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In Vitro Effect of *n*-Hexane and Its Metabolites on Selected Enzymes in Glycolysis, Pentose Phosphate Pathway and Citric Acid Cycle

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The effect of *n*-hexane, 2-hexanol, 5-hydroxy-2-hexanone, 2,5-hexanediol, methyl *n*-butyl ketone (MnBK) and 2,5-hexanedione (2,5-HD) has been studied in vitro on crystalline glyceraldehyde-3-phosphate dehydrogenase (GAPDH), DL-glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating) EC. 1.2.1.12 and phosphofructokinase (PFK) ATP: D-fructose-6-phosphate-1-phosphotransferase; EC. 2.7.1.11 and lactic dehydrogenase (LDH) L-lactate: NAD^+ oxidoreductase, EC. 1.1.1.27. MnBK and 2,5-HD both inhibited GAPDH and PFK activities selectively. *n*-Hexane and 2-hexanol had no effect on GAPDH and PFK activities; 5-hydroxy-2-hexanone and 2,5-hexanediol exhibited a slight inhibitory effect on these enzymes. Neither metabolites of *n*-hexane have any effect on LDH activity. 2,5-Hexanedione did not inhibit transketolase (D-sedoheptulose-7-phosphate: D-glyceraldehyde-3-phosphate glycolaldehyde transferase, EC. 2.2.1.1) and succinate dehydrogenase (succinate: 2,6-dichlorophenol-indophenol oxidoreductase, EC. 1.3.99.1) activities. The levels of ATP were reduced in 2,5-HD-treated cat sciatic nerves and returned to normal levels by exposing the nerve to sodium pyruvate.

INTRODUCTION

Central-peripheral distal axonopathy is the pathological substrate of many neurotoxic diseases which induce in man and animals the clinical picture of distal symmetrical polyneuropathy[25,26]. Some of these diseases, such as those induced by repetitive administration of acrylamide, *n*-hexane or its oxymetabolites, induce a multifocal accumulation of 10-nm neurofilaments in distal axons prior to overt nerve-fiber breakdown. The underlying biochemical mechanism of these morphological changes is not understood, although toxic inhibition of selected glycolytic enzymes is associated with nerve fiber degeneration in acrylamide and hexacarbon neuropathies[8–10,15,16,21,22,24].

Since glycolysis is the primary pathway for the oxidation of glucose, inhibition of glycolytic enzymes by 2,5-HD in nerve tissue would lead to decreased ATP, resulting in blockade of the energy-dependent axonal transport system[18]. However, it is not known whether glycolytic enzyme inhibition by 2,5-HD is selective, and other enzymes in intermediary metabolism are also inhibited by this neurotoxin. To examine this, the effect of 2,5-HD on the activity of transketolase, an enzyme of the pentose-phosphate pathway, and succinic dehydrogenase, an enzyme of the citric acid cycle, has been studied. In addition, the in vitro effect of *n*-hexane and its metabolites on glycolytic enzymes has been studied and the data are presented in this communication. A preliminary account of this study has been presented[20].

MATERIALS AND METHODS

Materials

The following chemicals were obtained from Sigma Chemicals (St. Louis, MO): a suspension of crystalline glyceraldehyde-3-phosphate dehydrogenase

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(GAPDH) in 2.6 M $(NH_4)_2SO_4$ from rabbit muscle: a suspension of crystalline phosphofructokinase (PFK), type III from rabbit muscle in 1.4 M $(NH_4)_2SO_4$; a suspension of crystalline lactate dehydrogenase (LDH), type II, from rabbit muscle in 2.1 M $(NH_4)_2SO_4$, triose phosphate isomerase, aldolase, alpha-glycerophosphate dehydrogenase, nicotinamide adenine dinucleotide (NAD), reduced NAD (NADH), sodium pyruvate and DL-glyceraldehyde-3-phosphoric acid (G-3-P). 2,5-Hexanedione (2,5-HD), *n*-hexane, 2-hexanol, 2,5-hexanediol and 1,6-hexanediol (1,6-HDiol) were obtained from Eastman Kodak, Rochester, NY. Methyl *n*-butyl ketone (MnBK) (commercial grade) was obtained from Tennessee Eastman, Kingsport, TN. All other reagents were of analytical grade.

In vitro treatment of enzymes with hexacarbons

In a typical experiment, 500 μl of the enzyme solution of either GAPDH, PFK or LDH, 1 mg/ml, were mixed with 500 μl of the test agent diluted to a desired concentration with appropriate buffer and incubated at 37 °C for 20 min in a shaking water-bath. In a control experiment, the test agent was replaced with an equivalent amount of buffer. Following preincubation, the samples were cooled on ice for 5 min and the enzyme activity determined immediately.

Rat liver, brain homogenates or mitochondria were pretreated with 2,5-HD as follows: liver and brain homogenates (5% w/v) were prepared in ice-cold 0.25 M sucrose. The tissue homogenates and mitochondrial suspension (500 μl) were pretreated for 20 min with 2,5-HD at the desired concentration. In the control experiment, brain or liver homogenate was pre-incubated with buffer alone. After incubation, the tubes were cooled on ice, and the enzyme activity determined immediately.

Assay for transketolase activity

Transketolase activity was determined spectrophotometrically by a slightly modified method of Datta and Racker[1]. This assay of transketolase activity is based on the formation of G-3-P which is measured spectrophotometrically by coupling the reaction with NADH. The reaction mixture contained the following reagents in a final volume of 1.0 ml: 465 μl of buffer 'B' (containing 0.028% NaCl, 0.925% KCl, 0.029% $MgSO_4$ and 0.273% K_2HPO_4) (pH 7.4), 100 μl of 2 mM xylulose-5-P, 100 μl of 4 mM ribose-5-phosphate, 100 μl of 0.2 mM thiamine pyrophosphate, 100 μl of 0.17 M disodium arsenate, 25 μl of tissue homogenate, 10 μl of crystalline GAPDH, 1 mg/ml. The enzyme reaction is started by the addition of 100 μl of 0.02 M NAD and the increase in the optical density due to formation of NADH is measured at 340 nm for 2 min.

Assay for succinic dehydrogenase

Succinic dehydrogenase activity in rat brain homogenate and crude mitochondria was assayed spectrophotometrically by the method of Veeger et al.[28].

The crude mitochondria were prepared as follows: an adult male rat was decapitated and the brain rapidly removed. A 5% (w/v) homogenate was prepared in ice-cold 0.25 M sucrose. The homogenate was centrifuged at 2000 *g* for 10 min at 4 °C. The supernatant was spun at 12,000 *g* for 15 min and the pellet suspended in 0.25 M sucrose. The suspension was layered on 0.85 M sucrose and centrifuged at 75,000 *g* for 30 min. The crude mitochondria in the pellet was suspended in 0.1 M sodium phosphate buffer (pH 7.6). Fresh mitochondria were prepared just before each experiment.

The enzyme reaction was carried out in test tubes in a final volume of 3.0 ml of the following composition: 0.5 ml of 0.3 M phosphate buffer (pH 7.6); 0.3 ml of 0.4 M sodium succinate (pH 7.6); 0.1 ml of 3% (w/v) bovine serum albumin; 0.1 ml of KCN; 30 mM 2,6-dichlorophenol-indophenol; 0.2 ml of tissue homogenate or mitochondrial suspension and 1.5 ml distilled water. The tubes were incubated for 5 min at 37 °C. Following incubation, samples were cooled on ice for 5 min, centrifuged at 100,000 *g* for 15 min and the optical density (O.D.) of 2,6-dichlorophenol indophenol determined at 600 nm. Tissue samples heated in boiling water for 5 min were used in control experiments. The difference in the O.D. between the heated and unheated sample was used to calculate enzyme activity. In this assay, 1 mole of succinate reduces 1 mole of dye.

Assay for GAPDH activity

GAPDH activity was assayed by the procedure of Sabri et al.[16]. The incubation medium (3.0 ml) had the following composition: 2.52 ml of 0.03 mM sodium pyrophosphate buffer (pH 8.5), 23.5 μg of crys-

talline GAPDH in 10 μl buffer, 0.05 ml of 0.02 M NAD, 0.1 ml of 0.1 M cysteine-HCl in pyrophosphate buffer, and 0.3 ml of 0.17 M disodium arsenate. The enzyme 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 optical density at 340 nm due to NADH formation was monitored for 2–3 min in a Gilford 250 spectrophotometer equipped with a recorder. The temperature of the cuvette compartment was maintained at 37 ± 1.0 °C during the course of the reaction by a constant-temperature circulating bath. The enzyme activity was calculated in the first 30 s of the reaction where it was linear. The enzyme activity was expressed as specific activity (spec. act.) (units/mg protein). One unit of enzyme is equivalent to 1.0 μmol of glyceraldehyde-3-phosphate oxidized per min.

Assay for PFK activity

The assay for PFK was based on the phosphorylation of fructose-6-phosphate to form fructose 1,6-diphosphate, which was determined enzymatically by coupling the reaction to the oxidation of NADH with the use of aldolase, triose phosphate isomerase (TPI) and alpha-glycerophosphate dehydrogenase (GPDH)[11,15]. The incubation medium (3.0 ml) had the following composition: 0.5 ml of 0.2 M Tris-HCl buffer (pH 8.0), 0.05 ml of 0.02 M ATP (pH 7.0), 0.05 ml of 0.2 M $MgSO_4$, 0.5 ml of 0.2 M KCl, 0.03 ml of 0.1 M dithiothreitol and 0.1 ml of an auxilliary enzyme solution (containing 0.2 ml of aldolase, 10 mg/ml; and 0.2 ml of the mixture of GPDH, (97 units/mg protein) and TPI, (744 units/mg protein), 9.8 mg/ml, dissolved in 4.6 ml of 0.02 M Tris-HCl (pH 8.0), 0.1 unit of PFK in 3 μl, 1.517 ml water and 0.2 ml of 2.4 mM NADH. The enzyme reaction was started by the addition of 50 μl of fructose-6-phosphate (0.02 M). The assay was run at 37 °C in a Gilford spectrophotometer, and the reaction monitored by the decrease in O.D. at 340 nm for 1 min.

The enzyme activity was expressed as spec. act. (units/mg protein). One unit will convert 1.0 μmol of fructose-6-phosphate to fructose-1,6-diphosphate per min, and each mole of fructose-1,6-diphosphate formed leads to the oxidation of 2 moles of NADH.

Assay for lactate dehydrogenase (LDH) activity

LDH activity was determined spectrophotometrically at 340 nm[16,27]. The reaction mixture (3.0 ml) had the following composition: 2.79 ml 0.1 M phosphate buffer (pH 7.2), 10 μl of enzyme containing 1 μg of LDH and 100 μl of freshly-prepared 2.4 mM NADH. The reaction was started by the addition of 100 μl of 0.66 mM pyruvate into the cuvette and the decrease in O.D. was monitored for 1 min at 340 nm.

The enzyme activity was expressed as spec. act. (units/mg protein). One unit LDH will reduce 1.0 μmol of pyruvate per min under experimental conditions.

Determination of ATP and CP levels in cat sciatic nerves

Two young adult cats were anesthetized with sodium pentabarbitol (30 mg/kg), and sciatic nerves with tibial branches removed bilaterally. The nerves were quickly transferred to an Erlenmeyer flask containing 50 ml of Krebs-Ringer (pH 7.4) with glucose, and the solution vigorously bubbled with 95% O_2 + 5% CO_2 for 30 min. Nerves were transferred to flasks containing 2,5-HD or 1,6-hexanediol (a non-neurotoxic hexacarbon) dissolved in Krebs-Ringer (pH 7.4). In one sample, 200 mM pyruvate was added along with 200 mM 2,5-HD and incubated for 2 h at 37 °C under oxygen. The control nerve sample was incubated with Ringer solution alone.

Following incubation, nerves were quickly removed and frozen in Freon-12 that had been cooled to just above its freezing point in liquid nitrogen. The frozen nerves were crushed to a fine powder in a mortar and pestle (pre-cooled in acetone chilled with dry-ice), 2.5 ml of 5% (v/v) perchloric acid (cooled in acetone–dry-ice mixture) added, and the frozen samples were transferred and homogenized in a Potter-Elvehjem homogenizer at 0 °C. The homogenate was centrifuged at 10,000 *g* at —10 °C for 10 min. The clear supernatant was neutralized with 6 M K_2CO_3 to pH 7.5, and the ATP and CP determined as described by Sabri and Ochs[18].

Protein determination

The protein determination was carried out by the method of Lowry et al.[12] using bovine serum albumin as the standard.

TABLE I

Effect of n-hexane and its metabolites on glycolytic enzymes in vitro

Crystalline GAPDH, PFK and LDH (1 mg/ml) from rabbit muscle were exposed to hexacarbons as described in Methods. The test compound was diluted in appropriate buffer (final concentration 25 mM). In the control, the hexacarbon was replaced by an equivalent amount of buffer. Enzyme activities of GAPDH, PFK and LDH were determined as described. Each value is the mean ± standard deviation (S.D.) from 3 separate experiments.

Hexacarbon	*μmol/min/mg protein*		
	GAPDH	*PFK*	*LDH*
Buffer control	9.9 ± 1.3	247.8 ± 10.2	80.6
n-Hexane	9.5 ± 0.4	256.2 ± 13.6	91.4
2-Hexanol	8.5 ± 0.7	246.3 ± 11.6	75.3
2,5-Hexanediol	7.4 ± 0.4	262.8 ± 22.6	88.7
5-Hydroxy-2-hexanone	8.5 ± 0	194.4 ± 16.5	90.5
Methyl *n*-butyl ketone	3.8 ± 0	175.6 ± 21.6	86.0
2,5-Hexanedione	5.4 ± 0	116.7 ± 17.7	80.6

RESULTS

In vitro effects of hexacarbons on glycolytic enzymes

The in vitro effect of the 6 interrelated metabolites of *n*-hexane on glycolytic enzymes is given in Table I. *n*-Hexane and 2-hexanol had no inhibitory effect on GAPDH, PFK or LDH activities. Only 2,5-HD and MnBK were found to inhibit GAPDH and PFK activities (Table I). After 20 min of pre-incubation, MnBK inhibited GAPDH activity by 62% and PFK activity by 30%, whereas an equivalent concentration of 2,5-HD inhibited GAPDH and PFK activities by 46% and 53%, respectively. 2,5-Hexanediol produced 25% inhibition of GAPDH activity but had no effect on PFK activity. 5-Hydroxy-2-hexanone induced a 22% inhibition of PFK activity but had no effect on GAPDH activity. LDH activity was not inhibited by any of the metabolites.

Effect of 2,5-HD on transketolase activity

The in vitro effect of 2,5-HD on the transketolase activity in rat tissue homogenates is given in Table II. 2,5-HD does not inhibit transketolase activity in brain or liver homogenates pre-incubated for 20 min at 37 °C with 25–100 mM toxin.

Effect of 2,5-HD on succinic dehydrogenase activity

The effect of 2,5-HD on succinic dehydrogenase activity in rat brain homogenate and crude mitochondrial suspension is given in Table III. After 20 min of pre-incubation with 5–25 mM, 2,5-HD does not alter succinic dehydrogenase activity of homogenate or mitochondria. These data strengthen the notion that 2,5-HD inhibition may be restricted to glycolysis. A 3-fold enrichment of succinic dehydrogenase activity is evident in the mitochondria relative to that in the homogenate (Table III).

TABLE II

Effect of 2,5-hexanedione on transketolase activity in vitro

Adult male rat brain and liver homogenates (5% w/v) were prepared in cold saline. 200 μl of tissue homogenate were preincubated with an equivalent volume of 2,5-HD (final concentration 25–100 mM) for 20 min at 37 °C. Transketolase activity was determined in triplicate in 25 μl aliquots. Each value is the mean ± standard deviation (S.D.) from three separate experiments.

Tissue	*μmol/min/mg protein*
Brain	
Buffer (control)	0.228 ± 0.020
2,5-HD (25 mM)	0.285 ± 0.025
2,5-HD (50 mM)	0.226 ± 0.040
2,5-HD (100 mM)	0.214 ± 0.012
Liver	
Buffer (control)	0.260 ± 0.015
2,5-HD (25 mM)	0.230 ± 0.011
2,5-HD (50 mM)	0.232 ± 0.010
2,5-HD (100 mM)	0.232 ± 0.014

TABLE III

In vitro effect of 2,5-hexanedione on succinic dehydrogenase activity in rat brain

500 μl brain homogenate or crude mitochondria prepared from it, were preincubated with an equivalent volume of 2,5-HD (final concentration 5–25 mM) for 20 min at 37 °C. In the control experiments, 2,5-HD was replaced by buffer. After preincubation samples were cooled on ice for 5 min and succinic dehydrogenase activity determined immediately in 200 μl aliquots. Each value is the mean ± standard deviation (S.D.) from three experiments.

Toxin concentration	*μmol/min/mg protein*	
	Crude mitochondria	*Homogenate*
Buffer (control)	0.038 ± 0.003	0.014 ± 0.002
2,5-HD (5 mM)	0.041 ± 0.003	0.013 ± 0.001
2,5-HD (10 mM)	0.040 ± 0.003	0.018 ± 0.002
2,5-HD (25 mM)	0.040 ± 0.003	0.020 ± 0.002

TABLE IV

In vitro effect of neurotoxic 2,5-HD and non-neurotoxic (1,6-HDiol) on ATP and CP levels in cat sciatic nerve

Cat sciatic nerves were treated with 2,5-HD, sodium pyruvate or 1,6-hexanediol in an atmosphere of 95% O_2 5% CO_2 as described in Methods. Following exposure, the nerves were frozen in Freon-12 cooled in liquid nitrogen and ATP and CP levels determined. Each value is the mean of four sciatic nerves from 2 animals.

Treatment	*μmol/100 g nerve*	
	ATP	*CP*
Buffer (control)	44.4	17.5
2,5-HD (50 mM)	39.2	—
2,5-HD (100 mM)	37.6	18.7
2,5-HD (200 mM)	25.4	12.5
2,5-HD (200 mM) + Pyruvate (200 mM)	46.4	14.0
1,6-HDiol (100 mM)	41.7	—
1,6-HDiol (200 mM)	40.8	21.5

Effect of 2,5-HD on ATP and CP levels

The levels of ATP and CP in control cat sciatic nerves are given in Table IV. ATP, but not CP, was decreased in nerves exposed to 2,5-HD, and the degree of decrease was dependent on 2,5-HD concentration. Thus, 50 mM 2,5-HD produced a 10% decrease and 200 mM 2,5-HD caused a 42% decline in ATP levels. Addition of 200 mM sodium pyruvate to the incubation medium restored the concentration of ATP to normal levels, even in the presence of 200 mM 2,5-HD. By contrast, 100–200 mM 1,6-hexanediol, a non-neurotoxic hexacarbon[23] had no effect on ATP or CP levels in the nerve. This indicates that the locus of inhibition might lie in glycolysis.

DISCUSSION

The present study demonstrates that MnBK and 2,5-HD, the neurotoxic metabolites of *n*-hexane, are inhibitory to the glycolytic enzymes GAPDH and PFK. While the concentration of the metabolites used in the present study are higher than those likely to be found in animals exposed to hexacarbons, our recent studies have shown that if the time of pre-incubation of enzyme and toxin is extended to 2 h, concentrations as low as 1 mM 2,5-HD will inhibit GAPDH activity[22]. Thus, in vitro inhibition of GAPDH by 2,5-HD appears to be progressive in nature. At equimolar concentrations, 2,5-HD and MnBK were the most potent compounds for the induction of axonal degeneration in tissue culture[29]. Data on the relative neurotoxicity of *n*-hexane and its metabolites in rats (O'Donoghue and Krasavage[14]) likewise showed that *n*-hexane was the least and 2,5-HD the most effective neurotoxic compound in rats and that the onset of hexacarbon neuropathy was correlated with the serum concentration of 2,5-HD.

The inhibition of GAPDH by 2,5-HD is similar to the inhibition with IAA[17] in cat sciatic nerve. IAA is known to inhibit GAPDH activity by reacting with thiol groups essential for enzyme activity; it is therefore possible that 2,5-HD-induced inhibition of GAPDH activity is also mediated by a similar mechanism. Earlier studies[16] showing inhibition can be prevented by dithiothreitol support this notion. Other investigators[2,5,6] have reported that 2,5-HD forms stable conjugated Schiff bases or forms dimethyl pyrroles with tissue proteins. Another hypothesis suggested a defect in sterol synthesis by 2,5-HD[3,4].

This study demonstrates a relatively selective in vitro inhibition of glycolytic enzymes (GAPDH and PFK) but not of pentose phosphate shunt (transketolase) or tricarboxylic acid cycle (succinic dehydrogenase) by 2,5-HD. Reduction in nerve ATP levels following 2,5-HD treatment and restoration of ATP by treatment with sodium pyruvate, strengthen the notion that 2,5-HD inhibition may be restricted to certain enzymes in glycolysis. A non-neurotoxic hexacarbon, 1,6-hexanediol[23], neither decreases the levels of ATP in the nerve nor inhibits nerve GAPDH activity[16] suggesting a relatively selective inhibition of glycolytic enzymes by 2,5-HD.

The inhibition of GAPDH activity by 2,5-HD and consequent drop in ATP levels in the nerve would be expected to block energy-dependent fast axoplasmic flow[18]. This prediction is borne out by data reporting that the rate of fast axonal transport is reduced in experimental animals exposed to hexacarbons[13,19]. Griffin et al.[7] observed that the fast transported material was impeded maximally in the passage across swellings which appeared in distal axons in 2,5-HD neuropathy. It remains to be demonstrated, however, if interference with glycolytic enzymes underlies nerve fiber degeneration in hexacarbon neuropathy.

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