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EFFECT OF ACRYLAMIDE INTOXICATION ON PYRIDINE NUCLEOTIDE CONCENTRATIONS AND FUNCTIONS IN RAT CEREBRAL CORTEX*

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Abstract—Male rats were given cumulative doses of 428 mg/kg (low dose) and 668 mg/kg (high dose) of acrylamide. Concentrations of oxidized pyridine nucleotides were determined in samples of cerebral cortex from treated and control rats. The concentration of NAD^+ was significantly increased ($P < 0.01$) in brains from the high dose animals. The function of pyridine nucleotides was evaluated by measuring substrates of pyridine nucleotide-dependent enzymes both in immediately frozen cerebral cortex and in cortices subjected to ischemia, by measuring total high-energy phosphate and the rate of its use during ischemia, and by determining the cytoplasmic redox state of pyridine nucleotides from enzyme equilibrium constants and measured substrate levels. α-Glycerophosphate was significantly elevated in the brains from the high dose animals ($P < 0.05$), while other measured substrates were unchanged. Changes in substrate concentrations during ischemia were similar in cerebral cortices from controls and treated rats and did not suggest interference by acrylamide with coenzyme function. Total high-energy phosphate sources were unchanged in treated animals and the rate of their use during ischemia was the same as that of controls. Calculation of the redox state of cytoplasmic pyridine nucleotides suggested that both NAD and NADP may be more in the reduced form in cerebral cortices of treated animals than in controls; however, these changes were small compared to redox changes under other conditions. While acrylamide increased the concentration of NAD^+ in cerebral cortices from treated rats, there was little suggestion of an interference with pyridine nucleotide function.

Acrylamide causes both acute and chronic neurotoxicity in humans and in experimental animals [1–8]. Fullerton and Barnes [9] identified the principal lesion in the chronic toxicity as a "dying back" type of peripheral neuropathy. Characteristically, the longest and largest diameter myelinated axons are affected first in acrylamide intoxication, but Schaumburg *et al.* [10] have reported that the earliest histological evidence of injury occurs in some of the shorter axons. The chronic form of intoxication is cumulative. Kuperman [11] showed that a cumulative dose of 102 mg/kg produced neurotoxic symptoms in the cat over a dosage range of 1–50 mg/kg/day. Similar cumulative action has been reported in dogs, rats and monkeys [5, 9, 12, 13].

Histological and ultrastructural studies have revealed the morphological changes which accompany acrylamide intoxication [7, 10], but little is known about the biochemical changes which accompany the intoxication or the mechanism by which the chemical causes axons to degenerate.

Kaplan *et al.* [14] suggested that the biochemical lesion leading to acrylamide neuropathy might involve an interference with pyridine nucleotide metabolism or function. This hypothesis was based on two observations. First, the cat, the most susceptible species to the toxic action of acrylamide, is deficient in its ability to convert tryptophan to nicotinamide [15] and might be expected to be more sensitive to an interference in the synthesis or function of pyridine nucleotides. Second, they suggested that a dimer of acrylamide might be produced by the nucleophilic attack of the β-carbon of one acrylamide monomer by the α-carbon of another. Such a dimer resembles the nicotinamide portion of the pyridine nucleotide molecule (Fig. 1) and might disrupt synthesis of normal concentrations of coenzyme or form an analog which could interfere with the molecule's normal coenzymatic functions. This second mechanism of toxicity is known to occur with two analogs of nicotinamide which produce neurotoxic symptoms, 3-acetyl pyridine [16] and 6-aminonicotinamide [17]. This investigation was undertaken to test this hypothesis.

Interference with pyridine nucleotide metabolism was tested by measuring concentrations of oxidized pyridine nucleotides in brain extracts from acrylamide-intoxicated rats. Impairment of nucleotide function was evaluated in three ways. First, substrates of pyridine nucleotide-dependent enzymes in both quick-frozen brains and brains subjected to periods of ischemia were measured. During a period of ischemia, glycolytic flux is nearly maximal [18] and an impairment of flow through a pyridine nucleotide-dependent enzymatic step should be apparent by the accumulation of substrate for that step. Second, concentrations of potential high-energy phosphate sources

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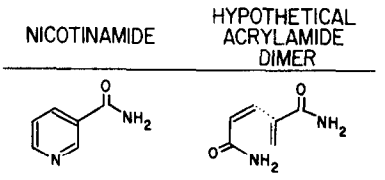

Fig. 1. Structural similarity between nicotinamide and two acrylamide molecules after Kaplan *et al.* [14].

and the rates of their use during ischemia were compared. Finally, the redox states of cytoplasmic NAD and NADP were determined using the equilibrium constants of lactate dehydrogenase (EC 1.1.1.28 D-lactate:NAD^+ oxidoreductase), malic enzyme (EC 1.1.1.40 L-malate:$NADP^+$ oxidoreductase[oxalacetate-decarboxylating]), and measured levels of pyruvate, lactate and malate.

EXPERIMENTAL

Animals and treatments. Male Holtzman rats (260–300 g) were used. The animals were housed in air-conditioned rooms and supplied with food (Ralston Purina Lab Chow) and water *ad lib.* Acrylamide (Eastman special grade for electrophoresis) was purchased from Eastman Organic Chemical Co., Rochester, NY. Initially, 4 mg/kg/day was administered in aqueous solution by i.p. injections in a volume equivalent to 0.1 per cent of the body weight. As no neurological symptoms were apparent after 1 week, the dosage was increased to 40 mg/kg/day and animals were sacrificed when the total cumulative doses were 428 mg/kg (low dose) and 668 mg/kg (high dose). The low dose animals were beginning to show signs of intoxication while animals receiving the high dose were characterized as severely affected. Weight records were maintained during the course of treatment. Impairment of neurological function was tested by suspending the rats by their hind limbs from a wire rack held vertically. Acrylamide-treated rats were more likely to fall from the rack while controls usually swung their bodies forward and grasped the rack with their forepaws. This procedure was termed the vertical rack test and an animal was scored successful in this test if it remained attached to the rack on two of three trials.

Tissue preparation. All assays were performed on extracts from cerebral cortical samples since studies on experimental animals [3, 11, 7, 19] and humans [1, 4] indicated that the central as well as the peripheral nervous system is affected in acrylamide intoxication. Also, Hashimoto and Aldridge [20] have shown that label from administered [^{14}C]acrylamide is found in the brain as well as the spinal cord and peripheral nerve. Another important consideration in selecting brain was the difficulty in freezing the spinal cord quickly enough to minimize postmortem changes in concentration of labile intermediates.

Rats were sacrificed by decapitation and the heads frozen in liquid nitrogen either immediately or after time intervals of 10, 30 or 60 sec at 37° for the ischemia data. Tissue was stored at −80° until dissection. Brains were dissected from the surrounding tissue while being maintained at −50° in a chest loaded with dry ice. Superficial areas of cortex were removed and weighed on a balance chilled to −50°. Perchloric acid extracts were made from 100-mg tissue samples according to Lowry *et al.* [18] as modified by Kauffman and Albuquerque [21].

Biochemical assays. All enzymes except lactate dehydrogenase were purchased from Boehringer Mannheim, Indianapolis, IN. Lactate dehydrogenase and all substrates and cofactors were purchased from Sigma Chemical Co., St. Louis, MO. All substrates and cofactors were measured fluorometrically using a Farrand fluorometer. ATP, dihydroxyacetone phosphate, glucose, glucose 6-phosphate, glutamate, α-glycerophosphate, phosphocreatine and pyruvate were measured as described by Lowry and Passonneau [22]. Lactate was measured according to Harkonen *et al.* [23]. Glyceraldehyde 3-phosphate was measured in the reagent for dihydroxyacetone phosphate with added triose phosphate isomerase (20 μg/ml) after the reaction for dihydroxyacetone phosphate was complete. 6-Phosphogluconate was measured by enzymatic cycling as described by Kauffman and Albuquerque [21]. Malate was measured according to Goldberg *et al.* [24]. Glycogen was measured by the method of Lowry *et al.* [18]. Oxidized NAD and NADP were determined in unneutralized aliquots of the perchloric acid extracts. NAD^+ was measured in the same reagent as that used for lactate except that 0.2 mM lactate was substituted for NAD^+. $NADP^+$ was determined by enzymatic cycling according to Lowry *et al.* [25]. Before measuring glyceraldehyde 3-phosphate, dihydroxyacetone phosphate, α-glycerophosphate and 6-phosphogluconate, the extracts were filtered through acid-washed charcoal according to Lowry and Passonneau [22] to remove fluorescent impurities which increase the blank. In most cases, bovine serum albumin was added to the reagents to a concentration of 0.01% to improve enzyme stability.

Statistics. The results of the vertical rack test were analyzed using the chi-square test. Dunnett's modification of Student's *t*-test was used for comparing the two acrylamide treatment dosages with the control groups. The dose dependency of the pyridine nucleotide changes in response to acrylamide treatment and the rates of change of substrate during ischemia were calculated by linear regression [26, 27].

RESULTS

Characteristics of intoxicated animals. Rats given the lower cumulative dose (428 mg/kg) showed mild symptoms of intoxication characterized by decreased body tone and increased excitability. Animals receiving the higher dose (668 mg/kg) were severely affected. They dragged their hind limbs with the plantar surface turned up, some had bladder incontinence and there was some wasting of the hind limbs. In the vertical rack test, 3/22 low dose animals were successful compared to 20/20 successes in controls (χ^2, $P < 0.001$). None of the high dose animals were successful (0/6, χ^2, $P < 0.001$). Over the course of the experiment, control animals gained an average of 42.0 ± 13.0 g while low and high dose acrylamide-treated rats gained 5.9 ± 14.9 and 0.11 ± 0.07 g respectively.

Table 1. Oxidized pyridine nucleotides in brains of normal and acrylamide-treated rats

	Control	Acrylamide 428 mg/kg*	Acrylamide 668 mg/kg*
NAD^+	374 ± 20†	431 ± 19	455 ± 12‡,§
$NADP^+$	5.23 ± 0.62	4.24 ± 0.48	4.67 ± 0.74

* Cumulative dose of acrylamide.

† Values are expressed as mean ± S.E.M. of concentrations (nmoles/g of cerebral cortex) for four, four and six rats in control, low and high dose rat groups.

‡ $P < 0.01$, compared to control values.

§ $P < 0.01$ linear regression.

Biochemical assays of pyridine nucleotides. Total oxidized pyridine nucleotides were measured in cerebral cortical extracts from control and both treatment groups. The data are summarized in Table 1. While $NADP^+$ concentrations remained constant, NAD^+ concentrations showed a dose-related increase (slope of the regression line, $P < 0.01$) in the treated rats. NAD^+ concentrations were significantly higher than controls in the high dose animals ($P < 0.01$). Reduced pyridine nucleotides were not measured because of their instability at acid pH during extraction [28].

Substrates. Measured concentrations for nine substrates of pyridine nucleotide-dependent enzymes are shown in Table 2. Only the increase in α-glycerophosphate in the high dose rats was statistically significant ($P = 0.05$). Concentrations of all measured substrates, except glutamate, were determined in the cerebral cortices from low dose rats and controls after various periods of ischemia, i.e. prior to freezing in liquid nitrogen. Differences in the pattern of substrate use during the ischemic intervals might reveal an interference in pyridine nucleotide coenzymatic function that would not be apparent by measuring substrate concentrations in the immediately frozen tissues. However, no significant differences were seen (Table 3).

Potential high-energy phosphate. Sources of high-energy phosphate were calculated as the sum of the measured molar concentrations of phosphocreatine

Table 2. Substrates of pyridine nucleotide-dependent enzymes in cerebral cortices of control and acrylamide-treated rats

Substrate	Control	Acrylamide 428 mg/kg	Acrylamide 668 mg/kg
α-Glycerophosphate	112 ± 6*	104 ± 10	155 ± 13†
Lactate	3750 ± 420	3500 ± 240	5190 ± 450‡
Dihydroxyacetone phosphate	43.1 ± 11	51.1 ± 4.4	57.9 ± 7.2
Glyceraldehyde 3-phosphate	42.1 ± 6.9	30.7 ± 5.3	39.1 ± 5.0
Glucose 6-phosphate	36.5 ± 6.1	30.2 ± 2.6	34.7 ± 3.0
Glutamate	8860 ± 480	9180 ± 300	9720 ± 330
Malate	278 ± 30	303 ± 30	349 ± 26
6-Phosphogluconate	6.74 ± 0.87	7.19 ± 0.41	8.15 ± 0.77
Pyruvate	204 ± 22	157 ± 24	204 ± 29

* Values are expressed as means ± S.E.M. of concentrations (nmoles/g of cerebral cortex) for four, four and six rats in control, low and high dose rat groups.

† $P = 0.05$, compared to controls.

‡ $0.05 < P < 0.10$, compared to controls.

Table 3. Rates of change of substrates of pyridine nucleotide-dependent enzymes during ischemia

Substrate	Control	Acrylamide*
α-Glycerophosphate	84.0 ± 20.2†	90.3 ± 37.8
Lactate	2270 ± 1207	2430 ± 930
Dihydroxyacetone phosphate	32.1 ± 21.1	58.7 ± 15.8
Glyceraldehyde 3-phosphate	10.9 ± 15.3	9.67 ± 13.9
Glucose 6-phosphate	23.3 ± 15.1	18.7 ± 8.1
Malate	−91.1 ± 56.4	−110 ± 75
6-Phosphogluconate	−1.86 ± 1.98	1.08 ± 1.30
Pyruvate	−110 ± 46	−111 ± 60

* Cumulative dose, 428 mg/kg of acrylamide.

† Values are expressed as nmoles/g of cerebral cortex/min as determined from the slope and S.E.M. of the regression line computed from the measured substrate concentrations in cerebral cortices from groups of four rats at 0, 10 and 30 sec of ischemia. No significant differences were observed between control and acrylamide-treated groups.

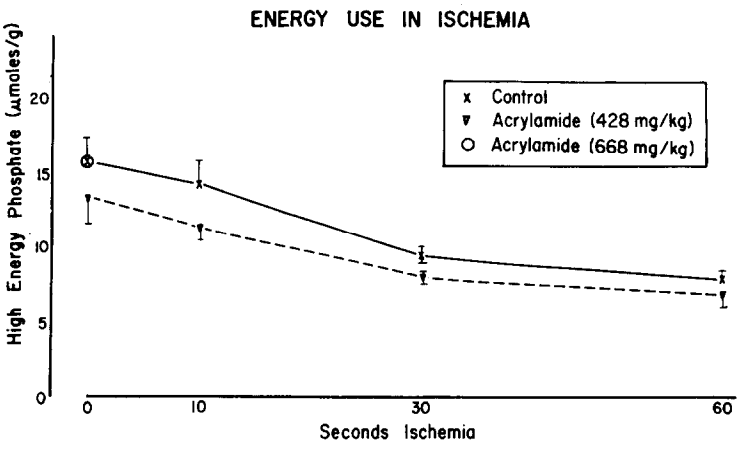

Fig. 2. Calculated initial concentration of potential high-energy phosphate and its use during ischemia in control (×) and acrylamide-treated (▽) rat cerebral cortices. Each point represents a mean ± S.E.M. derived from four animals. The ordinate represents the calculated sum of high-energy phosphate which could be derived from ATP, phosphocreatine, glucose and glycogen. The circle (○) represents the mean value at 0 sec for six rats receiving a cumulative dose of 668 mg/kg of acrylamide.

glycogen × 2.9, ATP × 2, and glucose × 2 [18, 21]. This sum gives the total high-energy phosphate which could be derived from these sources. The results of these calculations as well as the rate of use of high-energy phosphate in the low dose and control groups during the ischemic period are shown in Fig. 2. A comparison of the values at the zero time point shows that there was no difference in the original total of high-energy phosphate between the three groups. The sum of high-energy phosphate was 15.8 ± 1.3, 13.3 ± 1.6, and 15.9 ± 0.6 μmoles/g of cerebral cortex in control, low and high dose rats respectively. There was no apparent difference in the rates of high-energy phosphate use during ischemia between control and low dose rat cerebral cortices. Based on the slope of the regression line calculated using 0-, 10- and 30-sec values, these rates of use were 12.9 ± 3.3 and 11.0 ± 2.8 μmoles/g/min in the control and low dose cortices respectively. Concentrations in μmoles/g of the individual components of the total high-energy phosphate in the control, low and high dose rats were respectively: for ATP—1.4 ± 0.18, 1.57 ± 0.12, and 1.60 ± 0.10; for glucose—0.305 ± 0.089, 0.182 ± 0.044, and 0.220 ± 0.052; for glycogen—3.75 ± 0.29, 2.99 ± 0.39, and 3.67 ± 0.13; and for phosphocreatine—0.985 ± 0.165, 1.040 ± 0.159, and 0.965 ± 0.101. There were no significant differences between control and treated rats for any high-energy phosphate source.

The ratios of oxidized to reduced cytoplasmic pyridine nucleotides were calculated using the measured concentrations of pyruvate, lactate and malate and the equilibrium constants for lactate dehydrogenase (1.11×10^{-4}) and malic enzyme (3.44×10^{-2}) [29]. The results of these calculations are shown in Table 4. While the mean ratios determined for treated animals are lower than those for controls, there differences were not significant ($P > 0.05$).

DISCUSSION

The effect of acrylamide intoxication on the concentrations of pyridine nucleotides in brain was studied by measuring oxidized pyridine nucleotides in cerebral extracts. The concentration of NAD^+ was increased 22% ($P < 0.01$) in rats given a cumulative dose of 668 mg/kg of acrylamide. This indicates that acrylamide, either directly or indirectly, affects the regulation of total (bound and free) NAD^+. This increase could be the result of increased NAD synthesis or decreased degradation or it could reflect an increase in the ratio of total oxidized to total reduced NAD.

The effect of acrylamide on pyridine nucleotide function was studied first by comparing the concentrations of substrates of pyridine nucleotide-dependent enzymes in cerebral cortices and their pattern of change during ischemia. In other studies this

Table 4. Calculated cytoplasmic pyridine nucleotide ratios in cerebral cortices of control and acrylamide-treated rats*

	Control	Acrylamide 428 mg/kg	Acrylamide 668 mg/kg
NAD^+/NADH	460 ± 48†	405 ± 41	363 ± 45
$NADP^+$/NADPH	0.0250 ± 0.0018	0.0177 ± 0.0026	0.0203 ± 0.0029

* Ratios are calculated from measured values for pyruvate, lactate, malate and the equilibrium constants of lactate dehydrogenase (1.11×10^{-4}) and malic enzyme (3.44×10^{-2}) [29].

† Expressed as mean ± S.E.M. of calculated values for four, four and six rats in control, low and high dose rat groups.

approach proved to be a sensitive method for determining the antimetabolite action of 6-aminonicotinamide [30]. While the concentration of α-glycerophosphate in cortices of rats given 668 mg/kg of acrylamide was increased ($P = 0.05$), no significant differences in concentrations of the other substrates measured were observed. Cerebral cortical α-glycerophosphate concentrations in control rats and rats given 428 mg/kg (cumulative dose) of acrylamide increased at the same rate during ischemia, indicating that there was no change in the activity of α-glycerophosphate dehydrogenase (EC 1.1.1.8 L-glycerol-3-phosphate:NAD oxidoreductase).

Impairment of pyridine nucleotide function at enzymatic steps other than those specifically studied might be revealed by a failure to maintain high-energy phosphate concentrations or an impairment of their use during ischemia. However, calculated potential cerebral cortical high-energy phosphate at sacrifice and the rate of its use during ischemia did not differ between control and acrylamide-treated rats. A possible source of error in interpreting these data was the fact that the treated animals weighed less at the time of sacrifice than did controls. If heads from the treated rats froze faster than controls, then higher concentrations of high-energy phosphate would be expected in the treated animals at the onset of ischemia. This was not observed (see Fig. 2).

Pyridine nucleotide function was further evaluated by calculating the ratios of the oxidized to reduced forms of pyridine nucleotides in the cytoplasm. This was done using the equilibrium constants for lactate dehydrogenase and malic enzyme and the measured concentrations of their substrates and products, as these are thought to be in equilibrium in the brain [31]. These ratios are informative because a large percentage of total pyridine nucleotides are protein bound and do not affect thermodynamic characteristics of enzymes such as the direction of reversible reactions or the effectiveness of NADPH as a reducing agent for fatty acid synthesis [29]. Furthermore, this approach may reveal compartmental differences in the redox state not apparent from measurements of total pyridine nucleotide concentrations in tissue extracts. Calculated ratios of free cytoplasmic pyridine nucleotides were not significantly different from controls, although the mean values were slightly lower in treated rats. The observation that total NAD^+ was increased while the cytoplasmic ratio of free NAD^+/free NADH decreased slightly suggests that total NADH may also be increased or that the relationship between bound and free forms is changed.

In conclusion, this study suggests that while there was little evidence of a significant effect of acrylamide on pyridine nucleotide functions, there was an increase in the brain concentrations of NAD^+ and α-glycerophosphate. These increases appear to be relatively specific since the survey of metabolites and energy use showed no other differences between the experimental and control groups. NAD^+ has been shown to be a stimulator of phospholipid synthesis *in vitro* [32], and α-glycerophosphate provides the glycerol backbone for phospholipids. These observations suggest that lipid metabolism may be affected in acrylamide intoxication.

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