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Effects of neurofilamentous axonopathy-producing neurotoxicants on in vitro production of ATP by brain mitochondria

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The site and mode of action of acrylamide (ACR), γ-diketone hexacarbons and 3,3′-iminodipropionitrile (IDPN) in producing a neurofilamentous axonopathy are unknown. Whether the neuropathy is caused by reductions in axonal transport produced by energy depletion is under investigation. Reductions in the quantity of proteins fast transported following a single dose of ACR or neurotoxic γ-diketones have been reported[25,26]. The current study examines the in vitro effects of these toxicants upon ATP production by mitochondria. Isolated rat brain mitochondria incubated for 30 min at 37 °C with neurotoxic doses of ACR (0.7 mM) or 3,4-dimethyl-2,5-hexanedione (0.25 mM) retained similar capacities for synthesis of ATP from pyruvate and endogenous concentrations of ATP compared to controls. 2,5-Hexanedione (2,5-HD; 4 mM) and IDPN (0.1%) significantly reduced the rate of synthesis (–22.5% and –15%, respectively); but only 2,5-HD decreased the endogenous concentration of ATP (–21.6%) following a single 30 min exposure. Toxicant action on ATP production is limited to 2,5-HD; the lack of correlation between the toxicant-induced changes in axonal transport and mitochondrial ATP production demonstrate the necessity to evaluate other structures as the critical site of action in producing axonal transport changes.

INTRODUCTION

Peripheral neuropathy of the neurofilamentous axonopathy type is the consequence of inadvertant exposure to a diversity of chemicals. Included in the list of environmental contaminants which produce this disorder are the prototypic chemicals acrylamide (ACR), 2,5-hexanedione (2,5-HD), 3,4-dimethyl-2,5-hexanedione (DMHD) and 3,3′-iminodipropionitrile (IDPN). The common manifestation of this neural disorder is the appearance of swellings at specific locations along the nerve which are filled with neurofilaments, the neuron-specific intermediate filament[31]. Following repeated exposure to ACR, 2,5-HD or DMHD, degeneration of the distal nerve fiber occurs; axonal atrophy, *without degeneration* is the result of IDPN exposure. While significant differences between the neuropathy produced by these chemicals have been reported, it is frequently implied or directly stated that the mechanism of action is similar for these neurotoxicants[22], at least for those toxicants which produce the degeneration.

Potential mechanisms of action of these chemicals receiving primary attention are the 'neurofilamentous' and the 'energy' hypotheses. The former predicts that these chemicals produce chemical modification of the neurofilament cytoskeletal proteins[4–6,22]. The energy hypothesis predicts that these toxicants inhibit neuron-specific isoenzymes of energy transformation pathways, producing depletion of high energy phosphates[7–9,30].

Both hypotheses suggest that a block of axonal transport is the consequence of these actions. Slow anterograde transport is altered by ACR, γ-diketones and IDPN but, it is difficult to determine whether these are primary or secondary (see refs. 25 and 26 for more details). Retrograde axonal transport is reduced at both acute and chronic time frames[11,12]. Although earlier reports demonstrated little change in fast anterograde axonal transport, use of acute time points of study have revealed significant reductions in protein transport following single injections of ACR and γ-diketones, but not by IDPN and non-neurotoxic analogues of the former[25,26,29]. These data demonstrate a correlation between the toxicant-induced compromise of fast axonal transport with distal nerve degeneration by the same toxicants.

Changes in fast axonal transport could be the result of depletion of high energy phosphates caused by inhibition of oxidative enzymes. Inhibitions of oxidative enzymes (NADH-tetrazolium reductase) by single doses of ACR have been reported[23,27,28]. However, the degree of inhibition was minor and, in some instances, not neuron-specific[24]. Instead of laborious trial-by-trial enzyme analyses of toxicant effects, the prudent approach of mea-

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suring the effects of these neurotoxicants (ACR, 2,5-HD, 3,4-DMHD, IDPN) on neural mitochondria ATP production from pyruvate was employed. The same time and dose of exposure to the toxicants which produced the axonal transport changes was chosen to permit determination of a cause-effect relationship between changes in ATP production and axonal transport deficiencies.

MATERIALS AND METHODS

Rat brain mitochondria isolation[17]

Male, adult Sprague–Dawley rats of 250–300 g body weight were decapitated without anesthesia, the brains were removed immediately, stripped of pia mater and homogenized with a teflon/glass homogenizer for 30 s in 10 volumes of cold (4 °C), 10 mM potassium phosphate buffer (pH 7.4) containing 300 mM mannitol and 1 mM EDTA. All subsequent steps were done at 4 °C. The homogenate was centrifuged at 600 *g* for 8 min. The supernatant was recentrifuged at 10,000 *g* for 10 min. The pellet was resuspended in the same solution, centrifuged at 10,000 *g* for 10 min, the pellet resuspended and diluted, as specified in the method of LeMasters and Hackenbrock[14], to a concentration of 1 mg protein/ 10 μl of solution (protein assay of Bradford[3]; using bovine serum albumin as standard) with the same buffer. Initially, an additional cycle of centrifugation and suspension was used to further purify the mitochondrial fraction. Even though the resultant fraction contained a significantly greater proportion of mitochondria (verified with electron microscopy), the oxidative phosphorylation capacity of the resultant fraction was inadequate for use. Proper function of mitochondrial fractions was verified by addition of 1 mM dinitrophenol (DNP; uncoupler of oxidative phosphorylation) and omission of the substrate (pyruvate).

Neurotoxicant exposure

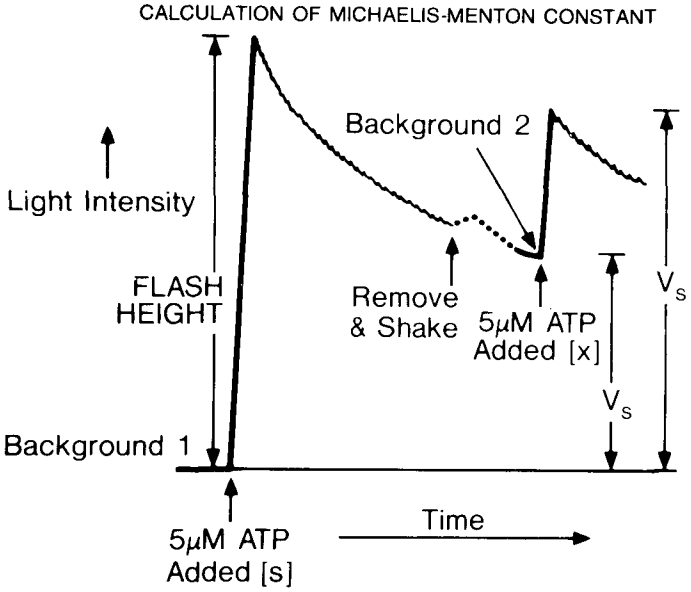
Aliquots of the fraction were diluted with an equal volume of buffer containing the neurotoxicants such that final concentrations of 0.7 mM ACR, 4 mM 2,5-HD, 0.25 mM DMHD, and 8.12 mM (0.1%) IDPN were reached. Control aliquots were diluted with an equal volume of buffer only; one control and one experimental was done each day. Both controls and experimentals were incubated at 37 °C for 30 min, then immediately returned to an ice bath.

ATP assay

ATP concentration and production were determined using a firefly luciferin-luciferase bioluminescence assay developed by LeMasters and Hackenbrock[14] and modified by us for use in a Beckman LS1801 liquid scintillation counter. This is the only known method for direct measurement of ATP production rate; in addition, the ATP concentrations with this method are equivalent to more recent HPLC methods (unpublished results). Purified luciferin-luciferase containing $MgSO_4$ and EDTA in a human albumin base in glycine buffer was used exclusively (Sigma product no. L0633). The assay media contained: 155 mM sucrose, 5 mM $MgCl_2$, 10 mM K_2HPO_4, 10 mM KH_2PO_4, 40 μg luciferin-luciferase and 1 mg mitochondrial protein (proper quantity for easily measured response) in a total of 1 ml of assay media. The scintillation counter was set on single photon monitor mode (single set of photomultiplier tubes) and an interval counting time of 6 s. All assays were conducted with the room lights off, a small quantity of light straying in from the hallway was adequate for the investigator to operate the instrument without interfering with the assay.

Calculation of Michaelis–Menton constant

The Michaelis–Menton rate constant for the luciferase enzyme activity was calculated under control and experimental conditions in order to adjust for direct toxicant effects upon the assay. Control vials contained all of the components of the assay media listed

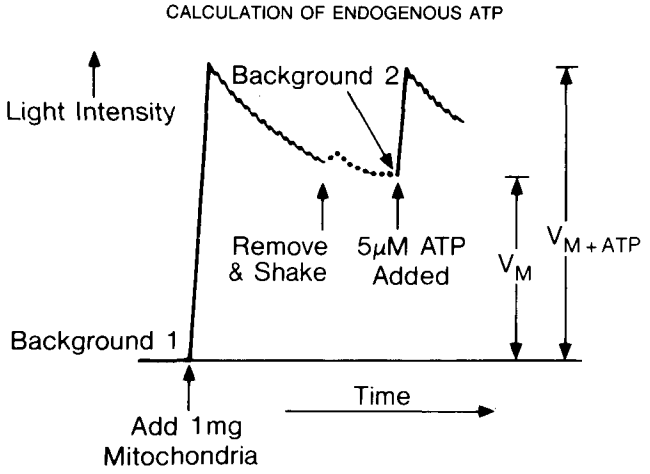


$$K_S = \frac{[s]\,(1-V_S/V_{S+X})}{V_S/V_{S+X} - [s]/([s]+[x])}$$

$$V_S = \text{Background 2} - \text{Background 1}$$

$$V_{S+X} = \text{Peak 2} - \text{Background 1}$$

Fig. 1. Diagram of the changes in cpm during the procedures used to determine the Michaelis–Menton constant of the bioluminescence reaction and the equations used to calculate the constant (see text for details).



$$[ATP_M] = \frac{5\mu M\,(V_M/V_{M+ATP})}{(V_S/V_{S+X})}$$

or

$$[s] = \frac{-(K_S+[x])}{2} + \sqrt{\frac{V_S/V_{S+X}\,(K_S-[x])^2 - (K_S+[x])^2}{4\,(V_S/V_{S+X}-1)}}$$

Fig. 2. Diagram of the changes in cpm during the procedures used to determine the concentration of ATP in rat brain mitochondria and the equations used to calculate the concentration (see text for details).

above; the experimentals differed from controls by containing mitochondria which had been previously exposed to each toxicant. Therefore, the effects of any neurotoxicant, carried over from the preincubation step, were measured. The constant was determined as follows (see Fig. 1): the background cpm was measured, then 5 μM of ATP was added to the vial; this interacts with luciferin/luciferase to produce light by the following reaction.

$$\text{Luciferase} + \text{luciferin} + \text{ATP} \xrightarrow{Mg} \text{luciferase-luciferin-AMP-PPi}$$

$$\text{luciferase-luciferin-AMP-PPi} + O_2 \rightarrow \text{Luciferase} + CO_2 + \text{AMP} + \text{oxyluciferin} + \text{LIGHT}$$

The light produced is measured by the scintillation counter. With the introduction of the bolus of ATP ([s]), the cpm rapidly increased to a peak. As the concentration of oxyluciferin (inhibitor) increased, the cpm slowly declined. When this occurred, the vial was removed, shaken and returned to the instrument for measurement of background no. 2. The vial was again removed and another 5 μM ATP ([x]) added. The cpm rose rapidly to a second peak and began to fall slowly with the further increase in oxyluciferin. Knowing: (1) the initial ATP concentration added (abbreviated as [s]), (2) the second ATP concentration (abbreviated as [x]), (3) the cpm of background before addition of [x] (second 5 μM ATP) minus background prior to addition of [s] (first ATP bolus)] known as V_s, and (4) the cpm at second peak minus background cpm prior to addition of initial ATP known as V_{s+x}; the Michaelis–Menton constant (K_s) may be determined using a rearrangement of the Michaelis–Menton equation (shown in Fig. 1).

Endogenous mitochondrial ATP concentration

The ATP content of the isolated mitochondrial fraction was determined by a method similar to that used to establish the Michaelis–Menton constant (Fig. 2). The difference is replacement of the first ATP addition, used in the K_s determination, with an aliquot of mitochondria (1 mg protein). Since the K_s for the reaction was previously determined, rearrangment of Michaelis–Menton equation solving for the initial concentration of ATP added ([s] in previous equation) permitted calculation of the initial concentration of ATP added which, in this case, was the ATP in 1 mg of isolated mitochondria (Fig. 2, eqn. 1). In addition to determining the endogenous ATP concentration of each control and experimental sample which was necessary for calculations of the ADP/AMP ratio to add for oxidative phosphorylation (see below), we determined the effect of the preincubation of each toxicant upon the ATP content in the same mitochondrial sample. Mitochondria were isolated as above, an aliquot of each was preincubated with buffer only (control) or buffer with 0.7 mM ACR, 4 mM 2,5-HD, 0.25 mM DMHD or 0.1% IDPN for 30 min at 37 °C. The endogenous ATP concentration of control and each experimental was determined for 3 different mitochondrial samples. This was a more controlled experiment than extracting these figures from the daily experiments since daily variations in percentage of mitochondria in the isolated fraction could introduce variation in endogenous ATP.

Oxidative phosphorylation

The concentration of AMP, ADP and ATP in mitochondria remains in a constant equilibrium defined by the equation: $[ADP]^2 = [ATP]/[AMP]$ (equilibrium constant is approx. 1). Isolated mitochondria will produce ATP if supplied with a substrate (e.g. pyruvate) and by the addition of ADP and AMP, forcing the equilibrium towards the production of ATP. The subsequent increase in ATP concentration can then be measured as an increase in light intensity with the bioluminescence assay. In order to prevent the activity of adenylate kinase, which synthesizes ATP by conversion of 2 moles of ADP into ATP and AMP, the ADP and AMP were added in a proportion based upon the endogenous ATP concentration. The [AMP] added was fixed at 20 μM, the proper ADP concentration to be added was calculated using 20 μM AMP, the endogenous ATP concentration of the mitochondrial fraction (previously determined) and the above equation. The sequence of events and equations used for calculating the oxidative phosphorylation rate and capacity are provided in Fig. 3. The background was

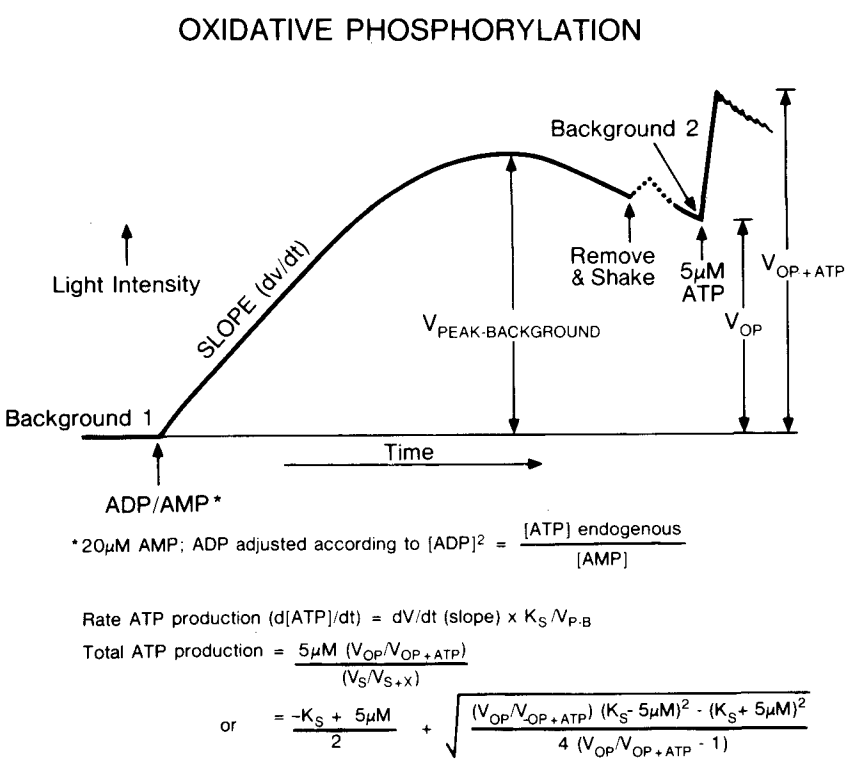


Fig. 3. Diagram of the changes in cpm during the procedures used to determine the effects of neurotoxicants upon mitochondrial ATP formation and the equations used to calculate total ATP production and rate of formation (see text for details).

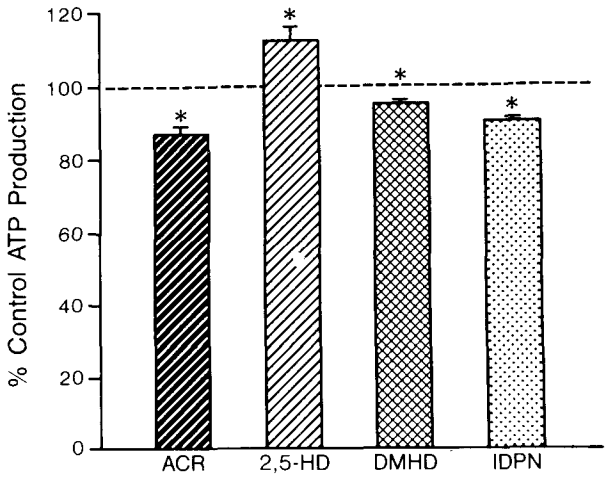


Fig. 4. Total quantity of ATP produced by mitochondrial fractions following incubation with acrylamide (ACR), 2,5-hexanedione (2,5-HD), 3,4-dimethyl-2,5-hexanedione (DMHD) or IDPN expressed as percent of buffer-incubated control (see text for details).

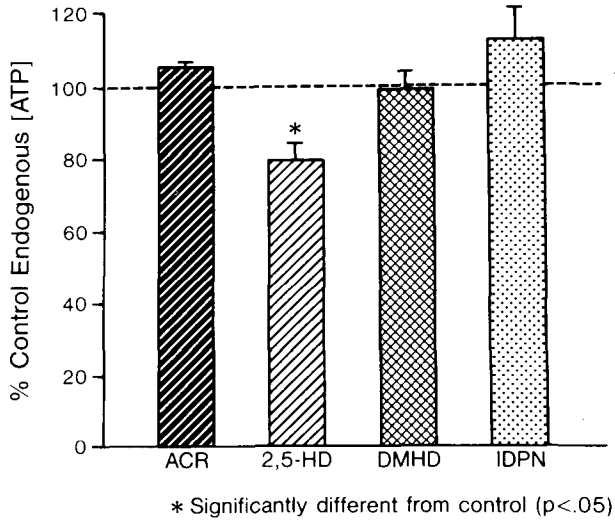


Fig. 6. Concentration of ATP in the mitochondrial fractions of rat brain following incubation with acrylamide (ACR), 2,5-hexanedione (2,5-HD), 3,4-dimethyl-2,5-hexanedione (DMHD) or IDPN expressed as percent of control (buffer-incubated) (see text for details).

measured in each vial containing the complete assay media including control and/or neurotoxicant-preincubated mitochondria and 5 mM pyruvate as substrate. ADP and AMP are added to the vial and the rate and peak of increasing cpm was followed. From the time of introduction of the AMP/ADP, the cpm increased linearly for approximately 3–5 min. Once the peak was reached, the vial was then removed, shaken and returned to the counting chamber where the second background was measured. Then 5 μM ATP was added and the second peak cpm was determined. The rate of ATP production is calculated from the slope of the increase in cpm per unit time, the Michaelis–Menton constant and the peak minus background (Fig. 3); the total ATP production was calculated according to the second formula provided in Fig. 3. The procedure was done in triplicate for each control and experimental for each toxicant.

Statistical analysis

In daily successive experiments, the differences between experimentals and controls were similar. However, variation in the daily percentage of mitochondria in the mitochondrial fractions prepared on successive days resulted in data variation from day to day. Since the nature of the experiment permitted only one control and one experimental to be assayed each day, the daily variation was eliminated by using a one-way, paired *t*-test ($P < 0.05$); paired as control vs experimental from the same daily mitochondrial fraction. This test was used to determine significant differences in the Michaelis–Menton constant, total ATP production and rate of ATP production between control (buffer-incubated) and toxicant-incubated mitochondria. Statistically significant changes in the endogenous ATP concentration of the same mitochondrial fraction after control and all four toxicant preincubations, were determined with a randomized block ANOVA and a post-hoc Dunnett's ($P < 0.05$). For illustration purposes, the results were standardized as percent of control (paired) ± S.E.M.

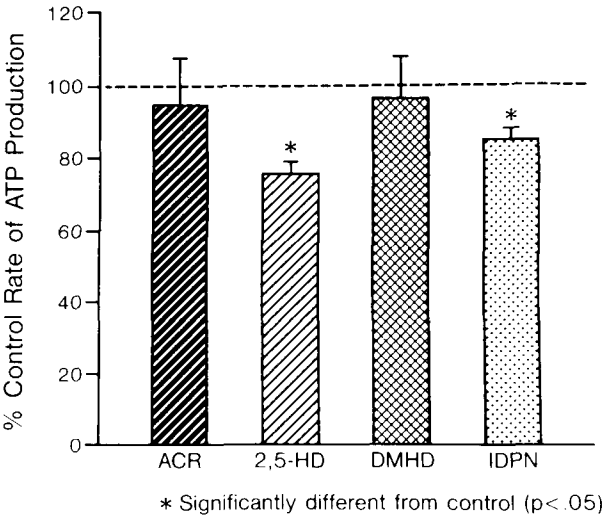


Fig. 5. The rate of ATP production by mitochondrial fractions following incubation with acrylamide (ACR), 2,5-hexanedione (2,5-HD), 3,4-dimethyl-2,5-hexanedione (DMHD) or IDPN expressed as percent of buffer-incubated control (see text for details).

RESULTS

Mitochondria isolation

Mitochondria isolated from rat brains were extremely fragile; alterations in the methodology yielded fractions incapable of conducting oxidative phosphorylation. In fact, methods developed for isolation of liver mitochondria were totally inadequate for obtaining functional brain mitochondria. With the methods outlined, we were able to consistently obtain a mitochondrial fraction capable of synthesizing ATP from pyruvate. Aside from mitochondria, other membranous elements including synaptic vesicles are included in the assay. Further purification resulted in a more pure fraction of mitochondria; however, oxidative phosphorylation was considerably reduced. Therefore, we chose to use partially purified fractions. Obviously, the specific concentrations and rates of formation of ATP are underestimated due to contamination by non-mitochondrial proteins. Preincubation of the mitochondrial fraction with 1 mM DNP

resulted in total loss of ATP synthesis and addition of DNP during the oxidative phosphorylation assay resulted in instantaneous cessation of ATP production. In addition, omission of the pyruvate substrate resulted in a lack of ATP synthesis.

Michaelis–Menton constant

The Michaelis–Menton constant for the luciferin/luciferase bioluminescence reaction was not significantly affected by the presence of any of the neurotoxicants except 2,5-HD. The average control K_s was 12.70 ± 0.70. Constants obtained from toxicant-exposed were 12.51 ± 1.17, 13.97 ± 0.79, 12.22 ± 1.05 and 12.55 ± 1.14 for ACR, 2,5-HD, DMHD and IDPN-exposed mitochondrial fractions, respectively. Even though slight toxicant-induced changes in the rate constant for the bioluminescence were observed, it is critically important to realize that the Michaelis–Menton constant was calculated for each control and experimental sample. Therefore, adjustments were automatically made for any changes in conditions which would influence the measured bioluminescence.

ATP production and endogenous ATP content

ACR, DMHD and IDPN produced only a few changes in mitochondrial ATP production, all of which were minor in comparison to 2,5-HD. ACR had no effect upon endogenous ATP concentration (1.24 ± 0.12 control vs 1.29 ± 0.15 nmol/mg; Fig. 6) or the rate of production (4.63 ± 0.53 control vs 4.54 ± 0.65 nmol/mg protein/min; Fig. 5), but slightly decreased the total quantity of ATP produced by 6.3% (Fig. 4; 5.91 ± 0.07 vs 5.54 ± 0.13 nmol/mg protein). DMHD had no significant effect on any of the parameters examined in this study (Figs. 4–6) except in total ATP production. However, the change was less than 5%; significance was observed due to extremely small errors in this measurement. IDPN caused a slight decrease in the rate (15%; Fig. 5) and total quantity of ATP produced (10.3%; Fig. 4). Endogenous ATP concentration of mitochondria preincubated with IDPN was unchanged (Fig. 6). In contrast to the minor changes caused by ACR, DMHD and IDPN, exposure of mitochondria to 2,5-HD produced a significant change in rate and total quantity of ATP production as well as the endogenous ATP content (Figs. 4–6). While the rate of ATP production was decreased by 22.5% (4.09 ± 0.32 vs 3.18 ± 0.28 nmol/mg protein/min; Fig. 5), the total time of production was much longer; the net result was an increase in the total quantity of ATP produced of 12% (6.43 ± 0.53 vs 7.20 ± 0.41 nmol/mg protein; Fig. 4). Endogenous ATP content of the mitochondria was decreased an average of 21.6% after the 30 min exposure to 4 mM 2,5-HD (Fig. 6).

DISCUSSION

The approach used in this study analyzes the effects of the neurotoxicants upon all mechanisms involved in the conversion of pyruvate to ATP. This includes all of the enzymes of pyruvate dehydrogenase complex, the citric acid cycle, electron transport, ATPase synthetase as well as measuring any functional changes in the mitochondria caused by changes in ion fluxes and membrane permeability, fluidity or structure. Addition of AMP and ADP in equilibrium concentrations with endogenous mitochondrial ATP concentrations precludes involvement of adenylate kinase activity[14]. If the neurotoxicant had a pathologically significant effect upon any of the components, it would have been reflected as a decreased rate or content of ATP. Only 2,5-HD caused a significant reduction in both the rate of ATP formation and the endogenous ATP content of the mitochondrial fraction. Aside from these reductions, a surprising observation was the prolongation of synthesis of ATP in the 2,5-HD exposed mitochondria which resulted in a net increase in total ATP produced. When 10 mM pyruvate (rather than the 5 mM used in the current study) was added to the assay media, the time of synthesis and total ATP produced were increased (unpublished data) similar to 2,5-HD, suggesting that 2,5-HD may act as a substrate supply to the mitochondria. The minor decrease in production of ATP caused by exposure of the mitochondria to ACR is considered insignificant functionally, especially since the endogenous ATP content was unchanged. The significance of the 15% decrease in rate of ATP production caused by IDPN is more difficult to assess in terms of the mode of action. However, based upon the lack of effect of this toxicant upon the endogenous ATP content, we predict that IDPN would not significantly affect oxidative metabolism. Obviously, DMHD has no effect upon the synthesis of ATP from pyruvate.

The lack of effect of most of the tested neurotoxicants on the production of ATP from pyruvate is evidence against oxidative energy transformation enyzmes as the critical site of action of these chemicals in producing the neuropathy. The current study has determined that only 2,5-HD has a significant effect upon these mitochondrial functions. The similarities in the neuropathy produced by exposure to these chemicals[31-33] and the absence of a consistent deleterious effect of the neurotoxicants upon ATP generation, indicate that this site and mode of action is not a common critical factor in precipitation of the axonopathy. DMHD is believed to act similarly to 2,5-HD due to its similar chemical structure and the fact that specifically substituted 2,5-HD's cause an entire temporal and spatial distribution of pathologies [1,2]. The

total lack of effect of the more neurotoxic γ diketone (DMHD) on ATP synthesis and the exclusive action by the weaker neurotoxicant (2,5-HD) is evidence against this site as being critical to production of the neuropathy by the γ-diketones. The action of 2,5-HD upon high energy phosphate production may either be inconsequential to the axon, or it may contribute to differences in the action of 2,5-HD compared to the other neurotoxicants studied.

Inhibitions of both glycolytic and oxidative enzymes have been demonstrated to occur following exposure to acrylamide and 2,5-HD[7–9,19,23,24,27,28]. Previous reports have provided evidence which shows that the glycolytic enzyme inhibitions by acrylamide and 2,5-HD have an insignificant effect upon the flux of metabolites through glycolysis[15,20,21]. In addition, administration of pyruvate is an ineffective tool to prevent the acrylamide neuropathy[34]. The current work indicates that the ACR-induced oxidative enzyme inhibitions[23,24,27,28] are inconsequential to the flow of metabolites through oxidative metabolic pathways. In light of the current evidence and the additional evidence demonstrating no change in high energy phosphates in the peripheral hindlimb nerves of animals treated with ACR, at times when significant changes in axonal transport are observed [25,26,29], indicates that a depletion of energy to the axonal transport mechanisms is not responsible for the ACR-induced block of transport. It remains to be tested whether or not 2,5-HD and/or DMHD lower nerve high energy phosphate levels at the time which they block transport. Based upon the current study, we would predict that only 2,5-HD would have a statistically significant effect upon nerve high energy phosphate levels. Therefore, if axonal transport block is the critical site of action of the neurotoxicants in producing nerve degeneration[25,26], the mode of action does not appear to be a block of energy production. The results of the current study can be added to the growing list of evidence indicating that energy metabolism is not a critical site of action of these neurotoxicants.

Certain reservations regarding the approach of the current study require discussion. First, the use of brain mitochondria in the study of chemicals which produce a peripheral neuropathy may seem inappropriate until one considers that CNS axons are equally vulnerable to these toxicants as PNS axons[31]. However, the mitochondria isolated in the current study are contributed by both neurons and glia; a specific action upon neuronal proteins may be obscured by the predominant glial population. This complication is a common problem to this research and is unavoidable.

Second, one could argue that the use of a limited exposure of the toxicant (single exposure to a neurotoxic dose for only 30 min at 37 °C) may have missed significant effects which may occur with longer exposure or multiple exposures. However, the time and temperature selected for this study were based on previous reports indicating that in vitro 37 °C incubations of 30 min inhibit oxidative as well as glycolytic enzymes[19,23,24,28]. In vivo studies also support the use of these time and temperatures. Maximal oxidative enzyme activity inhibition in rat motoneurons occurred 1 h following an injection of ACR[28]. Absorption and distribution of ACR is anticipated to require approximately 20–30 min; therefore, 30 min preincubation was calculated to most closely represent the in vivo exposure. The appropriate dose of exposure was chosen on two criteria. One, optimal conditions for demonstrating an effect were desired. Although the intra-neuronal and/or intra-axonal toxicant concentration following systemic exposure is unknown, concentration of the toxicant has not be shown. Therefore, the single daily dose of each toxicant (shown to produce the neuropathy and the axonal transport changes) would be expected to produce the maximal response. Two, the dose and time frame selection was critical to interpreting a relationship between enzyme inhibitions and axonal transport compromise. If inhibition of oxidative enzymes is critical to alterations in fast anterograde transport which occurs in min and lasts for at least 3 h [25,26,29], the production of ATP would have been reduced at the time point and dose used in this study. Inhibition of oxidative enzymes by other poisons has been shown to decrease the level of high energy phosphates in nerve to one-half control levels and block transport in 20 min[16,18]. No changes were observed in ATP and CP levels in the nerves of animals treated with ACR during the same time frame of action on transport[29]. We are currently determining the effects of the γ-diketones and IDPN upon ATP and CP content of rat sciatic nerves at times of axonal transport changes. However, based upon the data from the current study it appears that only 2,5-HD is likely to produce alterations in energy metabolism sufficient to alter fast anterograde or retrograde axonal transport.

Finally, the approach taken in the current study has not considered metabolism of IDPN to a more potent metabolite as necessary to produce the neuropathy. Subepineurial injection of IDPN produces dramatic reorganization of the cytoskeleton suggesting that metabolism is not essential (Griffin et al.[6a]). However, recent reports have hypothesized the transfer of a cyanoacetylaldehyde group from dehydro-IDPN to ε-amino groups and altering protein function[10] as a mode of action. Furthermore, a higher potency of *N*-hydroxy-IDPN than IDPN has been demonstrated[13]. Whether conversion to these metabolites occurs intraneurally or within our mitochondrial preparations is unknown.

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