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STUDIES ON THE BIOCHEMICAL BASIS OF DISTAL AXONOPATHIES—I. INHIBITION OF GLYCOLYSIS BY NEUROTOXIC HEXACARBON COMPOUNDS

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Abstract—Neurotoxic hexacarbon compounds 2,5-hexanedione (2,5-HD) and methyl *n*-butyl ketone (M*n*BK) inhibit crystalline and endogenous CNS and PNS glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Preincubation of the enzyme with the toxin was necessary for inhibition. The enzyme activity of GAPDH was preserved by the addition of dithiothreitol in the presence of either neurotoxin. By contrast, lactate dehydrogenase (LDH) activity was not inhibited by these neurotoxic chemicals. Neurologically inactive compounds 1,6-hexanediol and acetone failed to inhibit GAPDH. The present data indicate that 2,5-HD and M*n*BK block energy metabolism by inhibiting glycolysis at the site of GAPDH. These observations may account for the known failure of GAPDH-dependent axonal transport and the axonal degeneration which occurs in hexacarbon neuropathy.

DISTAL axonopathy describes a group of human and experimental diseases which are characterized clinically by polyneuropathy, and pathologically by distal and retrograde ('dying-back') axonal degeneration occurring in long and large fiber tracts both in the central (CNS) and peripheral nervous systems (PNS) (SPENCER & SCHAUMBURG, 1976).

Experimental models of distal axonopathies are readily produced in a number of laboratory species by chronic exposure to a variety of neurotoxic compounds. The neurotoxic hexacarbon ketonic solvents, methyl *n*-butyl ketone (MnBK) and 2,5-hexanedione (2,5-HD), have proven especially useful in studying the spatial–temporal pattern of nerve fiber degeneration in this type of distal axonopathy (SPENCER & SCHAUMBURG, 1977). These studies have shown that the nerve fiber breakdown is heralded by focal accumulations of 10 nm neurofilaments which produce axonal swellings above nodes of Ranvier in the distal parts of vulnerable axons (Fig. 1). These multifocal paranodal accumulations of neurofilaments are associated with a blockade of fast axonal transport (MENDELL *et al.*, 1977; GRIFFIN *et al.*, 1977, in press). Because fast axonal transport is known to be an energy consuming process, requiring the activity of glyceraldehyde-3-phosphate dehydrogenase (SABRI & OCHS, 1971, 1972), the present study examines the effect of neurotoxic (M*n*BK, 2,5-HD) and non-neurotoxic (1,6-hexanediol and acetone) solvents on this enzyme. Preliminary results from this study have been reported elsewhere (SABRI *et al.*, 1978*a*, *b*).

Abbreviations used: 2,5-HD, 2,5-hexanedione; M*n*BK, methyl *n*-butyl ketone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; PNS, peripheral nervous system; G-3-P, DL-glyceraldehyde-3-phosphoric acid; IDPN, β-β'-iminodipropionitrile.

MATERIALS AND METHODS

Materials. Crystalline glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH), NAD, NADH, sodium pyruvate and DL-glyceraldehyde-3-phosphoric acid (G-3-P) were obtained from Sigma Chemical Co., St. Louis, MO. 2,5-Hexanedione, methyl *n*-butyl ketone (neurotoxic hexacarbons), and 1,6-hexanediol (non-neurotoxic hexacarbon) were obtained from Eastman Kodak Co., Rochester, NY, and acetone (a non-neurotoxic solvent with a dimeric relationship to 2,5-HD) from Fisher Scientific Co., Springfield, NJ. All other reagents including acetone were of analytical grade.

Assay for GAPDH activity. The effect of neurotoxic and non-neurotoxic solvents on GAPDH activity was studied using both the crystalline enzyme and the endogenous enzyme present in nerve tissue. Enzyme activity was assayed by measuring the rate of reduction of NAD at 340 nm in the presence of GAPDH. The procedure used was similar to that described by SABRI & OCHS (1971). For the study of crystalline GAPDH, the incubation medium had the following composition: 2.5 ml of 0.03 M-sodium pyrophosphate buffer (pH 8.5), 3.5 μg of crystalline GAPDH in 10 μl buffer, 0.05 ml of 0.02 M-NAD, 0.1 ml of 0.1 M-cysteine in pyrophosphate buffer, and 0.3 ml of 0.17 M-disodium arsenate (total incubation medium 3.0 ml). The reaction was started by adding 2 μmol of DL-glyceraldehyde-3-phosphate (G-3-P) in 20 μl of pyrophosphate buffer directly into the cuvette. The increase in extinction at 340 nm resulting from NADH formation was monitored for 3 min in a Gilford 250 spectrophotometer equipped with a recorder. The temperature of the cuvette was maintained at 37 ± 1.0°C during the course of the reaction.

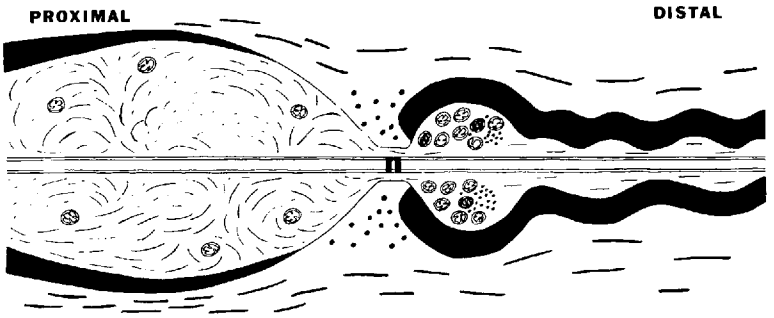

FIG. 1. Diagram to show the early pathological changes occurring towards the end of a single nerve fiber removed from a calf muscle branch of the tibial nerve of a rat intoxicated orally for 4–6 weeks with 0.5% (v/v) 2,5-HD. On the proximal side of the node of Ranvier (*n*), an abnormally increased number of 10 nm neurofilaments have accumulated causing the axon to swell focally and the myelin sheath (black) to retract away (to the left) from the nodal region. On the distal side, the axon has few neurofilaments, is shrunken and the myelin sheath corrugated.

Determination of GAPDH activity in rat desheathed peripheral nerves or brain homogenate was carried out by a semi-micro method using an incubation medium of the following composition: 700 μl of buffer, 30 μl of 0.01 M-dithiothreitol (instead of cysteine), 100 μl of arsenate, 50 μl of NAD, and 100 μl of nervous tissue homogenate (total incubation medium 1.0 ml). The reaction was initiated by the addition of 20 μl of G-3-P into the cuvette. To determine GAPDH activity in rat brain, only 10 μl of homogenate was used and the incubation medium was made up to 1 ml by the addition of pyrophosphate buffer.

Assay for lactate dehydrogenase (LDH) activity. LDH activity in the presence of hexacarbons or acetone was determined using crystalline and endogenous enzymes. Enzyme activity was monitored spectrophotometrically by the rate of decrease in extinction at 340 nm (STOLZENBACH, 1966). The reaction mixture for the crystalline enzyme had the following composition: 2.5 ml 0.1 M-phosphate buffer (pH 7.2), 10 μl of enzyme containing 1 μg of protein and 100 μl of freshly-prepared NADH (total incubation medium 3 ml). The reaction was started by the addition of 100 μl of pyruvate into the cuvette. The decrease in optical density at 340 nm at 37°C was monitored as before. The enzyme activity in nerve and brain homogenates was determined by a semi-micro method using an incubation medium composed of 800 μl of buffer, 50 μl of nerve homogenate and 50 μl of NADH (total incubation medium 1 ml). The reaction was started by the addition of 100 μl of pyruvate into the cuvette. For rat brain, only 10 μl of homogenate was used to determine LDH activity.

Preincubation of crystalline GAPDH and LDH with neurotoxic and non-neurotoxic solvents. These experiments were designed to study the effects on GAPDH and LDH when these enzymes were incubated *in vitro* with the test compounds. For this purpose, rabbit muscle crystalline enzyme suspensions in 2 M-ammonium sulfate were diluted with water to give 700 μg/ml protein concentration. Test solvents were diluted with the appropriate buffer solutions to give a required concentration. In a typical experiment, 500 μl of the enzyme (GAPDH or LDH), containing 500 μg of enzyme protein, were mixed with 500 μl of the test agent in a 2 ml test tube and then incubated at 37°C for 20 min in a shaking water bath. A control experiment was run in which the test solvent was replaced with an equivalent amount of buffer solution. After the incubation period, the tubes were chilled in ice, and the enzyme activities determined as soon as possible.

Treatment of intact nerve with 2,5-hexanedione. A 3–4 cm length of sciatic nerve was removed from below the sciatic notch of normal, adult Sprague–Dawley rats following systemic perfusion with cold, isotonic saline. Nerve fascicle contents containing nerve fibers and collagen were plucked from the connective tissue sheath under a dissecting microscope. The extracted nerve tissue from four rats was pooled and immersed in cold, isotonic sucrose. 200 300 mg (wet weight) of nerve tissue was suspended in 25 ml of 50 mM-2,5-HD (pH 7.4) and incubated in a shaking water bath at 37°C for 30 min. As a control, comparable weights of intact tissue were incubated in buffered saline (pH 7.4) under identical conditions. Following incubation, tissue was quickly chilled and rinsed three times with cold 0.25 M-sucrose, and homogenized in the same medium to give a 10% (w/v) homogenate. The tissue homogenate was centrifuged at 2500 *g* for 10 min to remove unhomogenized collagen in the nuclear pellet. The resulting post-nuclear supernatant was used as the 'homogenate' for enzyme and protein determinations. The protein determination was carried out essentially by the method of LOWRY *et al.* (1951).

Treatment of rat brain homogenate with 2,5-HD and MnBK. Rats were decapitated without anesthesia and the brains rapidly removed. A 10% (w/v) homogenate was prepared in cold 0.25 M-sucrose. One ml of the homogenate was mixed with an equivalent volume of toxin solution in a test tube and incubated for 20 min at 37°C in a shaking water bath. Following the incubation period, the tubes were quickly immersed in ice, and enzyme activities and protein concentrations determined. A similar procedure was carried out with normal brain homogenate in which the toxin was replaced by buffer.

RESULTS

Inhibition of glyceraldehyde-3-phosphate dehydrogenase. DL-*Glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating) EC* 1.2.1.12

The neurotoxic compound 2,5-HD inhibited GAPDH activity as a function of the concentration of the toxin (Fig. 2). Preincubation of the enzyme with the toxin was mandatory and the degree of inhibition was also a function of the duration of incubation (Table 1). MnBK at 10 mg/ml concentration also in-

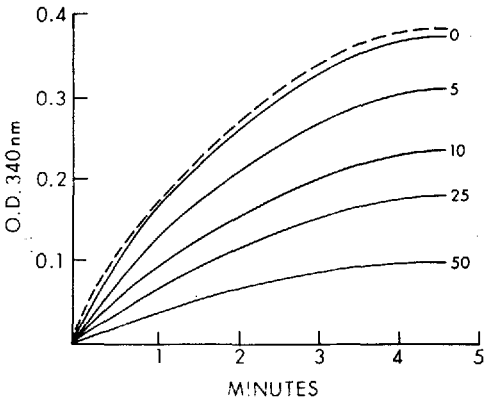

FIG. 2. Effect of different concentrations of 2,5-HD on crystalline GAPDH activity: 500 μl of crystalline GAPDH (0.7 mg protein per ml) and 500 μl of 2,5-HD solution in pyrophosphate buffer having 0–50 mg 2,5-HD per ml were pre-incubated for 10 min at 37°C in a shaking water bath. After pre-incubation, the tubes were immersed in ice and GAPDH activity was determined as described in text. Inhibition of GAPDH activity did not occur if the enzyme was not pre-incubated with 2,5-HD (broken line) indicating that preincubation with the toxin was required for enzyme inactivation to occur.

terfered with GAPDH activity as shown by the kinetic data in Fig. 3. 1,6-Hexanediol, a neurologically inactive hexacarbon,, and acetone, which has a dimeric relationship with 2,5-HD, were each found to have no effect on GAPDH activity, even after prolonged incubation at 50 mg/ml concentration (Fig. 3).

Another set of experiments compared the relative inhibition of GAPDH activity produced by equimolar concentrations of 2,5-HD and M*n*BK. The data shown in Table 2 revealed that M*n*BK produced a greater inhibition than 2,5-HD. However, GAPDH activity was preserved at normal levels by the addition of 0.02 M-dithiothreitol to the incubation medium

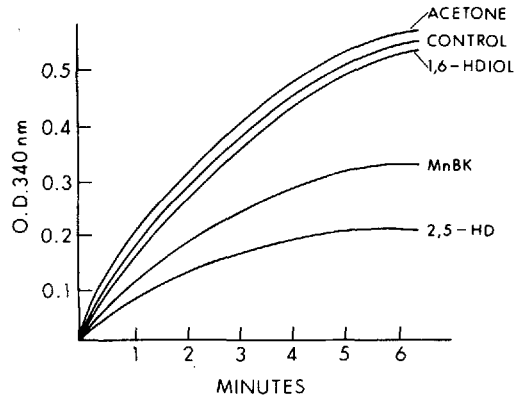

FIG. 3. Effect of the neurotoxic hexacarbons M*n*BK (10 mg/ml) and 2,5-HD (50 mg/ml) and non-neurotoxic solvents 1,6-hexanediol (1,6-HDIOL 50 mg/ml) and acetone (50 mg/ml) on GAPDH activity. 500 μl of crystalline GAPDH was pre-incubated with 500 μl of the solvent for 10 min at 37°C. GAPDH activity was determined as described in the text.

TABLE 1. EFFECT OF 25 mM 2,5-HD ON CRYSTALLINE GAPDH

Pre-incubation (min)	% Enzyme activity
0	100
10	88.3
20	61.1
30	32.5

100 μl of crystalline GAPDH (0.7 mg protein/ml) were preincubated with 100 μl of 50 mM-2,5-HD at 37°C in a shaking water bath. Aliquots of 10 μl each were taken out at the indicated times to determine residual enzyme activity. Each value represents the mean of two determinations.

containing either 2,5-HD or M*n*BK (Table 2). These results indicated that 2,5-HD and M*n*BK might be inhibiting GAPDH activity by interfering with the sulfhydryl groups of the enzyme.

To study further the type of inhibition of GAPDH produced by 2,5-HD and M*n*BK, enzyme activities were determined in the presence of different concentrations of the substrate G-3-P, with and without pretreatment with either toxin. Plots of reciprocal velocities against reciprocal G-3-P concentrations revealed in both cases no relationship between the degree of inhibition and the concentration of the substrate. However, inhibition was a function of the concentration of either 2,5-HD or M*n*BK. Both the slope and the intercept ($1/V_{max}$) were increased from normal values in the presence of either toxin (Fig. 4). These inhibitors altered both velocity (V) and the Michaelis constant (K_m) indicating that a mixed type of inhibition was taking place with 2,5-HD and M*n*BK.

Lactate dehydrogenase. (*EC* 1.1.1.27)

Since the formation of lactate from glycolysis depends on the action of LDH through a coupled oxidation-reduction with GAPDH, it was of interest to study the action of 2,5-HD and M*n*BK on LDH activity. The results revealed that there was no inhibition of LDH activity, even after prolonged preincubation with these neurotoxic solvents (Fig. 5). This contrasted with the results obtained for GAPDH and suggested that neurotoxic hexacarbons display specific enzyme inhibition properties.

Effect of 2,5-HD and MnBK on nerve fiber enzymes

The specific activities of GAPDH and LDH in toxin-treated and untreated nerve homogenates are given in Table 3. These results showed that the activity of GAPDH was lowered by 2,5-HD. As expected, LDH activity was not affected by the toxins. These results are in accord with those obtained for crystalline GAPDH and LDH.

Since M*n*BK and 2,5-HD affect the central nervous system, as well as the PNS, the effect of these toxins on enzymes in rat brain homogenates was also determined. The results showed that both MnBK and 2,5-HD effectively blocked GAPDH activity of rat

TABLE 2. EFFECT OF 2,5-HD AND MnBK ON CRYSTALLINE GAPDH

Sample	μmol of NADH formed per min per mg of protein
GAPDH + buffer (control)	16.77 ± 1.1
GAPDH + 2,5-HD	7.17 ± 0.67
GAPDH + 2,5-HD + DTT	14.33 ± 1.5
GAPDH + MnBK	2.87 ± 0.19
GAPDH + MnBK + DTT	19.64 ± 3.4

50 μl of crystalline GAPDH (0.7 mg protein/ml) were preincubated with 17 mM-2,5-HD or 17 mM-MnBK for 20 min at 37°C with and without the addition of 0.02 M-dithiothreitol (DTT). In a control experiment, the toxin was replaced by an equivalent amount of buffer containing 0.02 M-DTT. After pre-incubation, 10 μl of the enzyme were used to determine enzyme activity. Each value is a mean and S.D. of three determinations.

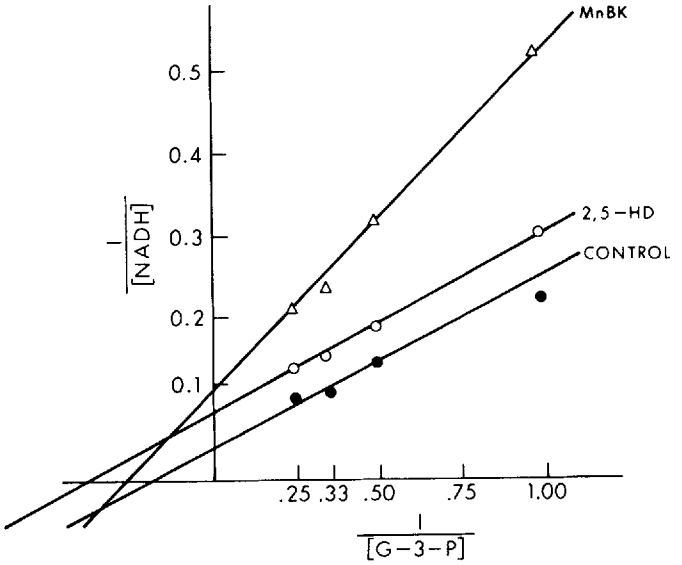

FIG. 4. Lineweaver–Burk plots for GAPDH. 500 μl of crystalline GAPDH and 500 μl of neurotoxic solvent or buffer were pre-incubated for 10 min at 37°C. Following pre-incubation, GAPDH activity was determined using different concentrations of the substrate G-3-P. Reciprocal rates of reaction (1/μmol NADH formed) were plotted against the reciprocal concentrations of the substrate (1/μmol G-3-P) (△) 12.5 mM-MnBK, (○) 12.5 mM 2,5-HD, (●) buffer control.

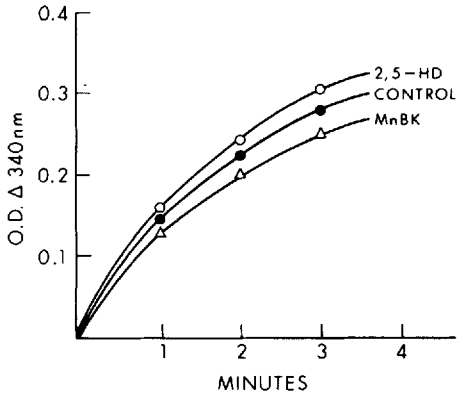

FIG. 5. Effect of 2,5-HD (50 mg/ml) and MnBK (10 mg/ml) on crystalline LDH activity. 500 μl of crystalline LDH (1 mg protein/ml) were pre-incubated with 500 μl of neurotoxic solvents. Following pre-incubation, LDH activity was determined as described in the text.

brain (Table 4). Under identical conditions, MnBK was more inhibitory than 2,5-HD, the latter being effective only at a 50 mM-concentration. By contrast, MnBK inactivated GAPDH activity completely at this concentration. Neither of these toxins inhibited LDH activity in rat brain homogenates.

DISCUSSION

This study demonstrates that the neurotoxic hexacarbon compounds 2,5-HD and MnBK inhibit the activity of GAPDH when either the crystalline enzyme or endogenous enzyme in brain or nerve tissues is incubated *in vitro*. By contrast, enzyme activity was unaffected by the neurologically inactive compounds 1,6-hexanediol and acetone (SPENCER *et al.*, 1978). The specificity of GAPDH inactivation was demonstrated by the failure of either 2,5-HD or MnBK to affect the activity of LDH.

The protective action of dithiothreitol on GAPDH function in the presence of MnBK or 2,5-HD suggests

TABLE 3. ENZYME ACTIVITIES IN RAT DESHEATHED NERVE EXPOSED TO 2,5-HD *in vitro*

Sample	μmol NADH formed per min per mg protein (GAPDH)	μmol NADH oxidized per min per mg protein (LDH)
Buffer control	0.067	0.403
2,5-HD	0.048	0.520

Desheathed sciatic nerves from four rats were pooled, pre-incubated with 50 mM-2,5-HD, homogenized and enzyme activities determined as described in the text. Each value is a mean of two analyses.

TABLE 4. EFFECT ON ENZYMES OF RAT BRAIN HOMOGENATES INCUBATED *in vitro* WITH 2,5-HD AND M*n*BK

Sample	μmol NADH formed per min per mg protein (GAPDH)	μmol NADH oxidized per min per mg protein (LDH)
Buffer control	0.80 ± 0.09	0.49 ± 0.03
2,5-HD (25 mM)	0.72 ± 0.06	0.49 ± 0.05
2,5-HD (50 mM)	0.64 ± 0.03	—
M*n*BK (25 mM)	0.51 ± 0.02	0.57 ± 0.06
M*n*BK (50 mM)	0.02 ± 0	—

Freshly prepared rat brain homogenate (10% w/v) was pre-incubated with 2,5-HD or M*n*BK for 10 min at 37°C. Controls were run in which the toxic compounds were replaced by buffer solutions. Following the incubation, tissue was quickly chilled and the enzyme activities determined as described in the text. Each value indicates the mean and S.D. of three experiments.

that neurotoxic hexacarbon compounds interfere with sulfhydryl groups of GAPDH required for enzyme activity. Whether the observed enzyme specificity of neurotoxic hexacarbons is associated only with GAPDH, or is linked to a number of other sulfhydryl enzymes is presently under investigation. The sulfhydryl reactivity of neurotoxic hexacarbons may be highly significant because many other compounds which induce axonal degeneration are also sulfhydryl reagents. For example, acrylamide which likewise produces a neurofilamentous distal axonopathy in experimental animals, causes a depression of glutathione when incubated with brain tissue *in vitro* (HASHIMOTO & ALDRIDGE, 1970). Furthermore, the neurotoxic action of acrylamide on chick ganglia tissue cultures, reported by SHARMA & OBERSTEINER (1977), is rendered ineffective if glutathione is incorporated into the culture medium. These investigators suggested that glutathione might be reacting with acrylamide, thereby reducing the effective concentration of the neurotoxic agent in the nutrient fluid exposed to the tissue culture.

Although the neurotoxic properties of hexacarbons have been studied extensively, and the pattern of CNS and PNS nerve fiber degeneration characterized in detail (see review by SPENCER & SCHAUMBURG, 1976), the locus of toxic damage and metabolic etiology of neurotoxic action have not been addressed experimentally. Prior to the morphological observations of SPENCER & SCHAUMBURG (1977), it was widely assumed that retrograde or 'dying-back' axonal degeneration resulted from toxic damage to the anabolic machinery of the neuronal perikaryon. Study of the spatial–temporal pattern of nerve fiber damage revealed the pattern of degeneration to be more compatible with an *axonal* locus of toxic damage. The present observations demonstrate for the first time that these neurotoxic compounds do interfere directly with the metabolism of the nerve fiber. Although it remains to be demonstrated that neurotoxic interference with glycolysis *in vitro* is associated with the causation of nerve fiber degeneration in man and experimental animals, these data are consistent with local axon toxicity of these compounds.

A notable feature of the inactivation of GAPDH by neurotoxic hexacarbon compounds is the high concentration and extended preincubation time required to achieve maximal enzyme inhibition. These are important observations which may relate to the time course of the disease process in animals or man following exposure to these compounds. Experimental studies have consistently demonstrated that neurotoxic hexacarbons must be administered repeatedly over a period of many weeks before nerve fiber changes begin to appear. It is conceivable that during this period of silence, the neurotoxins are progressively inactivating GAPDH activity in the nerve fiber axon. Presumably, enzyme would be resupplied from the neuronal perikaryon via the axonal transport system. As the inactivation of GAPDH progressed, axonal demand for enzyme replacement might increase beyond the capacity of the neuron to respond; proximal portions of the axon would be resupplied with GAPDH, leaving inadequate amounts of enzyme available for distal regions. Such events would presumably occur first in large neurons maintaining the greatest volume of axoplasm and therefore the largest metabolic load. These ideas

may explain the morphological observation that the distal regions of the largest diameter nerve fibers degenerate before equivalent regions of smaller diameter fibers, and longer fibers degenerate before shorter fibers of similar diameter (SPENCER & SCHAUMBURG, 1976).

The observed inhibition of GAPDH would be expected to block fast axoplasmic transport, since this is an energy consuming phenomenon dependent on the normal function of this glycolytic enzyme (SABRI & OCHS, 1971, 1972). This prediction is consistent with the observation in experimental hexacarbon neuropathy that the rate of fast axonal transport decreases progressively as the front travels down affected nerve fibers (MENDELL *et al.*, 1977). Fast transported material is impeded maximally in its passage across the axonal swellings, which develop just above nodes of Ranvier in the distal axon and herald the onset of axonal degeneration (GRIFFIN *et al.*, 1978, in press). GRIFFIN *et al.* (in press) suggest that the slowing of fast transport is a secondary event caused by the presence of a large number of 10 nm neurofilaments in the swellings. This view is strengthened by the observation that fast transport is normal prior to development of analagous neurofilamentous axonal swellings following intoxication with β-β'-iminodipropionitrile (IDPN). The neurofilament accumulations in IDPN neuropathy are associated with a selective blockade in the proximal axon of neurofilament proteins which are carried in a slow phase of axonal transport (GRIFFIN *et al.*, 1978). Since it is possible that impeded slow transport also accounts for the neurofilamentous swellings in hexacarbon neuropathy, it seems clear that this phenomenon must be illuminated before the etiology of hexacarbon neuropathy can be understood. Whether all types of axonal transport mechanisms are energy dependent (KOENIG, 1969), such that inhibition of glycolysis also causes a blockade of slowly transported materials, is an issue which remains to be resolved.

It is widely believed that the neurotoxicity of *n*-hexane and M*n*BK results from the neurotoxic activity of 2,5-hexandione, the metabolite to which both solvents are oxidized *in vivo*. This view is strengthened by the observation that the neurological activity of metabolically related neurotoxic hexacarbons is positively correlated with measured amounts of 2,5-HD in the serum of intoxicated animals (KRASAVAGE *et al.*, 1978). The present finding that M*n*BK, rather than 2,5-HD, shows a greater degree of GAPDH inhibition *in vitro* qualifies these earlier findings. Our biochemical observations, coupled with recent preliminary data obtained from nerve tissue cultures exposed to hexacarbon metabolites, suggest that M*n*BK is neurologically more active than 2,5-HD (B. Veronesi, unpublished observations). These findings are not inconsonant with those of KRASAVAGE and co-workers because M*n*BK is known to be rapidly oxidized *in vivo* to the more persistent neurotoxic compound 2,5-HD (DI VINCENZO *et al.*, 1976).

In summary, these studies demonstrate that the neurotoxic hexacarbons 2,5-HD and M*n*BK are capable of inhibiting the glycolytic enzyme GAPDH. Although the relationship of these observations to the causation of nerve fiber damage is uncertain, the known dependency of axonal transport function on glycolysis, coupled with the known failure of fast axonal transport in distal axons during hexacarbon intoxication, suggests that these observations are more than coincidental, and may be related to the mechanism of nerve fiber degeneration (SPENCER *et al.*, in press).

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