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Influence of 2,5-Hexanedione, Acrylamide, tri-o-totyl Phoshate, Leptophos and Methylmercury on Endogenous Levels of Tryptophan, Serotonin and 5-Hydroxyindoleacetic Acid and Serotonin Turnover Rates in Rat Brain

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INFLUENCE OF 2,5-HEXANEDIONE, ACRYLAMIDE, TRI-O-TOLYL PHOSPHATE, LEPTOPHOS AND METHYLMERCURY ON ENDOGENOUS LEVELS OF TRYPTOPHAN, SEROTONIN AND 5-HYDROXYINDOLEACETIC ACID AND SEROTONIN TURNOVER RATES IN RAT BRAIN

by

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Craig H. Farr

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by

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Several industrial and environmental chemicals cause distal and/or central neuropathy among other diverse toxic effects. Spague-Dawley derived rats were fed doses of 2,5-hexanedione, acrylamide, tri-o-tolyl phosphate, Leptophos and methylmercury via gavage. The dose levels and administration periods were established in previous experiments designed to assess clinical neuropathy using rats trained to walk on a rotorod apparatus fitted with an electrode floor. After intravenous injections of ^{3}H-Tryptophan, whole rat brain homogenates were analyzed using liquid scintillation and spectrofluorometric techniques for levels of tryptophan, serotonin and 5-hydroxyindoleacetic acid. Serotonin turnover rates were calculated using the specific activities of

tryptophan and serotonin at two different time periods. The levels of serotonin as well as the serotonin turnover rates were unaffected by dosages of 5 to 50 mg acrylamide/kg given daily for five days or 30 to 300 mg 2,5-hexanedione/kg given in seven daily doses, while whole brain concentrations of 5-hydroxyindoleacetic acid increased significantly in a dose-dependent manner. The rise in 5-hydroxyindoleacetic acid levels coupled with no effects on the other levels in acrylamide and 2,5-hexanedione-fed animals suggests a possible inhibition of the energy-dependent 5-hydroxyindoleacetic acid efflux system in the brain. Animals given five doses of Leptophos (4.5 to 45 mg/kg) or six doses from 30 to 300 mg/kg tri-o-tolyl phosphate, administered every third day, showed slightly elevated, non-significant, serotonin turnover rates while levels of serotonin and tryptophan remained unchanged with a slight decrease in 5-hydroxyindoleacetic acid levels at the highest dosages. Levels of endogenous indole compounds in methylmercury treated rats showed no significant differences from control values; however, the turnover rates and levels of serotonin were slightly lower in the two lower treatment levels, while the highest dose level had no apparent effect on turnover rates or concentrations. Further studies involving longer treatment periods, alternate species or examination of discrete brain areas, may further clarify the effects of these chemicals on brain biochemistry.

(79 pages)

INTRODUCTION

In the past century, the chemical and pharmaceutical industries have synthesized thousands of new chemicals. More than 1,000 new compounds are developed each year in addition to the 40,000 chemicals and 2,000,000 mixtures and formulations presently in industrial use (Landrigan *et al.*, 1980). Among the diverse array of compounds causing toxic effects, a number of chemicals cause central and/or peripheral neuropathic effects. Often the neurotoxic properties of new compounds have not been recognized before their introduction to the market. Workers in the pesticide and chemical industries are particularly susceptible to chemically-induced neurologic disease while the general population is also susceptable to increased exposure to these and other chemicals in the environment.

Several neurotoxic chemicals have been shown to affect the metabolism of biogenic amines in the central nervous system (CNS). Sharma (1976) reported an increase in 5-hydroxyindoleacetic acid (5-HIAA) in dieldrin treated mice while levels and turnover rates of 5-hydroxytryptamine (serotonin or 5-HT) were unchanged, suggesting a possible effect of 5-HIAA efflux from the brain. Changes in the metabolism of 5-HT and norepinephrine (NE) may be responsible for DDT-induced hyperthermia (Hrdina *et al.*, 1973). Other chemicals, including metals, alter brain biogenic amine metabolic activity. Manganese and nickel treatments depress levels of 5-HT, NE, and dopamine (DA) in whole rat brain (Neff *et al.*, 1969; Mustafa and Chandra, 1971) while lead exposure increases NE synthesis rates

coupled with lower levels of NE in brain tissues (Michaelson *et al.*, 1974; Hrdina *et al.*, 1976).

The chemicals used in this study, including 2,5-hexanedione (2,5-HD), acrylamide, tri-o-tolyl phosphate (TOTP), O-4-bromo-2, 5-dichlorophenyl O-methyl phenylphosphonothioate (Leptophos) and methylmercuric chloride (ME-Hg), represent a broad spectrum of chemicals causing distal and/or central neuropathy. The literature concerning their histopathological effects is very extensive (Spencer and Shaumburg, 1974a, 1974b; Chang, 1977; Davis and Richardson, 1980; Spencer *et al.*, 1980) but they also influence brain enzyme activities. Acrylamide reduces levels of 5-HT, NE and DA in rat brain (Dixit *et al.*, 1980), while mercury influences biogenic amine metabolism (Hrdina *et al.*, 1976) and amine uptake in synaptosomal preparations (Bracken *et al.*, 1981). Availability of tryptophan, the precursor for serotonin synthesis is reduced after exposure to lead (Lorenzo and Gewirtz, 1977) with Me-Hg (Yoshino *et al.*, 1966) and acrylamide (Schotman *et al.*, 1977a) both inhibiting CNS uptake of leucine. Several intermediate glycolytic enzymes in the brain are also affected by acrylamide, 2,5-HD and Me-Hg (Damstra and Bondy, 1980). Moreover, leptophos and TOTP also show central effects through inhibition of brain neurotoxic esterase activities (Hussain and Oloffs, 1979; Davis and Richardson, 1980).

Delayed neurotoxicity is difficult to visually assess in a quadrapedal animal. Although various psychological and physiological procedures have been used for these studies (Tilson and Cabe, 1978a), accurate assessments of acrylamide-induced clinical neuropathy have been made with a rotorod device which is inexpensive and easily

operated (Kaplan and Murphy, 1972).

This investigation was undertaken to evaluate individual effects of 2,5-HD, acrylamide, TOTP, Leptophos and Me-Hg on various aspects of serotonin synthesis and metabolism (i.e. endogenous levels of Trp, 5-HT and 5-HIAA and 5-HT turnover rates) in rat brains using established spectrofluorometric and liquid scintillation techniques (Neff *et al.*, 1971; Marini *et al.*, 1979).

Rotorod observations provided the basis for establishing the dosing regimes for the brain biochemistry studies. Neuromuscular deficiencies were quantified based on set dose levels administered for prescribed periods of time.

REVIEW OF LITERATURE

Serotonin Metabolism in the Central Nervous System

The biosynthesis of 5-HT is dependent upon the uptake of Trp through the blood-brain barrier from the plasma. The first biochemical transformation involves the hydroxylation of Trp by tryptophan-5-hydroxylase to form 5-hydroxytryptophan (5-HTP) which is decarboxylated by aromatic-L-amino acid decarboxylase to form 5-HT (Cooper *et al.*, 1974). Several authors have provided evidence that serotonin exerts product inhibition over its own synthesis at the tryptophan-5-hydroxylase step (Macon *et al.*, 1971; Millard *et al.*, 1972). Although Trp hydroxylation is regarded by many as the rate-limiting step in 5-HT synthesis, observations that suggest that the amount of Trp available to the brain actually controls 5-HT synthesis include: 1) daily and parallel rhythm in the brain 5-HT and Trp; 2) a high Michaelis constant for Trp hydroxylase relative to the whole brain Trp concentration; and 3) a large increase of brain 5-HT levels and its metabolite, 5-HIAA, after a greater systemic dose of Trp (Wurtman and Fernstrom, 1972).

The principle catabolic pathway of 5-HT involves oxidative deamination catalyzed by monoamine oxidase (MAO). The reactant product, 5-hydroxyindole acetaldehyde, is then oxidized by aldehyde dehydrogenase to 5-HIAA (Cooper *et al.*, 1974). The existence of an alternative route by 6-hydroxylation has been demonstrated (Lemberger *et al.*, 1971), and whether or not this metabolite may

interfere with normal function of the 5-HT-containing terminals remains to be explored.

Many compounds alter 5-HT metabolism. When administered systemically, p-chlorophenylalanine (PCPA) selectively decreases the level of 5-HT in the brain by inhibiting Trp hydroxylase activity (Koe and Wiessman, 1966; Gal *et al.*, 1970), whereas 5-HT synthesis from 5-HTP remains normal (Pujol *et al.*, 1971). The methyl xanthines, caffeine, theophylline and theobromine, are some of the most widely consumed drugs. A single dose of caffeine elicits a significant increase in both 5-HT and 5-HIAA levels in rat brains, after either one or five hours, while theophylline is less potent in elevating brain 5-HT although a significant increase is observed (Berkowitz and Spector, 1973). The mode of action of several mild tranquilizers has been hypothesized to be through a reduction of 5-HT synthesis and metabolism. Dominic (1973) reported decreases in levels of brain 5-HT coupled with increases in 5-HIAA after exposure to chlordiazepoxide (Librium), diazepain (Valium) or flurazepain (Dalmane). Other drugs alter 5-HT metabolism by increasing the turnover rate as is seen with morphine (Yarbrough *et al.*, 1973) or by decreasing 5-HT while increasing 5-HIAA levels as with ethanol treatment (Gothoni and Ahtee, 1980).

Several pesticides have cause changes in the 5-HT metabolic pathway. Dieldrin-treated mice show no change in brain 5-HT levels while 5-HIAA values increase suggesting a possible influence of dieldrin on 5-HIAA efflux from mouse brain (Sharma, 1976). Conflicting reports indicate both significant increases and decreases of rat brain 5-HT after similar doses of dieldrin (Kohli

et al., 1977; Wagner and Greene, 1978). Activity of MAO is reduced in dieldrin treated hamster brain homogenates with increased levels of 5-HIAA *in vivo* while 5-HT values increased (Willhite and Sharma, 1978). Increases in rat brain concentration of 5-HIAA without affecting that of 5-HT, which the investigators attributed to enhanced 5-HT turnover, are seen after DDT exposure (Hrdina *et al.*, 1973). Other studies show increased levels of rat brain 5-HT with similar increases in 5-HIAA in carbaryl-treated rats which the investigators suggested may be due to an effect on the energy-requiring membrane transport for the acid metabolite (Hassan and Santolucito, 1971).

Metals, including manganese, nickel and mercury, influence the 5-HT biosynthetic and metabolic pathways. A significant decrease in 5-HT levels with an accompanying lower activity of L-aromatic amino acid decarboxylase has been reported in rats fed subacute doses of manganese (Kimura *et al.*, 1978), while nickel-fed hooded rats showed a significant lowering of serotonin levels in both the cerebral cortex and basal ganglia (Ali *et al.*, 1980). Rat pups exposed *in utero* to methylmercury (Me-Hg) and sacrificed as 28-day-old weanlings were reported to have a significant reduction of 5-HT concentration in the midbrain-diencephalon with decreased but non-significant levels of 5-HIAA. Pons-medullary 5-HT values were reduced but did not reach an acceptable level of significance (Sobotka *et al.*, 1974).

Several methods have been utilized to determine turnover rates of 5-HT. All are based on the assumption that 5-HT and 5-HIAA are in a steady-state, with rates of formation equal to rates of

metabolism and elimination. Probenecid reduces the renal excretion of organic acids including that of 5-HIAA (Despopoulos and Weissbach, 1957), and injection of probenecid increases the brain concentrations of 5-HIAA (Neff *et al.*, 1964). The accumulation of 5-HIAA after probenecid treatment is used as an index of serotonin turnover rates (Neff *et al.*, 1967) but a recent study (van Wijk *et al.*, 1979) suggests that probenecid also increases 5-HT formation by elevating Trp levels in rat brain.

The decline of brain 5-HIAA after MAO inhibition has been used as an estimator of 5-HT turnover rates. Tozer and co-workers (1966) demonstrated that after monoamine oxidase activity was blocked with either parglyine or tranylcypromine, brain levels of 5-HIAA declined exponentially.

A third widely used method involves the injection of radioactive Trp and calculating turnover rate of brain 5-HT from the accumulation of ^{3}H-5-HT coupled with the decline of the labelled Trp (Neff *et al.*, 1971). This method also allows the investigator to determine levels of Trp, 5-HT and 5-HIAA in the same animals.

Overview of Clinical Neuropathy Assessment

Numerous methods for detecting neurotoxicity have been reported in the literature. General motor activity is often used as an indication of neurotoxic status. Observational methods, both qualitative and quantitative, and automated techniques have been developed to assess general activity (Reiter and Macphail, 1979).

Peripheral neuropathy, in bipedal animals such as chickens, is readily assessed by observation and applying an ataxic index for

quantification (Watanabe and Sharma, 1977). An early manifestation of mercury-induced rat peripheral sensory neuropathy is "tail rotation", characterized by a sustained vigorous circling movement of the tail when held by the body. This syndrome is seen two to three weeks prior to the onset of crossing and/or ataxia of the hind legs commonly seen in Me-Hg poisoning (Ohi *et al.*, 1978). Other motor-involvement indices include fatige, measured by swim endurance (Campbell, 1976) and frequency of tremor occurrence (Remington and Anisman, 1976). Tilson and Cabe (1978b) have developed an inclined screen procedure in which the rat is picked up by the tail and, from a distance of 3 m, is gently tossed with an upward motion toward the middle of a rectangular screen. The animals are then rated in ability to cling to the screen on a 0 to 5 point scale. Forelimb grip strength and hindlimb extensor response tests are procedures which utilize strain gauges to monitor muscular strength by isolating either the front or rear extremities (Cabe and Tilson, 1978; Cabe *et al.*, 1978).

The ability of rats to maintain their balance on a rotating rod (rotorod) has been used as an index to assay clinical effects of neurotoxic chemicals (Dunham and Miya, 1957). Kaplan and Murphy (1972) described a rotorod assembly with a scrambled shock device, in contact with an electrode floor, to discourage jumping from the rotorod. Investigations by them showed that acrylamide (50 mg/kg/day) produced mean rotorod failure times of 5.3 days. The authors concluded the rotorod was a sensitive indicator of clinical neuropathy. Another study of acrylamide-induced motor dysfunction, as measured by the hindlimb extensor response and inclined screen

tests, showed hindlimb impairment while no effect was seen on forelimb grip (Tilson and Cabe, 1979). The cumulative dose required to induce these effects was 100 mg/kg compared to the 180 mg/kg required for changes in ambulation and rearing in the open field (Gipon *et al.*, 1977), the 420 mg/kg reported to produce failure on the rotorod (Kaplan and Murphy, 1972) and similar levels used in a procedure which measured the spread of the hindlimbs upon landing in rats which were dropped from a standard height (Edwards, 1977).

Several of the procedures described require a large investment of time and resources. The choice of tests to use in neurobehavioral toxicology is dependent upon several factors related to the characteristics of the animal model, the availability of technology and training for measuring a specific function, and the cost effectiveness/time efficiency factors associated with the number of animals and doses of compounds under study (Tilson and Cabe, 1978a).

Aspects of Hexacarbon Neurotoxicity

Methyl-*n*-butyl ketone (MNBK) and *n*-hexane have been found to cause neurotoxicity following prolonged human exposure. A few cases of neuropathy have been attributed to methyl-isobutyl ketone (AuBuchon *et al.*, 1979) and to cyclohexane (Lande *et al.*, 1976), but these compounds have failed to produce neuropathy in experimental animals (Spencer *et al.*, 1975). Exposure to MNBK was initially suspected as a peripheral neuropathogenic agent subsequent to an outbreak of neuropathy in a plant producing plastic-coated and color-printed fabrics (Billmaier *et al.*, 1974; Allen *et al.*, 1975). Mallov (1976) found evidence to suspect MNBK as the causative agent

in neuropathy in spray painters. Humans exposed to *n*-hexane as cabinet finishers (Herskowitz *et al.*, 1971, shoe factory workers (Franchini *et al.*, 1978) and paper adhesive workers (Paulson and Waylonis, 1976) have also exhibited varying degrees of polyneuropathy.

The most common cause of hexacarbon neuropathy has been the deliberate inhalation vapors of laquers, solvents or glues containing *n*-hexane (Gonzales and Downey, 1972; Prockop *et al.*, 1974, Korobkin *et al.*, 1975). Hexane mixtures free of *n*-hexane gave no evidence of neurotoxic effects (Egan *et al.*, 1980).

Serum from guinea pigs dosed with MNBK showed three major gas chromatography-mass spectrometry peaks which were identified as MNBK, 5-hydroxy-2-hexanone (5-OH-2H) and 2,5-hexanedione (2,5-HD) while *n*-hexane-exposed animals produced the same metabolites (DiVincenzo *et al.*, 1976). In the rat, MNBK has been reported to be metabolized to 2-hexanol, 5-OH-2H and 2,5-HD (DiVincenzo *et al.*, 1977). The principal metabolites of *n*-hexane in the rat are reported to be 1-hexanol and 2-hexanol which are further metabolized to 2,5-HD (Peribellini *et al.*, 1978) while the primary urinary *n*-hexane metabolite in man has been identified as 2,5-HD with smaller amounts of 2-hexanol, suggesting that *n*-hexane in men and animals follows the same metabolic pathway (Perbellini *et al.*, 1980). Since 2-hexanol is a common metabolite of *n*-hexane and MNBK, this alcohol may be considered a neurotoxic product connecting the metabolic pathways of *n*-hexane and MNBK. Krasavage *et al.*, (1980) investigated the relative neurotoxicity of MNBK, *n*-hexane and their metabolites by administering equimolar doses of each compound by gavage. Clinical evidence of severe hindlimb weakness or paralysis was the endpoint

used to determine neurotoxicity. All test compounds produced both clinical and histological neuropathy. The relative neurotoxicity of the test compounds in decreasing order of potency was: 2,5-HD, 5-OH-2H, 2,5-hexanediol, MNBK, 2-hexanol and *n*-hexane. These researchers also found that the neurotoxic potency was directly related to the amount of 2,5-hexanedione produced by each compound. The magnitude of body weight changes paralleled the neurotoxic potency of each compound. Other studies have confirmed these results (Spencer *et al.*, 1978; Eben *et al.*, 1979).

Neurotoxic hexacarbons produce distal axonopathies in the longer and larger axons in both the peripheral (PNS) and central nervous system (CNS). Early changes show axonal swelling with a decrease in axonal transport (Griffin *et al.*, 1977). As the dying-back process continues, the axonal swelling ascends the nerve fiber with a resultant focal demyelination followed by remyelination (Powell *et al.*, 1978).

Neurotoxic hexacarbon compounds, 2,5-HD and MNBK, have been shown to inhibit brain glycolytic enzymes including phosphofructo-kinase (Sabri *et al.*, 1979a), glyceraldehyde-3-phosphate dehydrogenase Sabri *et al.*, 1979b) and enolase, while neither compound affected lactate dehydrogenase (Howland *et al.*, 1980). These results support the hypothesis that neurotoxic hexacarbon compounds inhibit the activity of enzymes required for energy production. Gilles and co-workers (1981a, 1981b) recently reported significant decreases in the *in vitro* incorporation of [1-^{14}C] acetate into ubiquinone in sciatic nerve and brain of rats fed 1% 2,5-HD in drinking water for 6 weeks while acetate incorporation into phospholipids, fatty

acids and cholesteryl esters was similar in tissues of 2,5-HD-treated rats and pair-fed controls. Loss of body weight induced by 2,5-HD was similar to that seen in pair fed control rats. These authors hypothesized that since ubiquinone has been shown to transfer electrons between NADH dehydrogenase and cytochrome *b* in the electron transport chain of the mitochondria (Ramasarma, 1968), an inhibition of oxidative-phosphorylation could result from decreased ubiquinone biosynthesis.

Endogenous levels of brain indoleamines in neurotoxic hexacarbon-treated animals have not been reported in the literature.

Aspects of Acrylamide Neurotoxicity

The major use of acrylamide is as a vinyl monomer in the production of high molecular polymers which are useful flocculators. Acrylamide became commercially important in the early 1950's with applications in mining operations, purification of water supplies and disposal of industrial waste. About the same time acrylamide was used as a strengthener in the manufacture of paper and cardboard (McCollister *et al.*, 1964), as well as a grouting agent in mining and tunnel construction (Fullerton and Barnes, 1966).

The neurotoxic characteristics of acrylamide were discovered soon after the substance was manufactured. Observations showed that the acrylamide monomer was neurotoxic, but when polymerization occurred the product was no longer toxic (Spencer and Schaumburg, 1974a), which limits the exposed population of workers to those involved in manufacture of the monomer or in the polymerization process (Garland and Patterson, 1967). Intoxications have occurred

during grouting operations conducted in confined spaces when the monomer is pumped into the soil and, with the addition of catalysts, *in situ* polymerization occurs. Auld and Bedwell (1967) provided the first clinical description of a worker intoxicated in this way and, more recently, four men working together in a tunnel were similarly affected (Kesson *et al.*, 1977). Spencer and Schaumburg (1974a) concluded that most instances of industrial toxicity have resulted from dermal absorption rather than inhalation. The only report of toxicity unrelated to work was to a family in Japan who developed acute toxicity after their well water became contaminated with acrylamide used in nearby underground construction (Igisu *et al.*, 1975). Following removal from exposure, which is the only effective remedy known, affected persons showed a reduction of symptoms. In patients with mild neuropathy, complete recovery may be expected (Garland and Patterson, 1967; Kesson *et al.*, 1977) while in those more severely affected, there may be residual abnormalities (Fullerton, 1969).

Kuperman (1958) published one of the first reports of acrylamide toxicity in animals. He investigated intoxication in cats and reported the cumulative nature of acrylamide and demonstrated the effect was not dependent on the route of administration. Kuperman also studied changes in cerebral electrical activity, but pathological studies showed no abnormalities in the central nervous system. In 1964, McCollister and coworkers did further work with acrylamide on rabbits, cats, monkeys, guines pigs and rats. The functional effects were similar to those seen before, and again no central nervous system pathology was seen.

Repeated acrylamide exposure produces a bilateral, distal neuropathy. More distal axons innervating the hindlimbs are affected by acrylamide before those more proximal which correlates with motor deficits first seen in the hindlimbs (Spencer and Schaumburg, 1975). Though systematic study of the pattern of nerve damage in experimental animals demonstrated that distal regions of nerves are more severely affected than proximal areas, distal fiber degeneration is seen in conjunction with proximal preservation (Fullerton and Barnes, 1966). The term "dying-back" is used to describe the pattern of slow, progressive retrograde degeneration seen with acrylamide intoxication (Prineas, 1969; Schaumburg *et al.*, 1974). Long and large myelinated fibers are affected more than short and thin fibers with the most severe damage near blood vessels (Suzuki and Pfaff, 1973). Initial motor nerve terminal degeneration is widespread and not restricted to the terminals of the longest axons (Jennekens *et al.*, 1979). Schaumburg and co-workers detected the earliest morphological changes in nerve terminals of Pacinian corpuscles in the foot pad followed by degeneration of nerve endings in the muscle spindles (1974). It is also known that in dying-back syndrome, axononal degeneration occurs in long fiber nerve tracts of the central nervous system including regions of the medulla, spinal cord and cerebellum.

Depletion of brain biogenic amines, including NE, DA and 5-HT has been observed following nine daily 50 mg acrylamide/kg intraperitoneal injections (Dixit *et al.*, 1980). Acrylamide-treated chick ganglia cell cultures showed morphological alterations of neurons and neuroglia which could be prevented by addition of various nucleotides or cofactors (Sharma and Obersteiner, 1977).

Several biochemical effects are seen in acrylamide intoxication. A significant depression of leucine incorporation into brain proteins has been correlated with the progress of acrylamide neuropathy (Schotman *et al.*, 1977a), while similar findings were observed in the incorporation of labelled lysine and methionine in the spinal cord (Hashimoto and Ando, 1973). A decrease in spinal cord and brain stem protein synthesis rates has been demonstrated with acute and chronic acrylamide intoxication (Schotman *et al.*, 1977b). Acrylamide has also been shown to reduce the activities of brain glyceraldehyde-3-phosphate-dehydrogenase, phosphofructokinase and neuronal specific enolase in the glycolytic pathway. These studies were based on the proposal by Spencer and Schaumburg (1975) that the multiple axonopathy seen after acrylamide intoxication is caused by 1) impaired axonal transport, 2) failure of the synthetic machinery in the neuronal cell bodies, and/or 3) changed in axonal metabolism.

Aspects of Organophosphorus Neurotoxicity

Organophosphorus (OP) compounds have been shown to affect both the central and peripheral nervous systems. Inhibition of acetylcholinesterase (AChE), with the resultant effects, has been widely studies and reviewed (Eto, 1974). Many OP compounds have been shown to produce a central-peripheral distal axonopathy similar to the axonal degeneration seen with acrylamide and neurotoxic hexacarbons (Cavanagh, 1973; Bouldin and Cavanagh, 1979). This effect is not dependent upon the inhibition of AChE but a strong correlation has been made involving the inhibition of another

esteratic enzyme, neurotoxic esterase (NTE), a membrane bound nerve-cell protein. Neurotoxic esterase is present in various brain regions, and in spinal cord and sciatic nerve, and is inhibited by phosphorylating a serine residue within its active site (Johnson, 1975). Reactions of NTE with several carbamyl and phosphinyl esters produce NTE inhibition without the resulting neurotoxic effects. Johnson (1976) suggested that these compounds may provide a protective function by occupying the NTE active site. Although NTE has been identified as the target enzyme for neurotoxic OP compounds in the nervous system, the physiological role of this enzyme is unknown.

Alterations of central neurotransmitter levels have been resported after exposure to some OP compounds. Cerebral cortex levels of DA and NE in parathion-fed rats were reduced (Fiscus and Van Meter, 1977) while disulfoton reduced rat hippocampal NE levels (Holt and Hawkins, 1978).

Several widespread human intoxications have occurred with TOTP, also referred to as tri-o-cresyl phosphate (TOCP), which is commonly used as a lubricating oil additive. The most serious human exposure to TOTP was in 1930 when thousands of people in the southern U.S.A. exhibited symptoms of paralysis. All had ingested varieties of "Jamaica ginger" to which substances had been added to enhance the potency (Davies, 1963) and subsequent analysis showed that all compounds which caused paralysis were found to be contaminated with TOTP (Smith *et al.*, 1930; Burley, 1931) which gave this syndrome the name, Ginger Jake. Many poisonings relating to the accidental or deliberate addition of tricresylphosphates to edible oils occurred

during the following several years (Mednikyan and Mirzoyan, 1936). Smith and Spaulding (1959) described a mass poisoning in Meknes, N. Africa affecting 10,000 people when they ingested a mixture of olive oil and lubricating oils containing a high amount of TOTP. Other cases of TOTP neuropathies have occurred in the Fiji Islands (Sorokin, 1969) and in workers of the Spanish shoe industry (Bermejillo, 1971). An outbreak of acute polyneuropathy affected more than 20 young females in Sri Lanka during 1977-78. The illness was seen only in girls, soon after menarche and is related culturally to the custum of restricting meat and fish in the diet during that period and substituting raw eggs and gingili oil which is not normally eaten because of its cost. Each woman was given one or two doses of TOTP-contaminated oil daily for two weeks (Senanayke and Jeyaratnam, 1981). Unfortunately, the prognosis for recovery from organophosphate neuropathy is poor due to the degeneration of long-tracts in the spinal cord (Morgan and Penovich, 1978).

The ability of TOTP to cause neuropathy is dependent upon the α-hydroxylation of a ring sidechain followed by the formation of a cyclic phosphate ester in conjunction with the expulsion of a tolyl ring. If the side chain is in the para or meta position, cyclization does not occur, but, instead further oxidation to an acetyl group takes place (Eto *et al.*, 1962) rendering these compounds inactive for neuropathogenic activity (Aldridge, 1954; Hine *et al.*, 1956).

Leptophos or Phosvel (o-4-bromo-2,5-dichlorophenyl O-methyl phenylphosphonothioate) has been implicated in the death and/or paralysis of a large number of water buffalo in Egypt (Abou-Donia

et al., 1974) as well as possible human paralysis (Abou-Donia and Graham, 1978). Leptophos has been reported to be an inhibitor of NTE (Hussain and Oloffs, 1979). These same researchers reported that rats fed doses of 5.0 mg Leptophos/kg showed neither signs of ataxia nor histological alterations but did have a significant weight decrease. Chickens dosed orally or by topical application to the comb were affected by paralysis, loss of weight, plasma cholinesterase inhibition and increased activity in plasma acid phosphatase (Abou-Donia and Preissig, 1976; Abou-Donia and Graham, 1978). Herin and co-workers (1978) reported that mallard ducklings showed a similar response after Leptophos exposure.

The neuropathology of Leptophos is similar to that seen after TOTP exposure *i.e.* paralysis correlated with the degeneration of the anterior descending tract of the spinal cord and degeneration of the sciatic nerve (Preissig and Abou-Donia, 1978).

Aspects of Methylmercury Neurotoxicity

Industrial processes account for over 20,000 tons of mercury being released into the environment each year (Magos, 1975). Among mercury compounds, Me-Hg is known to be more neurotoxic than inorganic mercury (Berlin and Ullberg, 1963). Initial indications of a public health threat caused by Me-Hg exposure came after an epidemic in Japan had affected 126 people, with 46 deaths, mostly fishermen and their families. The cause was traced to Minamata Bay effluents from a chemical factory utilizing mercuric chloride as a catalyst in the manufacture of vinyl chloride. Consumption of Me-Hg-treated seed grain has also produced several

epidemics of neurotoxicity in humans. The most catastrophic episode occurred in 1972 in Iraq where thousands were hospitalized and 459 people died after eating contaminated grain (Marsh, 1979).

Impairment of the blood-brain barrier has been demonstrated after injection of trypan blue dye or fluorescein-labelled albumin into the nervous system of mercury-treated rats (Steinwall and Klatzo, 1966). Chang and Hartman (1972) have demonstrated the disposition of mercury within many biological membraines after mercury exposure. Several studies show that the increased permeability and dysfunction of the blood brain barrier after mercury intoxication is probably due to damage of the endothelial and glial membranes by the mercury ions forming cross-linkages with a cell membrane protein moiety, resulting in the leakage of plasma solutes (Ware *et al.*, 1974) and a reduction of the uptake of amino acids (Steinwall and Klatzo, 1966).

Distribution studies showed that after chronic, continuous administration of radio-labelled methylmercuric hydroxide to rats, the spinal dorsal ganglia contained the highest concentration of mercury, followed closely by the cerebral cortex and the cerebellum, then by the subcortical part of the forebrain (Somjen *et al.*, 1973). Cerebellar changes in rats, after exposure to Me-Hg *in utero*, have shown disintegration in the endoplasmic reticulum and Golgi complex in Purkinje cells (Spyker and Chang, 1974). The sensory neurons of the dorsal spinal ganglia were found to be extremely sensitive to the toxicity of mercury. Changes included the disintegration of endoplasmic reticulum and ribosomes (Herman *et al.*, 1973) which supports the discovery of a reduction of RNA (Chang

et al., 1972). Pathological examination of peripheral nerves of Me-Hg-treated rats showed swelling and degeneration of Schwann cells with notable changes in both myelin sheaths and axons which tend to begin at the nodes of Ranvier (Miyakawa *et al.*, 1970).

Myelinated cultures of cerebellum were sensitive to exposure to methylmercuric acetate. Severe vacuolar degeneration was observed in nerve cells, particularly in granule and Purkinje cells while axons and myelin sheaths also underwent a considerable degree of degeneration (Kim, 1971). Sharma and Obersteiner (1981) have also reported a dose-dependent cell growth inhibition in mercury-exposured chick ganglia cultures.

Several glycolytic enzyme activities are affected by Me-Hg. Using histochemical techniques, Chang *et al.* (1973) demonstrated a decrease in the activities of succinic dehydrogenase. Levels of glucose-1-phosphate and glyceraldehyde phosphate have been shown to be increased one hour after mercury administration (Patterson and Usher, 1971).

Other biochemical effects of methylmercury show decreased levels and turnover rates of acetylcholine (Kobayashi *et al.*, 1980). In 28 day old rat pups exposed *in utero* to 2.5 mg methylmercuric chloride/kg from day six through day five of gestation, decreased cholinesterase activity was found in the telencephalon and cerebellum while reduced levels of serotonin and norepinephrine were seen in the midbrain-diencephalon region (Sobotka *et al.*, 1974).

METHODS

Experimental Animals

Male Sprague-Dawley derived rats [Sim:(SD)sBR], weighing 120 to 140 g were obtained from Simonsen Laboratories, Inc. of Gilroy, CA. Upon arrival the animals were randomly assigned to groups, earmarked, and placed in polycarbonate rat cages with screened pine shavings as bedding. Laboratory feed (Wayne Lab-Blox, Allied Mills, Chicago, IL) and water were provided ad libitum. All animals were acclimated at least 10 days to the animal care facility maintained under constant environmental conditions (22±2°C, 45-60% relative humidity and illuminated 12 hr/day beginning at 7 a.m.)

Distal Neuromuscular Pathology Assessment

The rotorod apparatus (Figure 1) was similar to one described by Kaplan and Murphy (1972). The dimensions were 0.92 X 0.46 X 0.61 M (length X width X depth) with the enclosure divided into three compartments of equal size. An acrylic-plastic front wall was used for ease of observation. A wooden dowel (7.62 cm diameter) was placed 33.0 cm above the floor, which was fashioned of stainless steel rods (0.64 cm diameter) placed 1.57 cm apart at the centers. The electrode floor was supplied with an elecritcal potential through a scrambled shock device. The speed of the belt-driven rod was varied using different combinations of pulleys on the shaft of the dowel and, a 25 rpm, constant-speed motor.

For the first few days, a speed of 12 rpm was utilized to train the rats, while the final testing speed was 20 rpm. Rats with normal neuromuscular status could perform well without distractions or attempts to jump from the rotorod. After a 7-10 day training period, rats were randomly assigned to four groups of five animals each. To be considered normal in neuromuscular function, each rat was required to walk on the rotorod for at least 1 min during any of three attempts on the testing days.

All compounds were administered by gavage with a 3 in., 16 gauge feeding needle, bent slightly at the ball end. In each case, intubuation was after the rotorod testing. The dosages were designed to elicit a clinical neuropathy in the highest dose groups with no observable effects in the lowest dose groups. Three dose levels and a control were used in each study, while the vehicle varied according to the solubility of the test chemical. Dosing solutions were prepared such that each animal received 1 ml/100 g body weight with the doses measured to the nearest 0.1 ml.

Levels of 3.0, 1.0 and 0.3 g 2,5-hexanedione (Eastman grade, Eastman Kodak Co., Rochester, NY)/kg made up in propylene glycol and acrylamide (Eastman grade, Eastman Kodak Co.) doses of 50,15 and 5 mg/kg prepared with water, were administered daily along with the appropriate vehicle controls. Solutions of Leptophos (98% analytical standard, Chem Service Co., Westchester, PA), tri-o-tolyl phosphate (practical grade, Eastman Kodak Co.) and Methylmercuric (II) chloride (95%, Ventron Corp., Danvers, MA) were given at three day intervals. Leptophos was dissolved in propylene glycol in doses of 90, 30, and 9 mg/kg. One hour prior to receiving Leptophos, the animals were

injected IP with 50 mg/kg (0.5 ml of a 20 mg/ml solution) atropine sulphate (Sigma Chemical Co., St. Louis, MO). Solutuions of TOTP were solubilized in oil and administered at levels of 1.0, 0.3 and 0.1 g/kg. Dosages of 10.0, 3.3, and 1.0 mg/kg Me-Hg were made by dissolving 10 mg Me-Hg/ml in 95% ethanol. The final mercury solutions were prepared in corn oil containing 95% ethanol.

Biochemical Studies

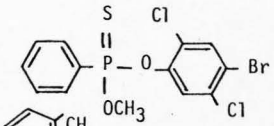
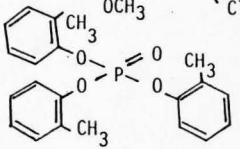
From the results of a rotorod testing, the dosages and duration of dosing in a separate set of animals for biochemical evaluations were established as shown in Table 1. Each of the dose groups for the various compounds had 14 rats, except acrylamide, which had 16 in each group. On the day following the last treatment, tail vein injections of 0.050 mCi (0.20 ml) L-[^{3}H(G)]-tryptophan (7.88 Ci/mmol, obtained through New England Nuclear, Boston, MA) were administered. The specific activity of ^{3}H-Trp shows linear decline over time (Neff *et al.*, 1971), and after one hour, half of the animals in each dose level were guillotined, while the remainder were decapitated after two hours. The data showing the decline of ^{3}H-Try specific activity was reconfirmed to establish sacrifice periods.

Brains were quickly removed, frozen on dry ice, and stored at -80°C until assayed. The acrylamide-treated animals were similarly sacrificed in two groups after 40 min and two hours, respectively. The injections were consistently timed for sacrifice to coincide with the third and fourth hours of the light cycle.

Column Separation procedures followed in the separation of Trp, 5-HT and %-HIAA were based on a method developed by Costa *et al.*

TABLE 1

COMPOUNDS AND DOSAGES USED IN BIOCHEMICAL STUDIES

Compound	Structure	Dosages (mg/kg) high	med.	low	Vehicle	Frequency	Total Number of Doses
2,5-hexanedione	$CH_3CH(CH_2)_2CHCH_3$ (O, O)	300	100	30	propylene glycol	daily	7
Acrylamide	$CH_2 = CHCONH_2$	50	15	5	H_2O	daily	5
Leptophos		45	15	4.5	propylene glycol	every 3rd day	5
TOTP		300	100	30	corn oil	every 3rd day	6
methylmercuric chloride	CH_3-Hg-Cl	10.0	3.3	1.0	10% ethanol in corn oil	every 3rd day	5

(1968) and later modified by Marini *et al.* (1979) to include 5-HIAA. Columns constructed from Pyrex glass, measuring 6 mm x 120 mm, were fused to a reservoir with a capacity of 10 ml. The column outlet was narrowed to accomodate a 25 cm length of Tygon tubing (2 mm ID), used to regulate the flow rate depending on the height of the tubing. Dowex 50X-X4, 200-400 mesh size, a strongly acidic cation exchange resin, was obtained from Sigma Chemical Co. (St. Louis, MO), subjected to a thorough washing and packed into the columns which had been loosely plugged with a small piece of cotton to support the resin. The columns were packed to a height of 35 mm with resin in the hydrogen form.

All chemicals were reagent grade unless otherwise indicated. Water was purified by deionization and reverse osmosis. Whole brains were homogenized in 0.4 N $HClO_4$ with 1 mg/ml Na_2S_2O, at 0°C, using a Teflon and glass apparatus. Five ml of $HClO_4/Na_2S_2O$ solution per gram of brain tissue were used in each case. The homogenate was centrifuged (14,000 x g at 0°C for 15 min), after which the supernatant of each sample was decanted into individual 50 ml plastic centrifuge tubes followed by pH adjustment to 2.0-2.5 with 5 N KOH. During a five min storage in an ice bath, $KClO_4$ was precipitated after which the mixture was centrifuged (8.000 x g at 0°C for 10 min) to allow separation. An aliquot (4.9 ml) of each supernatnat, as well as a 1 ml distilled water rinse, were added to the column reservoir. The supernatnat was passed through the resin followed by a series of eluting solutions (Table 2). Internal standards of Trp, 5-HT and 5-HIAA (all obtained through Sigma Chemical Co., St. Louis, MO) were processed in a similar fashion using a mock homogenate consisting of 1.31 g NaCl and 100 mg

TABLE 2

FLOW CHART FOR SEPARATION OF INDOLE COMPOUNDS WITH DOWEX 50-X4 RESIN

Elution solutions	Compounds in Effluent
1. Supernatant, pH 2-2.5 and 1 ml H_2O rinse.	1. Supernatant. Discard.
2. 7 ml 60% methanol - H_2O.	2. 5HIAA for assay. Discard first 0.5 ml.
3. 9 ml 0.1 m sodium phosphate buffer, pH 6.5.	3. Trp for assay. Discard first 2 ml.
4. 2 ml 1 N HCl.	4. HCl wash. Discard.
5. 6 ml 4 N HCl.	5. HCl wash. Discard.
6. 2 ml H_2O.	6. H_2O wash. Discard.
7. 6 ml 0.5 m Na_3PO_4.	7. 5HT for assay.
8. 5 ml resin washes with H_2O, 4 N HCl and sodium phosphate buffer.	8. Resin washes. Discard.

$Na_2S_2O_5$ prepared to a total volume of 100 ml with 0.4 N $HClO_4$.

Fluorometric assays were performed using an Aminco-Bowman Spectrofluorometer with the slits set at 4 mm and the photomultiplier set at 4 for all assays. A modification of the o-pthaldialdehyde-condensate method of Atack and Lindquist (1973) was used to assay *5-HIAA*. Two thoroughly mixed, 1 ml portions of each sample effluent, one for the sample, the other for the oxidized blank, were taken. After addition of 0.1 ml H_2O to the sample, 0.1 ml of cysteine-potassium ferricyanide solution (equal volumes of 6% cysteine and 0.2% $K_3Fe(CN)_6$) was added and mixed. When ready for assay, 1.4 ml conc. HCl and 50 µl 0.3% o-pthaldialdehyde (OPT) were added and mixed. The oxidized blank was then formed by mixing 0.1 ml H_2O and 0.2 ml conc. HCl to the second 1 ml sample aliquot. Noting the time, 50 µl of the 0.2% $K_3Fe(CN)_6$ solution was added and mixed followed after 10 min by 50 µl of the 6% cysteine. After the solution was mixed, 1.2 ml conc. HCl and 50 µl of the OPT solution were added and shaken. The solutions were heated for 20 min in a water bath set at 78°C after which they were cooled in room temperature water for 3 min, mixed thoroughly and read at excitation and emission wavelengths of 360 nm and 480 nm, respectively.

L-tryptophan assays involved an extraction of the fluorescent derivative as outlined by Marini *et al.*, 1979. A 2.6 ml aliquot was taken from each of the pH 6.5 phosphate buffer column effluents and placed in a 8 ml screw-cap tube. Blanks consisted of 2.0 ml of the phosphate buffer. A 50 µl aliquot of 18% formaldehyde solutions was mixed into each tube followed by 50 µl of 0.01 M $FeCl_3$ in conc. HCl. The tubes were mixed, covered with marbles and placed in a 100°C oil

bath. After one hr. the tubes were removed from the bath and placed in cold tap water for a few minutes, followed by a thorough mixing, addition of 0.2 ml 5 N KOH and a second mixing.

Washed ethyl acetate (4.5 ml) was added to each tube. The tubes were tightly capped, shaken for 10 min and centrifuged 5 min at 2500 rpm. A 3.0 ml aliquot of the ethyl acetate layer was transferred to an 8 ml screw-cap tube containing 1.5 ml 0.1 N HCl. These tubes were capped, shaken 10 min, and centrifuged 5 min at 2500 rpm after which the ethyl acetate layer was carefully aspirated from the tubes. The HCl extract was assayed directly with excitation and emission wavelengths of 376 nm and 458 nm, respectively.

Serotonin is not stable in strong base (Na_3PO_4) so the extraction procedure (Marini *et al.*, 1979) was performed shortly after the samples were eluted from the columns. After a thorough mixing, 4 ml of each sodium phosphate effluent was taken for assay, placed in a 50 ml polypropylene tube and mixed after each addition of 1.5 g NaCl, 2.0 ml borate buffer and 15 ml ethyl acetate. The samples were shaken 10 min, and centrifuged 5 min after which 12 ml ethyl acetate were transferred to tubes containing 1.5 ml 0.1 N HCl and 25 ml cyclohexane. After shaking 10 min and centrifuging, the combined organic phase was removed by aspiration. The resulting HCl extract was assayed as outlined by Karasawa *et al.*, 1975. A 1.0 ml portion of the HCl solution was combined with 0.05 ml 6% L-cysteine hydrochloride, vortexed and mixed with 1.5 ml OPT solution (10 mg % in conc. HCl). An oxidized blank was made by combining a 1 ml aliquot with 0.02 ml of 0.1 M ethanolic iodine for 15 min at room temperature. This was then treated, in a similar fashion, as a sample. These solutions were

all heated at 75°C for 10 min. After cooling in tap water for 2 minutes, the solutions were assayed on the spectrofluorometer at 355 nm and 480 nm as the excitation and emission wavelengths, respectively.

Radiometric assays. Radioactive samples of ^{3}H-Trp and ^{3}H-5-HT were made up to 30% aqueous suspensions using 0.6 ml of the respective phosphate effluent and 1.2 ml H_2O with 4.2 ml fluor solution consisting of scintillation grade toluene:Triton X-100 (2:1) and 7 g/l 2a70 fluor (98% PPO, 2° bis-MSB, Research Products International, Elk Grove Village, IL). The vials were mixed, placed in a 40°C water bath for 30 min, mixed again and placed in a Packard 2660 tri-carb scintillation counter to equilibrate to the lower temperature (12°C) and darkness for at least six hours prior to counting. The samples were counted 10 min. using a sample channels ratio efficiency correction program which was calibrated with mini-vial standards.

Data Analysis

Levels of Trp, 5-HT and 5-HIAA expressed as nmol/g whole wet brain and 5-HT turnover rates (nmol/g/hr) were calculated using external standards in conjunction with derivatization procedures. These numbers were then multiplied by the inverse of the fractional recovery and by the inverse of the grams of tissues per sample to calculate the nmol/g values for each compound as follows:

$$\text{nmol indole compound per gram of brain tissue} = \text{nmol compound in sample} \left(\frac{1}{\text{fractional recovery}}\right) \left(\frac{1}{\text{g tissue per sample}}\right)$$

The specific activities (DPM/nmol) for Trp and 5-HT were

calculated and used in the determination of turnover rate constants for 5-HT based on the equation of Neff *et al.* (1971). The turnover rate was determined by the steady state equation for the change in 5-HT concentration, d5-HT/dt = K (Trp-5HT). This equation can be rearranged to:

$$K = [(5HT_{t_2} - 5HT_{t_1})/(t_2 - t_1)]/([(Trp\text{-}5HT)_{t_1} + (Trp\text{-}5HT)_{t_2}]/2)$$

where:

K = constant for the turnover rate of 5HT

$5HT_{t_1}$, $5HT_{t_2}$ = 5HT specific activity at 1 and 2 hours, respectively, after 3H-Trp injection

t_1, t_2 = 1 and 2 hours, respectively

5-HT turnover rate = k[5-HT]

A single-factor analysis of variance was employed to analyze the effect of the various test compounds. Significant differences among treatment means ($p < 0.05$) were calculated by a Tukey's - HSD multiple mean comparison test (Neter and Wasserman, 1974).

RESULTS

The objective of this study was to assess the biochemical status of the brain 5-HT pathway after *in vivo* exposure to a series of occupational neurotoxic compounds including 2,5-hexanedione, acrylamide, tri-o-tolyl phosphate, Leptophos and Me-Hg. Preliminary investigations of neurotoxic indices, as measured by performance on the rotorod, were necessary to establish optimal dose periods and levels for each compound.

Rotorod Studies

In animals gavaged with 2,5-hexanedione, a high mortality rate was seen in the 3.0 g/kg/day group, with 80% of the rats dead by the third day while 1 rat in the 1 g/kg group died on day five (Fig. 2). Because of these deaths, dosing was discontinued in the 3.0 g/kg group after day three and in the 1.0 g/kg animals after day 4. The animals given daily doses of 0.3 g/kg showed a gradual loss of coordination on the rotorod with complete failure seen in all animals after the fifth day. No tremors were observed in the animals at any dose level and the 0.3 g/kg/day dosed-group showed a slightly higher weight gain than those animals given equal volumes of propylene glycol only (Table 3).

Acrylamide-treated rats at the lower dose levels had little difficulty in maintaining their balance on the rotorod (Fig. 3), while animals in the 50 mg/kg group exhibited a time-related decrease in performance with signs of tremors by the fourth day,

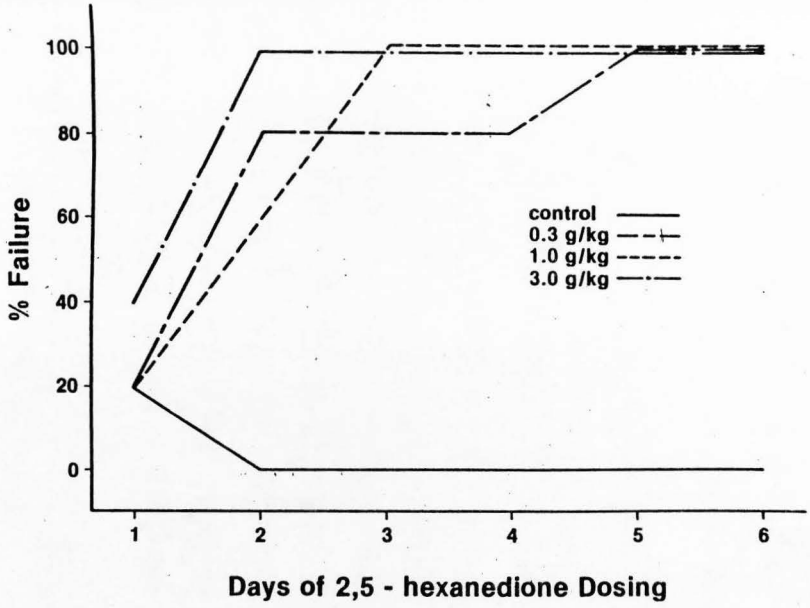


FIG. 2. Percentage of rats unable to remain on the rotorod for 60 seconds at 20 RPM prior to 2,5-hexanedione dosages (n = 5 in each group). The animals were dosed daily. Two animals in the 3g/kg group died on both days 2 and 3 while one died in the 1g/kg group on day 5. Dosing was discontinued in the 3.0 g/kg group after day 3 and in the 1.0 g/kg group after day 4.

TABLE 3

RAT WEIGHT CHANGES FROM DAY OF THE FIRST DOSE TO THE DAY OF SACRIFICE

	Duration of Treatment (Days)	Animal Weights (g)[b]		% change
		Initial	Final	
2,5 hexanedione (mg/kg)	8			
Control		192 ± 3	224 ± 6	+ 17
30		187 ± 3	226 ± 4	+ 21
100		191 ± 4	219 ± 5	+ 15
300		186 ± 4	211 ± 4	+ 13
Acrylamide	6			
Control		236 ± 1	272 ± 3	+ 15
5		234 ± 4	267 ± 4	+ 14
15		237 ± 5	278 ± 3	+ 17
50		241 ± 4	271 ± 3	+ 12
TOTP	17			
Control		182 ± 3	283 ± 4	+ 55
30		183 ± 4	282 ± 4	+ 54
100		185 ± 2	288 ± 4	+ 56
300		174 ± 3	272 ± 5	+ 56
Leptophos	14			
Control		234 ± 6	322 ± 9	+ 38
4.5		228 ± 5	311 ± 6	+ 36
15.0		233 ± 3	311 ± 9	+ 33
45.0		235 ± 5	293 ± 6	+ 25
Me-Hg	14			
Control		218 ± 3	285 ± 4	+ 30
1.0		223 ± 5	293 ± 7	+ 31
3.3		226 ± 3	296 ± 6	+ 31
1.0		226 ± 3	275 ± 5	+ 22

[a] 14 animals in each treatment group except acrylamide which had 16 animals per group.

[b] Weights in grams are shown as mean ± S.E.

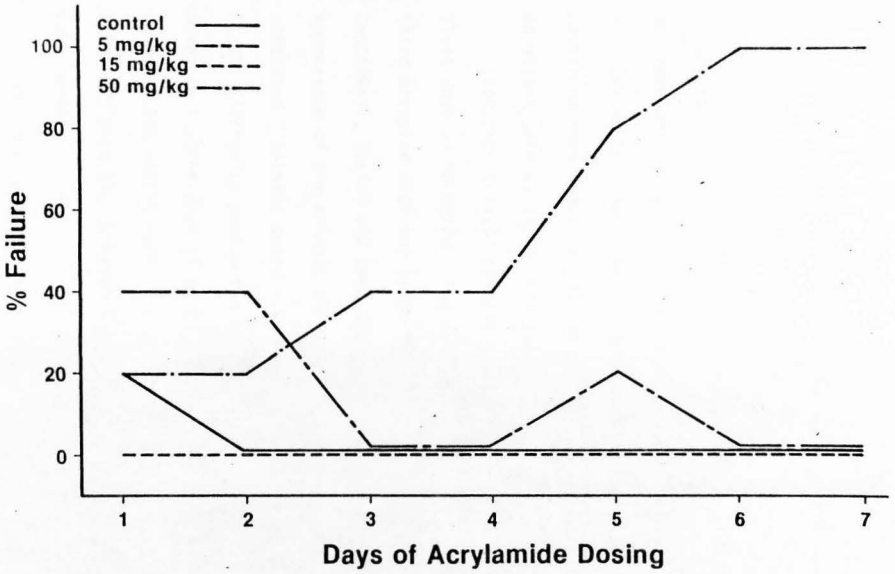


FIG. 3. Percentage of rats unable to remain on the rotorod for 60 seconds at 20 RPM prior to acrylamide dosages (n = 5 in each group). The animals were dosed daily.

until they were unable to walk on the rotorod by day six. Weight gains in the acrylamide-treated animals were similar to gains in 2,5-hexanedione-fed rats with a 12% increase in the high-dose group compared to a 15% gain in control animals while the other dose groups increases were similar to control values (Table 3).

TOTP had a severe effect on animals given oral doses of 1.0 g/kg. The entire group died within two days after the first dose was given. Rats in the 0.33 g/kg group were affected in their attempts at the rotorod and by the sixteenth day, all showed slight tremors and none were able to perform satisfactorily on the rotorod (Fig. 4). During the same time period, treatments with 0.1 g TOTP/kg caused no impairment in rotorod performance. TOTP had no effect on weight gain at any of the dose levels studied (Table 3).

Leptophos-treated animals showed signs of tremors after the first dose of 90 mg/kg. Two animals in that group died after the third atropine dose but prior to oral administration of the Leptophos. Dosing was then discontinued because the general appearance of the animals showed they were very lethargic and emaciated. Animals dosed with either 30 mg/kg or 9 mg/kg had little difficulty performing the rotorod test for a total of six doses in sixteen days (Fig. 5). Animals in the 45 mg/kg dose group showed a 25% weight gain in sixteen days compared to 38% in control animals (Table 3). Leptophos had no effect on weight gain at lower dose levels.

Time- and dose-related responses were seen in methyl mercury-treated animals. By the sixteenth day, animals given doses of 10 mg/kg failed completely while attempting to walk on the rotorod

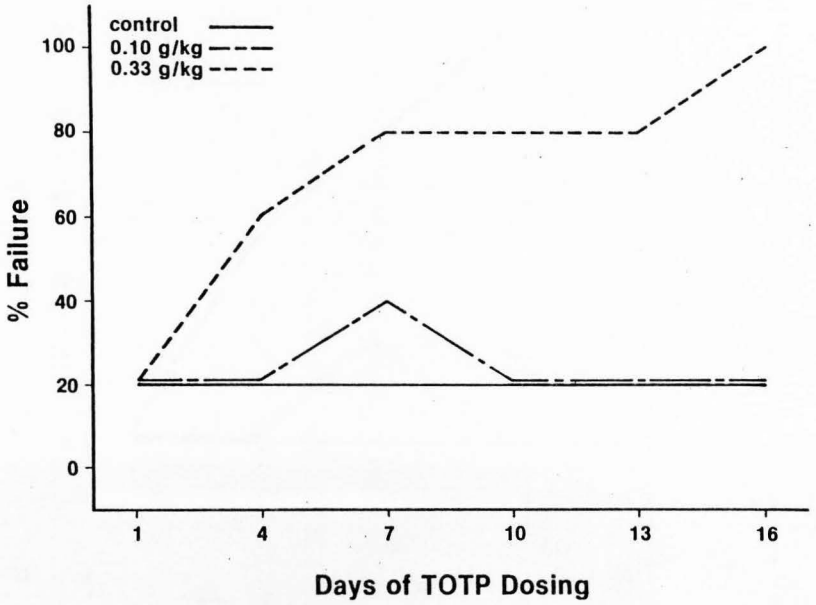


FIG. 4. Percentage of rats unable to remain on the rotorod for 60 seconds at 20 RPM prior to TOTP dosages (n = 5 in each group). The animals were dosed every third day.

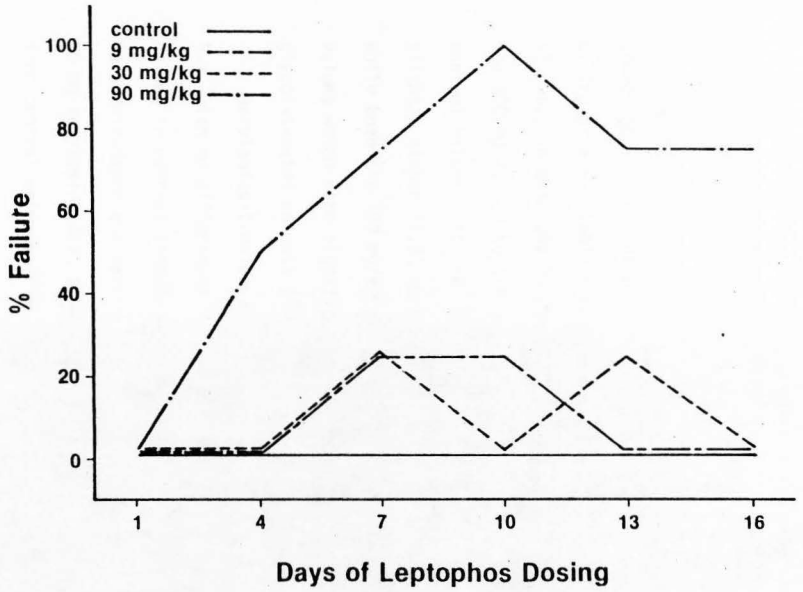


FIG. 5. Percentage of rats unable to remain on the rotorod for 60 seconds at 20 RPM prior to Leptophos dosages (n = 4 in each group). The animals were dosed every third day. Dosing was discontinued in the 90 mg/kg group after day 7.

(Fig. 6). At the same time rats in the 5.0 mg/kg and 2.5 mg/kg treatment groups showed 60% and 40% failure rates, respectively. A 22% weight gain was seen in the 1.0 g Me-Hg/kg group compared to gains in control and other treated animals of 31% (Table 3).

Biochemical Studies

These studies were done with brains of a separate group of animals treated *in vivo* with various chemicals at the doses established by rotorod testing. Animals dosed with 2,5-hexanedione showed no significant differences from control animals in levels of Trp or 5-HT (Table 4). Serotonin turnover rates declined slightly in both the 30 mg/kg and 100 mg/kg dose levels, but the 300 mg 2,5-HD/kg-treated animals had turnover rates equal to control values. At the 30 mg/kg dose, levels of 5-HIAA were slightly higher (1.81 nmol/g) than control values, (1.59 nmol/g), while both the 100 mg/kg and 300 mg/kg treated animals had 5-HIAA values which were significantly different ($p < 0.05$) from propylene glycol-treated animals (Fig. 7).

Acrylamide-treated animals showed constant levels of Trp and 5-HT with no differences in serotonin turnover rates compared to values in control animals (Table 5). Again, 5-HIAA levels increased in a dose-dependent manner with the two highest dose levels, 15 and 50 mg acrylamide/day, showing significant differences ($p < 0.05$) from control animals (Fig. 8).

Levels of endogenous indole compounds in the brains of TOTP-treated animals showed no effect (initial 6%, non-significant increase) on Trp levels in the 30 and 100 mg TOTP/kg dose groups while

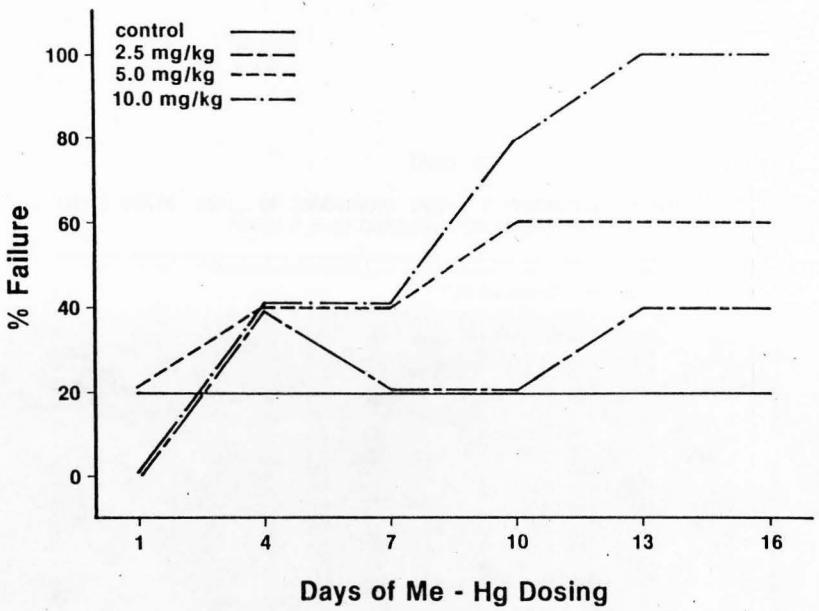


FIG. 6. Percentage of rats unable to remain on the rotorod for 60 seconds at 20 RPM prior to Me-Hg dosages (n = 5 in each group). The animals were dosed every third day.

TABLE 4

WHOLE BRAIN LEVELS OF ENDOGENOUS INDOLE COMPOUNDS AND 5-HT TURNOVER RATES AFTER 2,5-HEXANEDIONE TREATMENT *in vivo*[a]

	2,5-hexanedione (mg/kg)			
	Control	30	100	300
Tryptophan[b]	24.03 ± 0.75	23.78 ± 0.57	24.42 ± 0.83	24.87 ± 0.90
5-HT[b]	2.75 ± 0.07	2.69 ± 0.07	2.82 ± 0.05	2.67 ± 0.09
5-HIAA[b]	1.59 ± 0.11	1.81 ± 0.07	1.98 ± 0.10[d]	2.02 ± 0.08[d]
5-HT turnover rate[c]	2.01 ± 0.08	1.90 ± 0.11	1.82 ± 0.12	1.98 ± 0.08

[a]14 animals in each treatment group.

[b]Concentrations are expressed as nmol/g. Data are shown as means ± S.E.

[c]Rates are expressed as nmol/g/hr. Data are shown as mean ± S.E.

[d]Significantly different ($p < 0.05$) from control values.

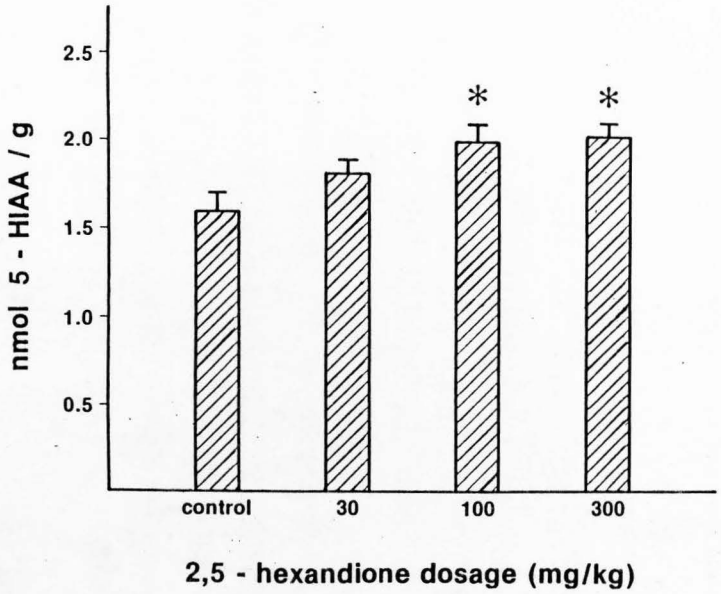


FIG. 7. Levels of rat brain 5-HIAA levels after 2,5-hexanedione administration for 7 consecutive days. The vertical bars represent the standard error of the mean; n = 14 in each dose level. Asterisk indicates a significant difference ($p < 0.05$) from control values.

TABLE 5

WHOLE BRAIN LEVELS OF ENDOGENOUS INDOLE COMPOUNDS AND 5-HT TURNOVER RATES AFTER ACRYLAMIDE TREATMENT *in vivo*[a]

	Acrylamide (mg/kg)			
	Control	5	15	50
Tryptophan[b]	23.38 ± 0.54	22.78 ± 0.72	23.00 ± 0.57	23.05 ± 0.63
5-HT[b]	2.56 ± 0.05	2.60 ± 0.04	2.69 ± 0.05	2.58 ± 0.04
5-HIAA[b]	1.68 ± 0.03	1.90 ± 0.07	2.16 ± 0.08[d]	2.30 ± 0.05[d]
5-HT turnover rate[c]	1.88 ± 0.07	2.02 ± 0.10	1.94 ± 0.08	1.95 ± 0.10

[a] 14 animals in each treatment group

[b] Concentrations are expressed as nmol/g. Data are shown as mean ± S.E.

[c] Rates are expressed as nmol/g/hr. Data are shown as mean ± S.E.

[d] Significantly different ($p < 0.05$) from control values

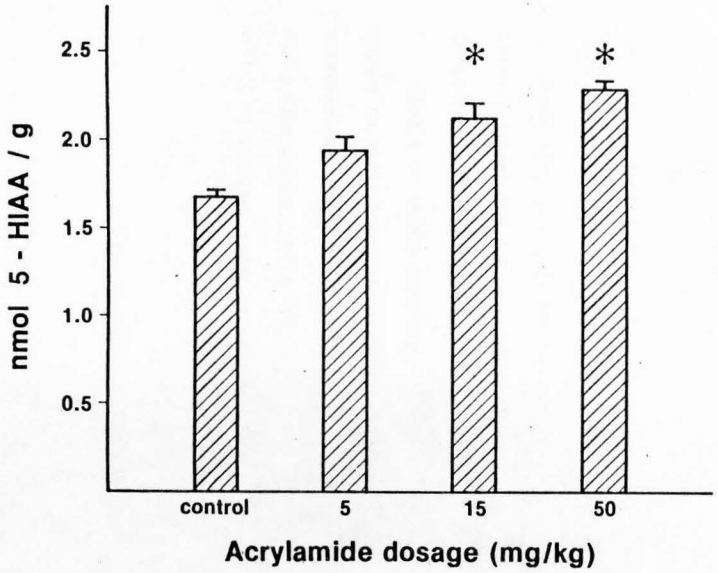


FIG. 8. Levels of rat brain 5-HIAA levels after acrylamide administration for 5 consecutive days. The vertical bars represent the standard error of the mean; n = 16 in each dose level. Asterisk indicates a significant difference ($p < 0.05$) from control values.

the 300 mg/kg-treated animals had values very close to those of control animals (Table 6). Serotonin levels were unchanged throughout the doses studied and 5-HIAA levels were slightly (6%) lower in the two higher dose groups. Serotonin turnover rates showed a non-significant, dose-dependent increase to a maximum of 10% over control values.

In rats gavaged with Leptophos, levels of tryptophan and 5-HT remained unchanged. Serotonin turnover rates were similar to those seen in TOTP-treated animals with values slightly, but non-significantly, higher than control values (Table 7). A bimodal trend was seen in the 5-HIAA levels with an initial increase in animals dosed with 4.5 mg and 15 mg Leptophos/kg followed by concentrations less than control values at 45 mg/kg.

Levels of indole compounds in Me-Hg treated rats (Table 8) showed no significant differences from control values; however, the turnover rates and levels of 5-HT were lower in the 1.0 and 3.3 mg/kg dose groups, while the highest mercury dose level had no effect on turnover rates or concentration.

TABLE 6

WHOLE BRAIN LEVELS OF ENDOGENOUS INDOLE COMPOUNDS AND 5-HT TURNOVER RATES
TRI-O-TOLYL PHOSPHATE TREATMENT *in vivo*[a]

	Tri-O-tolyl phosphate (mg/kg)			
	Control	30	100	300
Tryptophan[b]	22.62 ± 0.81	24.01 ± 0.72	24.12 ± 0.87	22.98 ± 0.93
5-HT[b]	2.67 ± 0.05	2.61 ± 0.05	2.73 ± 0.08	2.63 ± 0.07
5-HIAA[b]	1.72 ± 0.08	1.78 ± 0.12	1.67 ± 0.07	1.61 ± 0.08
5-HT turnover rate[c]	1.95 ± 0.11	1.99 ± 0.06	2.05 ± 0.09	2.15 ± 0.12

[a]14 animals in each treatment group.

[b]Concentrations are expressed as nmol/g. Data are shown as means ± S.E.

[c]Rates are expressed as nmol/g/hr. Data are shown as mean ± S.E.

TABLE 7

WHOLE BRAIN LEVELS OF ENDOGENOUS INDOLE COMPOUNDS AND 5-HT TURNOVER RATES AFTER LEPTOPHOS TREATMENT *in vivo*[a]

	Leptophos (mg/kg)			
	Control	4.5	15	45
Tryptophan[b]	23.07 ± 0.97	24.87 ± 0.82	22.92 ± 1.04	23.23 ± 0.86
5-HT[b]	2.72 ± 0.07	2.63 ± 0.09	2.69 ± 0.05	2.78 ± 0.11
5-HIAA[b]	1.52 ± 0.08	1.67 ± 0.15	1.59 ± 0.07	1.43 ± 0.10
5-HT turnover rate[c]	1.88 ± 0.09	1.96 ± 0.05	1.58 ± 0.05	2.07 ± 0.10

[a]14 animals in each treatment group.

[b]Concentrations are expressed as nmol/g. Data are shown as means ± S.E.

[c]Rates are expressed as nmol/g/hr. Data are shown as mean ± S.E.

TABLE 8

WHOLE BRAIN LEVELS OF ENDOGENOUS INDOLE COMPOUNDS AND 5-HT TURNOVER RATES AFTER Me-Hg TREATMENT *in vivo*[a]

	Me-Hg (mg/kg)			
	Control	1.0	3.3	10.0
Tryptophan[b]	22.63 ± 0.77	23.05 ± 0.65	22.87 ± 0.92	22.79 ± 0.75
5-HT[b]	2.65 ± 0.07	2.77 ± 0.09	2.56 ± 0.07	2.69 ± 0.05
5-HIAA[b]	1.70 ± 0.06	1.62 ± 0.08	1.77 ± 0.14	1.60 ± 0.09
5-HT turnover rate[c]	1.90 ± 0.15	1.78 ± 0.11	1.75 ± 0.08	1.95 ± 0.11

[a]14 animals in each treatment group.

[b]Concentrations are expressed as nmol/g. Data are shown as means ± S.E.

[c]Rates are expressed as nmol/g/hr. Data are shown as mean ± S.E.

DISCUSSION

Rotorod Studies

The initial training period of 7 to 10 days was accomplished with little difficulty in most rats. Occasionally, a rat in a group could not walk on the rotorod for the prescribed period of time, but because the animals were selected randomly, all animals were used in the analysis which accounts for many of the failures reported in control and low dose groups.

In the 2,5-HD study, 80% of the rats in the 3.0 g/kg/day treatment group died by the third day. The 1.0 g/kg/day group lost all coordination in walking the rotorod by the third day. By day five they were showing slight signs of tremor and one animal died. The 0.3 g/kg dose group exhibited a gradual decline in their ability to walk the rotorod and on the fifth day none of the animals in this group could remain on the rotorod for the prescribed time period. A level of 300 mg/kg/day to be administered seven consecutive days was designated as the high dose group with 100 mg/kg and 30 mg/kg doses given concurrently.

Cumulative doses of 380 mg/kg acrylamide (Gipon *et al.*, 1977) and 420 mg/kg acrylamide (Kaplan and Murphy, 1972) have been reported to produce deficits on the rotorod with accompanying muscular tremors. These experiments used dosing regimes similar to the present study, which showed consistent rotorod failures at a cumulative dose of 300 mg/kg.

The acrylamide-induced muscular weakness observed in these experiments could be secondary to decreases in body weight. Schotman *et al.* (1977a) found that pair-fed controls had higher weight gains than acrylamide treated rats. Although food intake is reduced during acrylamide intoxication (Gipon *et al.*, 1977), tryptophan levels in the present study were consistent with control values at all dosages of test chemicals.

Acrylamide-treated animals (50 mg/kg/day) showed tremors on the fourth consecutive day of treatment and could not satisfactorily complete the rotorod testing by the fifth day. These results indicated an acrylamide regime, lasting five days with the same dose levels used in the rotorod testing, would be satisfactory for biochemical studies.

All TOTP-treated animals in the high dose group (1.0 g/kg) died after the first gavage. At 0.33 g/kg, given every three days, the animals showed a gradual decline in rotorod performance with complete failure at day 16 while the 0.10 g/kg group had no problems with rotorod performance. The 300 mg/kg level was selected as the highest dosage for the biochemical studies with 100 mg/kg and 30 mg/kg given as the medium and low doses to be given in six doses every third day.

Leptophos animals did not do well physiologically at the 90 mg/kg level. Tremors were seen in all animals before the second dose, while two animals of that group died after atropine injections on the seventh day. The animals in the 30 mg/kg group were still doing rather well in the rotorod testing by the sixteenth day. A slightly higher dose of 45 mg/kg, to be given in five doses every

third day, was chosen as the highest level for the brain chemical studies with levels of 15 mg/kg and 4.5 mg/kg also given through the same time period.

Methylmercury-treated animals exhibited a dose-related response in rotorod performance. At a dose of 10 mg methylmercury/kg, given every 3 days, the animals reached 100% failure on the rotorod before the fifth dose on the thirteenth day, while the 5 mg/kg and 2.5 mg/kg animals had failure rates of 60% and 40%, respectively, after five doses. These same dose levels were chosen for the brain chemical assays and were administered in five doses every third day.

The rotorod seemed to be a reliable indicator of neuromuscular deficiency. As a tool for assessment of clinical neuropathy, it requires very little familiarity by the operator or training such as is required by more indepth psychological studies. Other tests such as hindlimb extensor tests and forelimb grasp tests seem to be somewhat more sensitive and specific (Tilson and Cabe, 1979; Tilson *et al.*, 1980), but also require specialized expensive equipment. The purpose of the rotorod testing was to establish doses for the subsequent biochemical studies and the rotorod has provided sufficient information to answer those questions.

Biochemical Studies

Whole brain levels of endogenous indole compounds as well as 5-HT turnover rates compared very closely with reported literature values. The average values in this laboratory for Trp were 23.14 ± 0.56 nmol/g, while 5-HT averaged 2.67 ± 0.08 nmol/g and 5-HIAA levels were 1.64 ± 0.06 nmol/g. Smith *et al.* (1975) have

reported values for Trp, 5-HT and 5-HIAA of 15.6 nmol/g, 2.5 nmol/g and 1.7 nmol/g, respectively, while Marini *et al.* (1979) reported average values of 22.5 nmol/g for Trp, 2.4 nmol/g for 5-HT and 1.95 nmol/g for 5-HIAA. Rates of 5-HT turnover have been reported as 1.5 nmol/g/hr in whole rat brain by Neff *et al.* (1971) and as 2.32 nmol/g/hr (Tozer *et al.*, 1966).

A consideration in establishing the dosing regime for the biochemical studies was that they not have a prolonged time period because of the difficulty involved in tail vein injections of rats exceeding 350 g. However, the dosing durations used were sufficient to produce clinical neuropathy, in at least one dose level. While Gilles *et al.* (1981a, 1981b) demonstrated an inhibition of lipid metabolism and sterologenesis in rats after a 6 week exposure to 1% 2,5-HD in drinking water, 5-HT and 5-HIAA levels in rats and hamsters increased after single exposure to dieldrin followed by sacrifice either a day or a week later (Kohli *et al.*, 1977; Willhite and Sharma, 1978) and within 2-6 hrs after a single carbaryl dose (Hassan and Santolucito, 1971). These studies indicate the ability of the 5-HT pathway to respond rapidly after toxic chemical exposure. Nevertheless, lack of biochemical activity at the dose levels used in these studies doesn't ruleout the possibility that an effect may be seen at lower dose levels over a longer period of time.

Normally, brain 5-HT is almost quantitatively converted to 5-HIAA (Tozer *et al.*, 1966). The pial lining of the adult brain offers little resistance to the passage of acid metabolites to the cerebrospinal fluid (CSF) compartments (Cserr, 1974 and Fenstermacher

et al., 1974). The CSF system has low concentrations of acid metabolites relative to high levels in brain interstitial fluid. This relationship was proposed to serve as a "diffusional sink" or "quasi-lymphatic system" for the elimination of 5-HIAA from brain (Meek and Neff, 1973; Oldendorf and Davson, 1967). In the 30 day old rat, the rate of 5-HIAA formation is about 4.2 ng/brain/min and CSF concentrations of 5-HIAA are 126 ng/ml. Complete clearance of the acid metabolite would require a bulk flow rate of 33 µl/min, whereas the circulatory rate for CSF can account for no more than 6% of the 5-HIAA elimination (Bass and Lundberg, 1976). These researchers showed that the transfer of the organic acid to ventricular CSF and their subsequent active transport to blood via the choroid plexus is not the major route for removal of 5-HIAA from the whole brain, although those areas situated proximal to the ventricle may depend on this route. They found the efflux rate from the CSF was too slow to account for the entire 5-HIAA clearance and demonstrated an active transport system at the glia-capillary interphase which accounted for the efflux of 75% of the 5-HIAA formed in the brain.

Since the levels of Trp and 5-HT as well as the 5-HT turnover rates were not significantly changed with any of the dosages of acrylamide and 2,5-HD used in this study, and while the 5-HIAA levels increased in a dose-dependent manner, it is possible the energy-dependent carrier-mediated acid transport efflux system from the brain to the cerebral spinal fluid or the blood stream via glial cells may have been affected. Aldous *et al.* (1981) found a similar significant increase in 3,5-dihydroxyphenylacetic acid (DOPAC), a

major DA metabolite, with the same regime of acrylamide administration used in this study, while no apparent changes in levels or turnover rates of DA were seen. Based on the results of several studies of glycolytic enzymes, Spencer and co-workers (1979) have hypothesized neurotoxic compounds (acrylamide and neurotoxic hexacarbons), that cause central-peripheral distal axonopathy, deplete energy supplies in the nerve fiber by inhibiting enzymes required for energy synthesis leading to a blockage of energy-dependent axonal transport.

The organophosphorus-dosed animals showed no significant changes in Trp, 5-HT and 5-HIAA levels when compared to control animals although there were dose-related increases in 5-HT turnover rates and endogenous 5-HT with a decrease of similar magnitude in 5-HIAA levels, indicating a possible MAO inhibition. Chickens or cats are much more sensitive to neurotoxic organophosphate compounds than rats (Davis and Richardson, 1980) and perhaps the methods employed in this study should be adapted to one of these species for further clarification.

Methylmercury-treated rats were not significantly different from control animals with respect to levels of indole compounds, although turnover rates were slightly lower in animals fed either 1.0 or 3.3 mg/kg. Other studies have shown methylmercury causes damage in the rat blood-brain barrier (Ware *et al.*, 1974) with a resultant reduction of amino acid uptake in the brain (Steinwall and Klatzo, 1966), as well as reduced brain levels of 5-HT in neonatal rats.

The results indicate that at the dose levels and period of administration used in the present study, methylmercuric chloride has no effect on Trp uptake or synthesis and metabolism of 5-HT.

Dietary availability of Trp and interference with the Trp transport processes at the blood-brain barrier both alter brain 5-HT synthesis (Fernstrom, 1979). Acrylamide (20 mg/kg) has been shown to reduce brain membrane protein levels (Agrawal *et al.*, 1981). Since whole brain Trp levels were not significantly changed with any of the test compounds in this study, the availability of amino acid precursors was not a factor, even in those animals with weight gains much less than controls. Animals with muscle tremors apparently consumed enough food to maintain a proper amino acid balance. No demonstrated loss of functional integrity was observed in the blood-brain barrier with regard to Trp transport.

Discrete brain areas show varied concentrations of 5-HT with representative values (μg/g) for various brain areas being 2.03 in the hypothalamus, 1.11 in the pons-medulla, 1.10 in the midbrain, the caudate-putamen area is 1.05, the hippocampus values average 0.57 while the cortex and cerebellum values are 0.49 and 0.33, respectively (Jacobowitz and Richardson, 1978). Gothoni and Ahtee (1980) found a significant decrease in 5-HT concentrations in the section of the brain containing the pons and medulla oblongata with an increase in 5-HIAA levels in these areas. The values in the cortex remained unchainged. Nickel also decreases 5-HT levels in the basal ganglia (Ali *et al.*, 1980) with similar changes in the cerebral cortex. The present study involving whole brain showed no significant changes in 5-HT concentrations or 5-HT turnover rate. A study involving 5-HT-rich brain areas may show some changes which were possibly masked by whole brain values.

SUMMARY AND CONCLUSIONS

This investigation was initiated to determine if *in vivo* exposure to several industrial and/or environmental chemicals affects endogenous whole brain levels of Trp, 5-HT and 5-HIAA as well as 5-HT turnover rates in rats. Preliminary experiments using a rotorod apparatus, fitted with an electrode floor, were performed to establish dosage levels and duration of exposure. The rotorod procedure used in this study, provided an adequate clinical indication of neuropathy although discrimination between fore- and hind-limb deficits were not possible.

Levels of endogenous indole compounds were determined spectrofluorometrically and specific activities of Trp and 5-HT were determined at both one and two hours after ^{3}H-Trp IV injection.

The data show a dose-dependent increase in 5-HIAA levels while 5-HT and Trp values as well as 5-HT turnover rates showed no significant differences from control levels in animals treated with either acrylamide or 2,5-hexanedione. These results suggest an inhibition of the energy-dependent acid efflux system from the brain to the cerebral spinal fluid or to the blood stream.

None of the chemicals used in this study altered the whole brain levels of Trp, indicating no effect on dietary status or subsequent Trp transport across the blood brain barrier, although some animals did show weight gains much less than controls.

Animals dosed with either TOTP, Leptophos or Me-Hg had no significant changes in the parameters studied although TOTP and

Leptophos treated animals showed slight, dose-dependent, increases in 5-HT turnover rates with insignificant decreases seen in 5-HIAA levels. Since chickens or cats are more sensitive to organophosphorus neuropathy than rats, investigation in these species may provide evidence for influence of indole metabolism.

No significant changes in 5-HT synthesis in metabolism were seen in animals treated with Me-Hg. Since Trp levels in these rats were similar in control and treated rats, there is no evidence of commonly observed Me-Hg-induced blood-brain barrier damage at the dose levels used in this study.

Further studies involving longer dose periods or examination of discrete brain areas may further clarify the effects of these chemicals on brain biochemistry.

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