Published in final edited form as:

Toxicology. 2008 November 20; 253(1-3): 104–109. doi:10.1016/j.tox.2008.08.019.

The acute lethality of acrylonitrile is not due to brain metabolic arrest

E. Cristian Campian and Frederick W. Benz*

Department of Pharmacology and Toxicology, University of Louisville School of Medicine, Health Sciences Center, Louisville, KY 40292

Abstract

Acrylonitrile (AN) is an organic compound produced in large quantities by the chemical industry and is acutely toxic. One mechanism proposed to explain the toxicity of AN is metabolism by P450 into cyanide (CN). Although blood and brain levels of CN in rats following an LD90 dose of AN are consistent with acute toxicity, blocking CN formation with P450 inhibitors does not prevent lethality. Another mechanism implicated in toxicity is covalent binding of AN to cysteine residues in tissue proteins. Previous work in our laboratory has shown that AN can irreversibly inactivate the catalytically active cysteine-149 in glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Inactivation of GAPDH by AN would be expected to impair glycolytic ATP production and, when coupled to the inhibition of mitochondrial ATP synthesis by the AN metabolite CN, would result in metabolic arrest, particularly in brain. In this study we have measured the high energy metabolites Phosphocreatine (PCr), ATP, ADP and AMP by HPLC and compared their levels in the brains of rats treated with an LD90 dose of AN, when respiration ceased, vs. controls. Two methods of rapid brain freezing in liquid nitrogen were used: Funnel Freezing (FF) and Head Immersion (HI). AN administration resulted in large decreases in PCr of 74% (FF) and 80% (HI) but relatively minor decreases in ATP of 5% (FF) and 21% (HI) and Energy Charge of 6% (FF) and 10% (HI). Thus, although substantial depletion of PCr was observed, possibly due to inhibition of creatine kinase by AN, we found no evidence that brain ATP is depleted when respiration ceases in AN-intoxicated rats.

Keywords

Acrylonitrile; Cyanide; ATP; Phosphocreatine; Energy Charge; Brain

1. Introduction

Acrylonitrile (AN) is a vinyl monomer used extensively in the production of acrylic fibers, plastics, synthetic rubber and acrylamide. Almost 9 billion pounds of AN are produced yearly around the world and the production in the USA was almost 3 billion pounds in 2001 (Anonymous, 1999; Anonymous, 2002). Workers may be exposed to AN in an industrial setting during production and polymerization, while other people may be exposed during transportation of AN or through industrial pollution or cigarette smoking.

© 2008 Elsevier Ireland Ltd. All rights reserved.

*Corresponding author, Email: E-mail: benz@louisville.edu, Phone: 502-852-5611, Fax: 502-852-7868.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

AN is acutely toxic (Gut et al., 1981). The temporal sequence of symptoms observed following acute AN intoxication is as follows (Benz and Nerland, 2005). Initially, intoxicated rats go through an excitatory phase and the animals appear agitated. Cholinergic symptoms follow, such as salivation, lacrymation, urination and defecation. Subsequently the animal typically will experience tremors and mild clonic seizures followed by a prolonged terminal stage preceding death where the animal is moribund and displays labored breathing. Following an LD90 (115mg/kg) dose of AN subcutaneously, death usually occurs within 3–3.5 h, immediately preceded by severe clonic convulsions and generally a terminal tonic extensor convulsion. Similar signs have been observed in humans intoxicated with AN (Buchter and Peter, 1984; Sartorelli, 1966; Van Luijt, 1963; Vogel and Kirkendall, 1984).

Despite having been studied for over 60 years, the mechanism responsible for the acute toxicity of AN has not been established. Several mechanisms have been proposed. AN undergoes both oxidative and non-oxidative metabolism (Kedderis et al., 1993; Sumner et al., 1999). One of the products of the oxidative pathway is cyanide, which is acutely toxic. Although the metabolically released cyanide undoubtedly contributes to AN-induced lethality, as it is 10 times more potent than AN on a molar basis, metabolism to cyanide cannot solely explain the acute toxicity of AN. This is evidenced by the fact that neither inhibition of AN metabolism to cyanide by the P450 inhibitor 1-benzylimidazole nor by the alternative substrate ethanol was able to protect rats against AN-induced mortality, despite the fact that both agents reduced blood cyanide to insignificant levels (Benz and Nerland, 2005).

However, the parent AN molecule has also been suggested to be acutely toxic (Ahmed and Patel, 1981; Bolt et al., 1986; Gut et al., 1981). AN is a direct alkylating agent that can undergo Michael addition reactions with nucleophiles (Friedman et al., 1965). Previously, we have shown that AN is highly reactive with rat tissue proteins *in vivo* (Benz et al., 1997). Blood was the most reactive tissue studied and hemoglobin was the most reactive protein in blood. Of the five possible cysteine residues in the rat hemoglobin alpha-beta dimer, only beta-Cys125 was labeled by AN (Li, 1999). This high and selective reactivity was explained by the low pKa of this cysteine residue (Rossi et al., 1998). However, since blockade of both beta-Cys125 residues with GSH did not affect rat hemoglobin function (Rossi et al., 1998), it is unlikely that alkylation of these same residues by the smaller and uncharged AN molecule would either.

These results suggested, however, that other more critical proteins with highly reactive cysteine residues would likely be candidates for covalent bonding by AN. Indeed, previous research in our laboratory has shown that AN reacts with the critical glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) *in vitro* with subsequent loss of enzymatic activity by alkylating Cys149 at the active site (Campian et al., 2002). Covalent labeling of this cysteine residue by other agents such as iodoacetate or iodoacetamide is known to cause irreversible inhibition of enzyme activity and therefore glycolytic ATP synthesis (Harris and Walters, 1976). Thus inactivation of GAPDH by AN should also impair glycolytic ATP production and, when coupled with the inhibition of mitochondrial ATP synthesis by the AN metabolite cyanide, would be expected to result in severe impairment of ATP production leading to metabolic arrest. The brain can withstand metabolic arrest for only a few minutes thus these combined actions may account for the acute toxicity of AN *in vivo*. These experiments were conducted to test this hypothesis.

2. Materials and methods

2.1. Chemicals

Acrylonitrile (>99% pure), containing 35–45 ppm hydroquinone monomethyl ether as a polymerization inhibitor, was purchased from Aldrich Chemical Co. (Milwaukee, WI).

2.2. Animal treatment

Male Sprague-Dawley rats, weighing between 250–350 g were obtained from Harlan (Indianapolis, IN). Animals were housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and all experimental procedures were approved by the University of Louisville Institutional Animal Care and Use Committee. All animals were maintained on a 12-h light-dark cycle at 22 ± 2 °C. The rats were acclimated prior to use and were allowed Purina Rat Chow (Ralston Purina Co., St. Louis, MO) and water ad libitum. Prior to initiating experiments, the rats were weighed and randomly assigned to the various treatment groups. Animals were injected with AN subcutaneously in the scruff of the neck (Benz et al., 1997). AN was dissolved in distilled water. The dose administered, 115 mg/kg (LD90), was based on extensive dose-response analyses conducted previously (Benz et al., 1990; Benz et al., 1997). Control rats were injected with distilled water. All injection volumes were 5 ml/kg. Three experimental groups were used, each consisting of 3–5 AN-treated rats and the corresponding controls. The brains of AN-treated rats were frozen when respiration ceased. Death was typically accompanied by a terminal tonic extensor convulsion (Benz and Nerland, 2005). Control rats were anesthetized with diethyl ether 3–3.5 hours post injection, which approximates the average time to death in AN-treated animals (Benz and Nerland, 2005). Control anesthetized rats were breathing normally at the time of brain freezing. Brain energy reserves are exhausted rapidly post mortem. Thus several different techniques were utilized to measure brain high energy phosphate levels (Ponten et al., 1973). Group 1- Decapitation Technique: The rats in this group were decapitated with the aid of a guillotine. The heads were then immersed in liquid nitrogen at predetermined delay intervals between 0–7.5 minutes. Group 2-Immersion Technique: The rats in this group were immersed head-first up to their shoulders in liquid nitrogen for a total of four minutes, until thorough freezing of the brain occurred. After freezing, the head was quickly detached from the rest of the body and kept frozen in liquid nitrogen. Group 3-Funnel Freezing Technique: The scalp of rats was incised longitudinally with a scalpel and the skin was retracted, exposing the bony skull. A plastic funnel was placed on the top of the scalp and liquid nitrogen was poured through the funnel, for three minutes. Subsequently, the rats were immersed head-first into liquid nitrogen for an additional 2–3 minutes, to ensure freezing of the whole head. The head was then detached from the rest of the body and stored in liquid nitrogen.

2.3. Tissue sampling

Tissue sampling and processing was performed in a walk-in freezer (–15 °C), to prevent thawing of the samples. The skin and skull bone were incised using a rotary saw (Dremel, Racine, WI) and then removed, exposing the underlying brain. The brain was chiseled out using pre-cooled instruments under intermittent irrigation with liquid nitrogen and ground in a liquid nitrogen cooled mortar. The finely ground brain tissue was either stored in 2 ml vials at –80° C or used immediately for further processing and analysis.

2.4. Tissue processing

To determine the exact weight of the sample, vials containing approximately 0.5 g of powdered frozen brain were weighed before and after emptying their contents into 2 ml of ice-cold 0.76 M perchloric acid. After homogenization, the protein precipitate was removed by centrifuging the homogenate at 3000 g for 5 min at 4 °C. The supernatant, 2 ml, was removed and was neutralized by the addition of approximately 0.2 ml of 6N KOH, controlling the pH with pH indicator strips (colorpHast®, EM-Reagents, Merck, Darmstadt, Germany). The exact volume of KOH used for neutralization was taken into account when calculating energy metabolite levels. Any further precipitate appearing after neutralization was removed by centrifuging the sample again at 3000 g for 5 min at 4 °C. The supernatant was filtered with a 13 mm disposable

nylon syringe 0.45 μm filter (Xpertek®, P.J. Colbert Associates, St. Louis, MO) and used for direct analysis of energy metabolites by HPLC, after an optional dilution step.

2.5 HPLC assay of energy metabolites

Energy metabolites were assayed by HPLC, using a modified procedure of Sellevold et al., (1986). Analysis was performed on a Beckman HPLC system (Beckman, Arlington Heights, IL) consisting of a Model 126 Programmable Solvent Module and a Model 166 Programmable detector. Solvent programming, data collection and analysis were accomplished using Beckman Gold Nouveau software. For separation of metabolites, a reverse phase 15 cm Supelcosil LC-18-T column (Supelco, Bellefonte, PA) with 5 μm particle size and 4.6 mm internal diameter was used, preceded by a 3 cm guard column with an identical stationary phase. Mobile phase A contained KH_2PO_4 100 mM, tetrabutylammonium hydrogen sulfate (Sigma, St. Louis, MO) 4 mM with the pH adjusted to 6 with KOH. Mobile phase B contained a mixture of 70% mobile phase A and 30% methanol (v/v). Adenylate phosphates and phosphocreatine (PCr) were measured in the same run. The HPLC program was optimized to achieve the maximum separation of the peaks of interest in a minimum amount of time. Before each run, the column was equilibrated in 95% mobile phase A and 5% mobile phase B for 30 min. Flow rate was set at 1 ml/min. A volume of 20 μl of sample was injected each time. After injecting the sample, the concentration of mobile phase B was maintained at 5% for an additional 2.5 min, followed by a 2.5 min linear increase to 30% mobile phase B. A second 5-minute linear gradient was employed to increase mobile phase B concentration to 60%. At minute 10 of the run, a third 3-minute linear gradient increased the concentration of mobile phase B to 95% and these conditions were maintained for 20 minutes until the end of the run. Total run time was 33 minutes. The UV detector was set at 206 nm for detection of PCr; at minute 7 of the run, after the elution of PCr, the UV detector was switched to 259 nm to detect adenylate nucleotides. After each use, the column was washed with a 50% v/v solution of methanol in water. The same methanol concentration was used to store the column overnight. Weekly, the guard column and the main column were each reversed and washed with isopropanol.

2.6 Calibration Curves

Standard solutions of adenylate phosphates and PCr were dissolved in 0.5 M perchloric acid and neutralized with KOH. Five standard concentrations were used to establish standard curves for quantitative measurements of tissue extracts. The concentrations of adenylate phosphates and PCr were expressed as micromoles per gram wet tissue weight. The energy charge (EC) was calculated using the equation: $\text{EC} = [\text{ATP}] + \frac{1}{2} [\text{ADP}] / [\text{ATP}] + [\text{ADP}] + [\text{AMP}]$. Statistical evaluation was performed using Student's 't' test, $p < 0.05$.

3. Results

3.1. HPLC of energy metabolites

The effects of AN on brain energy metabolism were studied in male Sprague-Dawley rats that were injected subcutaneously with an LD90 (115 mg/kg) dose of AN (Benz et al., 1997) and sampled when respiration ceased. Energy metabolites were assayed by reverse phase high performance liquid chromatography. Standard solutions of the compounds of interest, PCr, ATP, ADP, AMP and the internal standard adenosine 5'-tetrphosphate [AP4] were run on the HPLC to determine the exact retention times (Fig 1a). Various amounts of energy metabolite standards were assayed by HPLC and standard curves were drawn for each metabolite. A typical curve for ATP is shown in Fig 1b. Standard curves for the other analytes of interest were similar.

3.2. HPLC of tissue extracts from control and AN-treated rats

Tissue extracts were obtained from frozen rat brains as described in the Methods and analyzed by HPLC. Individual nucleotide peaks were identified by elution times corresponding to the standard solutions (Fig 2). Recovery of the energy metabolites from tissue samples was studied by three methods. First, the precipitate resulting from homogenization in perchloric acid was re-homogenized in perchloric acid and the extraction procedure was repeated. Upon HPLC analysis, the corresponding peaks for the energy metabolites were absent, indicating complete removal of the metabolites during the first extraction. Second, to identical tissue samples known amounts of standards were added and recovery measured. Recovery rates between 94–105 % were found. Third, an internal standard, adenosine 5'-tetraphosphate, was used with each analyzed sample. This allowed direct monitoring of recovery in each individual sample. The purity of the peaks of interest was assessed by peak shifting. The PCr peak was completely abolished in the presence of creatine kinase, ADP and magnesium, confirming the identity and purity of the peak. The ATP peak was completely abolished by storing the brain sample at room temperature for 30 minutes prior to homogenization and extraction.

3.3. Lability of ATP in control and AN-treated rats following decapitation

Due to their high lability, several different methods were employed to determine the levels of energy metabolites. In one set of experiments, the rats were decapitated and the heads were kept at room temperature for varying times before freezing in liquid nitrogen. This allowed the determination of the time course of ATP decay in decapitated brains and the determination of ATP levels at time zero in control and AN-treated animals at the time of death by extrapolation. For each time point duplicate injections of samples obtained from individual rats were used. Five to six animals were used in each group, one at each time point.

ATP levels decreased rapidly postmortem in both control and AN-treated animals. The data were fitted to a single exponential decay curve using the software Slide Write Plus version 5.0 for Windows. A half-life for postmortem ATP depletion was calculated from the best fit to the exponential decay curve. ATP decreased in an exponential manner with a half-life of 1.85 min in brains of control animals and 1.76 min in brains of AN-treated rats. The correlation coefficients were 0.97 and 0.89, respectively. Extrapolation to time zero indicated that, at the time of death in AN-treated animals, the ATP level was 1.45 $\mu\text{mol/g}$ wet tissue. This represents a 26% reduction from control ATP levels of 1.95 $\mu\text{mol/g}$ wet tissue.

3.4. Energy metabolite levels determined via in situ freezing techniques

In a second set of experiments, rats were again treated with a LD90 dose of AN and the brains were frozen in situ. Two in situ methods of freezing were used: head immersion in or funnel freezing with liquid nitrogen. In the literature, the two methods are described as having similar effectiveness in preserving adenine nucleotides (Ponten et al., 1973). In our hands, funnel freezing resulted in higher ATP levels in control rats, but immersion yielded generally better reproducibility. AN treatment resulted in a decrease in ATP concentration of 21% from control levels in the immersed group (Table 1). In the funnel frozen group, the observed decrease in ATP was only 5%. The decrease in ATP levels observed in the immersed group correlates very closely to the 26 % decrease in ATP that was calculated by extrapolation in the decapitated group. The level of PCr decreased 74% from control level in the funnel frozen group and 80% in the immersed group. No difference in Energy Charge was observed between control and AN-treated animals in the funnel frozen group but a small decrease was observed in the head-immersed group.

4. Discussion

The main energy-consuming process of the brain is the maintenance of ionic gradients across the plasma membrane, a condition that is crucial for excitability. Maintenance of these gradients is achieved predominantly through the activity of ionic pumps fueled by ATP, particularly the Na,K-ATPase (Clarke and Sokoloff, 1998; Lipton, 1999). Because of the brain's high metabolic rate, coupled with its low energy reserves, the brain is very susceptible to disturbances in energy metabolism (Ames, 2000). When the concentration of high energy phosphates decreases to a critical value, neurons lose their ability to maintain normal ionic gradients (Knickerbocker and Lutz, 2001). Intracellular sodium increases and intracellular potassium decreases and a profound membrane depolarization occurs (Lipton, 1999; Verhaegen et al., 1995). This depolarization results in a cytotoxic increase in intracellular calcium, the release of neurotoxic amounts of glutamate, which initiate a cascade of intracellular processes leading to neuronal death (Dugan and Kim-Han, 2006, Lutz and Milton, 2004; Lipton, 1999).

The mechanism of AN acute toxicity is undoubtedly complex. Despite a considerable amount of research, the mechanism directly responsible for acrylonitrile's acute toxicity is still not clear. In this paper we tested the hypothesis that depletion of brain ATP could be the immediate cause of acute AN-induced lethality. This hypothesis was based on the knowledge that AN is oxidatively metabolized into cyanide, which itself is acutely toxic. Blood and brain levels of cyanide following an LD90 dose of AN have been shown to be at a level expected for severe cyanide poisoning (Ballantyne, 1987; Benz et al., 1997) and thus for severe impairment of mitochondrial respiration and ATP production. However, under such conditions, cells can undergo the Pasteur Effect and shift to a dependence on glycolysis for ATP production (Dugan and Kim-Han, 2006). Thus in order to insure complete metabolic arrest, glycolysis must also be inhibited with agents such as iodoacetate (Doll et al., 1991), which inhibits the glycolytic enzyme GAPDH. For AN, however, this is not necessary since the parent AN molecule has been shown to irreversibly inhibit GAPDH in vitro and is predicted to be equally effective in vivo (Campian et al., 2002). Thus AN alone, via its metabolite cyanide and its direct inhibition of GAPDH, would be expected to induce complete metabolic arrest and consequentially the depletion of brain ATP. Despite the logic of this argument and although significant depletion of PCr was observed, we found no evidence for toxicologically significant depletion of brain ATP or reduction in energy charge when respiration ceased in AN-treated animals.

Because of the lability of the high energy phosphates in brain, we used several techniques to measure their levels. Head immersion and funnel freezing are two methods that have been shown to produce reliable results (Ponten et al., 1973) and our observations confirmed this. The control levels of the adenylate nucleotides, PCr and energy charge found in our experiments (Table 1) agree well with those observed by other investigators (Gupta et al., 2000; Gupta et al., 2001; Verhaegen et al., 1995; Wagner and Lanier, 1994). On the other hand, the extrapolation method (Fig 3) was unsatisfactory but served to reinforce the fact of the extreme lability of ATP post mortem.

The brains of AN-treated animals were frozen when respiration ceased, which was typically accompanied by a terminal tonic extensor convulsion. These signs signal the death of the animal and generally occurred 3–3.5h after receiving an LD90 dose of AN subcutaneously. No AN-treated animal has ever been observed to recover subsequent to these signs. It was at this precise moment that the brains were rapidly frozen to assess high energy phosphate levels and energy charge. The timing of this sampling was critical for testing the hypothesis that depletion of brain ATP could be the immediate cause of acrylonitrile lethality. Delayed sampling would result in erroneous depletion of brain ATP due to its extreme lability post mortem (Fig 3) and earlier sampling likely would result in normal ATP levels as it is known with other toxic insults,

such as severe hypoxia, that brain ATP is well maintained until about one minute before death (Ueda et al., 1988).

Our results indicate that, when respiration ceased in AN-treated animals, ATP levels were 95% of control in the funnel-frozen animals and 79% of control in the head-immersed animals, respectively (Fig 2 and Table 1). On the other hand, the PCr level was only 26% and 20% of controls in the same treatment groups, respectively (Fig 2 and Table 1). The substantial reduction in PCr with relatively unchanged levels of ATP could be a result of inhibition of creatine kinase (CK) by AN. Recent unpublished results in our laboratory indicate that AN is covalently bound to rat brain CK *in vivo*. This binding is quite likely at the active site, based on the high reactivity of AN with sulfhydryl groups and the reactive cysteine residue at the active site of CK. With the funnel freezing technique, however, we cannot rule out a methodological explanation for the decreased PCr in AN-treated animals vs. controls. PCr is known to be highly labile and very sensitive to blood oxygenation, decreasing rapidly with the onset of hypoxia and even faster in the case of ischemia. This has been clearly demonstrated experimentally (Ponten et al., 1973). Specifically, Ponten showed that the highest brain PCr levels were measured using the funnel freezing technique vs. whole head immersion or decapitation. The reason is that in the funnel freezing technique, the animal continues to breathe as the brain is frozen, while in the other two techniques, breathing ceases immediately. Our control data (PCr-funnel frozen vs. PCr-head immersion) in Table 2 confirmed this observation. As for the AN-treated animals, they were not funnel-frozen until respiration ceased, while the control animals were anesthetized and breathing normally during the funnel freezing process. Thus we cannot rule out the possibility that some of the 74% decrease in PCr observed between AN-treated and control animals, both funnel-frozen, was due to the methodological requirement that the AN-treated animals had ceased breathing and thus were hypoxic during the freezing process, while the control animals were not. This methodological difference in breathing time was considerably less pronounced when AN-treated and control animals were both frozen by head immersion and cannot account for the 80% depletion in PCr observed using this freezing technique. Thus inhibition of CK by AN remains a viable explanation. With regard to Energy Charge, which is a parameter useful for furnishing a quantitative estimate of the energy state of the cell (Atkinson, 1968), it was unchanged in the funnel-frozen animals and only slightly decreased in the head-immersed animals.

Although statistically significant decreases in ATP and Energy Charge were observed in AN-treated vs. controls with the head-immersion technique (Table 1), the small changes observed are not considered toxicologically significant based on the following points. Experiments in rats indicate that ATP levels are in the range of 13–18% and Energy Charge is 26–31% of controls at the time of brain membrane depolarization (Verhaegen et al., 1995), whereas our ATP levels were 79–95% and energy charge was 90–94% of control, respectively when respiration ceased. In addition, in experiments where status epilepticus was induced in rats treated either with acetylcholinesterase inhibitors or kainic acid, brain ATP levels measured one hour after treatment were reduced to the range of 40–51% and PCr to 34–49% of controls in certain brain regions, yet similarly treated animals survived these treatments (Gupta et al., 2000; Gupta et al., 2001). Based on these data in the literature, we conclude that the small changes in ATP and Energy Charge that we observed are not consistent with brain metabolic arrest and the depletion of ATP being the immediate cause of acute AN lethality. Of course, because our measurements were made in whole brain, we cannot rule out the possibility that the level of ATP in certain critical brain regions was dramatically reduced but the effect was lost when averaged over the whole brain.

In summary we found substantial depletion of whole brain PCr but no toxicologically relevant depletion of ATP at the time when respiration ceased in AN-intoxicated rats. The depletion of PCr, although considerable and possibly due to direct inhibition of CK by AN, cannot be

considered the cause of the cessation of respiration since CK knockout mice have no detectable brain PCr but normal brain ATP, and are essentially normal albeit with some behavioral and learning problems (in 't Zandt et al, 2004). Thus the mechanism responsible for the acute lethal actions of AN must be sought elsewhere.

Acknowledgements

This research was supported by the National Institute of Environmental Health Science Grant ES06141 to F.W. Benz.

References

- Ahmed AE, Patel K. Acrylonitrile: in vivo metabolism in rats and mice. *Drug. Metab. Dispos* 1981;9:219–222. [PubMed: 6113929]
- Ames A III. CNS energy metabolism as related to function. *Brain Res. Rev* 2000;34:42–68. [PubMed: 11086186]
- Anonymous. Acrylonitrile. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. 1999;71(Pt 1):43–108.
- Anonymous. *Chem. Eng. News* 2002 June 24;:61.
- Atkinson DE. The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. *Biochemistry* 1968;7:4030–4034. [PubMed: 4972613]
- Ballantyne, B. Toxicology of cyanides. In: Ballantyne, B.; Marrs, TC., editors. *Clinical and Experimental Toxicology of Cyanide*. Bristol, England: IOP Publishers; 1987. p. 41-126.
- Benz FW, Nerland DE. Effect of cytochrome P450 inhibitors and anticonvulsants on the acute toxicity of acrylonitrile. *Arch. Toxicol* 2005;79:610–614. [PubMed: 15864551]
- Benz FW, Nerland DE, Corbett D, Li J. Biological markers of acute acrylonitrile intoxication in rats as a function of dose and time. *Fundam. Appl. Toxicol* 1997;36:141–148. [PubMed: 9143483]
- Benz FW, Nerland DE, Pierce WM, Babiuk C. Acute acrylonitrile toxicity: studies on the mechanism of the antidotal effect of D- and L-cysteine and their N-acetyl derivatives in the rat. *Toxicol. Appl. Pharmacol* 1990;102:142–150. [PubMed: 2296765]
- Bolt HM, Peter H, Wiegand HJ. Biological reactive metabolites in human toxicity. *Adv. Exp. Med. Biol* 1986;197:1013–1016. [PubMed: 3766252]
- Buchter A, Peter H. Clinical toxicology of acrylonitrile. *G. Ital. Med. Lav* 1984;6:83–86. [PubMed: 6534784]
- Campian EC, Cai J, Benz FW. Acrylonitrile irreversibly inactivates glyceraldehyde-3-phosphate dehydrogenase by alkylating the catalytically active cysteine 149. *Chem. Biol. Interact* 2002;140:279–291. [PubMed: 12204582]
- Clarke, DG.; Sokoloff, L. Circulation and Energy Metabolism. In: Siegel, GJ.; Agranoff, BW.; Albers, RW.; Fisher, SK.; Uhler, MD., editors. *Basic Neurochemistry*. Vol. 6th edn. Philadelphia, PA: Lippincott Williams & Wilkins; 1998. p. 637-669.
- Doll CJ, Hochachka PW, Reiner PB. Effects of anoxia and metabolic arrest on turtle and rat cortical neurons. *Am. J. Physiol* 1991;260:R747–R755. [PubMed: 1849377]
- Dugan, LL.; Kim-Han, JS. Hypoxic-ischemic brain injury and oxidative stress. In: Siegel, GJ.; Albers, RW.; Brady, ST.; Price, DL., editors. *Basic Neurochemistry*. Vol. 7th edn. Burlington, MA: Elsevier Academic Press; 2006. p. 559-573.
- Friedman M, Cavins JF, Wall JS. Relative nucleophilic reactivities of amino groups and mercaptide ions in addition reactions with alpha, beta-unsaturated compounds. *J. Amer. Chem. Soc* 1965;87:3672–3682.
- Gupta RC, Milatovic D, Dettbarn WD. Depletion of energy metabolites following acetylcholinesterase inhibitor-induced status epilepticus: protection by antioxidants. *Neurotoxicology* 2001;22:271–282. [PubMed: 11405258]
- Gupta RC, Milatovic D, Zivin M, Dettbarn WD. Seizure-induced changes in energy metabolites and effects of N-tert-butyl-alpha-phenylnitron (PNB) and vitamin E in rats. *Pflugers. Arch* 2000;440:R160–R162. [PubMed: 11005654]

- Gut I, Kopecky J, Nerudova J. Relationship between acrylonitrile biotransformation, pharmacokinetics and acute toxicity. A Short Review. *G. Ital. Med. Lav* 1981;3:131–136.
- Harris, JL.; Walters, M. Glyceraldehyde-3-phosphate Dehydrogenase. In: Boyer, PD., editor. *The Enzymes*. NY: Academic Press; 1976. p. 1-49.
- Kedderis GL, Sumner SC, Held SD, Batra R, Turner MJ Jr, Roberts AE, Fennell TR. Dose-dependent urinary excretion of acrylonitrile metabolites by rats and mice. *Toxicol. Appl. Pharmacol* 1993;120:288–297. [PubMed: 8511799]
- Knickerbocker DL, Lutz PL. Slow ATP loss and the defense of ion homeostasis in anoxic frog brain. *J. Exp. Biol* 2001;204:3547–3551. [PubMed: 11707503]
- Li, J. Ph.D. dissertation. University of Louisville, ProQuest Digital Dissertations database; 1999. Acrylonitrile acute intoxication: Toxicodynamics and the effect of antidotes. (Publication No. AAT 9951327).
- Lipton P. Ischemic cell death in brain neurons. *Physiol. Rev* 1999;79:1431–1568. [PubMed: 10508238]
- Lutz PL, Milton SL. Negotiating brain anoxia survival in the turtle. *J. Exp. Biol* 2004;207:3141–3147. [PubMed: 15299035]
- Ponten U, Ratcheson RA, Salford LG, Siesjo BK. Optimal freezing conditions for cerebral metabolites in rats. *J. Neurochem* 1973;21:1127–1138. [PubMed: 4761701]
- Rossi R, Barra D, Bellelli A, Boumis G, Canofeni S, Di Simplicio P, Lusini L, Pascarella S, Amiconi G. Fast-reacting thiols in rat hemoglobins can intercept damaging species in erythrocytes more efficiently than glutathione. *J. Biol. Chem* 1998;273:19198–19206. [PubMed: 9668107]
- Sartorelli E. Acute poisoning due to acrylonitrile. *G. Ital. Med. Lav* 1966;57:184–187.
- Sellekvold OF, Jynge P, Aarstad K. High performance liquid chromatography: a rapid isocratic method for determination of creatine compounds and adenine nucleotides in myocardial tissue. *J. Mol. Cell Cardiol* 1986;18:517–527. [PubMed: 3723598]
- Sumner SC, Fennell TR, Moore TA, Chanas B, Gonzalez F, Ghanayem BI. Role of cytochrome P450 2E1 in the metabolism of acrylamide and acrylonitrile in mice. *Chem. Res. Toxicol* 1999;12:1110–1116. [PubMed: 10563837]
- Ueda H, Hashimoto T, Furuya E, Tagawa K, Kitagawa K, Matsumoto M, Yoneda S, Kimura K, Kamada T. Changes in aerobic and anerobic ATP-synthesizing activities in hypoxic mouse brain. *J. Biochem* 1988;104:81–86. [PubMed: 2851589]
- Van Luijt E. Fatal acrylonitrile poisoning. *Ned. Tijdschr. Geneesk* 1963;107:2186–2188. [PubMed: 14094563]
- Verhaegen M, Iaizzo PA, Todd MM. A comparison of the effects of hypothermia, pentobarbital, and isoflurane on cerebral energy stores at the time of ischemic depolarization. *Anesthesiology* 1995;82:1209–1215. [PubMed: 7741296]
- Vogel RA, Kirkendall WM. Acrylonitrile (vinyl cyanide) poisoning: a case report. *Tex. Med* 1984;80:48–51. [PubMed: 6729746]
- Wagner SR, Lanier WL. Metabolism of glucose, glycogen, and high-energy phosphates during complete cerebral ischemia. A comparison of normoglycemic, chronically hyperglycemic diabetic, and acutely hyperglycemic nondiabetic rats. *Anesthesiology* 1994;81:1516–1526. [PubMed: 7992921]
- in 't Zandt HJ, Renema KJ, Streijger F, Jost C, Klomp DW, Oerlemans F, Van der Zee CE, Wieringa B, Heerschap A. Cerebral creatine kinase deficiency influences metabolite levels and morphology in the mouse brain: a quantitative in vivo ^1H and ^{31}P magnetic resonance study. *J. Neurochemistry* 2004;90:1321–1330.

Fig. 1. HPLC chromatogram of (a) high energy phosphate standards and (b) ATP standard curve. Reversed-phase HPLC of a standard mixture of energy metabolites on a 5 μ m (150 mm X 4.6 I.D.) Supelcosil LC-18-T column using the chromatographic conditions described in the Methods section. The chromatogram (a) was obtained with a 20 μ l injection of the standard mixture containing 100 μ M PCr and 50 μ M each of AMP, ADP, ATP and internal standard AP4 (adenosine 5'-tetraphosphate) dissolved in 10 mM KHPO₄, pH 7. Flow rate was 1 ml/min. The UV detector was set at 206 nm for detection of PCr and at 259 nm for detection of AMP, ADP, ATP and AP4. ATP standard curve (b). Volumes of 20 μ l of solutions containing ATP concentrations ranging from 25 – 500 μ M in 10 mM KHPO₄, pH 7, were injected in random order. The correlation coefficient describing the linear relationship of peak area to nmoles injected is 0.99. Similar standard curves were prepared for ADP, AMP, PCr.

Fig. 2.

HPLC separation of energy metabolites in control (a) and AN-treated (b) rats. Rat brain tissue extracts were separated by reverse phase HPLC on a 5 μ m (150 mm $\times$ 4.6 mm) Supelcosil LC-18-T column. Rats were administered a LD90 dose of AN and assayed when respiration ceased. Control rats (a) were administered the same volume of distilled water and assayed at 3–3.5 h after injection, which approximates the time of death in AN-treated animals. In this particular experiment, the brains were frozen by immersing rats head-first in liquid nitrogen, harvested and homogenized as described in the Methods section. Adenosine 5'-tetraphosphate (AP4) was used as an internal standard. AN administration (b) resulted in a decrease in the concentrations of adenosine triphosphate (ATP) and phosphocreatine (PCr) with a resulting increase in the concentrations of adenosine diphosphate (ADP) and adenosine monophosphate (AMP).

Fig. 3.

ATP depletion post decapitation in control (a) and AN-treated rats (b). Treated rats were administered a LD90 dose of AN. Control rats were administered the same volume of distilled water. When respiration ceased in AN-treated rats, the animals were decapitated and the heads were kept at room temperature for a predetermined length of time and then immersed in liquid nitrogen. Each time point represents the time delay at room temperature prior to freezing and is the average of duplicate injections of samples obtained from a single rat. Control rats were anesthetized at 3–3.5 h, which approximates the time of death in AN-treated rats, and then processed as described above. ATP decreased in an exponential manner in both control and AN-treated groups. Extrapolation to zero time resulted in ATP levels of 1.95 and 1.45 μmol/g wet tissue for control and AN-treated animals, respectively.

Table 1

Brain levels of adenine nucleotides, energy charge (EC) and phosphocreatine after funnel freezing or head immersion in control and AN-treated (LD90) animals

Groups		ATP	ADP	AMP	EC	PCr
Funnel frozen	Control	2.27 ± 0.10	0.55 ± 0.07	0.09 ± 0.02	0.88 ± 0.01	5.92 ± 2.11
	AN-treated	2.16 ± 0.20	0.65 ± 0.06	0.20 ± 0.08	0.83 ± 0.03	1.55 ± 0.43*
Head immersed	Control	2.09 ± 0.07	0.74 ± 0.09	0.19 ± 0.07	0.82 ± 0.03	3.72 ± 0.67
	AN-treated	1.66 ± 0.10*	0.83 ± 0.01	0.31 ± 0.05	0.74 ± 0.02*	0.75 ± 0.23*

Values are given as means ± SE in μmol/g tissue wet weight. EC = ([ATP] + ½ [ADP]) / ([ATP] + [ADP] + [AMP]). Statistical evaluation was performed using Student's t test

* $P < 0.05$