was determined. Rats received daily doses of acrylamide (50 mg/kg/day i.p.) or saline. Animals were weighed daily and neuropathy was assessed by measuring the duration of response to a 1% zingerone solution applied to the eye one day prior to death. Animals (n=5/group) were killed by decapitation after receiving cumulative acrylamide doses of 50, 150, 250 and 350 mg/kg. Cerebral cortex, cerebellum, spinal cord, heart and liver were removed immediately and assayed for CoA content as previously described.

Diet Supplementation Studies

Rats were placed on a variety of dietary supplements to determine of these supplements had any effect on the development of acrylamide-induced toxicity. The drinking water of rats (n=4/group) was replaced with neutralized (pH 7.0) 0.2 M solutions of pyruvic acid, succinate or dl-β-hydroxybutyric acid. A control group of rats were maintained on a normal food and water diet. Rats were administered daily doses of acrylamide (50 mg/kg/day i.p.) or saline. Animal weights and consumption of drinking solutions were measured daily. Following a cumulative acrylamide dose of 150, 250, 400 or 500 mg/kg, animals were tested for neurotoxicity by timing the duration of response to a 1% zingerone solution applied to the eye. Rats maintained on pyruvate diets were also tested for alterations in foot-splay. Animals which received cumulative acrylamide doses of 250, 400 or 500 mg/kg were killed and cerebral cortex, cerebellum, spinal cord, liver and heart were removed and assayed for CoA content as previously described.

A second modified dietary supplement study was also performed. Rats (n=4/group) were maintained on a liquid diet consisting of Sego^{R} instant diet (Carnation Co., Los Angeles, CA) dissolved in whole milk. These diets were supplemented with either 0.1 M oxaloacetic acid or 0.1 M citric acid. Rats were administered daily doses of acrylamide (50 mg/kg/day i.p.) or saline. A control group of rats were placed on Sego^{R} diet alone. Diet consumption and animal weights were measured daily. After a cumulative acrylamide dose of 150, 250, 400 or 500 mg/kg, rats were tested for signs of acrylamide-induced intoxication by recording their response to a 1% solution of zingerone applied to the eye.

In Vitro Studies

Binding Studies

An attempt was made to synthesize an acrylamide-CoA adduct by reacting equimolar concentrations of 2,3-^{14}C-acrylamide and CoA (10 mM) in 0.1 M sodium phosphate buffer (pH 8.0). This mixture was incubated at 37°C for 4.5 hrs in a water bath. This protocol was previously used by Edwards (1975a) to synthesize an acrylamide-glutathione conjugate.

In a second experiment, equimolar concentrations of 2,3-^{14}C-acrylamide and CoA (0.2 mM, pH 7.5) were reacted in the presence of 15 mM $MgCl_2$, 0.15 M KF, 30 mM ATP, 30 mM GSH and 0.025 units (umoles/min) of S-acetyl-CoA synthetase. This reaction mixture was incubated at 37°C for 4.5 hrs.

An aliquot (50 ul) of the final reaction mixture from each experiment was chromatographed by high performance liquid chromatography (HPLC). Since an acrylamide-CoA adduct, if formed, would be radiolabelled, HPLC effluents were monitored by LSC to determine if any radiolabel eluted at times other than that of 2,3-^{14}C-acrylamide.

Inhibition Studies

The effect that acrylamide had on the ability of CoA to act as an acetyl group carrier was tested by utilizing the CoA assay procedure of Chan and Ebadi (1981) previously mentioned. Varying concentrations of acrylamide (0.04-40 mM) were added to the original reaction mixture containing 0.4 mM 1-^{3}H-acetic acid and 200 pmole CoA. The effect that acrylamide had on the amount of ^{3}H-N-acetyltryptamine formed in this reaction was determined.

A second experiment was performed to ascertain if acrylamide was reacting with CoA or some other substrate in this assay system. In this experiment, the reaction mixture contained non-labelled acetic acid (0.4 mM), 2,3-^{14}C-acrylamide (4.0 or 40 mM) and 200 pmole CoA. The organic layer from the final assay step was counted by LSC for any extractable ^{14}C-labelled material. It was previously determined that acrylamide is not extractable from aqueous solutions with chloroform.

Chromatographic Techniques

HPLC analysis was used to determine if CoA forms an adduct with acrylamide. A Whatman^{R} Partisil PXS 10/25 C-8 column and a solvent system consisting of methanol:water (55:45) with 0.01 M tetrabutylammonium phosphate (pH 5.5), added as an ion pairing agent, was used. The flow rate of the solvent system was 2.0 ml/min. This procedure was modified from that of Baker and Schooley (1981). CoA, acetyl-CoA and propionyl-CoA were used as standards in this system and were detected by UV absorbtion at 254 nm. A sample chromatogram is seen in Fig. 5. Acrylamide elutes with the solvent front in this system. It was presumed that an acrylamide-CoA adduct would elute at a retention time similar to that of propionyl-CoA since both of these compounds contain the same number of carbon atoms in the acyl-thioester side chain of CoA.

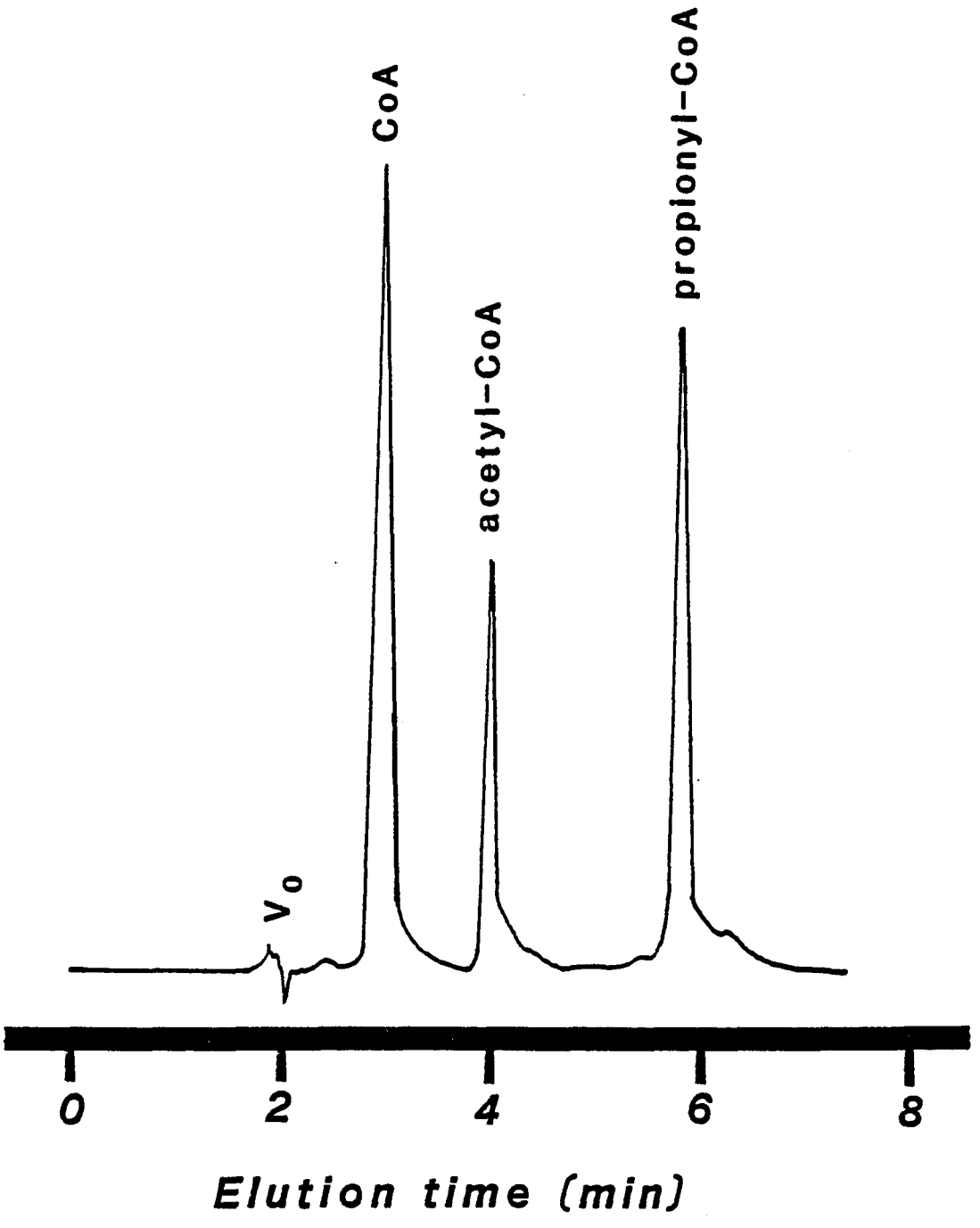

Figure 5. HPLC chromatogram of CoA and its acyl-thioester analogs.

Statistical Methods

All statistical comparisions were made using one way analysis of variance for each data group. Duncan's multiple range test was used to determine which groups were significantly different ($p<0.05$) from control. In order to determine the interaction that diet supplementation had on acrylamide-induced toxicity and alterations in CoA levels, two-way analysis of variance was performed.

RESULTS

Behavioral Testing

A variety of quantative behavioral testing methods were employed to test which sensory and motor parameters were affected by acrylamide. The results of this experiment are shown in Fig. 6. There was no change in response to mechanical or thermal pain as assessed by response to pin prick or tail flick, respectively. Sensitivity to pressure, as assessed with a Von Frey's anesthesiometer, was increased by 40%. There was a dramatic increase in response (260%) to the chemical irritant, zingerone. After a cumulative acrylamide dose of 500 mg/kg, motor dysfunction in both the hindlimbs and forelimbs of animals was apparent. Rats were markedly ataxic and foot-splay was increased by 46% over control values. Forelimb grip strength was decreased by 64%.

The sensitivity of the ocular zingerone response test and the dropping foot-splay test were compared. As seen in Fig. 7, a significant increase in the duration of response to a 1% solution of zingerone placed in the eye was seen after the animal had received a cumulative acrylamide dose of 150 mg/kg. Alterations in foot-splay were not detectable until a cumulative acrylamide dose of 225 mg/kg was reached.

The cumulative dose at which these alterations were first detectable was not affected by the magnitude of the daily acrylamide dose that rats received. Rats which were treated daily with 50 mg/kg

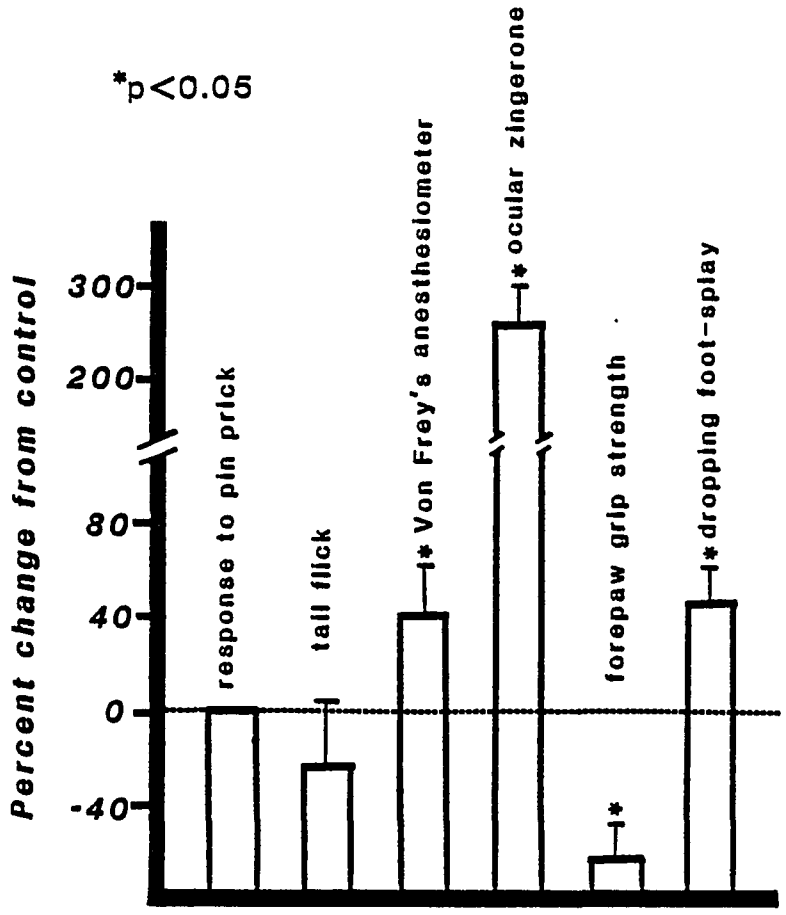

Figure 6. Effect of acrylamide (50 mg/kg/day x 10 days) on sensory and motor function tests.

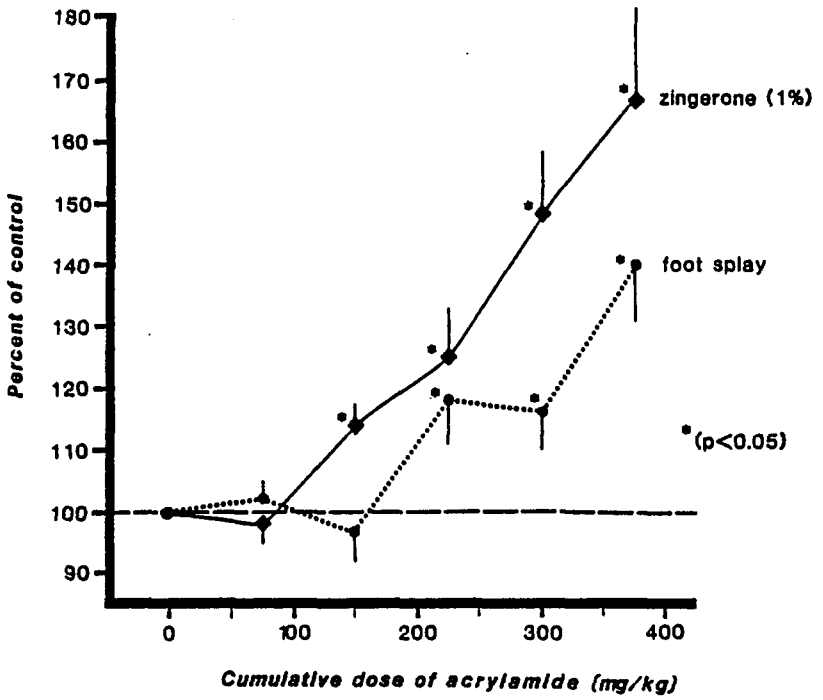

Figure 7. Response to ocular zingerone and foot-splay tests following administration of acrylamide (15 mg/kg/day i.p.). -- Control animals received daily doses of saline (i.p.). Statistical ($p<0.05$) changes from control are shown (*).

rather than 15 mg/kg of acrylamide, did not demonstrate changes in response to the ocular zingerone test and the dropping foot-splay test until cumulatative acrylamide doses of 150 and 250 mg/kg, respectively, were reached. The magnitude of the change in response observed was also identical.

Changes in the rate of weight gain was apparently dependent on the magnitude of the daily dose of acrylamide that the rats received (Fig. 8). Rats administered 50 mg/kg/day of acrylamide lost up to 5% of their original weight whereas rats which received 15 mg/kg/day of acrylamide gained weight. The weight gain observed in this latter group of animals was not as great as the weight increase observed in control groups (13 vs 24%, respectively).

Using a single-blind observation study, the observable degree of acrylamide-induced toxicity in rats was determined. The results of this study are shown in Table 1. (Refer to appendix A for individual animal data.) Although 67% of the rats tested demonstrated slight neurotoxic symptoms following a cumulative acrylamide dose of 75 mg/kg, a cumulative acrylamide dose of 300 mg/kg was required before all rats demonstrated neurotoxic symptoms. At this dose level, all rats demonstrated slight to moderate neurotoxic symptoms. Severe neurotoxic symptoms were not observed in any animal until a cumulative acrylamide dose of 375 mg/kg was reached.

Antichlolinergic Effects of Acrylamide

Atropine, a muscarinic cholinergic receptor antagonist, caused a dose-dependent increase in the response to the ocular zingerone test

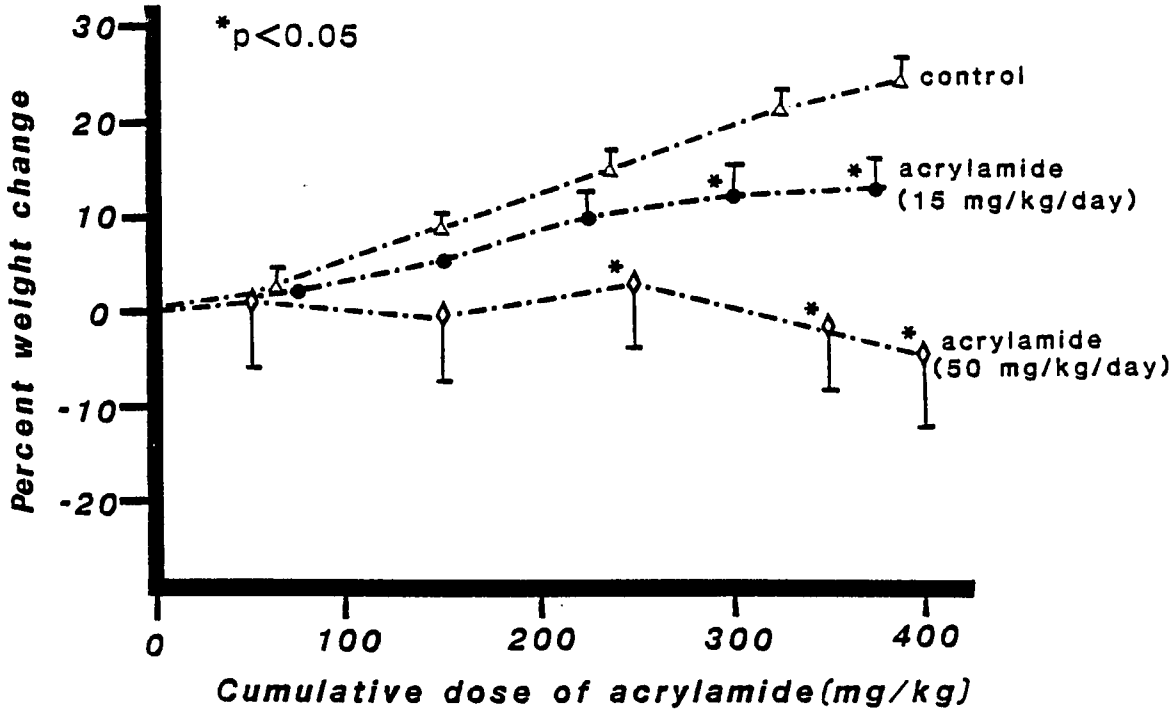

Figure 8. Percent change in animal weight following administration of acrylamide (15 vs. 50 mg/kg/day i.p.). -- Statistical ($p<0.05$) differences from saline treated controls are shown (*).

Table 1. Toxicity ratings for acrylamide treated rats.

Cumulative Dose[a] (mg/kg)	Toxicity Score[b] (x ± S.E.)	Percentage of Rats Demonstrating Neurotoxic Signs
0	1.0 ± 0.0	0
75	2.0 ± 0.4*	67
150	1.8 ± 0.3*	67
225	1.5 ± 0.2*	50
300	2.3 ± 0.2*	100
375	3.5 ± 0.3*	100

[a] 15 mg/kg/day i.p.; [b] refer to Methods section; * $p<0.05$ as compared to saline treated controls.

(Fig. 9). Both pilocarpine and neostigmine were effective in reversing the increased response to zingerone produced by atropine (Fig. 10).

Acrylamide also caused a dose-dependent increase in the response to ocular zingerone (Fig. 11). This increase in response was reversed in a dose-dependent manner with neostigmine but not pilocarpine (Fig. 12). As demonstrated in Fig. 13, neostigmine had a significant interaction with the increased response to zingerone produced by acrylamide.

Effects of Acrylamide on Tissue CoA Content

As seen in Fig. 14, acrylamide significantly increased basal CoA levels in all tissues except cerebral cortex. This increase in CoA levels was not observed until a cumulative acrylamide dose of 250 mg/kg had been reached. (Refer to appendix B for individual tissue CoA levels.) The animals used in this experiment exhibited significant increases in the response to the ocular zingerone test and significant decreases in weight after a cumulative acrylamide dose of 150 mg/kg (Table 2). Therefore, alterations in CoA levels occured at times corresponding to the development of neurotoxic symptoms.

Supplementary Diet Studies

As demonstrated in Fig. 15, only pyruvate and β-hydroxybutyrate significantly altered basal CoA levels. Pyruvate increased the basal CoA level in heart, cerebellum and cerebral cortex but not in liver or spinal cord. CoA levels in the cerebral cortex are also significantly increased by β-hydroxybutyrate.

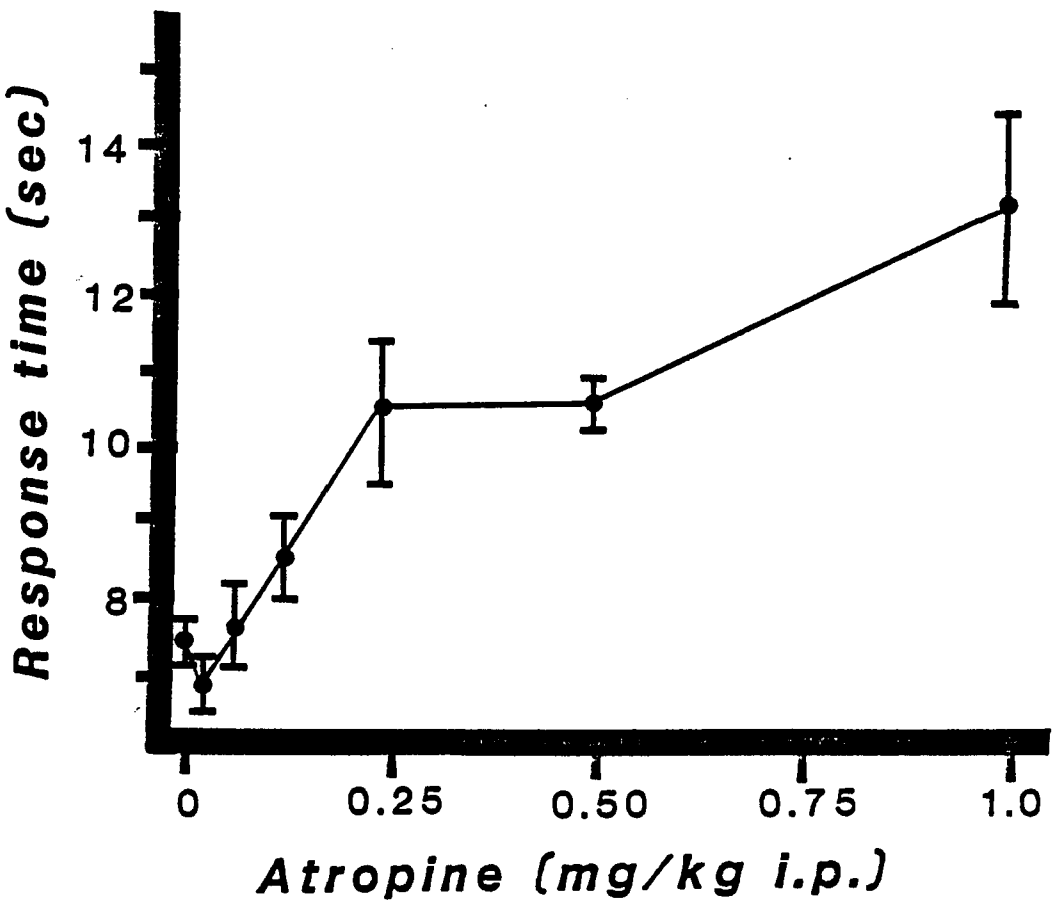

Figure 9. Effect of atropine on response to the ocular zingerone test.

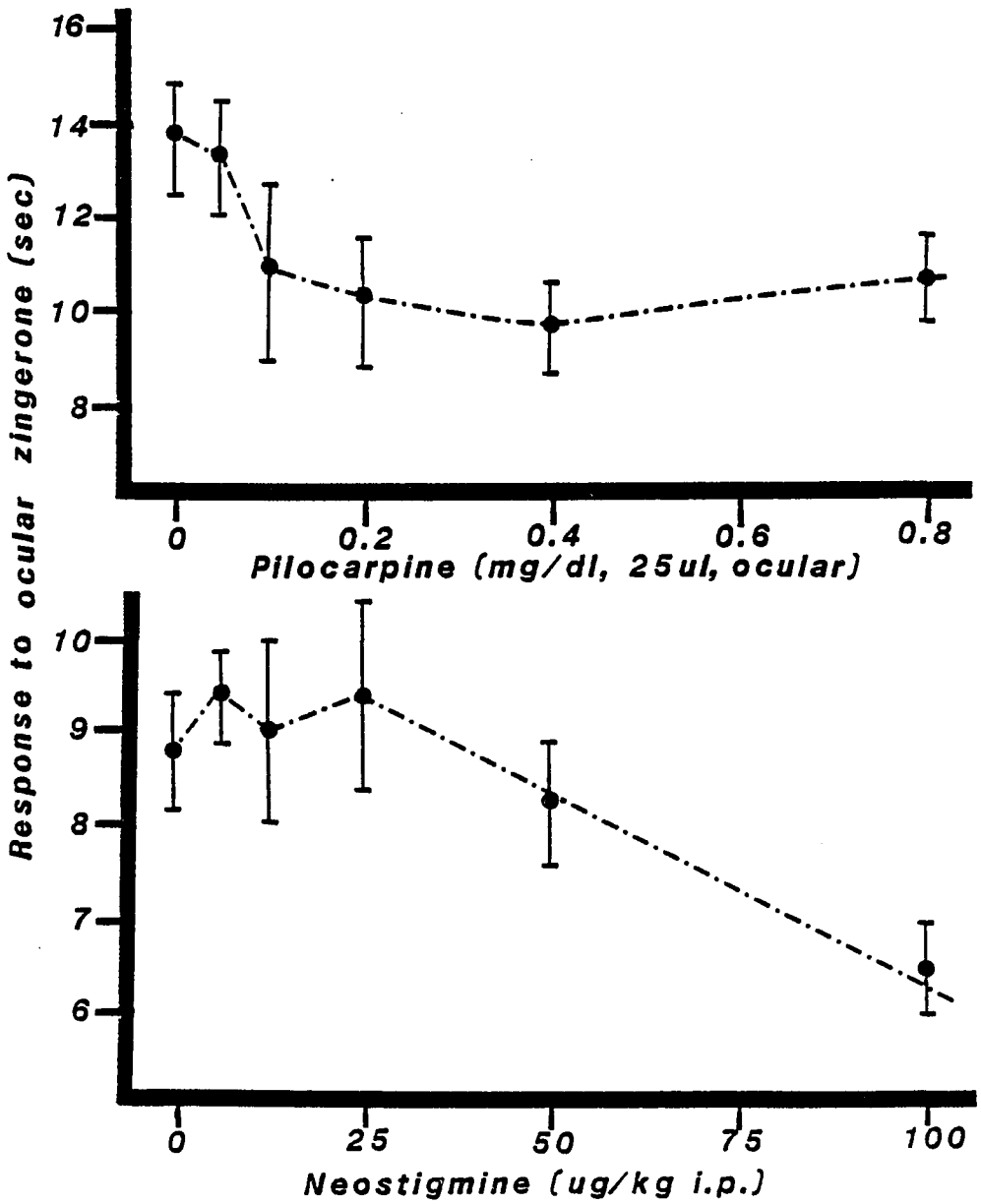

Figure 10. Response to the ocular zingerone test following the administration of pilocarpine and neostigmine.

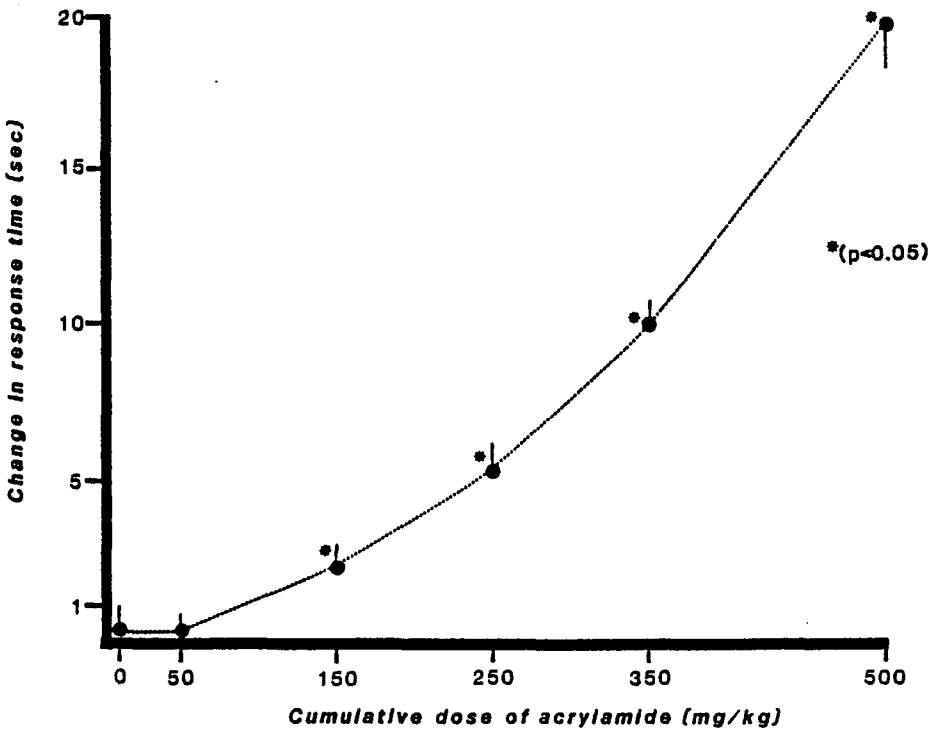

Figure 11. Change in response to the ocular zingerone test following the administration of acrylamide (50 mg/kg/day i.p.) as compared to saline treated controls.

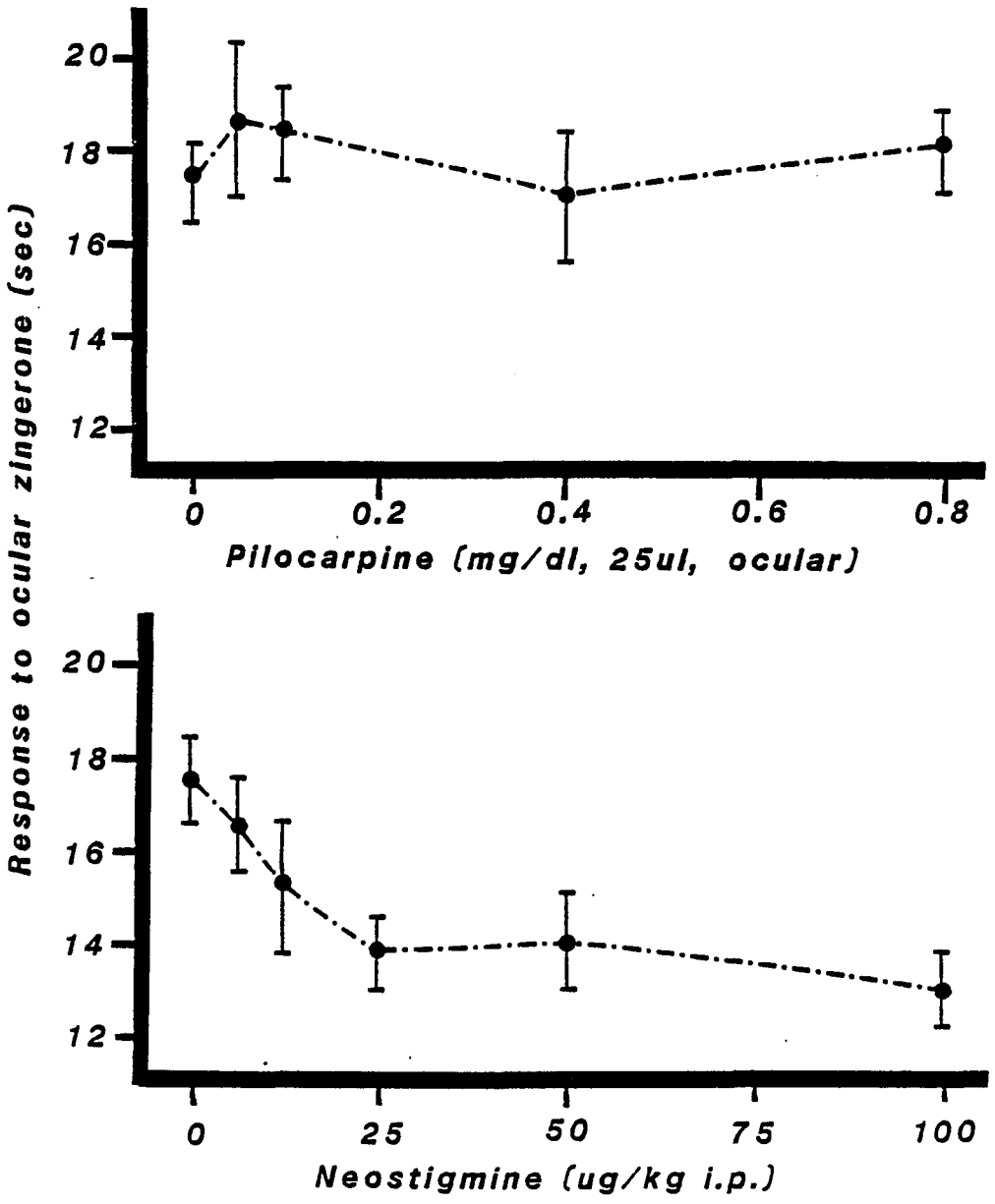

Figure 12. Effect of pilocarpine and neostigmine on acrylamide (400 mg/kg) treated rats as assessed by alterations in the ocular zingerone response.

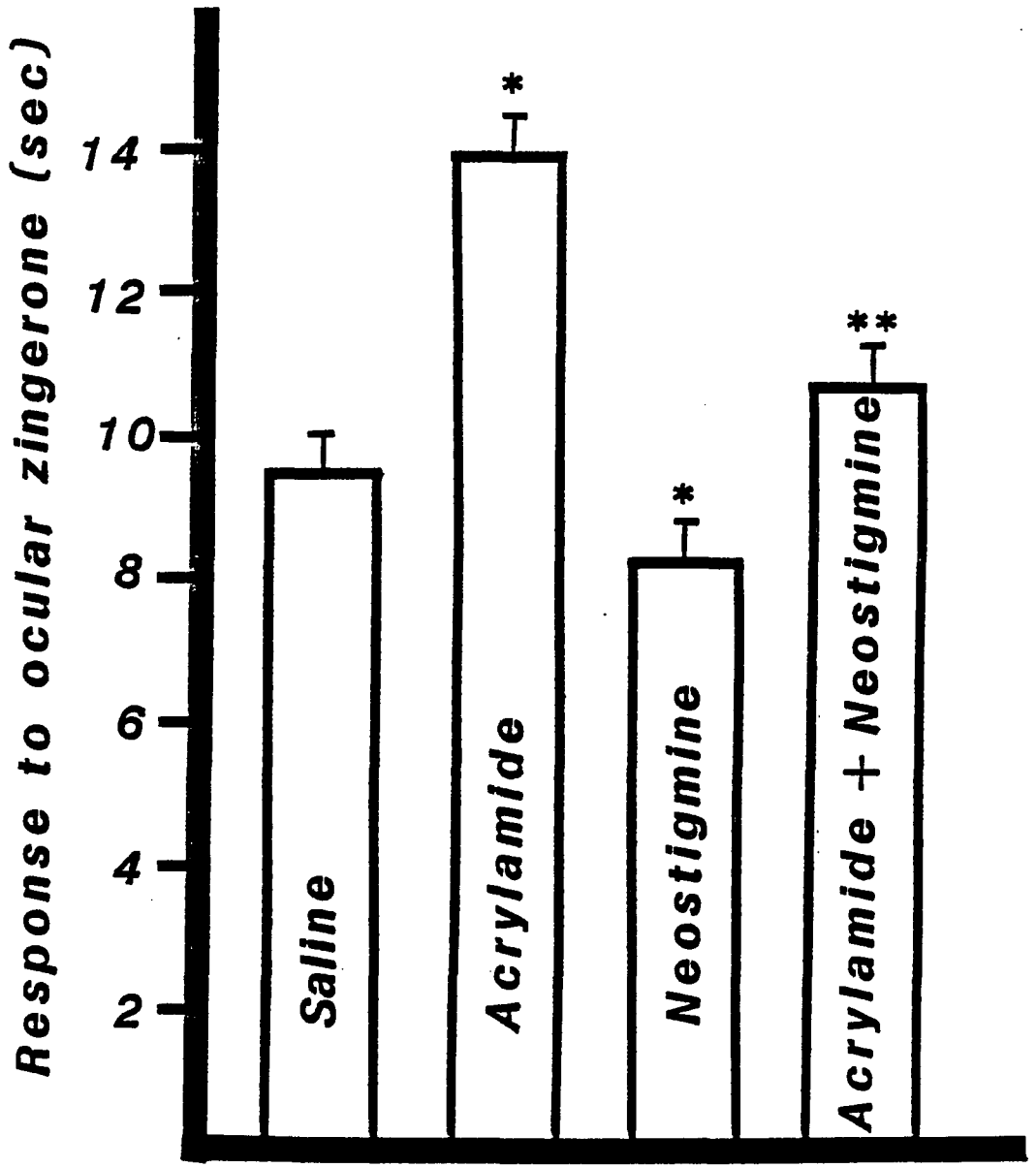

Figure 13. Response to the ocular zingerone test in saline treated, acrylamide treated (400 mg/kg), neostigmine treated (100 ug/kg) and acrylamide (400 mg/kg) plus neostigmine (100 ug/kg) treated rats. -- Significant ($p<0.05$) changes from saline treated controls are shown (*). Significant ($p<0.05$) interactions between acrylamide and neostigmine treatments are also shown (**).

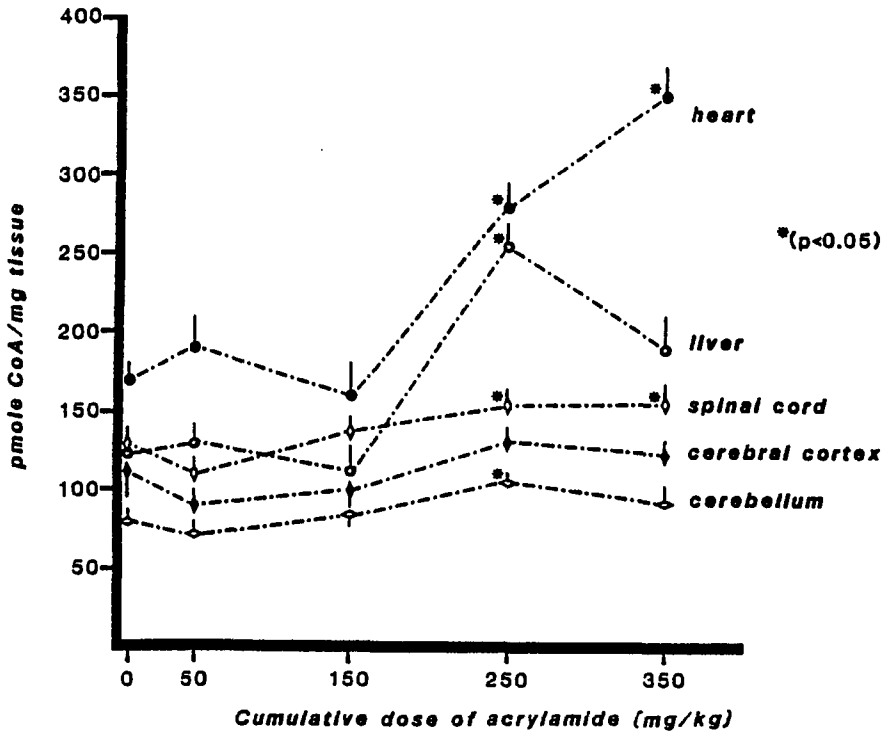

Figure 14. CoA levels in tissues following acrylamide administration (50 mg/kg/day i.p.). -- Significant ($p<0.05$) changes from saline treated controls are shown (*).

Table 2. Dose-response to the ocular zingerone test and weight changes in acrylamide treated rats.

Cumulative Dose[a] (mg/kg)	Duration of Response to Zingerone (sec)[b]	Body Weight (g)[b]
0	6.2 ± 0.5	277 ± 8.1
50	5.8 ± 0.4	273 ± 8.1
150	7.8 ± 0.6*	258 ± 9.8*
250	10.4 ± 0.5*	251 ± 10*
350	16.6 ± 1.1*	237 ± 11*

[a] 50 mg/kg/day i.p.; [b] mean ± S.E.; * $p<0.05$ as compared to saline treated controls.

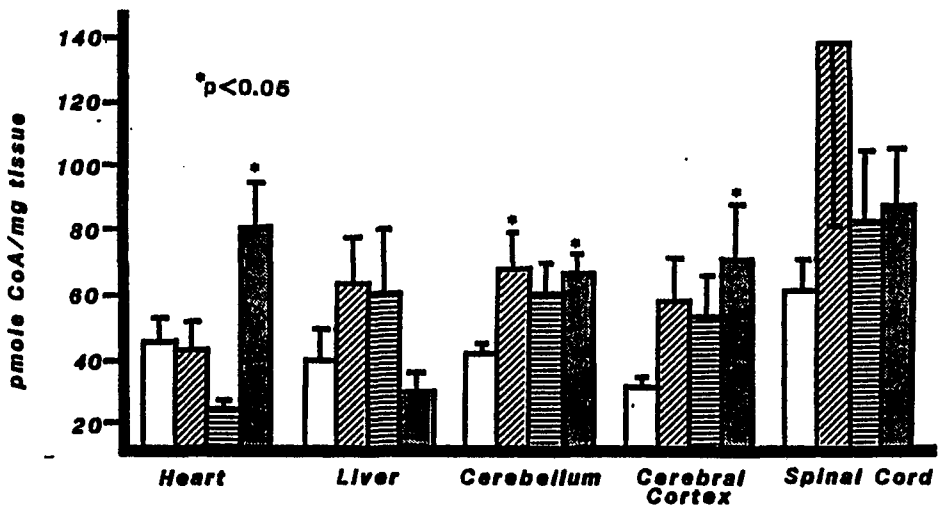

Figure 15. Effect of diet supplementation on tissue CoA levels. -- Diet supplements include: none (□), β-hydroxybutyrate (▨), succinate (▤) and pyruvate (▩). Significant ($p<0.05$) changes in CoA levels from animals maintained on non-supplemented diets are shown (*).

Figure 16 depicts the effect of pyruvate supplementation on alterations in CoA levels induced by 250 and 400 mg/kg of acrylamide. Following a cumulative acrylamide dose of 250 mg/kg, CoA levels were significantly increased in the heart and liver of non-supplemented animals. A similar trend was observed in neuronal tissues but this increase was not significantly different ($p<0.05$) from control values. No interaction was detected between pyruvate supplementation and altered CoA levels produced by acrylamide. The increases in CoA concentrations in the heart and liver of pyruvate supplemented rats treated with acrylamide (250 mg/kg) were additive effects. CoA levels in the cerebellum, cerebral cortex and spinal cord of pyruvate supplemented animals treated with acrylamide represented a cancellation of the effects that acrylamide and pyruvate alone had on CoA levels. Following a cumulative acrylamide dose of 400 mg/kg, CoA levels in the spinal cord and cerebellum of non-supplemented animals were significantly increased over controls. Pyruvate supplementation synergistically increased CoA levels in the heart only. CoA increases induced by acrylamide were significantly reduced by pyruvate in the cerebellum. No other statistically significant interactions between acrylamide and pyruvate were demonstrated.

Succinate supplemented in the diet of animals had a synergistic effect on CoA levels in the cerebellum of rats treated with 500 mg/kg of acrylamide (Fig. 17). No other statistically significant interactions between succinate and acrylamide at either dose level were observed. As seen in Fig. 18, no statistically significant interactions were seen in CoA alterations induced by 3-hydroxybutyrate supplemented in the diet of

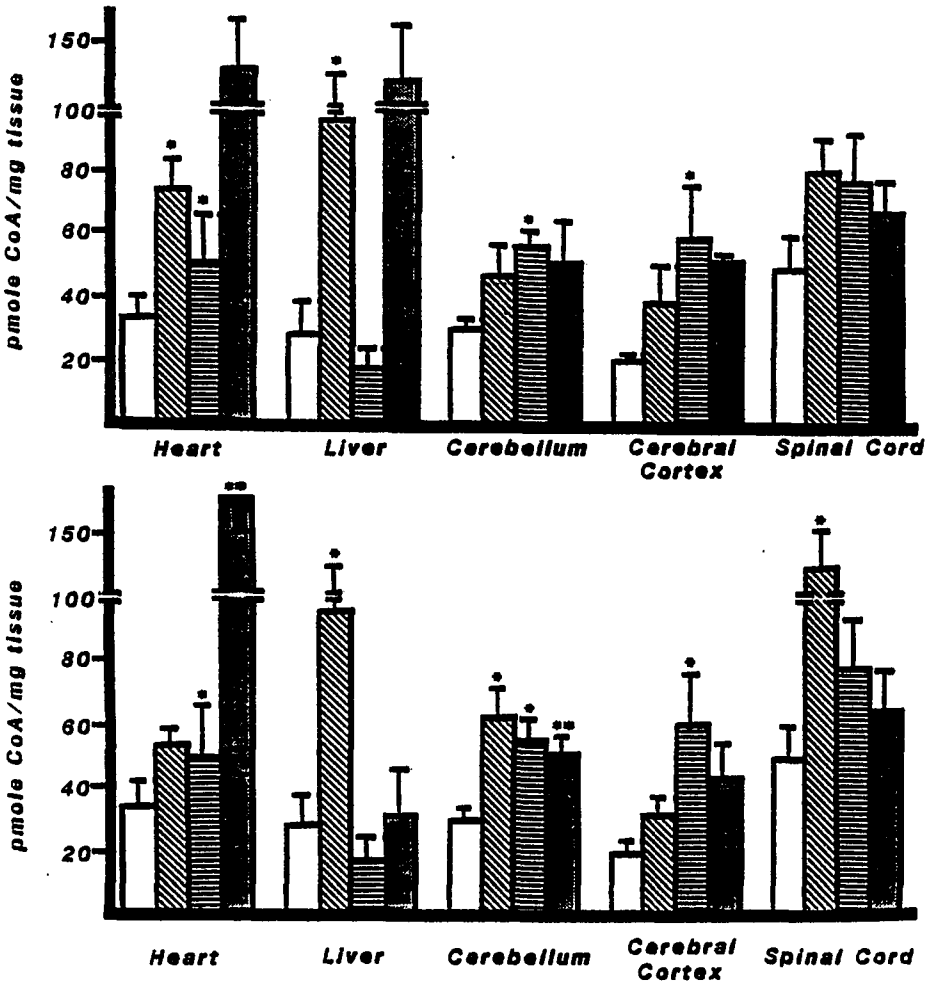

Figure 16. CoA levels in tissues from saline treated (□), acrylamide treated (▨), pyruvate supplemented (▤) and pyruvate supplemented plus acrylamide treated (▩) rats. -- The upper graph shows CoA levels in tissues from animals treated with 250 mg/kg of acrylamide. The lower graph shows CoA levels in tissues from animals treated with 400 mg/kg of acrylamide. Significant ($p<0.05$) changes from saline treated controls are shown (*). Significant ($p<0.05$) interactions between acrylamide and pyruvate supplementation treatments are also shown (**).

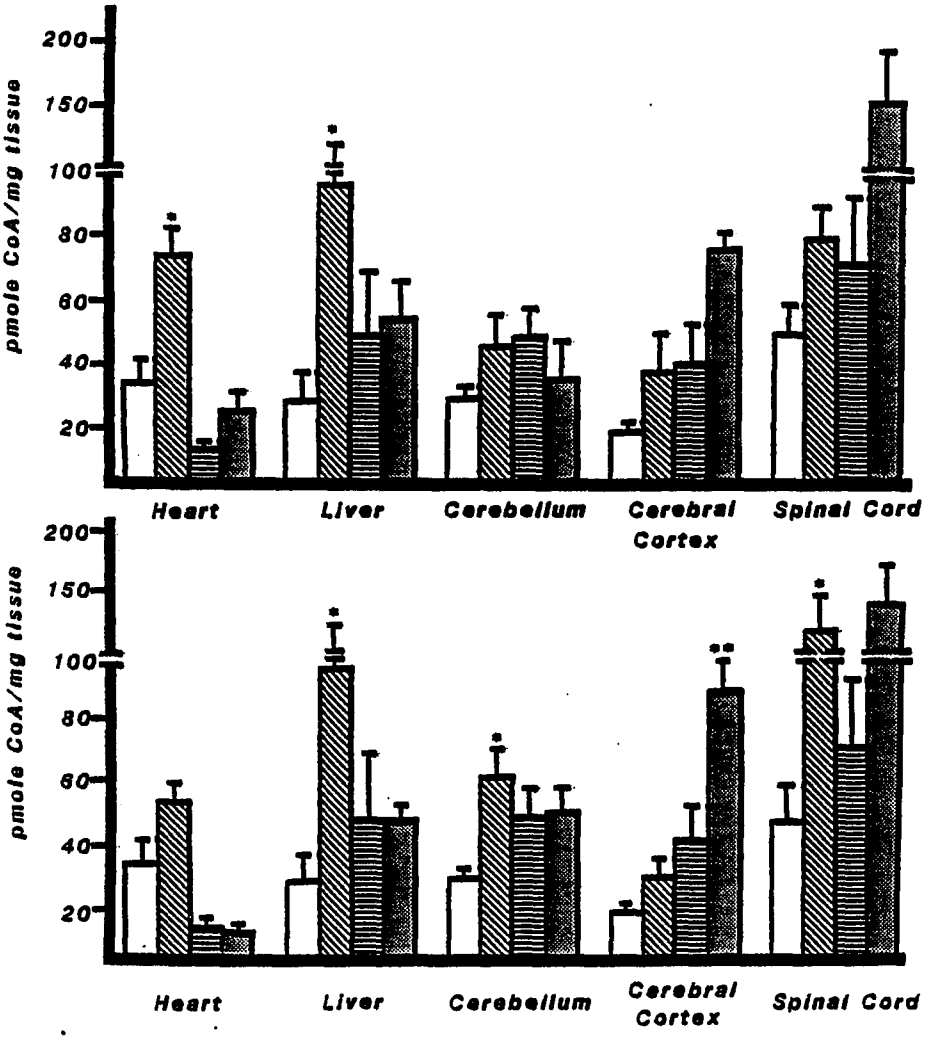

Figure 17. CoA levels in tissues from saline treated (□), acrylamide treated (▨), succinate supplemented (▤) and succinate supplemented plus acrylamide treated (■) rats. -- The upper graph shows CoA levels in tissues from animals treated with 250 mg/kg of acrylamide. The lower graph shows CoA levels in tissues from animals treated with 500 mg/kg of acrylamide. Significant ($p<0.05$) changes from saline treated controls are shown (*). Significant ($p<0.05$) intereactions between acrylamide and succinate supplementation treatments are also shown (**).

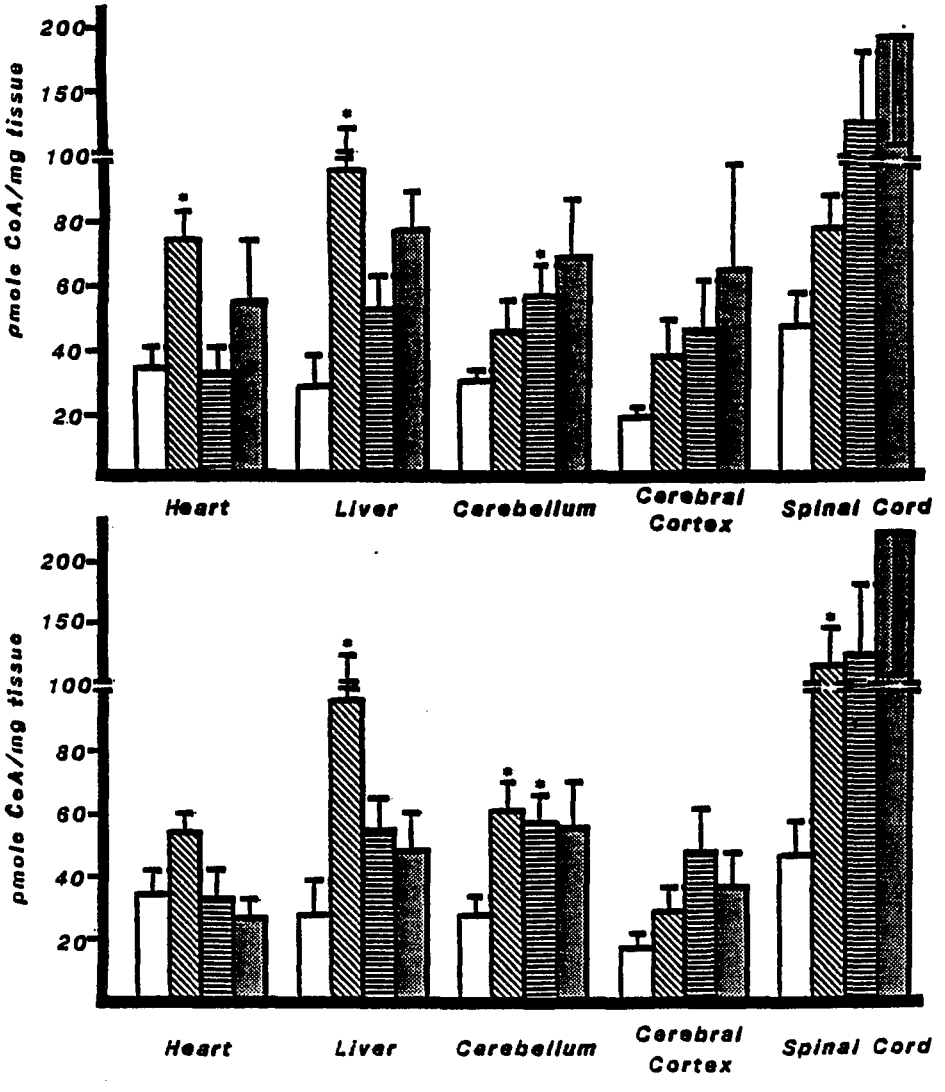

Figure 18. CoA levels in tissues from saline treated (□), acrylamide treated (▨), β-hydroxybutyrate supplemented (▤) and β-hydroxybutyrate supplemented plus acrylamide treated (■) rats. -- The upper graph shows CoA levels in tissues from animals treated with 250 mg/kg of acrylamide. The lower graph shows CoA levels in tissues from animals treated with 500 mg/kg of acrylamide. Significant ($p<0.05$) changes from saline treated controls are shown (*). Significant ($p<0.05$) interactions between acrylamide and β-hydroxybutyrate supplementation treatments are also shown.

animals treated with acrylamide at either dose level (250 mg/kg and 500 mg/kg). (Refer to appendix C for individual tissue CoA levels for diet supplementation studies.)

The effects that various supplements in the diet of animals had on acrylamide toxicity as assessed by the ocular zingerone test are shown in Fig. 19. None of the supplements tested altered the response time to a 1% solution of zingerone applied to the eye in control animals. Also, none of the supplements had any effect on either the onset or magnitude of response to the ocular zingerone test in acrylamide treated rats. Animals which were supplemented with pyruvate were also tested for acrylamide toxicity by using the dropping foot-splay test. No difference in the onset or degree of acrylamide toxicity was observed when compared to non-supplemented animals.

As demonstrated in Fig. 20, animals lost approximately 10% of their original weight after receiving a cumulative acrylamide dose of 250 mg/kg. This weight loss continued in dose-dependent manner. None of the dietary supplements had any effect on the dose-dependent weight loss observed in non-supplemented, acrylamide treated animals.

In Vitro Studies

All attempts to synthesize an acrylamide-CoA adduct *in vitro* were unsuccessful (refer to Methods section for experimental design). The only radiolabelled material recovered from reaction mixtures was unreacted acrylamide.

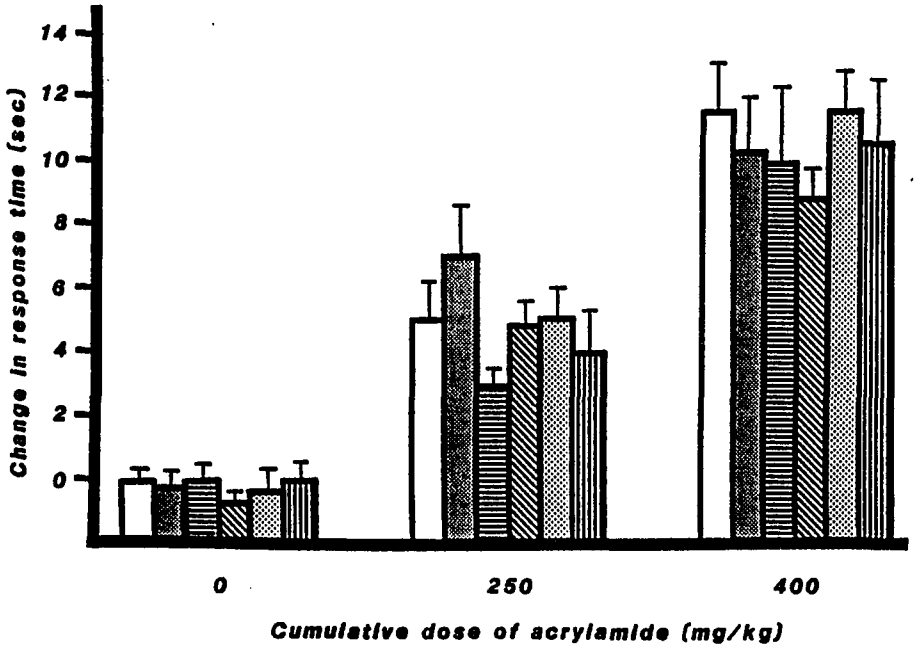

Figure 19. Effect of diet supplementation on acrylamide-induced alterations in response to the ocular zingerone test. -- Diet supplements include: none (□), pyruvate (▩), succinate (▤), β-hydroxybutyrate (▧), oxaloacetate (▦) and citrate (▥).

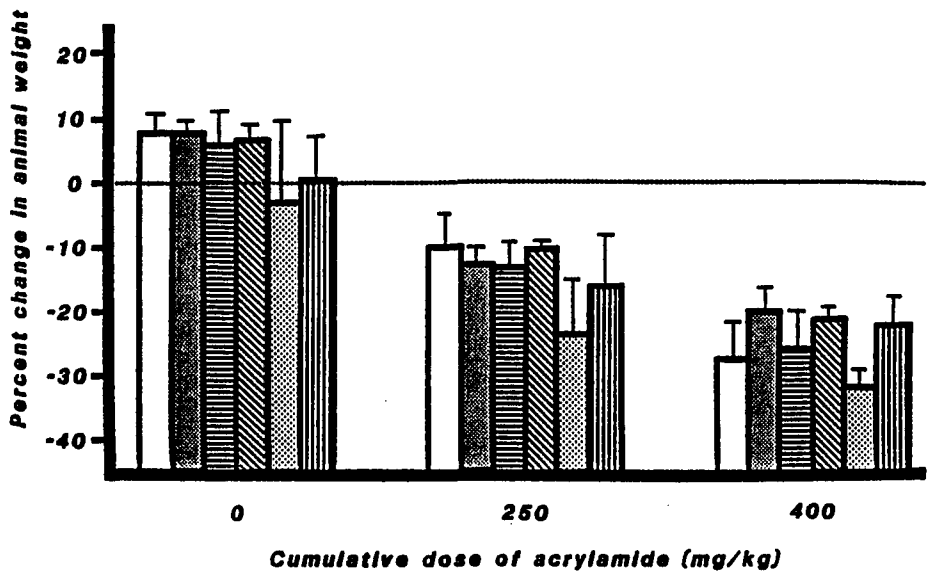

Figure 20. Effect of diet supplementation on acrylamide-induced weight changes. -- Diet supplements include: none (□), pyruvate (■), succinate (▤), β-hydroxybutyrate (▧), oxaloacetate (▩) and citrate (▥).

Acrylamide had no effect on CoA acyltransferase activity. As demonstrated in Table 3, the addition of acrylamide to CoA reaction mixtures had no effect on the amount of product formed. Also, when 2,3-^{14}C-acrylamide was added to reaction mixtures containing non-radiolabelled acetic acid and CoA, no ^{14}C-labelled products were detected in the final organic extract (refer to Table 3).

Table 3. *In vitro* effects of acrylamide on CoA reaction mixtures.

Reaction Mixture	Percent Product Formed
CoA, 0.4 mM ^{3}H-acetic acid	(0.18-0.20)
CoA, 0.4 mM ^{3}H-acetic acid, 0.04 mM acrylamide	(0.16-0.24)
CoA, 0.4 mM ^{3}H-acetic acid, 0.4 mM acrylamide	(0.17-0.20)
CoA, 0.4 mM ^{3}H-acetic acid, 4.0 mM acrylamide	(0.17-0.19)
CoA, 0.4 mM ^{3}H-acetic acid, 40 mM acrylamide	(0.16-0.20)
CoA, 0.4 mM acetic acid, 4.0 mM ^{14}C-acrylamide	none detected
CoA, 0.4 mM acetic acid, 40 mM ^{14}C-acrylamide	none detected

DISCUSSION

The underlying biochemical mechanism responsible for acrylamide-induced neuropathy is unknown. Schoental and Cavanagh (1977) suggested that acrylamide may bind directly to the active sulphydryl portion of CoA and thus decrease the available CoA content required for normal neuronal metabolism and maintenance of axon integrity. Our data suggest that acrylamide does not bind to CoA either *in vitro* or *in vivo*. An acrylamide-CoA adduct was not formed *in vitro* either with or without the addition of high energy compounds such as ATP and GSH. This finding was not predicted since acrylamide is known to actively react with sulphydryl groups on glutatione (Edwards, 1975a; Dixit, Husain et al., 1981) and n-acetylcysteine (Miller, Carter et al., 1982) under the identical conditions that we used in these experiments. It is possible that CoA is unable to react with acrylamide non-enzymatically (*in vitro*). However, an acrylamide-CoA adduct is also apparently not formed *in vivo*. This is evidenced by the fact that CoA levels in tissues increase after the administration of acrylamide. If an acrylamide-CoA adduct was being formed *in vivo*, then free CoA levels should decrease. The function of CoA is also not affected by acrylamide *in vitro*. CoA is still capable of reacting with acetate to form acetyl-CoA even when high concentrations of acrylamide (100 times that of acetate) are added to the reaction mixtures. Thus, acrylamide does not appear to inhibit the normal enzymatic function of CoA.

CoA levels in both neural and non-neural tissues increased at times corresponding to the development of acrylamide toxicity. Alterations in the steady-state concentration of CoA and its derivatives are indicative of metabolic disturbances (Tubbs and Garland, 1964; Guynn, Veloso and Veech, 1972; Voltti et al., 1979). Therefore, our data shows that some biochemical mechanism common to all tissues is altered by acrylamide in vivo.

The susceptibility of nervous tissue to acrylamide is most likely due to the unique structure and function of neural cells. The nerve cell consists of a cell body where proteins and enzymes are synthesized, and an elongated axonal process which is dependent on the transport of these synthesized materials for its function and integrity. Axoplasmic transport mechanisms present in neural tissues are not present in other tissue cells. Therefore, acrylamide may inhibit a biochemical mechanism common to all cells, which in turn selectively alters a unique transport system present only in neural tissues. This hypothesis is strengthened by recent reports that acrylamide inhibited retrograde transport systems which are present only in neural tissues (Miller, Burks et al., 1982). Acrylamide also inhibited neuronal specific enolase (Howland, 1981), a glycolytic iso-enzyme of enolase, which is found only in neural tissue. Other enolase iso-enzymes were not affected by acrylamide.

Several workers have postulated that acrylamide inhibits the glycolytic-TCA cycle pathway (Cavanagh, 1964; Spencer et al., 1979). Since this pathway provides virtually all of the energy supplies used by the nerve, a block in either glycolysis or the TCA cycle would

critically impair normal neuronal function. Although several glycolytic enzymes were inhibited by acrylamide (Howland, 1981), pyruvate levels in neural tissues were unchanged (Johnson and Murphy, 1977). However, lactate, NAD (Johnson and Murphy, 1977) and CoA levels were increased. A block in pyruvate dehydrogenase, the enzyme which converts pyruvate and CoA to acetyl-CoA, could account for the increases in lactate, NAD and CoA observed. Increased pyruvate levels would then be converted to lactate plus NAD.

Alternatively, acrylamide could inhibit the utilization of CoA in the formation of succinyl-CoA. Succinyl-CoA reacts with glycine in the initial steps of heme biosynthesis. Both acrylamide (Edwards et al., 1978) and a deficiency in pantothenic acid (DeMatteis and Remington, 1962), the precursor to CoA, caused an increase in liver porphyrins; this suggests an alteration in the heme biosynthesis pathway.

Increases in CoA levels could also be due to an alteration in the normal regulation of free CoA to acyl-CoA derivatives. For example, the conversion of CoA-glutathione (CoASSG) to free CoA might be increased by acrylamide. Acrylamide is known to actively bind with glutathione (Hashimoto and Aldridge, 1970; Edwards, 1975a). Thus, decreases in tissue glutathione levels may cause CoASSG to be cleaved to CoA and glutathione in an attempt to replenish glutathione supplies. As a result, CoA levels would increase (Dyar and Wilkens, 1972).

An increase in CoA synthesis or a decrease in CoA degredation could also account for the elevated CoA levels induced by acrylamide. CoA concentration is partially regulated by the rate of CoA degredation

(Voltti et al., 1979). These authors have shown that the synthesis of CoA follows zero-order kinetics whereas the rate of CoA degredation follows first-order kinetics. Therefore, CoA levels could be effectively increased if acrylamide was inhibiting the CoA degradation mechanism. Alternatively, CoA levels would increase if both synthesis and degredation mechanisms were affected by acrylamide.

Diet Supplementation Studies

We utilized a variety of dietary supplements designed to bypass the glycolytic pathway in an attempt to test the hypothesis that acrylamide inhibits glycolysis. These supplements included several TCA cycle intermediates (succinate, citrate, and oxaloacetate), β-hydroxybutyrate and pyruvate. TCA cycle intermediates were added to directly offset any putative blocks in glycolysis or in the TCA cycle. β-hydroxybutyrate was added as an alternate energy source which bypasses glycolysis and enters the TCA cycle as acetyl-CoA. Pyruvate was added to bypass any glycolytic blocks that may be present prior to the formation of pyruvate, and to offset any competative inhibition that acrylamide may have on pyruvate dehydrogenase. Figure 21 schematically demonstrates how these supplements would bypass any putative blocks in glycolysis induced by acrylamide. Also shown are the known effects that acrylamide has on glycolysis.

Dietary supplements alone cause alterations in basal CoA levels. Pyruvate causes an increase in heart and neural tissue CoA content. This would be expected since high levels of pyruvate will increase acetyl-CoA production (Harper, Rodwell and Mayes, 1977). As acetyl-CoA

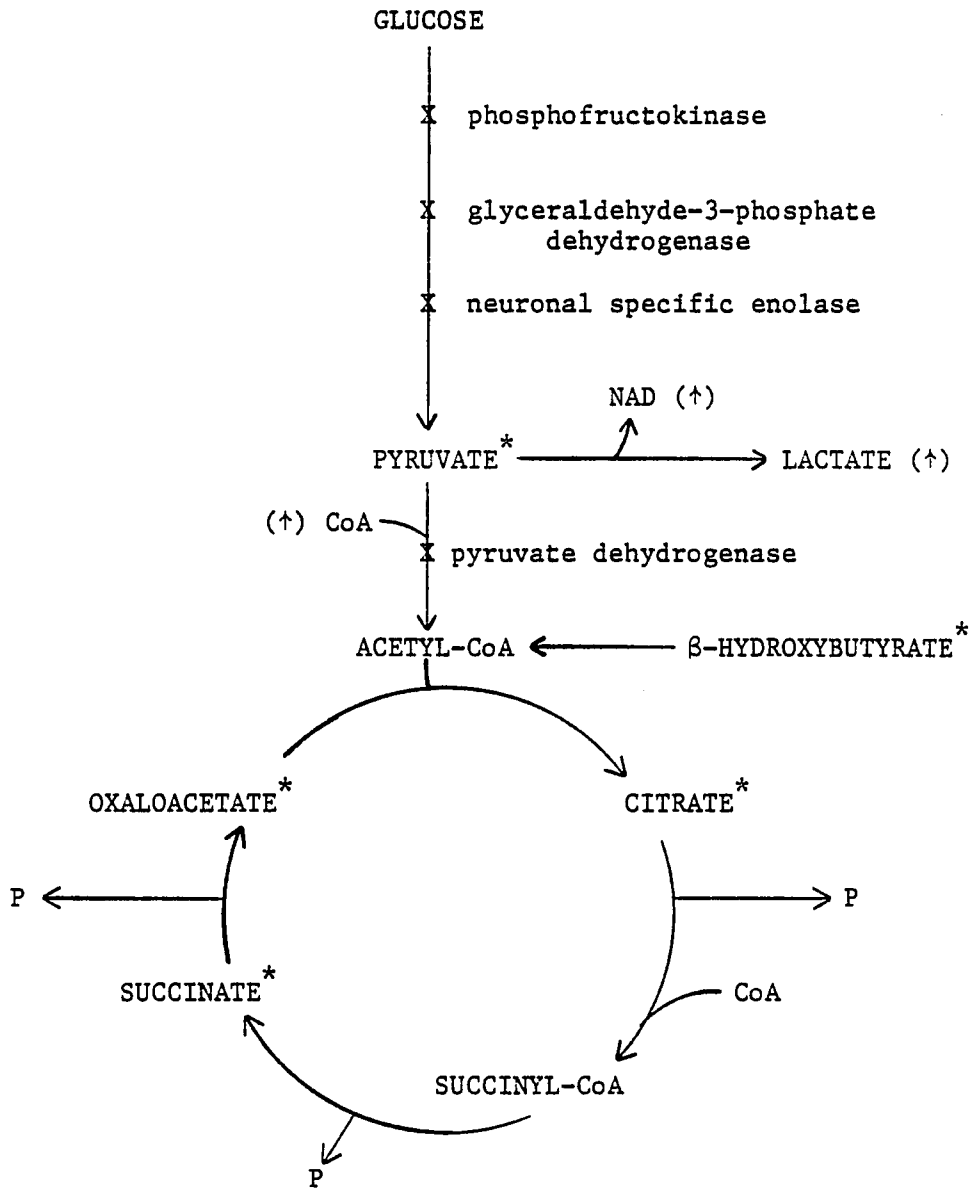

Figure 21. Diet supplementation studies designed to offset the effects of acrylamide on glycolysis. -- Putative sites at which acrylamide may inhibit glycolysis are indicated (X). Substrates used to attempt to bypass the putative glycolytic blocks produced by acrylamide are also indicated (*) as are sites at which high energy phosphate is generated (P).

builds up, pyruvate dehydrogenase is allosterically inhibited, thus keeping high levels of CoA immobile in the cytosol of these tissues. No increase in CoA levels is seen in the liver after pyruvate supplementation. In the liver, unlike in the neural tissue and heart, acetyl-CoA can be utilized to form cholesterol (Harper et al., 1977). Therefore, pyruvate dehydrogenase is not inhibited and all available pyruvate and CoA can be utilized.

Succinate supplementation has no effect on tissue CoA content. It might be expected that succinate would decrease free CoA levels in tissues in an effort to synthesize succinyl-CoA. Both succinate and CoA freely pass mitochondrial membranes where they are utilized in the TCA cycle. However, it is unknown if exogenous supplies of succinate are capable of passing cell plasma membranes. Succinate cannot pass intact liver cell membranes (Berry, 1976), however, an uptake mechanism for succinate is present in the kidney (Hassal, personal communication). Since succinate does not affect CoA levels, it may be speculated that succinate is also unable to pass neural or heart cell membranes.

Ketone bodies, such as β-hydroxybutyrate, are freely permeable to mitochondrial and cell membranes of extrahepatic tissues. During starvation, ketones are preferentially utilized by the heart and neural tissues in an effort to maintain glucose levels (Harper et al., 1977). Ketones can bypass glycolysis and enter the TCA cycle as an alternate source of energy production. Since ketones cannot be utilized by the liver, no hepatic CoA alterations should be, or were, seen. Although the CoA levels in cerebral cortex and spinal cord appear to increase, β-hydroxybutyrate statistically increased CoA levels only in

the cerebellum. This increase in CoA content was most likely due to a lack of utilization of CoA to form acetyl-CoA. Instead, β-hydroxybutyrate was being utilized to form acetyl-CoA for energy production via the TCA cycle.

None of the supplements used appeared to protect against acrylamide toxicity. All animals demonstrated neurotoxic signs such as weight loss, an increase in response to the ocular zingerone test and obvious ataxia following administration of acrylamide. The onset and magnitude of these neurotoxic symptoms were the same as for acrylamide treated animals whose diets were not supplemented. Two-way analysis of variance indicated that pyruvate, succinate and β-hydroxybutyrate were ineffective in altering the tissue CoA changes induced by 250 mg/kg of acrylamide. At this dose level, rats were obviously intoxicated and CoA levels in tissues were increased. Following a high dose of acrylamide (400 mg/kg), pyruvate appeared to interact with acrylamide in the heart and cerebellum. Succinate also had an interaction with high doses of acrylamide (500 mg/kg) in the cerebral cortex. However, these interactions are probably not biologically important since the rats are suffering from severe toxicity at these dose levels.

Several factors could explain the apparent ineffectiveness of these dietary supplements to protect against acrylamide-induced toxicity. Either the supplements were not reaching the TCA cycle or the amount of supplement which reached the TCA cycle was insufficient to offset the putative energy block produced by acrylamide. Another possibility was that acrylamide does not exert its actions via a block in glycolysis or in the TCA cycle.

All of the supplements used are able to pass mitochondrial membranes (Lehninger, 1977). Succinate, pyruvate and β-hydroxybutyrate pass mitochondrial membranes via passive diffusion. A specific mitochondrial uptake mechanism allows oxaloacetate and citrate to enter the TCA cycle. However, in order for the supplements to reach the mitochondria, they must first be able to enter the cell. Pyruvate and β-hydroxybutyrate can both effectively pass all cell membranes. As previously mentioned, it is unknown whether succinate can pass neuronal cell membranes. Neither oxaloacetate or citrate can pass hepatic cell membranes (Zuurendonk, Akerboom and Tager, 1976). Therefore, it is unlikely that these supplements are taken up by neural tissues. The probable inability of oxaloacetate, citrate and succinate to be taken up by tissue cells may account for the apparent ineffectiveness of these supplements to offset the neurotoxic actions of acrylamide.

Recently it has been suggested that pyruvate supplemented in the diet of animals delayed the onset of acrylamide-induced toxicity (Dairman,et al., 1981). However, we were unable to reproduce these results. There were some differences in our experimental design versus that of Dairman and collegues (1981). We gave animals pyruvate in the drinking water and administered 50 mg/kg/day (i.p.) of acrylamide. In contrast, Dairman's group mixed pyruvate in the food and administered lower daily doses of acrylamide (35 mg/kg/day s.c.). The difference in daily acrylamide doses administered to rats may account for the descrepancies between our results. The levels of pyruvate used in our experiments may not have been sufficient to offset the effects that higher doses of acrylamide produce. Also, it is possible that

pyruvate was not stable in the drinking water even though drinking solutions were shielded from light and prepared daily. These factors may account for our results which indicated that pyruvate did not protect against acrylamide toxicity.

β-hydroxybutyrate can freely enter extrahepatic tissues and be used as an alternate source of energy by directly entering the TCA cycle as acetyl-CoA. Therefore, supplementation of the diet of acrylamide treated animals with this compound should offset the toxicity produced by acrylamide. β-hydroxybutyrate did not appear to have any effect on the toxic actions produced by acrylamide. Therefore, either the amounts of β-hydroxybutyrate used were insufficient to bypass an energy block in glycolysis induced by acrylamide or the putative energy block occured at a point further down the energy producing pathways.

The possibility exist that the toxic actions of acrylamide may not involve the glycolytic-TCA pathways. If this is true, then none of the supplements used would be effective in reversing the toxicity of acrylamide. Unfortunately, our data do not conclusively demonstrate whether or not acrylamide exerts its actions by inhibiting the glycolytic-TCA pathway. The small test groups and large standard errors associated with many of our CoA results in the diet supplement studies make meaningful statistical interpretations of this data impossible. Also, we could not prove that dietary supplements were effectively being utilized by the glycolytic-TCA cycle pathway.

Behavioral Testing Methods

Although several quantitative assessments of behavioral changes induced by peripheral neurotoxins exist in the literature, most of these methods are either difficult to use or too insensitive to detect early behavioral alterations. We found that the ocular zingerone test and the dropping foot-splay test were the easiest methods available to reproducibly detect early behavioral changes in acrylamide treated animals.

The ocular zingerone test detected behavioral alterations produced by acrylamide following a cumulative acrylamide dose of 150 mg/kg, regardless of the magnitude of the daily dose administered. Response time in the ocular zingerone test increased in a dose-dependent manner. Therefore, this test is useful for assessing the relative degree of acrylamide toxicity.

In contrast, the dropping foot-splay test did not detect acrylamide-induced behavioral changes until a cumulative dose of 225 mg/kg was reached. Animals quickly reached a maximal foot-splay once acrylamide-induced neuropathy had occurred. This limits the usefulness of this test in severely intoxicated animals. Also, the results obtained using the dropping foot-splay test appeared to be dependent on how often the animals were handled (personal observation). Animals which were accustomed to being regularly handled tended to be more relaxed and had wider foot-splay than naive rats.

Our results demonstrate that the ocular zingerone test reproducibly detects early changes in behavior induced by acrylamide. This test has an advantage over other testing methods in that is easy to

use, gives reproducible dose-dependent results, and is not affected by inconsistencies in animal handling.

There are several reasons which may explain why animals demonstrate an increase in response time to the ocular zingerone test following acrylamide administration. Acrylamide may produce a heightened sensitivity of nerve terminals to chemical pain or acrylamide may increase the perception of pain. Alternatively, acrylamide may be eliciting an anticholinergic response which results in a decrease in lacrimation of the eye. This would cause the rat to wipe his eye longer in an effort to eliminate the irritant.

Other symptoms of autonomic changes seen during acrylamide treatment include retention of urine resulting in grossly enlarged and distended bladders and an apparent decrease in intestinal motility (personal observation). Although no intestinal motility studies have been reported, we have noted that when acrylamide intoxicated animals were killed, they had full intestines which appeared to be more engorged than the intestines of non-acrylamide treated animals.

Atropine administration had a similar effect on the ocular zingerone response as did acrylamide. The effects of atropine on the response to the ocular zingerone test were reversed in a dose-dependent manner with both pilocarpine and neostigmine. Pilocarpine, a muscarinic cholinergic agonist, did not reverse the increases in response time to the ocular zingerone test induced by acrylamide. However, neostigmine, an indirect acting cholinergic agonist, reversed the actions of acrylamide on the ocular zingerone test back to control levels. Two-way analysis of variance showed that neostigmine had a significant

interaction on the acrylamide-induced changes in the ocular zingerone test. These data suggest that acrylamide is not acting as a muscarinic cholinergic antagonist in the eye. Acrylamide has been shown to increase acetylcholinesterase activity (Bass and Goldberg, 1982) which would cause a decrease in acetylcholine levels. This decrease in acetylcholine would account for the apparent anticholinergic actions of acrylamide in the peripheral nervous system. Thus, acrylamide appears to produce an autonomic neuropathy in addition to its degenerative actions in the peripheral nervous system.

CONCLUSIONS

1. Acrylamide produces biochemical changes in both neural and non-neural tissues *in vivo*. Biochemical aberrations are seen as increased levels of CoA in tissues which occur at times corresponding to the development of acrylamide toxicity.

2. Acrylamide does not appear to bind to CoA *in vivo* or *in vitro*. Also, acrylamide does not alter the enzymatic function of CoA *in vitro*.

3. Dietary supplements (oxaloacetate, citrate, succinate, pyruvate and β-hydroxybutyrate) do not appear to protect against acrylamide toxicity. However, these results are not conclusive since we cannot prove if the supplements were being utilized in the glycolytic or TCA cycle pathways.

4. The ocular zingerone test provides a simple, sensitive, quantitative and reproducible technique for detecting acrylamide-induced behavioral changes.

5. Acrylamide may be acting as a cholinergic antagonist in the peripheral nervous system. The ocular zingerone test appears to be detecting these autonomic changes produced by acrylamide.

APPENDIX A

TOXICITY RATINGS FOR ACRYLAMIDE TREATED RATS - INDIVIDUAL ANIMAL DATA

Cumulative Acrylamide Dose[a] (mg/kg i.p.)	Individual Animal Toxicity Score[b]	Toxicity Score (X ± S.E.)
0	1,1,1,1,1,1	1.0 ± 0.0
75	1,2,3,3,2,1	2.0 ± 0.4
150	1,2,3,2,1,2	1.8 ± 0.3
225	1,2,1,2,2,1	1.5 ± 0.2
300	2,3,2,2,3,2	2.3 ± 0.2
375	2,4,4,4,3,4	3.5 ± 0.3

[a] 15 mg/kg/day i.p.

[b] refer to Methods section

APPENDIX B

CoA LEVELS IN TISSUES - INDIVIDUAL TISSUE DATA

Cumulative Acrylamide Dose[a] (mg/kg)	Tissue CoA Levels (pmoles/mg tissue)				
	Heart	Liver	Cerebellum	Cerebral Cortex	Spinal Cord
0	141	100	59	69	107
	154	282	70	119	138
	166	47	95	138	126
	200	147	103	137	137
	169	53	67	108	137
50	223	197	42	106	112
	145	145	70	65	112
	122	164	91	81	106
	274	69	80	100	100
	136	60	84	97	120
150	107	46	80	103	127
	91	107	75	103	119
	129	208	89	130	147
	227	155	89	87	152
	151	55	83	81	142
250	273	238	104	130	145
	221	257	86	127	168
	209	106	123	120	127
	396	339	114	152	172
	305	332	104	122	157
350	361	213	109	108	186
	368	240	83	122	156
	311	164	80	117	154
	419	294	91	133	141
	293	31	104	131	139

[a] 50 mg/kg/day i.p.

APPENDIX C

CoA LEVELS IN TISSUES FROM DIET SUPPLEMENTATION STUDY-
INDIVIDUAL TISSUE DATA

No Diet Supplementation

Cumulative Acrylamide Dose[a] (mg/kg)	Tissue CoA Levels (pmoles/mg tissue)				
	Heart	Liver	Cerebellum	Cerebral Cortex	Spinal Cord
0	47	8.6	34	18	103
	43	0.3	43	23	35
	28	61	37	15	48
	62	25	23	30	31
	4.2	54	20	27	59
	35	21	24	20	32
	26		38	18	40
250	117	44	84	31	93
	90	71	73	112	114
	94	186	63	37	100
	77	258	41	15	117
	50	45	23	34	75
	47	92	21	28	54
	66	60	24	14	23
	55	24			
400-500	60	28	55	6.3	72
	65	210	63	20	87
	58	141	34	27	134
	68	29	82	57	58
	45	114	26	25	113
	42	53	89	50	78
	36	84	82	29	310

[a] 50 mg/kg/day i.p.

Pyruvate Supplementation

Cumulative Acrylamide Dose[a] (mg/kg)	Tissue CoA Levels (pmoles/mg tissue)				
	Heart	Liver	Cerebellum	Cerebral Cortex	Spinal Cord
0	117	5.1	58	64	83
	63	11	68	23	47
	41	31	39	103	121
	64	28	59	50	57
250	212	29	71	53	55
	118	226	75	57	45
	166	150	43	50	74
	33	81	19	46	95
400	199	28	47	75	92
	175	8.5	63	32	51
	102	73	37	20	40
	234	17	55	47	78

[a] 50 mg/kg/day i.p.

Succinate Supplementation

Cumulative Acrylamide Dose[a] (mg/kg)	Tissue CoA Levels (pmoles/mg tissue)				
	Heart	Liver	Cerebellum	Cerebral Cortex	Spinal Cord
0	19	22	75	65	28
	12	28	42	24	134
	12	108	31	37	55
	14	43	50	42	73
250	23	80	25	82	80
	15	42	62	67	210
	38	43	24	84	187
	26	55	37	78	159
500	14	47	63	122	209
	13	38	54	49	119
	14	59	40	99	110
	13	48	52	90	146

[a] 50 mg/kg/day i.p.

β-hydroxybutyrate Supplementation

Cumulative Acrylamide Dose[a] (mg/kg)	Tissue CoA Levels (pmoles/mg tissue)				
	Heart	Liver	Cerebellum	Cerebral Cortex	Spinal Cord
0	30	46	40	30	39
	21	83	46	22	149
	49	31	54	91	40
	33	54	87	45	280
250	11	82	104	19	28
	70	105	43	168	426
	101	82	97	40	40
	42	45	33	33	282
500	13	78	50	24	24
	13	21	46	47	473
	40	54	30	17	30
	40	38	96	64	376

[a] 50 mg/kg/day i.p.

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