ABSTRACT

Title of Thesis: ACRYLAMIDE EFFECTS ON NERVE TERMINAL ENZYMES

Christopher S. E. Marlowe, Master of Science, 1984

Thesis Directed by: Richard D. Howland
Associate Professor of Pharmacology
University of Medicine and Dentistry
of New Jersey

and:

Richard B. Trattner, Professor of
Chemistry & Environmental Science
and Director of Academic Programs
Institute of Hazardous and Toxic
Waste Management
New Jersey Institute of Technology

Acrylamide causes a peripehral neuropathy. The biochemical mechanism by which it does so is the subject of intense investigation. One of the principal hypotheses for this mechanism centers on acrylamide's ability to inhibit glycolysis. This study examines the effects of in vivo exposure to acrylamide on the enzymes of the nerve terminal

particle, the synaptosome.

A method for measuring glycolytic enzyme inhibition in the synaptosome was developed. Acrylamide (500 mg/kg) was administered to rats intraperitoneally. Synaptosomes were prepared by the method of Dodd et al, and lysed by sonication. GAPDH activity and NSE concentration were measured. GAPDH inhibition measured in brain synaptosomes was similar to that measured in distal portions of peripheral nerve. One can infer from this data that NSE accumulates in the nerve terminal upon acrylamide exposure.

ACRYLAMIDE EFFECTS ON NERVE TERMINAL ENZYMES

by:

Christopher S. E. Marlowe

Thesis submitted to the Faculty of the Graduate School of the New Jersey Institute of Technology in partial fulfillment of the requirements for the degree of Master of Science in Environmental Engineering

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APPROVAL SHEET

Title of Thesis: ACRYLAMIDE EFFECTS ON NERVE TERMINAL ENZYMES

Name of Candidate: Christopher S. E. Marlowe
Master of Science

Thesis and Abstract Approved:

..... May 8, 1984

Richard D. Howland, PhD Date
Associate Professor of Pharmacology
University of Medicine and Dentistry
of New Jersey
and:

..... 5/8/84

Richard A. Trattner, PhD Date
Professor of Chemistry and Environmental Science
Director of Academic Programs
Institute of Hazardous and Toxic Wastes
New Jersey Institute of Technology

VITA

Name Christopher Sean Edward Marlowe

Permanent address:

Degree and date to be conferred:

Secondary Education: Columbia High School
Maplewood, NJ 1968

Collegiate Institutions	Dates	Degree	Date of Degree
Rutgers University (NCAS)	9/68-8/76	B. A.	1976
Rutgers Graduate School	9/77-6/82	none	
NJ Institute of Technology	9/82-6/84	M. S.	1984

Major: Environmental Engineering

Minor: Toxicology

Publications: none.

Positions Held:

Industrial Hygienist	USDOL OSHA 970 Broad Street Newark, N J 07102.
Quality Control Tech	Sealed Air Corporation 36 West End Road Totowa, NJ 78502.
Latex Operator	Adco Chemical Corporation Rutherford Street Newark, N J 07105.
Laboratory Assistant	Joseph Dixon Crucible Corporation 127 Wayne Street Jersey City, N. J. 07306.
Process Dev't Tech	National Starch & Chemical Corp. 1100 West Front Street Plainfield, N J 07060.

ACRYLAMIDE EFFECTS ON NERVE TERMINAL ENZYMES

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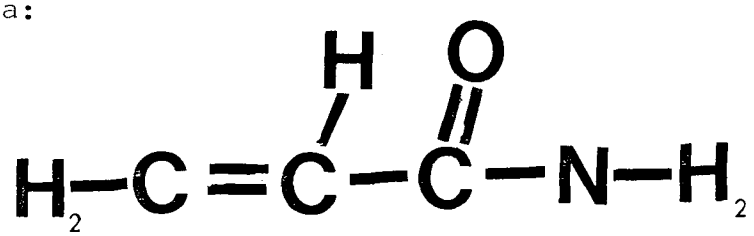
CHEMISTRY AND USES

Acrylamide is the common name for propenamide which has the formula:



Acrylamide is sold as flake-like white crystals, crystallized from benzene. Acrylamide has a specific density of 1.122, a molecular weight of 78.01, and a melting point of 84.5'C. Acrylamide degrades before boiling. Acrylamide is highly soluble in water. 215.5 grams of acrylamide can be dissolved in 100 ml of water at 30'C (Merck Index).

For a small molecule, acrylamide has several chemically active sites. The carbon - carbon double bond, the carbonyl carbon, the carbonyl oxygen, and the amine group all offer opportunities for chemical reaction. Most commercial and toxicologic reactions occur by addition to the carbon - carbon double bond.

Acrylamide is prepared by reacting acrylonitrile with sulfuric or hydrochloric acid (Bikales, 1963). Since acrylonitrile is a highly common chemical (1982 prod'n: 1 million tons, C & E News), this reaction may be performed right at the polymer plant. This means that production statistics for acrylamide may be unreliable. 1983 production was probably between 75 and 100 million pounds. (Personal estimate).

Acrylamide is used primarily because it can form water insoluble polymers in water solution. It is therefore used as a flocculator in mining, wastewater treatment, and in purification of water supplies. Acrylamide is used to strengthen paper and cardboard products. Much of this acrylamide is used as a grouting agent to stabilize soils in construction operations (LeQuesne, 1980).

ABSORPTION AND ELIMINATION

Acrylamide is very soluble in water. It is easily absorbed by inhalation, ingestion, dermal absorption, and injection. It demonstrates similar toxicity by all routes. It is distributed in total body water within a few minutes of exposure (Miller et al, 1982). It is not concentrated in the nervous tissue. Some concentration occurs in the red blood cells, where acrylamide binds covalently to hemoglobin (ibid).

Acrylamide disappears from blood plasma with a half life just under two hours. Its elimination, mostly in urine with less in feces and exhaled air, is biphasic with a first component half-life of 5 hours, and a second component of up to eight days (ibid). The acrylamide excreted in the second component is probably bound to protein and other macromolecules. Fifty percent of administered carbon-14 acrylamide is found in trichloroacetic acid insoluble precipitates (Hashimoto & Aldridge, 1970)

Sixty percent of acrylamide is excreted in the urine within three days. Two percent as acrylamide, forty-eight percent as a glutathione conjugate degradation product, and ten percent as an unknown metabolite (Miller et al, 1982). Acrylamide can inhibit the glutathione S-transferase that is

important in its own elimination. The importance of this inhibition has not been established (Das et al, 1982).

DESCRIPTIVE TOXICOLOGY

When acrylamide was first introduced in 1955, subchronic animal testing demonstrated its neurotoxic potential. At the same time, workers in a pilot plant experienced partial paresthesia. Fullerton & Barnes demonstrated that this effect was a peripheral neuropathy (1966). In the same year, the American Conference of Governmental Industrial Hygienists adopted a standard for acrylamide exposure of 0.3 milligram per cubic meter, A standard that both they and OSHA still maintain (ACGIH DocTLVs). Only 50 or so cases of acrylamide toxicity in humans have been reported in the literature. This is probably a gross underestimate, since studies of exposed workers often reveal undiagnosed cases (La Quesne, 1980).

Acrylamide has a one day LD50 of about 150 - 200 milligram per kilogram of body weight (mg/Kg). Exposure to 0.05 mg/Kg/day is held to be a no-ill-affect level (McCollister, 1964). Subacute exposure to levels between 10 and 100 mg/Kg/day cause neuropathies (Fullerton & Barnes, 1966). All mammalian species tested demonstrate this pattern, albeit at different dose levels. In the cat, ataxia occurs in 18 weeks at 1 mg/Kg/day (Kuperman, 1958). The same ataxia is achieved in the rat in 18 weeks at 10 mg/Kg/day (Fullerton & Barnes, 1966).

Acrylamide toxicity is manifested in the human by weakness and muscle wasting of the feet and hands, partial paresthesia (numbness or tingling in the feet and hands), truncal ataxia (a form of incoordination), dysarthria (incoordinated speech), and urinary retention. The hands and feet are often red and sweaty with exfoliative dermatitis (the skin peels off). The dermatitis is probably caused by contact effects. There is a loss of tendon reflexes. Combination of these symptoms with a history of acrylamide exposure is diagnostic. (LeQuesne, 1980). Laboratory animals manifest similar symptoms.

Acrylamide, 2,5 hexanedione, and carbon disulfide affect transport of materials within the nerve cell. Before we proceed further, let's define some terms. The basic nerve cell is called a neuron. It consists of dendrites, a soma, an axon, and a terminal (see Figure 1). The dendrite initiates electrical waves called action potentials when its chemoreceptors are exposed to a neurotransmitter from another neuron. The soma or perikaryon is the main cell body of the neuron. It conducts the genetic and synthetic work of the cell. The axon is a long (one micron to one meter or longer) tube that conducts the action potential. The nerve terminates in a structure specialized for the function of that nerve. For instance, the touch receptor is called a

pacinian corpuscle, and the muscle stretch receptors are called annulospiral endings.

The lumen of the axon contains the "axoplasm" and its membrane is called the "axolemma". The axon is partially enclosed in a Schwann cell. Slow fibers are enclosed in a single fold of their Schwann cells. Neurons which must conduct quickly are usually myelinated, which means that the Schwann cells wrap around the axon in multiple layers. There is usually a Scwann cell every millimeter or so. The area between the Schwann cells is called the Node of Ranvier. The part of the axon covered by the myelin is called an internode. Action potential conduction in the internode is by volume conduction. The Node of Ranvier is the site of intense metabolic activity, because the action potential is conducted by membrane action (see Figure 1, page 10).

The axon conducts material as well as electricity. The bulk of axoplasmic components is synthesized in the soma. These get to the terminal and axon by axoplasmic transport. There are five anterograde (towards the terminal) transport rates currently recognized (Spencer, 1984). Endoplasmic reticulum, plasmalemma, and vesicles containing amino acids and sugars are transported "fast" (410 mm/d). There are two intermediate rates which are not well

understood. Slow component A carries structural materials like tubules and neurofilaments at Ø.2-1mm. Slow component B transports constitutive enzymes (including glycolytic enzymes like enolase, and glyceraldehyde 3-phosphate dehydrogenase) and cytoskeletal proteins at 5-1Ø mm/d. Retrograde (towards the soma) transport is less well researched. There are two recognized rates of retrograde transport, but there may be a retrograde transport system for each anterograde system (Spencer, 1984). The concept of turn around in the terminal (conservation of mass demands it) is important in this study.

The pathology caused by subacute exposure to acrylamide is a peripheral neuropathy, of the class Spencer & Schaumburg (1977) call Central - Peripheral Distal Axonopathies. Other agents which cause this type of pathology include carbon disulfide, triorthocresylphosphate, and 2,5-hexanedione. Peripheral nerves from humans or animals exposed sub-acutely to these agents develop swellings in the axon on the proximal sides of nodes of Ranvier located in the distal regions of long nerve fibers. These swellings are characterized by fiber degeneration and regeneration, a tangled accumulation of 1Ø nm neurofilaments, abnormal, granulated mitochondria, and honeycombed, interdigitated Schwann cell/axon networks. Complete fiber breakdown is characterized by chains of these

ovoid swellings (Spencer & Schaumburg, 1977).

Acrylamide exposure reduces impulse conduction velocity in the nerve as a whole, but not in individual nerves. This happens because acrylamide is most toxic to long, large diameter, myelinated nerve fibers (Fullerton & Barnes, 1966). These fibers conduct faster than smaller or unmyelinated fibers. The distal portions of the axons are more severely affected than the proximal portions. Axon regeneration occurs at the same time as axon degeneration (ibid). Among the first organelles affected are the pacinian corpuscles in the hindfeet (Schaumburg, Wisniewski, & Spencer, 1974) and the muscle stretch receptors (Lowndes et al, 1978).

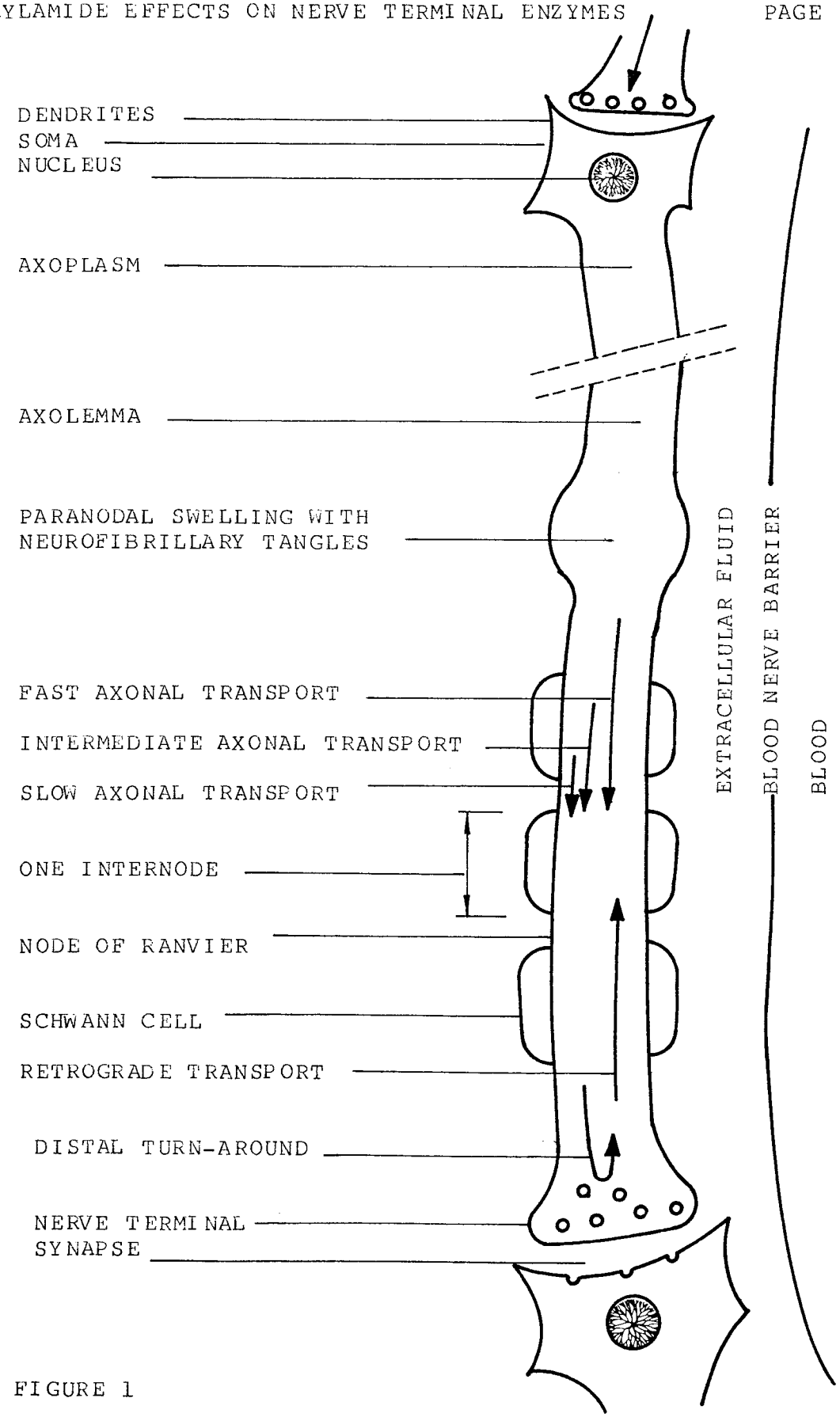

DENDRITES
SOMA
NUCLEUS
AXOPLASM
AXOLEMMA
PARANODAL SWELLING WITH
NEUROFIBRILLARY TANGLES
FAST AXONAL TRANSPORT
INTERMEDIATE AXONAL TRANSPORT
SLOW AXONAL TRANSPORT
ONE INTERNODE
NODE OF RANVIER
SCHWANN CELL
RETROGRADE TRANSPORT
DISTAL TURN-AROUND
NERVE TERMINAL
SYNAPSE
EXTRACELLULAR FLUID
BLOOD NERVE BARRIER
BLOOD

FIGURE 1

BIOMECHANISM

Investigation of the toxicodynamic mechanism of the central-distal peripheral axonopathies (CPDA) is a major thrust of current neurotoxicologic study. As discussed in the section on descriptive toxicology, these agents cause swellings at distal nodes of Ranvier in long axons with tangled neurofilaments and eventual death of the axon distal to the swelling.

The classical hypothesis suggested that neurotoxins interfered with neuron somal metabolism, resulting in the gradual decline in the production of materials needed for axon maintenance (Cavanagh, 1964). A corollary of this model was the idea that the terminal dies first, and degradation proceeds serially up the axon. Spencer & Schaumburg's observations using electron microscopy (1974 & 1977) demonstrated that the pathology commenced in simultaneous, multifocal, and random distribution along the length of the axon. J. W. Griffin has demonstrated that applying acrylamide to parts of the axon far removed from the soma can initiate this pathology (quoted in Spencer, 1984). Politis (1980) has demonstrated the same for 2,5 hexanedione.

When the swelling occurs at a more proximal location in the nerve fiber, the rest of the axon undergoes rapid degeneration (Schaumburg, 1974). This observation strongly suggests that loss of axoplasmic transport function is crucial in Central - Peripheral Distal Axonopathy. Since the swellings we observe contain accumulations of material that is transported (Pleasure et al, 1969) this seems to make sense. The question is: What causes the transport blockade?

Savolainen (1977) and Graham et al (198Ø) have suggested that these agents act by cross linking structural and motile proteins. While their experiments were conducted in vitro on purified proteins, this mechanism has the advantage of simplicity: A potent crosslinker directly causes the pathology we see.

We set out to evaluate this idea in vivo. These cross - linked protein products should demonstrate electrophoretic mobility quite different from the parent proteins. Our lab examined hundreds of axonal preparations by electrophoresis in hope of finding a protein whose mobility or concentration is changed by acrylamide or 2,5 hexanedione exposure. We have not found one yet (unpublished observations). We may not be looking for this cross linked product in the right place, but our inability to find it

encourages us to examine other mechanistic hypotheses.

Nerve tissue is particularly dependent on glucose as a source of ATP. Axonal transport and maintenance of transmembrane potential consume much of this energy (Sacks, 1957). One can cause failure of the fast axonal transport system within 2 hours by appplication of iodoacetic acid (Ochs, 1969). The same treatment takes the same time to deplete the axon's stores of high energy phosphate. Iodoacetic acid acts by blocking glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Application of pyruvate to the nerve restores fast axonal transport (Sabri, 1971). The same sort of transport blockade can be generated by blocking neuron specific enolase with fluoride ion. The blockade is also lifted by application of pyruvate (Edstrom & Mattsson, 1976 & Banks et al, 1973).

Glycolytic enzymes that are inhibited in vitro by acrylamide and 2,5 hexanedione include GAPDH, phosphofructokinase (PFK), and enolase (Howland et al, 1980, Graham & Abou-Donia, 1980). Measurements of the activity of these enzymes in control and acrylamide - intoxicated rats reveal in vivo inhibition of GAPDH, no affect on PFK, and a small affect on enolase activity (Howland, 1981).

There is an isozyme of enolase that is only found in

nerve tissue, neuron specific enolase (NSE) (Bock & Dissing, 1975, Marangos et al, 1978). We call the other isozyme non neuronal enolase (NNE). One can separately measure the activities of the two isozymes because NSE is stable, in the absence of magnesium ion, at 50'C while NNE is rapidly inactivated (Marangos et al, 1978). Howland (1981) found that NSE activity in the distal portion of the sciatic nerve of intoxicated cats was 30% of the activity of the control animals. Measurements of total enolase activity in the same sample revealed no significant difference.

The "glycolytic hypothesis" is an attractive explanation for these neuropathies. Reduced ATP production could explain a) impairment of axonal transport, b) the accumulations of neurofilaments, c) the reduced neurotransmitter releases shown by Lowndes & Goldstein (1979), and, ultimately, d) the degeneration of the axons distal to the blockage.

There are some problems with the hypothesis. In vitro inhibition of these two enzymes requires concentrations at least as high as the whole body concentrations of acrylamide and 2,5 hexanedione (Howland, 1984b). Significant decreases of oxygen consumption are not shown in acrylamide intoxicated animals. Neither GAPDH nor NSE is a limiting step in glycolysis. NSE, in particular,

represents 1.5% of the total brain soluble protein. NSE is not quite as abundant in peripheral nerve, but is still in excess of the concentration necessary to catalyze phosphoenolization (Marangos et al, 1975). The reductions shown might cause an increase in the concentrations of glyceraldehyde 3-phosphate or 2-phosphoglycerate, but one does not expect these normal metabolic products to be responsible for the kind of pathology we observe.

In defense of the glycolytic hypothesis, Sabri (1980) has observed that 2,5 hexanedione reduces ATP levels in nerve tissue in vitro. Addition of pyruvate restores ATP to control levels. Another interesting input is the observation that acrylamide and N-isopropyl acrylamide which are both quite neurotoxic, inhibit GAPDH in vitro (Vyas, 1984). N-hydroxyl acrylamide which demonstrates intermediate toxicity, inhibits GAPDH less than acrylamide does. Similarly, The neurotoxic agents 2,5 - hexanedione and methyl n-butyl ketone inhibit GAPDH while the non-neurotoxic agents 1,6 hexanediol and acetone have no affect (Sabri et al, 1979).

All of the glycolytic enzymes, together with actin, clathrin, myosin, and creatine phosphokinase travel down the the axon in slow component B (Brady & Lasek, 1982). The inhibition discussed above implies that neurotoxins bind

irreversibly to several of these proteins. Perhaps their affect is to disassociate an orderly arrangement of these enzymes in an operational complex. If this is so, 30% inhibition of two adjacent enzymes may cause more than 30% inhibition of the net energy production.

Droz and collaborators have reported that radiolabelled proteins and tubulo-vesicular structures accumulate in the more terminal portions of the sciatic nerves of chickens exposed to acrylamide (Souyri et al, 1981, Chretien et al, 1981). Sahenk and Mandell (1981) have suggested that acrylamide decreases the rate of turn-around of material at the nerve terminal. The significance of this turn-around loss is not yet well understood.

This study examined the affects of acrylamide exposure on some glycolytic enzymes in the brain and spinal cord nerve terminals. Since the nerve terminal is the portion of the neuron furthest removed from the soma, and not part of the axon, it may have a sensitivity to these agents different from the axon. Measurement of acrylamide's affect on the nerve terminal particle (synaptosome) yields information not otherwise available about turn-around and terminal accumulation. Since the terminals of peripheral neurons are located throughout the tissues, we had to use the nerve terminal preparation made from the brain and spinal cord.

METHODS

PREPARATION OF RATS FOR ASSAY

Male Sprague Dawley rats weighing 175 to 225 grams each were randomly divided into exposed and control groups. We injected them interperitoneally with a volume in microliters of an acrylamide solution equal to their mass in grams. The solution was 50 milligrams of acrylamide per ml of distilled water, so the dose equaled 50 mg / kg body mass per day. We injected an equivalent volume of sterile distilled water into the control rats each day.

We injected the rats each morning for 5 or 10 consecutive days (250 or 500 mg/kg, respectively). The rats were sacrificed one day after the last injection. The controls were sacrificed on the same day as the exposed rats, and at the same time for the whole brain studies.

All of the rats were housed in the same room, in flat bottom plastic cages, three per cage, lined with pine shavings. They were allowed free access to food and water. The temperature was maintained at 70'F. The lighting was operated on a 12 hour on and 12 hour off cycle.

All of the subject rats were killed by decapitation. Their brains were quickly removed and placed in 0.32 Molar sucrose buffer on ice. For the whole brain studies, each animal's brain was homogenized as soon as it was removed. In the brain region studies, brains were quickly dissected and the samples from each of the regions were pooled. Homogenization began as soon as all of the samples were collected.

The spinal cord was removed hydraulically. The spine was transected in the region of the iliac crest. An 18 gauge needle fitted to a 10 ml syringe was inserted into the intervertebral foramen. A ten milliliter aliqut of 0.32 Molar sucrose buffer was then vigorously injected. The spinal cord, minus the dura mater, and with some of the nerve trunks of the cauda equina, pops out of the spine at the cervical vertebra opened by decapitation. The spinal cord is then ready for homogenization. Synaptosomes were then made by the the following method.

SYNAPTOSOME PREPARATION by the method of Dodd et al (198)

SOLUTIONS

0.32 M Sucrose Buffer

Sucrose	320mM	54.75g
Potassium EDTA	1mM	0.185g
Tris(hydroxymethyl)aminomethane	10mM	0.60g
Distilled Water to:		500 cc

6 M HCl to pH = 7.4 (about 15 drops)

0.80 M Sucrose Buffer

Sucrose	800mM	27.38g
Potassium EDTA	1mM	0.037g
Tris (hydroxymethyl)aminomethane	10mM	0.12g
Distilled Water to:		100 cc

6 M HCl to pH = 7.4 (about 3 drops)

1.20 M Sucrose Buffer

Sucrose	1200mM	41.08g
Potassium EDTA	1mM	0.037g
Tris (hydroxymethyl)aminomethane	10mM	0.12g
Distilled Water to:		100 cc

6 M HCl to pH = 7.4 (about 3 drops)

METHOD

Mince rat brain tissue in 10 volumes of 0.32 M sucrose. Homogenize in a motor-driven glass Potter-Elvehjem mortar, using 7 up-and-down strokes of a tight fitting teflon pestle. Centrifuge 5 minutes at 800 g (2500 rpm) and 4'C in the Lourdes 1103 Rotor. The nuclei and whole cells deposit at the bottom of the tube. Place the supernatant (about 8 ml) into a polycarbonate centrifuge tube. Underlay with 4 ml of 1.2 M sucrose, and spin for 18 minutes on the Beckman SW 28 Ti rotor at 100,000 g (28,000 rpm), 4'C, and high acceleration / brake. The mitochondria sediment to the bottom, and soluble material remains in the 0.32 M sucrose buffer layer.

Remove the creamy material (about 2 ml) from the interface between the solutions. Dilute it with 7 ml of 0.32 M sucrose buffer. Homogenize gently in a 15 ml Dounce mortar with an A glass pestle. Underlay this suspension with 4 ml of 0.8 M sucrose solution. Spin for 18 minutes (SW 28 Ti, 100,000 g = 28,000 rpm, 4'C, accel/brake = high). The synaptosomes are in the pellet and myelin floats at the interface beween the layers.

LYSATE PREPARATION

LYSIS BUFFER

Tris (hydroxymethyl)aminomethane	10mM	0.12g
Cysteine	6mM	0.073 g
Distilled Water to:		100 cc
6 M HCl to pH = 7.4		
Triton X-100	1%	1g

Make up a fresh solution each day, because the cysteine oxidizes to cystine overnight.

Aspirate the sucrose solutions containing the myelin from the polycarbonate centrifuge tube mentioned above. Add the lysis buffer (10 ml for whole brain, mid brain, or cortex, 5 ml for cerebellum, brain stem, or spinal cord). Sonicate the contents of the tube for 40 seconds on the Branson 185 Sonifier (setting = 7, microtip, 50 milliamp). Transfer the contents to a 15 ml corex tube and centrifuge in the Lourdes 1103 rotor at 28,700 g (15,000 rpm) at 4'C for 20 minutes. Remove the topmost milliliter of the post-spin supernatant. This is the analyte for protein, GAPDH, and NSE measurement.

LOWRY PROTEIN ASSAY (Lowry, 1951)

SOLUTIONS

Reagent A		
Sodium Carbonate	190mM	20g
Sodium Hydroxide	100mM	4.0g
Sodium Potassium Tartrate	0.7mM	0.2g
Distilled Water to		1 liter

Reagent B		
Copper Sulfate (.5H20)	3mM	0.5g
Distilled Water to		100 ml

Reagent C	
Reagent A	50 ml
Reagent B	1 ml

mix immediately before use

Reagent E	
Folin & Ciocalteu's Phenol Reagent	1 ml
Distilled Water	1 ml

mix immediately before use

Standard BSA Solution (200 ug/ml)

Bovine Serum Albumin (BSA)	20 mg
Distilled Water to	100 ml

Lysis Buffer - see lysis procedure

Basic Reaction Mixture

Analyte	0.02 ml
Distilled Water	1.48 ml
Reagent C	1 ml
Reagent E	0.1 ml

Procedure

Run each determination in triplicate. Add 1.48 ml of distilled water to 20 ul of synaptosome lysate. Add 1 ml of reagent C. Vortex the tubes and place them in the dark for ten minutes. Add 0.1 ml of Reagent E. Vortex immediately and place in the dark for thirty minutes. Read the optical density at 750 nm in the Beckman 25 Spectrophotometer. Compare the average optical density to the standard curve developed using the same method. The standard curve of OD(750) vs concentration of BSA is linear in the range from 0 to 60 ng of protein.

Standard Curve for equivalent concentration in a 20 ul sample

Conc	Buffer	BSA	Water	Reagent C	Reagent E
0.5 g/l	20ul	50ul	1.43ml	1.0ml	0.1ml
1.0	20ul	100ul	1.38ml	1.0ml	0.1ml
2.0	20ul	200ul	1.28ml	1.0ml	0.1ml
3.0 g/l	20ul	300ul	1.18ml	1.0ml	0.1ml

GAPDH ACTIVITY ASSAY

(Schrier, 1963 & Amelunxen & Carr, 1967)

SOLUTIONS

Tris Acetate Buffer

Tris(hydroxymethyl)aminomethane	62.5mM	3.Ø28g
Dithiothreitol	1ØmM	Ø.617g
Distilled Water to		4ØØ ml

Acetic Acid to pH = 8.47

Add 55Ø mg of Sodium Arsenate

to 1Øml of buffer just before use.

NAD Solution

NAD	2Ø.7mM	72.4mg
Distilled Water		5 ml

Divide into 1Ø aliquots. Freeze at -7Ø'C until needed

3 Phospho Glyceraldehyde Solution

3ØØ mM 3-PG'hyde	Ø.5 ml
Distilled Water	4.5 ml

Divide into 1Ø aliquots. Freeze at -7Ø'C until needed

Reaction Mixture	Sample	Blank
Analyte	20 ul	20 ul
Tris - Arsenate Buffer	400 ul	400 ul
NAD Solution	20 ul	20 ul
3-PG'hyde Solution	25 ul	-----
Distilled Water	35 ul	60 ul
Total Volume	500 ul	500 ul

Procedure

Mix the analyte, the buffer, the NAD, and the water in a 1 ml cuvette. Make two samples and one blank for each lysate batch. Place the cuvette in the Beckman 25 Spectrophotometer, set at 30'C. Allow to equilibrate for 1 minute. Add the the 3-phosphoglyceraldehyde solution and mix. Return the cuvette to the Spectrophotometer and record the change in optical density at 340 nm with time using the recording chart accessory.

The reaction produces 3-phosphoglycerate and NADH. NADH has an optical density of 6.2 in a 1.0 mM solution. The change in absorbance in OD units per minute is divided by 6.2 and multiplied by 0.5 ml to yield the micromoles of NADH formed per minute. This is divided by the 20 ul of analyte to yield the uM NADH/min/ml of synaptosome lysate, which is proportional to the millimoles of 3-phosphoglycerate

produced per minute per liter of lysate. This is divided by the protein concentration to yield glyceraldehyde phosphate dehydrogenase activity in millimoles per minute per gram of protein.

RADIOIMMUNOASSSAY FOR NEURON SPECIFIC ENOLASE

(Marangos et al, 1979)

SOLUTIONS

Wash Buffer

Tris(hydroxymethyl)aminomethane	150mM	18.165g
MgSO4	2mM	0.240g
NaN3	0.1%	1.0g
Distilled Water to		1 liter

Set pH to 7.4 with H3PO4 (about 4ml)

Assay Buffer

Wash Buffer	500 ml
Bovine Serum Albumin	0.5 g

Ag*

Using tritiated N S E: (prepared in our lab May 1982)

current batch - 1.034g/l, 149,461,710 CPM/ML.

Dilute to 100 cpm/ul in Assay Buffer.

(Howland, 1984a)

NSE

Use unlabelled, purified NSE prepared May 14, 1982

dilute 10 ul in 6.27ml to yield 1 mg/liter

(Howland, 1984a)

Ab

Use sensitized rabbit serum, prepared Junel4, 1982, diluted 1:10,000 in Assay Buffer.

Goat Antibody

Suspend 1 mg of Bio Rad Immunobead goat anti-rabbit IgG beads in 1 ml of Assay Buffer

Reaction Mixture

0.1 ml Antibody Ab

0.035 ml tritiated N S E Ag*

20, 40, or 100 ul of synaptosome lysate

diluted 1:20 in Assay Buffer

345, 325, or 265 ul of Assay Buffer

.....METHOD.....

Prepare each of the reaction mixtures, which correspond to 1, 2, or 5 microliters of synaptosome lysate, in triplicate. Vortex gently. Incubate the reaction overnight at 4'C. Add 0.5 ml of Goat antibody. Vortex gently. Incubate at room temperature for two hours. Spin for 20 minutes at 900 g (2,000 rpm) and room temperature and pressure, in the IEC Model K centrifuge. Remove the supernatant by careful aspiration. Add 1.0 ml of Wash Buffer, vortex gently, and centrifuge as above. Remove the supernatant. Resuspend the pellet in 0.5 ml of distilled water and vortex. Add 3.0 ml of Aquasol Universal Liquid Scintillation Cocktail, and vortex gently. Transfer the suspension to a Liquid Scintillation Counter vial. Rinse the tube into the vial with another 3.0 ml of cocktail and count the vials in the Beckman 2800 Liquid Scintillation Counter on Auto Count.

Standard Curve

Run a set of serial dilutions to establish a standard curve covering the range from 10 - 100 ng of NSE in the 0.5 ml reaction. Use the exact same antibody and labelled antigen batches used for the lysate unknowns. Run a

standard curve for each batch of lysate unknowns. Prepare the following reaction mixtures in triplicate in disposable culture tubes:

NSE (mass)	Buffer	NSE	Ag*	Ab
labelled	-----	----	35 ul	------
unlabelled	400 ul	----	-----	100 ul
zero	365	----	35 ul	100 ul
10 ng	355 ul	10 ul	35 ul	100 ul
20 ng	345 ul	20 ul	35 ul	100 ul
40 ng	325 ul	40 ul	35 ul	100 ul
60 ng	305 ul	60 ul	35 ul	100 ul
80 ng	285 ul	80 ul	35 ul	100 ul
100 ng	265 ul	100 ul	35 ul	100 ul

The number of counts per minute in each tube should be proportional to the log of the mass of Neuron Specific Enolase present. Therefore there is a linear relationship between the counts in a tube, and the log of the number of microliters of cold NSE or unknown added to that tube. Calculate each of these lines by least square regression. Find the number of counts at one-half binding (Bl/2 = zero/2 + cold/2). Find the mass in nanograms of cold NSE (ng) that corresponds to Bl/2 from the standard curve relationship found above. Find the number of microliters of synaptosome lysate (ul) that corresponds to Bl/2 from the sample line. Divide ng by ul to yield the NSE Concentration in mg/l. Divide that by the protein concentration in g/l to yield an NSE concentration in milligrams of NSE per gram of protein.

COMPUTER PROGRAM FOR STATISTICAL ANALYSIS

```
10 REM * THIS PROGRAM TAKES DATA AS FLOATING DECIMALS, *
13 REM * FINDS THE STD DEVIATIONS
20 REM * AND YIELDS A POOLED ESTIMATE OF VARIATION AND
30 REM * STUDENTS T FOR THE TWO GROUPS
40 REM
50 REM * SET DATA TYPES AND ARRAY DIMENSIONS*
60 DEFDBL A - H, O - Z
70 DEFINT I - N
80 DIM X(100), Y(100), YCALC(100)
90 REM
100 REM * INITIALIZE REGISTERS *
110 XSUM = 0.0
120 YSUM = 0.0
130 XYSUM = 0.0
135 YYSUM = 0.0
140 XXSUM  = 0.0
150 REM
152 REM * READ DATA IN *
156 PRINT "ENTER THE TITLE OF THE DATA COLLECTION"
158 INPUT TITLE$
160 LPRINT TITLE$
162 PRINT "ENTER THE NAMES OF X AND Y"
164 INPUT X$, Y$
170 PRINT "ENTER X-Y PAIRS. USE X=200000 FOR END OF FILE"
173 FOR I = 0 TO 100
176 INPUT X(I), Y(I)
180 IF X(I) => 100000 THEN 300
190 REM
200 REM * FORM SUMS, ETC. *
205 LPRINT X$; " IS "; X(I), Y$; " IS "; Y(I)
210 XSUM = XSUM + X(I)
220 YSUM = YSUM + Y(I)
230 XYSUM = XYSUM + X(I) * Y(I)
240 XXSUM = XXSUM + X(I) [ 2
245 YYSUM = YYSUM + Y(I) [ 2
250 NEXT I
```

COMPUTER PROGRAM (CONTINUED)

```
300 REM
310 REM * DIVIDE THE SUMS BY i *
320 XMEAN = XSUM / I
330 YMEAN = YSUM / I
340 AVEXY = XYSUM / I
350 X2AVE = XXSUM / I
353 Y2AVE = YYSUM / I
360 REM
370 REM * COMPUTE POOLED VARIANCE FOR THE TWO SAMPLES
380 XDEVY = SQR((I * XXSUM - XSUM [ 2) / (I * (I - 1)))
390 YDEVY = SQR((I * YYSUM - YSUM [ 2) / (I * (I - 1)))
391 ESSQUARE = (I - 1) * (XDEVY [ 2 + YDEVY [ 2) / (I * 2 - 2)
395 T = (XMEAN - YMEAN) / SQR (ESSQUARE * 2 / I)
410 REM
420 REM * PRINT THE RESULTS *
434 LPRINT "THE AVERAGE OF "; X$; " EQUALS "; XMEAN
435 LPRINT "THE AVERAGE OF "; Y$; " EQUALS "; YMEAN
442 LPRINT "THE STD DEVIATION OF ";; X$; " IS:   "; XDEVY
443 LPRINT "THE STD DEVIATION OF "; Y$; " IS:   "; YDEVY
448 LPRINT "THE POOLED VARIANCE IS ", ESSQUARE
452 LPRINT "THE POOLED T EQUALS", T
456 LPRINT "DEGREES OF FREEDOM EQUALS ", (2 * I - 2)
460 END
```

MATERIALS

The following is a list of chemicals used in these studies, with their suppliers.

Dosing

1. Acrylamide - Electrophoresis Grade #32800
   US Biochemical Corporation Cleveland, Ohio
2. Distilled Water generated in house by double distillation with ion exchange polishing

Synaptosome Preparation

3. Tris(Hydroxymethyl) Amino Methane 99% #T1378
   Sigma Biochemical Buffer St. Louis, Mo.
4. Ethylene Diamine Tetraacetic Acid, Dipotassium Salt
   Sigma #ED2P
5. Sucrose Ultra Pure #821721
   Schwarz Mann Orangeburg, NY
6. Hydrogen Chloride (Concentrated) #A-144
   Fisher Scientific Pittsburgh Pa.

Synaptosome Lysis

7. Cysteine Sigma #C-7755
8. Triton X-100 source unknown

Lowry Protein Assay

9. Bovine Serum Albumin Sigma #A-7030
10. Sodium Carbonate #SX395 EM Science Gibbstown, NJ.

11. Sodium Hydroxide EM Science #SX590

12. Potassium Sodium Tartrate #3262

J. T. Baker Chemical Co. Phillipsburg, NJ

13. Copper Sulfate (5H2O) Baker #1843

14. Folin & Cioccalteu's Phenol Reagent Sigma #F-9252

GAPDH Assay

15. Glyceraldehyde Phosphate Dehydrogenase Sigma #G-0763

16. 3 Phospho Glyceraldehyde Sigma "G-5251

17. Imidazole Sigma #I-0125

18. D,L Dithiothreitol Sigma #D-0632

19. Arsenic Acid Disodium Salt Sigma # A-6756

20. Beta Nicotine Adenine Dinucleotide Sigma #N-7004

21. Acetic Acid (Glacial) Baker #3-9507

Radioimmunoassay

22. Immunobead Second Antibody #170-5604

Bio-Rad Labs Richmond, Calif

23. Rat Brain NSE - Prepared in our own lab

24. Radiolabelled NSE - Prepared in our own lab

25. Phosphoric Acid EM Science #PX995

26. Sodium Azide Sigma #s-2002

27. Magnesium Sulfate EM Science #MX70

The following equipment was used in These investigations:

1. Beckman L5-65 Ultracentrifuge
2. Beckman SW-28 Swinging Bucket Ultracentrifuge Rotor
3. Lourdes A-2 Centrifuge (Refrigerated)
4. Lourdes 1103 Centrifuge Rotor
5. IEC Size 2 Model K Centrifuge (Unrefrigerated)
6. IEC Model 2528-Q15 Centrifuge Rotor
7. IEC Model 2528-R40 Tube Carrier
8. Beckman Model 25 Spectrophotometer
9. Beckman 24-25 Spectrophotometer Recorder-Controller
10. Beckman LS 2800 Liquid Scintillation Counter
11. Beckman SS-2 Expandomatic pH Meter
12. Revco Freezer at 200'K
13. Potter Elvehjem 30 ml Tissue Grinder
14. Tight-fitting Teflon Pestle for above
15. Wheaton 200 Dounce Tissue Grinder
16. "A" Glass Pestle for above
17. Sartorius Model 2400 Analytical Balance
18. Ohaus Model 1500D Top-Loading Balance
19. Harvard Model 907 Infuser w. 30 ml BD Syringe
20. Branson Model 185 Sonifier, with Microtip

METHODS DEVELOPMENT

The dosing regimen used in these studies has been standard in acrylamide neuropathy studies since Hashimoto and Aldridge described it in 1970. The only alterations I attempted involved changing the ionic strength from 50 g/l (700 mM) to 22 g/l (isotonic: 310mM) to minimize the suffering of the rats. My subjective observation was that the additional volume injected hurt them more than the hyperosmotic solution

Methods of preparing synaptosomes are usually based on the original sucrose density gradient techniques of Gray and Whittaker (1962) or DeRobertis et al (1961). These methods take four to five hours from dissection to incubation. I originally tried to use method of Booth & Clark (1978), but the preparation proved unreliable as a source of metabolically active synaptosomes. I considered the methods of Wood & Wyllie (1981) and the method of Hajos (1975), but settled on the method of Dodd et al (1981) as the only method I could do quickly with the equipment in our lab.

The method of Booth and Clark involves a high speed spin through a discontinuous buffered sucrose gradient. The

method of Dodd used no buffering. I found that adding 1Ø mM Tris HCl to the sucrose buffers improved recovery by congealing the pellet from the first ultraspin into a consistency like custard. I verified that the Tris improved GAPDH recovery by experiment

Preparation	Protein	GAPDH
no buffer	1.9 g / l	Ø.187 mM/g/min
Tris Buffer	1.9 g / l	Ø.297 mM/g/min

Originally, I treated the synaptosome pellet by hypoosmotic lysis in 1Ø mM Tris and centrifuged immediately. I performed a series of experiments (each of the steps described in the METHODS section, on 12 exposed and 12 control rats). The GAPDH activities varied between Ø.15 and Ø.52 mM/g/min in the exposed rats and Ø.23 and Ø.76 mM/g/min in the controls. I needed to measure GAPDH more reproducibly if I wanted to reliably measure a sub-lethal inhbition level.

Since GAPDH is notoriously unstable, and is protected by sulfhydryl compounds. I added dithiothreitol to the lysis buffer to preserve GAPDH activity. I also tested the use of sonication as a means of improving my yield of enzyme. The product of a single homogenization was treated by a four way test. Results follow

Buffer	Lysis	Protein g/l	GAPDH mM/g/min
Regular	Time	1.033	0.226
Regular	Sonic	2.155	0.430
DTT	Time	1.016	0.298
DTT	Sonic	2.028	0.338

In addition to the benefit of sonication observed above, the sonified lysate showed less variation in the GAPDH assay. The duplicate measurements of GAPDH activity of sonified synaptosomes agree within 10%, but previous lysates varied by up to 40%.

Literature research into synaptosome preparations had revealed the work of Knull and Cottick, who measured glycolytic enzymes in synaptosome lysates. They lysed the synaptosomes with distilled water, and then brought the NaCl concentration to 100 mM to remove the enzymes from the cytoskeleton. I performed an experiment to evaluate the necessity for NaCl addition.

1st Add (4ml)	2nd Add (2ml)	Protein	GAPDH
Tris	Tris	2.245	0.539
Tris	NaCl	1.697	0.732
Tris	NaCl	1.650	0.696
Water	NaCl	1.895	0.351

The salted samples have about the same amount of GAPDH diluted by less protein. Knull did not use a buffer, or sonication. It seems likely that we are already accomplishing the goal that this technique achieves.

I dosed a dozen rats with 500 mg/kg of acrylamide, and prepared synaptosome lysates from their brains. Control GAPDH levels varied from 0.28/g to 0.56/g. Exposed GAPDH levels varied from 0.11 to 0.480. This was less spread than the earlier series, but it was not narrow enough for us to be statistically certain that acrylamide did or did not affect GAPDH levels in the synaptosome.

At this point, I realized that I did not have a way to assess the accuracy of the GAPDH assay. I ordered some yeast cell GAPDH from Sigma. When it arrived, I weighed 0.27 mg of it, and measured its activity, which should have been 10 mM/l min. It measured 0.043 mM/l/min. Amelunxen (1967) reccomends the use of cysteine in GAPDH assays to maintain GAPDH in its active D-form. I measured the activity of 0.16 mg in a solution containing 6mM of cysteine. Activity was 2.419 mM/l/min, A substantial improvement from 0.043.

I thenceforth put cysteine in the lysis buffers. That created another problem. A solution containing Tris and

cysteine but no protein yields a positive result in the Lowry Protein Assay. I conducted a series of Lowry standard curves with different concentrations of cysteine present. The results were interesting per se, but their consequence is that: the samples in the standard curve for the Lowry, which are prepared each time we perform the Lowry assay, must contain a volume of the lysis buffer equal to the volume of analyte in the unknowns.

I then tried to make synaptosomal proteins radiolabelled with 14C Acrylamide. The proteins acquired a label of only 600nCi / g. This was too low to allow autoradiography, which was our purpose. This project was cancelled.

The next series of exposures we conducted controlled for all of the error factors that we recognized. There were four sets of exposed and control rats. One batch of each was killed each day. Tris-Cys buffer was used for lysis. The Lowry assay was performed on all of the samples at the same time. The results are tabulated below.

CORTEX	Dose	Protein	GAPDH	RIAs
	mg/Kg	g/l	mM/g/min.	mg/g
Jan 3	----	1.607	0.351	7.81
Jan 4	----	1.679	1.187	5.73
Jan 5	----	1.238	0.684	7.67
Jan 6	----	2.3266	0.740	4.38
Jan 3	500	1.840	0.159	6.77
Jan 4	500	2.234	0.326	3.08
Jan 5	500	1.314	0.575	7.82
Jan 6	500	1.848	0.491	4.92

MID B	Dose	Protein	GAPDH	RIAs
	mg/Kg	g/l	mM/g/min.	mg/g
Jan 3	----	2.076	0.388	5.79
Jan 4	----	1.666	0.659	8.63
Jan 5	----	1.118	0.739	8.93
Jan 6	----	1.670	0.749	5.25
Jan 3	500	1.837	0.220	6.05
Jan 4	500	1.431	0.486	8.21
Jan 5	500	0.916	0.638	10.79
Jan 6	500	2.020	0.389	4.02

BELLA	Dose	Protein	GAPDH	RIAs
	mg/Kg	g/l	mM/g/min	mg/g
Jan 3	----	1.584	Ø.363	5.85
Jan 4	----	1.238	Ø.668	5.53
Jan 5	----	Ø.747	Ø.688	8.77
Jan 6	----	1.12Ø	Ø.594	6.Ø4
Jan 3	5ØØ	1.461	Ø.353	6.33
Jan 4	5ØØ	1.3Ø4	Ø.595	5.Ø4
Jan 5	5ØØ	Ø.993	Ø.7661	8.67
Jan 6	5ØØ	1.4Ø6	Ø.439	5.68

STEM	Dose	Protein	GAPDH	RIAs
	mg/Kg	g/l	mM/g/min	mg/g
Jan 3	----	1.73Ø	Ø.385	5.65
Jan 4	----	1.567	Ø.579	4.Ø1
Jan 5	----	Ø.233	1.255	22.66
Jan 6	----	1.231	Ø.819	4.29
Jan 3	5ØØ	1.265	Ø.263	3.81
Jan 4	5ØØ	Ø.9Ø7	Ø.456	4.59
Jan 5	5ØØ	Ø.34Ø	Ø.623	15.38
Jan 6	5ØØ	1.237	Ø.326	2.45

While there are strong differences between the

exposed and control values, the variation within each group kept these differences from being statistically significant. T - test results calculated using the program listed in the methods section follow:

JANUARY SYNAPTOSOME LYSATE ENZYME MEASUREMENTS

GAPDH LEVEL STATISTICAL COMPARISON

REGION	CONTROL	S. D.	EXPOSED	S. D.	T - VALUE
CORTEX	.741	.344	.388	.184	1.86
MIDBRAIN	.634	.169	.433	.175	1.65
CEREBELLUM	.578	.149	.537	.180	Ø.35
BRAIN STEM	.76Ø	.375	.417	.159	1.68

NSE LEVEL STATISTICAL COMPARISON

REGION	CONTROL	S. D.	EXPOSED	S. D.	T - VALUE
CORTEX	6.4Ø	1.65	5.65	2.Ø9	Ø.56
MIDBRAIN	7.15	1.9Ø	7.27	2.91	-Ø.Ø7
CEREBELLUM	6.55	1.5Ø	6.43	1.58	Ø.11
BRAIN STEM	9.15	9.Ø3	6.56	5.95	Ø.47

The degrees of freedom equals 6 in each of the above comparisons. The T - VALUE necessary to demonstrate statistically significant difference at p = Ø.Ø5 is 2.447. The highest T - VALUE shown by these data is 1.86. No statistically significant differences between exposed and control groups is shown.

I still felt that our problem was one of recovery from the prepared synaptosomes. Some of the synaptosomes were not bursting, or the GAPDH was precipitating with the pellet anyyway. I decided to add 1.0% Triton X-100 to the lysis buffer. I first had to establish whether Triton X-100 effects GAPDH activity. I made up two equivalent solutions of yeast GAPDH. One contained Triton X100 and the other did not. The one containing Triton had an activity 0.433 uM/ml/min and the control had an activity of 0.413. Two hours later, the TX100 one was 0.147, and the control was 0.135. Triton X100 has no effect on GAPDH activity.

Triton did effect the optical density readings obtained in the Lowry assay, but this was controlled for by the previously instituted practice of placing buffer into the standard curve samples. I felt that this addition would improve my precision. Other possible explanations for our problem were that the variation occurs because the samples are homogenized separately, or that the variation is inherent when using different animals.

To test these theories, I prepared synaptosomes from the brains of three rats, and lysed all six samples with lysis buffer. There were three homogenizations. Two consisted of half a brain from one rat and half from

another. Results follow.

Rat	Sample	Protein	GAPDH
A	1	3.36	Ø.577
A	2	3.42	Ø.522
1/2 B & 1/2 C	1	3.22	Ø.542
Same	2	3.5Ø	Ø.599
Same	1	3.55	Ø.57Ø
Same	2	3.82	Ø.574

These results were tightly grouped enough to give me confidence that the addition of Triton had overcome our problem. Since all of the GAPDH levels agree within 12%, We have enough data consistency to find an activity loss of 2Ø%. This established the methods reported in the Results section

EXPERIMENTAL RESULTS

I have reported only those final experiments which were performed using the techniques discussed in the METHODS section. Several earlier experiments using slightly different techniques are reported in the METHODS DEVELOPMENT section.

WHOLE BRAIN SYNAPTOSOME ASSAY RESULTS

In February of 1984, I dosed ten rats (mass about 275 grams) with 500 milligram of acrylamide per kilogram of body weight and six with water. I killed 3 & 3 on February 23rd, and 3 & 3 on February 24th. The protein assay of Feb 24th was unreliable because of cysteine precipitation. I ran another batch on March 14th as a substitute.

Group	rat #	Brain	Protein	GAPDH	NSE
		wt.g	g/l	mM/g/min	mg/g
control	1	1.93	2.59	0.529	3.32
control	2	1.95	2.15	0.539	3.69
control	3	1.97	1.84	0.503	5.01
control	4	1.80	2.38	0.316	3.98
control	5	1.77	2.31	0.462	4.33

control	6	1.99	2.48	Ø.429	4.31

Dose mg/kg		Brain grams	Protein g/l	GAPDH mM/g/min	NSE mg/g
Feb 23	1	1.75	2.47	Ø.393	5.12
Feb 23	2	1.83	2.55	Ø.341	5.81
Feb 23	3	2.Ø1	1.94	Ø.298	4.77
Mar 14	4	1.77	2.53	Ø.251	4.Ø5
Mar 14	5	1.75	2.6Ø	Ø.24Ø	4.17
Mar 14	6	1.59	2.72	Ø.262	4.67

BRAIN REGION SYNAPTOSOME ASSAY RESULTS

During March, 14 rats were treated with acrylamide and 9 with water as described in the METHODS section. They were sacrificed on the March 20th, 21st, and 22nd. Protein and enzyme levels were measured as described in the METHODS section. Results follow.

CORTEX	Dose	Tissue	Protein	GAPDH	RIAs
		wt, g.	g/l	mM/g/min.	mg/g
March 20	500	0.81	2.898	0.664	3.66
March 21	500	0.89	2.041	0.642	5.53
March 22	500	0.91	2.940	0.706	3.19
March 20	---	0.81	2.346	0.713	3.02
March 21	---	1.05	2.358	0.744	3.08
March 22	---	0.78	2.023	1.007	4.87

MID B	Dose	Tissue	Protein	GAPDH	RIAs
		wt, g.	g/l	mM/g/min.	mg/g
March 20	500	0.81	1.771	0.660	5.36
March 21	500	0.83	2.374	0.633	4.80
March 22	500	0.89	2.086	0.715	3.55
March 20	---	0.82	2.319	0.696	3.34
March 21	---	0.75	1.693	0.674	4.13
March 22	---	0.94	2.124	0.911	3.94

BELLA	Dose	Tissue wt, g.	Protein g/l	GAPDH mM/g/min.	RIAs mg/g
March 20	500	0.81	3.025	0.649	4.91
March 21	500	0.68	2.533	0.533	4.31
March 22	500	0.77	3.136	0.540	3.32
March 20	---	0.82	2.914	0.554	3.06
March 21	---	0.78	2.940	0.631	3.85
March 22	---	0.74	2.417	0.676	4.50

STEM	Dose	Tissue wt, g.	Protein g/l	GAPDH mM/g/min.	RIAs mg/g
March 20	500	0.68	1.371	0.526	4.00
March 21	500	0.75	1.849	0.425	3.00
March 22	500	0.79	1.877	0.505	1.99
March 20	---	0.77	1.227	0.506	1.46
March 21	---	0.62	1.439	0.588	3.11
March 22	---	0.53	1.200	0.706	3.08

SPINE	Dose	Tissue	Protein	GAPDH	RIAs
		wt, g.	g/l	mM/g/min.	mg/g
March 20	500	1.14	0.887	0.341	3.54
March 21	500	0.92	1.003	0.382	3.03
March 22	500	0.57	0.475	0.570	3.12
March 20	---	1.65	1.129	0.304	0.58
March 21	---	1.00	1.081	0.441	1.92
March 22	---	1.11	1.253	0.541	0.92

STATISTICAL INTERPRETATION

The GAPDH and NSE levels from each preparation were subjected to statistical analysis using the BASIC computer program listed in the METHODS section. We compared the group means for statistically significant difference at the $p = .05$ level. These results appear below.

SYNAPTOSOME LYSATE GAPDH LEVELS

REGION	CONTROL	S. D.	EXPOSED	S. D.	T - VALUE
WHOLE BRAIN	.463	± .Ø83	.298	± .Ø6Ø	3.97 ***
CORTEX	.821	± .162	.671	± .Ø33	1.58
MID BRAIN	.76Ø	± .13Ø	.669	± .Ø42	1.14
CEREBELLUM	.62Ø	± .Ø62	.574	± .131	Ø.9Ø
BRAIN STEM	.6ØØ	± .1ØØ	.485	± .Ø53	1.74
SPINAL CORD	.429	± .122	.431	± .119	Ø.Ø2

SYNAPTOSOME LYSATE NSE LEVELS

REGION	CONTROL	S. D.	EXPOSED	S. D.	T - VALUE
WHOLE BRAIN	4.11	± .586	4.765	± .647	-1.85
CORTEX	3.66	± .1.Ø5	4.13	± 1.24	-Ø.51
MID BRAIN	3.8Ø	± .412	4.57	± .927	-1.31
CEREBELLUM	3.8Ø	± .721	4.18	± .8Ø3	-Ø.61
BRAIN STEM	2.55	± .944	3.8Ø	± 1.Ø1	-Ø.56
SPINAL CORD	1.14	± .696	3.23	± .272	-4.84 ***

*** significant at the p = Ø.Ø5 level

The whole brain synaptosomes demonstrated a 36% loss in GAPDH per gram of protein. This was significant at the p = .005 level. They showed a 16% INCREASE in NSE per gram of protein. This was a very interesting trend, but it was not significant at p = .05.

The brain region synaptosomes demonstrated a loss in GAPDH activity of about 14% (ranged from 8 to 19%), but this was not significant at the p = .05 level. Spinal cord synaptosomes showed no loss of GAPDH activity at all. The brain region synaptosomes showed INCREASES of about 15% (ranged from 10 to 20 %) in neuron specific enolase per gram of protein. This was not significant. The spinal cord showed a 200% increase in NSE levels, which is statistically significant (p = .005), but highly suspect because of the low values measured.

DISCUSSION

The importance of axoplasmic transport in dying-back axonopathy has been recognized since Pleasure's work in 1969. Knowledge of these transport systems increases rapidly. The affect of acrylamide on retrograde rapid transport has been recognized quite recently (Miller et al, 1983 and Jakobsen & Sidenius, 1983). Our results tend to support this observation and to suggest a connection between this affect and the glycolytic hypothesis.

Axon transport consumes a great deal of energy (Sacks, 1957). Transport is dependent on temperature, extracellular calcium concentration, oxygen tension, and availability of high energy phosphate, but independent of axon length, diameter, electrical activity, and extracellular sodium or potassium concentrations. It is blocked by inhibitors that block energy transformation (Spencer, 1984).

A gradient of biochemical vulnerability seems to exist in the axon. Material being transported is deposited all along the length of the axon (Grafstein, 1980). This should establish a declining proximal-distal gradient of transported materials, consistent with the findings of

Howland (1981). The extent of this decrease may reach 60%, and is greater in long than in short axons (Lubinska, 1962). Nerve terminal mitochondria are crippled in that they cannot use alpha - ketoglutarate or succinate (Hajos, 1973). As a consequence, they are more sensitve to agents that affect pyruvate carboxylation. There is also evidence for a proximal-distal declining gradient of oxygen uptake in the axon (Majno, 1958).

The major conceptual objection to considering GAPDH inhibition a crucial step in the etiology of axonopathy is that no in vivo inhhibition greater than 50% has been shown. Enough survives to carry on glycolysis. If the nerve terminal indeed represents a low ebb in energy levels, the nerve terminal may be the one place where this is not so. Our results showed a 36% reduction in GAPDH activity in the whole brain synaptosome. This is not substantially different from measurements made in the distal sciatic nerve (Howland, 1981).

The experiments in which chemicals are applied to axons have not yet been extended as far as they might be. It is very interesting that applied pyruvate counteracts iodoacetate blockade of axonal transport, but what would be the effect of applying 3-phosphoglyceric acid or 3-phosphoglyceroyl phosphate? Such an experiment might more strongly implicate GAPDH inhibition as a crucial step in the

etiology of central peripheral distal axonopathies.

I must admit that this study involves measurement of enzyme activity in a SYNAPTOSOMAL PREPARATION, which is not necessarily the same as the synaptosome itself. We used the method of Dodd et al (1981) modified for our laboratory. We accepted the pellet this method produces as "synaptosomes," in full recognition that no synaptosomal preparation yields pure synaptosomes (Dodd et al, 1981, Booth & Clark, 1978). We did not examine the pellet by electron microscopy, or determine acetylcholine levels to evaluate contamination. We feel that the differences between one preparation and another is representative of differences within the nerve terminal.

Retrograde transport is blocked by low concentrations of acrylamide (Miller et al, 1983, Jakobsen & Sidenius, 1983). Several neuropathogens cause impaired distal turn-around early in the course of their pathology. Since the mass has to go somewhere, these conditions cause an accumulation of material in nerve terminals which then swell (Mendell & Sahenk, 198Ø, Blakemore & Cavanagh, 1969).

My results indicate increases of 15 (+/-5) % in synaptosome NSE levels. This difference was not

statistically significant within the individual groups (representing brain regions) and the groups could not be pooled for comparison, because the brain regions intrinsically differ in NSE levels. The fact that all six of the groups show an increase in NSE levels is, however, striking.

Howland (1984) showed that NSE is not transported by the rapid retrograde transport system. The magnitude of the NSE increase I observed is consistent with a loss of slow retrograde transport. This might have a significant contribution to the apparent disappearance of NSE from the distal axon shown by Howland (1981). One must, of course,be cautious when comparing NSE concentration and NSE activity. Acrylamide inhibits NSE by reversibly binding to it (Howland et al, 198Ø). This complex may still be recognized by the rabbit anti-rat NSE antibody we use in the radioimmunoassay.

One can conceive of some further experiments upon consideration of these results. If the increase of NSE levels in the synaptosome represents a failure of distal turn-around, and if that failure occurs early in the history of the pathology, rats dosed for twice as long with half as much acrylamide per day should show a greater increase in

NSE levels than that shown in this study. I did not measure NSE activities in the samples for which I had NSE concentrations. Measuring both would yield information about the level of inhibition of the NSE present.

The variation within each group of data remained a problem throughout the project. Careful methods development narrowed the range of values for GAPDH from 3:1 (highest value : lowest value) in the first series of experiments to 3:2 in the final series. How close this approximates the inherent biological variance, I can only guess. The method of adding salt to the synaptosome lysis buffer to free the GAPDH (Knull & Cottick, 1981) might improve precision. Our original tests of this method could not demonstrate its full advantage because they did not contain cysteine to protect the enzyme.

The synaptosomal preparations all involve ultracentrifugation. No swinging - bucket rotor can hold more than six samples at a time. This means that one can prepare no more than six samples at one time. When I was looking at enzyme levels in five brain regions, I had to compare preparations made on different days. A more reliable approach, albeit one that consumes more rats, would be to prepare three control and three exposed spinal cords (or cortices, or midbrains, etc.) all at one time. This would

improve the homogeneity of our data.

CONCLUSIONS

This study establishes a serviceable assay for the evaluation of nerve terminal enzyme levels. This method is fast, reliable, and rugged. Nerve tissue is homogenized. Synaptosomes are prepared by the method of Dodd et al (1981), with the addition of Tris buffer to improve recovery. The synaptosomes are lysed with a buffer containing Tris, cysteine, and Triton X100. The post 28,000g supernatant product of that lysis contains a fairly reproducible level of soluble enzymes in a solution that minimizes degradation.

GAPDH levels declined in acrylamide - exposed synaptosomal preparations to the same degree that they have been previously shown to decline in whole distal nerve preparations. Since the method of Dodd includes a centrifugal spin to separate the synaptosome and the myelin, this result casts doubt on the hypothesis (Howland, 1984b) that oligodendrocytes and Schwann cells mask the true extent of GAPDH inhibition in neurons. Astroglia may sediment like synaptosomes. If they do, They may present a source of contamination in our preparation. That contamination would be consistent with this masking hypothesis.

NSE levels increased in synaptosomes from all acrylamide - exposed brain regions we examined. This increase was not statistically significant. There is a 10% probability that each increase arose by chance alone. The trend, however, could lead one to infer that NSE accumulates in the nerve terminal of rats exposed to acrylamide. Experiments based on this observation may illustrate that the retrograde slow transport system is inhibited by acrylamide. This would provide another clue in the biochemical mystery of acrylamide neuropathology.

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