INHIBITION OF SYNAPTOSOMAL BIOGENIC AMINE TRANSPORT

BY A DIVERSE GROUP OF NEUROTOXIC CHEMICALS

by

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ABSTRACT

Inhibition of Synaptosomal Biogenic Amine Transport by a Diverse Group of Neurotoxic Chemicals

by

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Utah State University, 1980

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Synaptosomal membrane functions were monitored, after in vitro exposure to select environmental pollutants, in synaptosomal preparations originating from rat cerebral cortices. The uptake of NE and 5HT into the synaptosomes was monitored as was the K⁺-dependent phosphatase activity of the membrane. CH₃HgCl, Hg(NO₃)₂, CdCl₂, diisopropylfluorophosphate (DFP), paraoxon, acrylamide and Kepone were the test chemicals whose effects were studied.

CH₃HgCl, Hg(NO₃)₂ and Kepone had the greatest inhibitory effects on NE and 5HT uptake. The concentrations producing 50 percent inhibition (IC₅₀) were 1.4 x 10⁻⁴, 4.0 x 10⁻⁴, and 9.4 x 10⁻⁵ M, respectively, for NE uptake while the IC₅₀'s for 5HT uptake were 1.9 x 10⁻⁴, 6.0 x 10⁻⁴, and 3.3 x 10⁻⁴ M, respectively. Maximal inhibition was 60-100 percent at 10⁻³ M while the effective concentration range was between 10⁻⁴-10⁻³ M. The remaining test compounds produced no significant inhibition at concentrations up to 10⁻³ M.
K\textsuperscript{+}-dependent phosphatase was strongly inhibited by CH\textsubscript{3}HgCl, Hg(NO\textsubscript{3})\textsubscript{2}, CdCl\textsubscript{2}, and Kepone with IC\textsubscript{50}'s of 1.5 x 10\textsuperscript{-6}, 0.032 x 10\textsuperscript{-6}, 1.5 x 10\textsuperscript{-6}, and 13.0 x 10\textsuperscript{-6} M, respectively. The effective inhibitory concentrations for these chemicals ranged from 10\textsuperscript{-7}-10\textsuperscript{-3} M and suggested a specific high affinity inhibition. DFP, paraoxon and acrylamide did not produce a significant inhibition at concentrations between 10\textsuperscript{-5}-10\textsuperscript{-3} M.

A correlation of the phosphatase and monoamine uptake inhibitions, in search of a cause-effect relationship, was not suggested from the data. However, the low affinity inhibition (IC\textsubscript{50} greater than 10\textsuperscript{-5} M) of the NE and 5HT uptake by CH\textsubscript{3}HgCl, Hg(NO\textsubscript{3})\textsubscript{2} and Kepone, along with the general shape of the dose-response curve is suggestive of an all-or-none inhibition. The apparent high affinity inhibition (IC\textsubscript{50} less than 10\textsuperscript{-5} M) of the phosphatase demonstrates the specific influences these compounds can have on enzymatic processes. Such enzymatic inhibition could be of critical importance if these neurotoxicants were able to penetrate the synaptosomal or even neuronal membrane and gain access to the metabolic and synthetic machinery.

(107 pages)
INTRODUCTION

Federal regulations are restricting usage of many chemicals and preventing the use of others because of positive toxicological results in test systems. The manifestations of toxic effects assume various forms: cancer, neurotoxicity, metabolic poisoning, hepatic disease, etc. In recent years the neurotoxic potential of chemicals has become an increasing concern to health officials and federal agencies regulating their use. Mass poisonings by hexachlorobenzene, mercury, and tri-ortho-tolyl phosphate have resulted in victims with pathological and behavioral manifestations of neurological dysfunction (Allen et al., 1979; Harada, 1978; Smith et al., 1930). Widespread usage of neurotoxic pesticides has increased the exposure and risk of both the occupational worker and the general public. With thousands of chemicals now in use and the continual development of new ones, an examination of the pathological, biochemical and physiological effects of chemicals is important for both the assessment of their need for regulation and elucidation of their mechanism of action.

As will be detailed in a subsequent chapter, several neurotoxic chemicals have been shown to alter neurotransmitter concentrations and metabolism in brain tissue. Although this relationship is promising for the development of a definitive hypothesis for the cause of their neurotoxicity, several problems associated with the tissue preparation must be overcome before a complete mechanism can
be presented. Studies of this nature have been performed in either brain slices or brain homogenates making it difficult to identify specific types of target cells. In addition tissue homogenates mask the contribution of ionic gradients or intact plasma membranes in the expression of the toxicity. Disruption of the cellular compartmentalization in these preparations may also indirectly contribute to the observed changes by bringing together cellular constituents, not normally associated with the neurotransmitters, that in some non-specific manner may influence their metabolism. To overcome these deficiencies synaptosomal preparations can be used to monitor both metabolism and transport processes in intact structural units that are more indicative of the in situ conditions.

Synaptosomes are synaptic terminals which have been sheared, during fractionation, from their axonal connections and surrounding glial cells. After separation from the axon the terminal capsule reseals forming the intact structural unit (Jones, 1975). Often the inter-synaptic cleft and post-synaptic process are isolated as a portion of this unit. Utilization of synaptosomes as a model for the investigation of neurotransmission has several advantages. First, the integrity of the membrane and demonstration of its functional capacity is comparable to the in situ condition at the synapse. Secondly, the characterization of their metabolic activity indicates that all enzymes and co-factors are present as would be seen in vivo. Thirdly, the studies showing the viability of synaptosomes for several hours after removal from the brain allows for the time related studies. A final advantage would be the characterization of synaptic activity away from the diluting factors seen with tissue homogenates.
Behavioral change is seldom utilized as a tool for toxicity testing unless gross changes are apparent. Neurotoxicity is usually measured in terms of morphological damage rather than less apparent biochemical lesions. Subtle biochemical changes, although not as lethal initially, may ultimately be just as damaging. As will be outlined in the following chapter, behavioral modification has been associated with alterations of the biogenic amine neurotransmitter levels. Subtle changes in these levels may not produce acute modification but if these changes persist over long periods of time it is not known what behavioral alterations, if any, may take place. Detrimental changes in aggressive and reproductive behavior, as an example, would possibly have serious consequences for the survival of a species.

Investigation of the effects neurotoxic chemicals may have on neuro-transmitter function would yield valuable information concerning possible mechanisms of action. Parameters of concern would include metabolism, synthesis, release and uptake of the transmitters. Alteration in any of these processes could initiate a potentially hazardous result and can easily be monitored in a synaptosomal model system.

The present study is concerned with the examination of the effects various neurotoxic environmental pollutants, from several classifications, have on synaptic inactivation of norepinephrine and serotonin. An initial characterization of the synaptosomal preparations to determine optimum assay conditions along with a comparison of the obtained $K_m$ and $V_{max}$ values for the uptake processes
with known literature values was performed to assess the suitability of the synaptosomal preparations for the study. These synaptosomes, obtained from the cerebral cortex of the male rats were then used to determine if in vitro exposure to the test chemicals (CH$_3$HgCl, Hg(NO$_3$)$_2$, CdCl$_2$, diisopropylfluorophosphate (DFP), paraoxon, acrylamide and Kepone) modified the kinetics of the carrier-mediated membrane transport of the neurotransmitters. K$^+$-dependent phosphatase activity was monitored in the synaptosomal membrane to determine if effects the test chemicals have on the neurotransmitter uptake is specific in nature or a result of a more indirect membrane effect.

Since the mechanism of the toxicity of the neurotoxic agents under study is in all probability not dependent on synaptic transport phenomena, the present investigation will elucidate the potential these toxicants have for synaptic interactions and possible behavioral modification via an interruption of normal information flow in the central nervous system.
LITERATURE REVIEW

Pharmacological Modification of Central Nervous System Functions

The brain, as an organ, is an integrated composite of many distinct structures whose overall function is dependent on an accurate, reproducible but flexible information flow between individual components. The limbic system is an example of this interdependency that is commonly seen in the central nervous system (CNS). The total system is composed of units predominately in the brain stem and midbrain sections (hypothalamus, basal ganglia, amygdala, hippocampus and thalamus) joined by phylogenetically older cerebral cortical areas (medial and ventral portions) known as the limbic cortex. Together these units are involved with special types of behavior associated with emotions, subconscious motor and sensory drives and the intrinsic feelings of pain and pleasure (Guyton, 1976). Signals originating from parts of the limbic system other than the hypothalamus are funneled through the hypothalamus and give rise to various vegetative or behavioral effects that are known to occur after stimulation of the hypothalamus. At the same time the reticular activating system receives limbic signals to regulate the degree of wakefulness, alertness and attention while the non-limbic portion of the cerebral cortex receives behavioral signals directly from the limbic structures affecting cortical analytical function, sensory function, and at times even its motor function.
To facilitate information transfer to the appropriate areas of the brain, the synaptic junction or synapse regulates the passage of the signals from one neuron to the next. Two key functions imparted by this regulatory process are directionality and selectivity. Directionality provides an organized pattern for receiving and transmitting signals while selectivity adds a two-dimensional process that expands the range of interpretation for each signal. Without an inherent order in the signal processing, inefficiency in information flow would reduce the effectiveness of the integrated functions of the nervous system. The selective nature of the synapse occurs in the positive and negative feedback processes which filter out unnecessary stimuli while passing on other stimuli that is needed for normal nervous function.

With the central role the synapse has in the processing of signals within the CNS, it becomes a primary target for pharmacological manipulation. Koelle (1975) discussed the ability of selected drug types to elicit peripheral as well as central nervous system responses during drug therapy. Morphine and other opiates that produce analgesia, drowsiness, changes in mood and mental clouding when administered in humans, are primarily affecting higher levels of the CNS. Smith and Sheldon (1973) mentioned that morphine increases the release, synthesis, and turnover of catecholamines and more recently it has been found to compete for neuroactive peptide receptor sites (Hughes, 1978). Anti-psychotic drugs such as chlorpromazine (phenothiazine type) and haloperidol (butyrophenone type) similarly decrease depression and the psychotic states in mentally disturbed
patients. As yet unexplained changes are produced in normal patients given these drugs; a decrease in motor activity and minimal responsiveness to stimuli are known to occur while inhibition of performance on complex intellectual tests are suggested. Biochemical monitoring of synaptic activity showed that these anti-psychotic drugs inhibit inactivation of nonrepinephrine and serotonin, by preventing re-uptake into the synaptic terminals, producing a gradual decrease in monamine concentration (Brucke et al., 1969). Along with uptake inhibition chlorpromazine, at low concentrations, enhances membrane permeability in some systems suggesting that direct membrane effects may alter the Na$^+$ gradient required for membrane transport of the biogenic amines (Maoi et al., 1979). Tricyclic anti-depressants (i.e., imipramine and desipramine) which produce anti-psychotic responses similar to the phenothiazines and butyrophenones, are specific, highly potent inhibitors of serotonin and norepinephrine re-uptake (Wedley et al., 1978; Fuller and Wong, 1977).

Reserpine prevents the storage of biogenic amines in synaptic vesicles and subsequently causes a virtual depletion in monoamine concentration (Koelle, 1975). Monamine depletion occurs through an increased monamine oxidase activity since the intracellular concentrations of these neurotransmitters are initially increased on drug exposure. With time the amount of transmitter released into the synaptic cleft after membrane depolarization is decreased and this ultimately affects the degree of post-synaptic activation. Reserpine exposure in humans produces a state of indifference to environmental stimuli and a tendency towards sleep.
Benzodiazepines and related diazepam are the most commonly prescribed anti-anxiety drugs in use today. Animal behavior studies have indicated that a lowering of catecholamine turnover is responsible for the depressant action of these drugs while anti-anxiety responses may be attributed to decreasing the serotonin turnover (Stein et al., 1975). Rastogi et al. (1979) determined that chronic administration of diazepam to rats produced increased synaptosomal serotonin concentrations that were double the concentration found in acute exposure. Chronic exposure increased as well the uptake and the rate of synthesis of serotonin. From their studies Rastogi et al. (1979) concluded that diazepam exerts its central effects on mood and behavior by impairing the release of serotonin, and to a lesser extent catecholamines.

From the drug literature it is understood that modulation of synaptic function is a useful means for producing behavioral responses. In discussing these pharmacologically induced behavioral changes Koelle (1975) pointed out that modification of synaptic activity can be brought about by using drugs that affect neurotransmitter synthesis, metabolism and turnover as well as the membrane release and uptake mechanisms. Additional modification can occur by altering the synaptic membrane integrity, either increasing or decreasing its fluidity or plasticity or by using drugs that compete for post-synaptic membrane receptors thus enhancing or inhibiting the membrane response.
In a general discussion of the catecholamines, norepinephrine (NE) and dopamine (DA), and the indolamine, 5-hydroxytryptamine (5-HT), Synder (1976) pointed out that these biogenic amines are the only putative neurotransmitters which have been localized in discrete nerve tracts of the brain and whose relationship with specific animal or human behavior has been worked out. Stereotaxic mapping, utilizing a histochemical fluorescence method, reveals that NE and 5-HT, although widely distributed in low concentrations throughout the brain, congregate in cell bodies to the mutual exclusion of the other (Ungerstedt, 1971). NE cell bodies are extremely prominent in the locus coeruleus and give off both ascending and descending bundles which reach all levels of the brain and spinal cord. Cell bodies containing 5-HT concentrate in the raphe nuclei of the lower midbrain and upper pons. The serotonergic axons emanating from these nuclei ascend primarily into the forebrain from which they then disperse to all areas of the brain.

Termination of the effects of NE and 5-HT, after release into the synaptic cleft, occurs through a membrane transport phenomena that returns these neurotransmitters back into the presynaptic terminal. This process, which is energy and temperature dependent, obeys Michaelis - Menten kinetics and is believed to be highly specific for only one type of monoamine in any one terminal (Iverson, 1971). Na⁺ and K⁺, at physiological concentrations, are required for optimum activity and it is believed that the divalent
cations Mg$^{++}$ and Ca$^{++}$ as well have some function in maintaining this activity at peak levels (White, 1975).

Discovery of this re-uptake is credited to Axelrod and his co-workers (1965), who found that radiolabelled, intravenously injected, NE accumulated in tissues with dense sympathetic innervation. Homogenization of this tissue and subsequent use of density gradients revealed the label to be localized in the synaptic vesicles. Later work by Iverson and his coworkers (1967) revealed that the granular storage process could be separated from the actual uptake into the nerve terminals by using the drug resperine which enhances the release of the monoamines from the storage vesicles but does not disrupt membrane transport into the terminal.

A carrier-mediated model for the transport of biogenic amines across pre- and post-synaptic membranes and into glial cells has been suggested by Hammerschlag and Roberts (1976). In this model the uptake process has an absolute Na$^+$ requirement and operates at maximum velocity at physiologic Na$^+$ concentrations. Binding sites for the transmitter exist in the synaptic membranes, are specific in nature, and have an affinity with a Km of less than 1.0 $\mu$M for the monoamines. These amines bind much more readily in environments with high Na$^+$ concentrations (extracellular membrane surface) and much less readily in low sodium environments (intracellular membrane surface). As the transmitter is released into the synapse during neuronal activity, equilibration between receptor and transmitter occurs because of the high Na$^+$ concentrations in the synapse. Under this condition the transmitter readily binds to the receptor and in
a process that is not yet understood but may depend upon various asymmetries on the two sides of the membrane, such as oxidation-reduction potentials, degree of phosphorylation and concentrations of particular ions or metabolites, the transmitter is transported through the membrane into the intracellular compartment. Since intracellular Na\(^+\) concentration is low the transmitter rapidly dissociates from the receptor and becomes available for mitochondrial metabolism. Thus it is suggested that the assymmetrical Na\(^+\) concentration sets up the conditions necessary for transport of these transmitters. Although metabolically generated ATP is not directly required for this process there is an overall energy requirement for the transport to occur. It is felt that the potential energy of the Na\(^+\) gradient is probably the major source of energy for the carrier-mediated transport. ATP is, of course, required for operation of the Na\(^+\) pump (Na\(^+\), K\(^+\)-ATPase) that maintains the Na\(^+\) gradient.

Selected Aspects of Organophosphate Neurotoxicity

Acute toxicity of organophosphates is a product of the specific inhibition of acetylcholinesterase with the resulting accentuation of cholinergic activity. Although this esterase inhibition is the most acute effect, other enzyme inhibitions are known that are unrelated to the cholinesterase inhibition. Jovic et al. (1971) treated rats with 3.2 mg/kg DFP (1.3 x LD\(_{50}\)), after atropine pre-treatment, and demonstrated a significant reduction in the succinate
dehydrogenase, aldolase and Na$^+$, K$^+$-ATPase activities in brain slices. Qualitatively similar effects were also detected in vitro. Although the study was performed utilizing a lethal dose of DFP it none the less implicated this compound and possibly other structurally related organophosphates as inhibitors of cellular metabolism.

For sometime it has been known that organophosphates produce peripheral delayed neuropathies resulting in axonal degeneration and paralysis of affected limbs (Smith et al. 1930). Initially the foci at which the organophosphates initiated this response was thought to be the neuronal perikarya followed by retrograde axonal degeneration resulting from interference with somal metabolic functions (Cavanagh, 1964). Since then modern techniques have demonstrated this hypothesis to be inaccurate. Bouldin and Cavanagh (1979) have presented evidence that the initial lesion commencing axonal degeneration occurs within the axon itself. Utilizing a teased nerve fiber technique they were able to determine that the point of origin of the neuropathy was in the distal, but non-terminal, segment of the longest and largest nerve fibers. Subsequent to the initial lesion the axonal degeneration spread in a somatofugal direction to involve the entire distal axon. Prior to this degeneration fiber varicosities and demyelinated segments were identified.

In support of Bouldin and Cavanagh’s (1979) evidence for an initial axonal lesion preceeding the axonal degeneration is a study by Howland et al. (1980). The authors used a technique that allowed them to produce a mononeuropathy involving only one hind limb. By injecting radiolabelled DFP (2 mg/kg) into the left femoral
artery of a cat and sectioning the sciatic nerve in both the treated and contralateral sides, as well as spinal ganglia and dorsal and ventral roots, one and twenty-four hours post treatment, they were able to determine that DFP was restricted to the injected leg with very little migration of label to the contralateral side or into the roots or ganglia. The authors feel that if the perikarya were involved in the genesis of this mononeuropathy it would be manifested on both sides and significant levels of DFP would have been detected in the somal preparations.

In a study of the functional capacity of motor nerve terminals during DFP induced neuropathies Lowndes and Baker (1978) detected a decrease in the ability of soleus alpha terminals to generate stimulus-bound repetition. This depressed activity reached a maximum 21 days post exposure. Histologically the motor nerve terminal underwent marked degenerative changes but it appeared not to effect the conduction velocity or single impulse transmission, both of which were normal. At this point the animal demonstrated a high-fast step gait in the neuropathic limbs while the primary spindle frequency of firing at various lengths of stretch was decreased.

The above studies, for the most part, were concerned with the cholinergic parts of the nervous system. They provided evidence that DFP was capable of inducing degenerative changes in the nerve fibers after exposure to a concentration of DFP greater than that normally considered lethal. Little information is available on the affects produced after low level chronic exposure. Since DFP and other organophosphates are known to carry out membrane phosphorylation
(Johnson, 1969) and also since Jovic et al. (1971) reported the ability of DFP to inhibit CNS metabolic functions after in vivo treatment, it would be of interest to assess the membrane effects occurring within the CNS after organophosphate exposure. Of particular interest would be the effects phosphorylation might have on synaptic transmission and other synaptic membrane function.

**Selected Aspects of Heavy Metal Neurotoxicity**

Pathological changes within the nervous system, biochemical, functional and morphological, are documented expressions of heavy metal poisoning, of which lead and mercury have been the most intensely studied (Hrdina et al., 1976; Pentschew, 1965; Smith, 1976). Arsenic and cadmium exposure is also known to initiate neuropathology in experimental animals (Gabbiani et al., 1967; Hrdina et al., 1976; Krigman, 1978; Rastogi et al., 1977; Sato et al., 1978) but much less is known about their mechanisms of action. In the studies mentioned, as well as others, it is generally accepted that as a group heavy metals are able to effect both the peripheral and central nervous systems. Some of these metals are capable of injuring both systems while others are specific for one or the other. The critical factor in determining the type of injury is the solubility and distribution of a particular metal in the body. The type of injury produced is similar in both systems.
Oehme (1978) felt that in general terms the toxicity of heavy metals is principally a consequence of their high affinity for many ligands such as proteins and amino acids. Metal complexes are readily formed with sulfhydryl groups and to lesser degrees with amino, phosphate, carboxylate, imidazole and hydroxyl radicals of biologically significant compounds, often resulting in protein degeneration. With the high affinity many metals have for protein constituents and the significant role proteins have in membrane structure and function it is to be anticipated that membrane barriers might sustain considerable damage after heavy metal exposure.

In a review of the toxic effects of mercury, Chang (1977) stated that the neuropathology is a consequence of membrane degeneration followed by metabolic dysfunction that leads to functional changes in the neurophysiology of neuron and axon. This scenario is supported by information showing subcellular accumulation of mercury occurring in membrane fractions when characterizing the subcellular distribution by ultracentrifugation (Yoshino et al., 1966a). More recently electron-microscopic histochemical methods (metal-sulfide coupling) were able to demonstrate visually the close associations of mercury with membranous structures and the increasing amounts detected in the matrix of the cytoplasm and axoplasm as the intoxication progressed (Chang and Hartman, 1972a). Surprisingly this study indicated that very little mercury penetrates through the nuclear membrane.

It has been known for some time that mercury inhibits protein synthesis prior to development of neurologic symptoms while oxygen consumption, aerobic and anaerobic glycolysis and sulfhydryl enzyme
activities remain unchanged (Yoshino et al., 1966b). It subsequently was found that many enzyme activities were altered after mercury intoxication and the onset of neurologic symptoms. Succinic dehydrogenase, ATPase and alkaline phosphatase activities decreased indicating damage to mitochondria, cell membranes and the blood-brain barrier by mercury. Paterson and Usher (1971) detected a decrease in the ATP/ADP and ATP/AMP ratios as well as inhibition of enzymes towards the end of the glycolytic chain in mercury treated rats. Salvaterra et al. (1973) extended these metabolic studies and found changes in phosphocreatine and adenosine nucleotides as well as glycolytic intermediates. RNA synthesis is also altered after mercury intoxication but conflicting results have been obtained indicating that increased or decreased synthesis may be dependent on the susceptibility of specific cell types (Brubaker et al., 1973; Chang and Hartman, 1972a,b). Somjen et al. (1973) reported that the intracellularly recorded spike potential of many sensory ganglion neurons of mercury poisoned rats was significantly prolonged, indicating a retardation in membrane repolarization. These authors also indicated that the primary site of damage is the cell bodies of the sensory ganglion cells followed by axonal degeneration and that the largest myelinated fibers were affected to a greater extent than the smaller fibers.

Human sensitivity to cadmium induced neurotoxicity is so poorly documented that Krigman (1978) stated that neurotoxicity is not a feature of cadmium poisoning in humans. However, Vorobjeva (1957) examined 160 factory workers and reported CNS symptoms that included
headache, vertigo, and sleep disturbances. In 1961, Adams and Crabtree indicated that anosmia (loss of the sense of smell) occurs in humans exposed to heavy dust environments in alkaline battery factories.

In laboratory animals there is evidence of cadmium initiating neurological disturbances. Gabbiani et al. (1967) gave one subcutaneous injection of CdCl₂ to rats and rabbits at various time intervals after birth and found that lesions were detected in the nervous system only in animals treated at a very young age, mature animals appeared to be insensitive to the cadmium treatment. Parenteral administration of cadmium, at a low dose (10 ppm) to rats 5-6 weeks old with a gradual increase in treatment until the maximum dose (40 ppm) was reached at 14 months of age and maintained at this level for an additional 17 months, was found to produce slight leg weakness (Sato, 1975). Pathological changes were detected in the spinal peripheral nerves while being absent from the central nervous system. An additional study (Sato et al., 1978) that included the same treatment regimen and utilized electron microscopy revealed segmental demyelination in the sciatic nerve beginning at the nodes of Ranvier with active autophagocytosis of Schwann cells containing myelin remnants and dense bodies. Rastogi et al. (1977) examined the effects of cadmium administered to rats for 30 days, with exposure starting at birth, and detected an increase in tryosine hydroxylase and tryptophan hydroxylase activities in specific brain regions. At the low dose endogenous levels of NE, DA, and 5-HT failed to change in the various brain regions examined and the authors
suggested that the increase in synthesis of the neurotransmitters possibly led to an increased utilization of these compounds that produced the symptomatic effects. At the high dosage level a slight increase in turnover of the brain amines was noted as well as an enhancement of the DA and NE levels in several brain regions. The authors felt that their study indicated that cadmium acts at some point in the metabolism of the biogenic amines to increase intracellular synthesis and turnover. In contrast with this information Hrdina et al. (1976) found that cadmium exposure for 45 days at 1.0 mg/kg/day significantly decreased 5-HT concentration in the brain stem of treated rats as well as induced a transient enhancement of striatal DA levels. Of special significance was the finding that the 5-HT decrease persisted even after a 28-day withdrawal from treatment. All biochemical changes were noted prior to the onset of overt neurotoxic symptoms.

Although the studies of Rastogi et al. (1977) and Hrdina et al. (1976) have qualitative differences in the manner that cadmium affects the neurotransmitter levels, both studies provide evidence for cadmium affecting the nervous system under appropriate conditions. Whichever effect turns out to be correct or if both can actually be produced but under different conditions, it must be realized that the neurotransmitter system is of critical importance for normal nervous function.
Selected Aspects of Acrylamide Neurotoxicity

Exposure of experimental animals to acrylamide results in the development of delayed neuropathic lesions of the peripheral nervous system (Fullerton and Barnes, 1966; Prineas, 1969; Tilson and Cabe, 1979). As in other delayed neuropathies, acrylamide toxicity produces a dying-back syndrome in which the lesion ascends the nerve tract in a centripetal direction on continual exposure to the toxicant (Cavanagh, 1964). However, in acrylamide neurotoxicity the axonal lesions are multifocal in nature and are not restricted to the terminal of the longest and largest axons as is seen in other delayed neuropathies (Jennekens et al., 1979; Schaumberg et al., 1974).

In the histological examination of neuromuscular junctions after acrylamide exposure, Tsujihata et al. (1974) observed the disappearance of some axonal terminals from the pre-synaptic regions but the preservation of other terminals within the same endplate region. Extending this work Jennekens et al. (1979) attempted to correlate morphological changes at the terminal arborizations of the neuromuscular junction with behavioral changes in rats, as exemplified by ataxia and hindlimb paralysis. Although multifocal swellings were observed in the muscle groups examined, there was no correlation between the frequency of these focal swellings and the behavioral changes. To account for this the authors speculated that other factors such as the involvement of pre-terminal motor axons, peripheral sensory neurons and the central neurons may be of equal importance for the development of clinical signs of acrylamide.
neuropathy. They also felt that differences in the stages of various lesions were not due so much to the time of the initial insult as to the rate of repair at any one site. This is in agreement with Spencer and Schaumberg's (1977) theory that acrylamide induced distal axonal degeneration is the result of 1) failure of the synthetic machinery in the neuronal cell bodies, 2) impaired axonal transport, and/or 3) direct compromise of metabolic processes in the axons.

Additional information is becoming available to support this theory. Protein synthesis, as assessed by the rate of leucine incorporation, is known to be inhibited in the nervous system (Schotman et al., 1977a) although a causal relationship was not demonstrated (Schotman et al., 1977b, 1978). Bradley and Williams (1973) were among the first to demonstrate acrylamide inhibition of fast axonal flow in cats with acrylamide induced neuropathies. Although no supportive information has been found, Schoental and Cavanagh (1977) proposed that toxic substances such as acrylamide might gain entrance to the nerve fibers at the axonal terminals or the nodes of Ranvier and inactivate enzymes or other cofactors involved in axonal metabolism. If the speculated binding to enzymes or cofactors was to occur the resupply would be least adequate at points distal to such lesions.

Since any effects occurring in the axon or the neuronal cell body would ultimately be expressed in the nerve terminal it is possible that an examination of normal functions in the terminal might give an indication of the level of acrylamide exposure.
Selected Aspects of Cyclodiene Insecticide Neurotoxicity

Cyclodiene insecticides, the group to which Kepone belongs, are often considered to possess less acute toxicity but greater potential for chronic toxicity than the organophosphates and carbamates (Allen et al., 1979). Principal symptoms of acute poisoning are generally characteristic of central nervous system effects; hyperexcitability, tremors and convulsions, with the convulsion usually but not always preceding other signs of toxicity (Wooley, 1976; Allen et al., 1979).

Attempts have been made to correlate behavioral changes, produced after cyclodiene exposure, with concurrent changes in the levels of biogenic amines (McGeer, 1971). Sharma (1973) and Sharma et al. (1976) reported that dieldrin reduced levels of NE, DA, and 5-HT in mallard ducks at the same time that changes in aggressive and confrontation behavior were observed. Other studies have indicated a species difference in the reduction of brain amine concentrations, principally catecholamines; positive effects were seen in rats after α-chlordane exposure (Hrdina et al., 1974) while the levels of the biogenic amines were unchanged in mice after dieldrin treatment although 5-hydroxyindolaecetic acid (5-HIAA), a 5-HT metabolite, was increased suggesting an effect on 5-HIAA efflux from the brain (Sharma, 1976). At present there is too little information available to speculate whether an alteration in biogenic amine levels after treatment with cyclodiene insecticides has a causal relationship to the primary CNS lesion.

In spite of the well documented neurological signs observed in cyclodiene poisoning histological alterations in the CNS are relatively
slight or non-existent (Hodge et al., 1967). At the same time the cyclodienes are known to affect the conduction of the action potential without altering the resting membrane potential of the nerve fiber (Wooley, 1976). Together these studies suggest that the cyclodienes might produce a functional modification of normal synaptic activity leading to the expression of the neurological symptoms. To date very little information is available in the literature examining such a hypothesis.

Recently though, Kepone has been shown to stimulate the release of neurotransmitters from synaptosomes by preventing $\text{Ca}^{++}$ sequestration in mitochondria which normally would lead to reestablishment of the resting membrane potential (End et al., 1979a). End et al. (1979b) also reported that Kepone and KCN stimulated rat synaptosomal $\text{Ca}^{++}$ efflux by releasing mitochondrial stores. While KCN efflux proceeded via $\text{Na}^+-\text{Ca}^{++}$ exchange across the plasma membrane Kepone release of $\text{Ca}^{++}$ was independent of the $\text{Na}^+-\text{Ca}^{++}$ or $\text{Ca}^{++}-\text{Ca}^{++}$ channels suggesting that a direct membrane perturbation initiates this release. To support this hypothesis the study also found that Kepone releases synaptosomal lactate dehydrogenase as does KCN, electron microscopic examination of these synaptosomal preparations show the Kepone exposed samples undergoing membrane lysis while the KCN treated preparations incurring only minor swelling.

In an examination of the transport processes leading to inactivation of the biogenic amines released into the synapse, Ho et al. (1980) report a decrease in dopamine and norepinephrine uptake into mouse brain synaptosomes exposed to Kepone in vivo or in vitro.
Kepone was shown to decrease the affinity of DA for the ligand as well as the maximum amount of DA bound in both in vivo and in vitro treated preparations. The effect on NE was the same as DA in vitro but the NE affinity in vivo was not significantly different from the controls although the total uptake was reduced.

The above information indicates that at least one of the cyclodiene insecticides is capable of modulating synaptic function, although additional work is necessary to determine the relationship of this effect to the clinical symptoms.

**Metabolic and Functional Characterization of Synaptosomes**

Synaptosomes are synaptic terminals which have been sheared, during fractionation, from their axonal connections and surrounding glial cells. After separation from the axon the terminal reseals forming an intact structural unit (Jones, 1975). Often the inter-synaptic cleft and post-synaptic processes are isolated as a portion of the unit. Gray and Whittaker (1962) utilizing differential and density-gradient centrifugation were the first to isolate and identify synaptosomes. Synaptosomes sediment with mitochondria and because of their size were overlooked during early subcellular fraction studies on brain tissues.

Since the discovery of synaptosomes a considerable effort has gone into the elucidation and characterization of the biochemical mechanisms retained after separation. Johnson and Whittaker (1963) showed that synaptosomes contain a significant portion of the lactate dehydrogenase
and potassium of the tissue homogenates. This suggested that the synaptic terminal resealed after shearing and loss of soluble constituents was limited. Bradford (1969) incubated synaptosomes in vitro in appropriate media and demonstrated normal respiratory function. With glucose and pyruvate as substrates a high respiratory activity was seen. On exposure to hypotonic solution respiration diminished and became non-linear. ATP and phosphocreatine were synthesized with glucose as substrate. Synaptosomes showed an active accumulation of potassium from the media. In a comparison of rat synaptosomes and brain slices Bradford and Thomas (1969) showed that both preparations were able to carry out similar metabolic functions. Glucose was converted to lactate, aspartate, glutamate, glutamine, alanine, and γ-amino butyric acid (GABA). Pool sizes of these amino acids were greater in cortex slices than in synaptosomes while leakage of the amino acids from synaptosomes into the media was not seen providing additional evidence for the intactness of the membrane.

To qualify synaptosomes as models suitable for the study of neurotransmission it is necessary to demonstrate the existence of a trans-membrane potential. Due to their small size, 0.5 μm, direct measurement of membrane potential is difficult and it becomes necessary to establish its existence by inference. Application of electrical pulses to synaptosomal preparations gives rise to increased metabolic rates, loss of $K^+$ and differential loss of physiologically active amino acids (Bradford et al., 1973). This same study also showed the loss of synaptosomal $K^+$ and amino acids on
exposure to depolarizing concentrations of $K^+$. An active accumulation or extrusion of various ions supports the existence of a trans-membrane potential. Ling and Abdel-Latif (1968) demonstrated the active removal of $Na^+$ from synaptosomes while $K^+$ (Bradford, 1969) and $Ca^{++}$ (Lust and Robinson, 1968) are actively accumulated.

Whittaker (1969) in anticipation of the evidence revealing synaptosomes to be metabolically functional and to have physiologically active membrane activity described synaptosomes as "minature non-nucleated cells". Investigations of neurotransmitter release and uptake add support for this concept.

Electrical stimulation and depolarizing $K^+$ concentrations were shown to be responsible for the release of acetylcholine from synaptosomal suspensions (Haga, 1971). DeBellerroche and Bradford (1972) utilized synaptosomal beds (deposits of the nerve endings between nylon gauze to reduce tissue:fluid ratios) to support the evidence for acetylcholine release and found that this release was enhanced following electrical or $K^+$ stimulation. In $Ca^{++}$-free media acetylocholine released into the incubation medium was reduced. $Ca^{++}$-dependent norepinephrine loss from synaptosome to the medium was also observed on electrical or $K^+$ induced depolarization (Blaustein et al., 1972).

Membrane active transport processes have been demonstrated for cations, amino acids and biogenic amines. Kurokowa et al. (1965) in one of the first studies on synaptosomes, examined the $Na^+$, $K^+$-dependent adenosine-triphosphatase activity of the membrane. They showed guinea pig synaptosomes accumulated $K^+$ and extruded $Na^+$
against concentration gradients. Oubain and p-chloromercuribenzoate inhibited this enzyme function in a manner quantitatively similar to that seen in other tissues. L-Glutamate and GABA exhibited net uptake in depolarized synaptosomal fractions that are Na\(^+\) and temperature dependent (Roskoski, 1978). Depolarizing media was used for the assay to reduce the homoexchange of synaptosomal and exogenous L-glutamate or GABA. Depolarization of synaptosome preparations with KCl was shown to accelerate choline uptake over the control rates (Roskoski, 1977). The neurotransmitter biogenic amines undergo active uptake after release from synaptosomes. Garey and Heath (1974) prepared synaptosomes from post-mortem human brain tissue, 4-8 hours after death, and measured the levels of catecholamine uptake. Active uptake was seen in all samples. For comparison, synaptosomes prepared from rats, 1 or 12 hours after sacrifice, demonstrated active uptake at both time intervals. Kuhar et al. (1972) examined the transport of serotonin, dopamine and norepinephrine in rats with and without Raphe lesions. In all cases active accumulation was seen with serotonin being the only transmitter to have an altered uptake in treated animals.

Utilization of synaptosomes as a model for the investigation of neurotransmission has several advantages. First, the integrity of the membrane and demonstration of its functional capacity is comparable to the in situ condition at the synapse. Secondly, the characterization of their metabolic activity indicates that all enzymes and co-factors are present as would be seen in situ. Thirdly, the studies showing the viability of synaptosomes for several hours after
removal from the brain allows for time related studies. A final advantage would be the characterization of synaptic activity away from the diluting factors seen with tissue homogenates.
MATERIALS AND METHODS

Animals

Simonsen derived Sprague-Dawley male rats, 150-300 grams (Simonsen Laboratories, Inc., Gilroy, CA) were used for all synaptosomal preparations. Housing conditions consisted of a 12-hour day-night cycle and five animals per cage with food and water ad libitum.

Chemicals

Ficoll for the discontinuous gradient was obtained from Pharmacia (Uppsala, Sweden). The following chemicals were purchased from Sigma Chemical Company (St. Louis, MO); albumin recrystallized from Bovine serum, 3-O-methyl fluorescein, 3-O-methyl fluorescein phosphate, β-Nicotinamide adenine dinucleotide, reduced form (NADH), and ouabain. Chlorpromazine was obtained from Smith, Kline, and French Labs. (Philadelphia, PA). Sources, purity, and chemical names for all test compounds are listed in Table 1. De-ionized water was used for all procedures that required water as a diluent.

The radio-labelled amines were purchased from New England Nuclear Corporation (Boston, MA) and were supplied with the following specific activities, 5-1,2-3H(N)-Hydroxytryptamine creatinine sulfate, 29.6 Ci/mmol and DL-7-3H(N)-Norepinephrine, 15.2 Ci/mmol.
Table 1. Test compounds used for synaptosomal uptake and $K^+$-dependent phosphatase studies.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Chemical Name or Structure</th>
<th>Source</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl mercuric chloride</td>
<td>$CH_3HgCl$</td>
<td>Alfa Division</td>
<td>95+% pure</td>
</tr>
<tr>
<td>Mercuric nitrate</td>
<td>$Hg(NO_3)_2$</td>
<td>Fisher Scientific Co., Fair Lawn, NJ</td>
<td>Reagent grade</td>
</tr>
<tr>
<td>Cadmium chloride</td>
<td>$CdCl_2$</td>
<td>J, T. Baker Chemical Co., Phillipsburg, NJ</td>
<td>Reagent grade</td>
</tr>
<tr>
<td>Kepone</td>
<td>1-decachlorooctahydro-1,3,4-metheno-2H-cyclobutad(2H)-pentalen-2-one</td>
<td>U.S. Environmental Protection Agency, Toxicology Division, Research Triangle Park, NC</td>
<td>88% pure</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>$CH_2=CHCONH$</td>
<td>Eastman Kodak Co., Rochester, NY</td>
<td>Reagent grade</td>
</tr>
<tr>
<td>DFP (Diisopropyl fluoro phosphate)</td>
<td>phosphorofluoridic acid bis(l-methylethyl) ester</td>
<td>Sigma Chemical Co., St. Louis, MO</td>
<td>Analytical grade</td>
</tr>
<tr>
<td>Paraoxon</td>
<td>diethyl-p-nitrophenyl ester phosphoric acid</td>
<td>American Cyanamid Co., Agricultural Division, Princeton, NJ</td>
<td>Practical grade</td>
</tr>
</tbody>
</table>
Preparation of "Modified Crude Mitochondrial"

Fraction (MCM)

The preparation of synaptosomes followed the procedure of Cahill and Medzihradsky (1976) with the addition of a few modifications to increase the yield of synaptosomal fraction. All tissue procedures were carried out at 0-4°C in an ice-bath. Five rat brains were removed and placed in ice-cold 0.32 M sucrose, immediately after sacrifice. Cerebral cortices were dissected free from the whole brain, combined, weighed, minced, and homogenized in 0.32 M sucrose at a concentration of 10 percent (w/v) in a 50 ml Potter-Elvehjem homogenizer equipped with a teflon pestle. The homogenate was then centrifuged at 0-4°C for 15 minutes at 3,000 x g, in an International high speed refrigerated centrifuge model HR-1 equipped with a fixed angle rotor, to remove blood, nuclei and cell debris. The resulting supernatant was centrifuged at 15,000 x g for 25 minutes and subsequently divided into three fractions. Fraction 1, supernatant; fraction 2, upper portion of pellet characterized by a white layer having the appearance of a ring or "halo" on top of fraction 3, a firm yellow pellet. Fraction 2 and 3 when combined represented the crude mitochondrial fraction that contained mitochondria, synaptosomes and myelin fragments. Fraction 1 was removed with a pasteur pipet and saved, fraction 2 was also extracted and represents a modified crude mitochondrial fraction (MCM) enriched in synaptosomes (Cahill and
Medzihradsky, 1976), this fraction was also saved. Fraction 3 was then diluted with 10 ml of 0.32 M sucrose and re-centrifuged along with fraction 1 and 2 at 15,000 x g for 25 minutes. Supernatants from all three fractions were then removed and discarded while the "halo" that layered the pellet of fraction three was removed and combined with the pellet of fraction two. The combined fractions were re-centrifuged at 15,000 x g for 25 minutes after which the supernatant was discarded and the pellet was then combined with the previous pellet of fraction 1 which had been diluted with 0.32 M sucrose (0.5 ml/cerebral cortex). This final MCM suspension was then gently hand homogenized in a glass Dounce homogenizer in the final step of the preparation. In the procedures outlined by Cahill and Medzihradsky (1976) only the "halo" of the initial 15,000 x g pellet was saved while the supernatant and the firm yellow pellet were discarded. By retaining these fractions the ultimate MCM yield was increased indicating a more nearly complete recovery of synaptosomes. Total preparation time from removal of brain to final MCM was 3 hours. A flow diagram of the synaptosome preparation is given in Illustration 1.

Density-Gradient Separation of Synaptosomes, Mitochondria and Myelin Fragments

Where it was necessary to separate the homogenate into purified fractions of synaptosomes, mitochondria and myelin a procedure utilizing density-gradient centrifugation, was outlined by Kurokawa et al. (1965), was used. After tissue homogenization the homogenate was prepared as indicated above, through the first 15,000 x g
Illustration 1. Flow chart for isolation of MCM fraction from rat cerebral cortices.
centrifugation, at this point the entire pellet, crude mitochondrial fraction (fraction 2 and 3), was diluted with 0.32 M sucrose to a concentration of 3.0 ml/gram of original tissue. A 1.0 ml aliquot of this suspension was then layered on a discontinuous density-gradient, consisting of 2.0 ml of 3 percent (w/v) and 2.0 ml (w/v) Ficoll dissolved in 0.32 M sucrose, pre-chilled to 0-4°C. The gradients were then centrifuged at 20,900 x g for 15 minutes (International B-60 Ultracentrifuge with a #487 SB-283 titanium swinging bucket rotor) resulting in the formation of three distinct layers, A, B, and C. The liquid phases of A and C were nearly transparent, whereas that of B was hazy. The interface between layers A and B was white while that between B and C was greyish-white and the pellet, in layer C, was brown.

Electron microscopic studies of Kurokawa et al. (1965) showed A to consist mainly of fragments of myelin sheaths, also present were a few small synaptosomes as well as other vesicular membrane fragments. Subfraction B was principally composed of synaptosomes with a heterogenous mixture of membrane fragments as well as an occasional mitochondria. Subfraction C consisted mainly of mitochondria but an occasional synaptosome, as well as assorted membranous fragments, were also found to be present.

To separate the three layers each was extracted with a pasteur pipet and then centrifuged at 105,000 x g for 20 minutes. The supernatant was then discarded and the pellet resuspended in an appropriate medium.
Within 15 minutes of the final step of the MCM preparation the assay for measurement of NE or 5HT uptake was started. The MCM fraction was kept at 0.32 M sucrose (0-4°C) until just prior to its addition to the assay tubes. The assay was performed in 15 ml centrifuge tubes at 37°C with appropriate 0°C controls. Except for the actual incubation at 37°C all steps were carried out at 0-4°C.

As was needed for the uptake studies, 0.05 ml of the MCM suspension was transferred to 0.90 ml of assay buffer that included the compound to be studied, if appropriate. The assay buffer contained the following final concentrations (mM): Tris, 35; NaCl, 100; KCl, 10; MgSO$_4$·7H$_2$O, 1.2; KH$_2$PO$_4$, 1.2; NaHCO$_3$, 1.0; sucrose, 25; glucose, 10; and ascorbic acid, 1.0; the pH of the buffer was adjusted to 7.4 with HCl. To start the assay, 0.05 ml of a radiolabelled compound, the activity of which was adjusted to 0.03-0.10 μCi per 0.05 ml, (the final specific activity was adjusted by the addition of non-labelled NE or 5HT as needed), was added to the appropriate tubes which were then incubated at 37°C in a shaking water bath for a predetermined length of time. At the end of the incubation time the samples were placed into the ice-bath and the radio-labelled biogenic amine was then added to the 0°C controls and all samples were centrifuged at 8,000 x g for 5-7 minutes. After pelleting the synaptosomes the supernatant was removed and 1.0 ml of 0.4 N perchloric acid was gently added to each sample to extract the radio-labelled compound. After three hours duplicate 0.2 ml aliquots were prepared for liquid scintillation counting in a Packard Tri-Carb 2660 liquid scintillation counter.
Protein was determined by the method of Lowry et al. (1951) for each group of samples using crystallized bovine serum albumin as the standard.

**Assay for Lactate Dehydrogenase Activity**

Synaptosomal lactate dehydrogenase (E.C. 1.1.1.27) activity was determined by the method of Bergmeyer and Bernt (1974) in which the disappearance of NADH is monitored over time. The assay was carried out in a cuvette with a 1.0 cm light path and monitored at 340 nm in a Perkin-Elmer, Coleman 124 doublebeam spectrophotometer. The total volume for the assay solution was 3.15 ml and consisted of the following components and their final concentrations: phosphate, 48 mM; pyruvate, 0.6 mM; NADH, 0.18 mM; and 0.1-0.3 mg MCM protein. Triton X-100 (final concentration 0.1 percent) was used to determine intra-synaptosomal LDH activity while the LDH activity in the absence of Triton X-100 was considered to be extra-synaptosomal. The change in $E_{340}$ was recorded every 60 seconds for 3.0 minutes to obtain an average value in the presence or absence of Triton X-100. Activities were calculated according to the following equation:

$$\frac{\Delta E_{340}/\text{min}}{\varepsilon \times d \times \text{mg prot./ml}} = \mu \text{moles NADH disappearing/mg prot./min}$$

$E_{340}$ = change in extinction at 340 nm  
$d$ = light path, 1.0 cm  
$\varepsilon$ = NADH extinction coefficient, 6.2 cm$^2$/μmole
MCM was diluted with 250 mM Tris-HCl pH 7.4 to a concentration of approximately 0.1 mg/ml to give a final protein concentration of 1.5-2.0 μg/ml. The 3-0-methylfluorescein phosphatase assay developed by Huang and Askari (1975) was used to monitor K⁺-dependent phosphatase (E.C. 3.6.1.3) activity and is based on the determination of the amount of fluorescence due to the formation of 3-0-methylfluorescein. Fluorescence measurements were made with an Aminco-Bowman spectrofluorometer. The standard assay medium contained 1.0 μM 3-0-methylfluorescein phosphate, 1.0 mM MgCl₂, 10 mM KCl, and 50 mM Tris-HCl pH 7.4 in a final volume of 3.0 ml. The fluorometer was standardized with a 1.0 μM solution of 3-0-methylfluorescein, to obtain a 50 percent fluorescence, in the same buffer solution that was used for the assay. The excitation and emission wavelengths were 465 and 518 nm, respectively. All assays were carried out at 37°C by pumping water from a waterbath through the cuvette chamber which was designed for such a circulating water flow. Blank readings were determined for 0.05-0.10 ml MCM in the assay buffer without the substrate. On addition of the 3-0-methylfluorescein phosphate the change in fluorescence was monitored on a strip chart recorder for two minutes. The change in fluorescence per time period was calculated and used in the following equation to determine the activity of the phosphatase:

\[
\frac{dX}{50} \times \frac{1}{\text{prot.}} \times \frac{60}{\text{time}} = \text{nmoles phosphate liberated/mg prot./hour}
\]
\[dX = \text{change in fluorescence reading for a specified time period}\]

\[50 = \text{emission of standard (1 \mu M 3-O-methylfluorescein)}\]

\[\text{prot.} = \text{final concentration of protein in mg/ml}\]

\[\text{time} = \text{length of reaction in minutes}\]

**Estimation and Statistical Treatment**

of \(K_m\) and \(V_{max}\) Values

\(K_m\) and \(V_{max}\) constants were calculated according to the method of Wilkinson (1961) which incorporates the determination of the statistical parameters from the experimental data.
RESULTS

The objective of this study was to determine the influence of various environmental pollutants on synaptosomal inactivation of norepinephrine (NE) and 5-hydroxytryptamine (5-HT). In order to accomplish this it was necessary to characterize the kinetics of the transport process as well as to determine the optimum conditions for such a characterization. Chlorpromazine and ouabain were used as positive inhibitors of the uptake process to help determine the suitability of the synaptosomal preparations used in this study. A K⁺-dependent phosphatase was also monitored in the synaptosomal membrane to determine the effects the test chemicals have on the Na⁺, K⁺ gradient which is required for optimum transport of NE and 5-HT.

Characterization of NE and 5-HT Uptake

Uptake versus protein concentration

Since this study required the preparation of numerous MCM fractions it was necessary to determine if the NE and 5-HT uptake processes possessed a linear relationship over the range of protein concentrations used. Figures 1 and 2 indicated that such a relationship existed and that the uptake process had an initial linearity which was lost at higher protein concentration. All uptake studies were performed within the demonstrated range of linearity.
Figure 1. Representative presentation of the relationship between NE uptake into MCM fraction and MCM protein concentration. The uptake assay was performed with 0.5 μM NE at 37°C for 5.0 min.
Figure 2. Representative presentation of the relationship between 5HT uptake into MCM fraction and MCM protein concentration. The uptake assay was performed with 0.1 µM 5HT at 37°C for 3.0 min.
Uptake versus time

In assessing the optimum condition for the uptake study it was necessary to determine the time dependency of the transport processes in the MCM preparations. The specific activity of the NE uptake reached a maximum at 10 minutes while the 5-HT uptake reached its peak value at 4 minutes (Figure 3). NE uptake increased curve-linearly for the first 5 minutes at which time the linearity decreased and a plateauing occurred between 10 and 15 minutes. 5-HT uptake also had an early linear relationship between 1 and 4 minutes but by 5 minutes a decline in total uptake was observed. Since a time dependency existed for these two uptake systems the subsequent experiments were performed within the linear segment. With NE a 5 minute assay time was used while 3 minutes was used for the 5-HT assay. These time periods were used throughout the remainder of the study.

Estimation of kinetic constants

The transport of biogenic amines into the pre-synaptic terminal is an active process with identifiable kinetic constants. Table 2 lists the $K_m$ and $V_{max}$ values as determined for NE and 5-HT in the MCM preparations. 5-HT uptake was shown to have two transport processes while only one such process was identified for NE.

The biphasic nature of the 5-HT uptake is readily evident in Figures 4 and 5. Figure 4 is a standard plot of substrate concentration (5-HT) versus velocity (pmoles 5-HT/mg protein/min) and can be interpreted as showing the two phases of the 5-HT transport with the high affinity process occurring at concentrations
Table 2. $K_m$ and $V_{max}$ values for the 5HT and NE uptake into the MCM fraction.a,d

<table>
<thead>
<tr>
<th></th>
<th>5HT</th>
<th>NE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>high affinity</td>
<td>low affinity</td>
</tr>
<tr>
<td>$K_m$</td>
<td>$0.40 \pm 0.16$</td>
<td>3.64</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>$2.54 \pm 0.77$</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>1.11 $\pm 0.26$</td>
<td>4.65 $\pm 0.47$</td>
</tr>
</tbody>
</table>

a$K_m$ and $V_{max}$ values determined by the method of Wilkinson (1961)

b$\mu$M

cpmoles/mg protein/min

dEach value represents the mean ± s.d. obtained from 2 - 4 determinations involving 2 - 4 synaptosomal preparations except 5HT low affinity $K_m$ and $V_{max}$ values which were obtained from only one MCM preparation.
Figure 3. Time dependence of biogenic amine uptake into MCM fraction. The data presented for NE (O----O) and 5HT (•---•) are representative experiments performed with 0.5 μM NE and 0.1 μM 5HT.
Figure 4. Standard substrate vs velocity plot for $^3$H-5HT uptake into a MCM fraction. The data points indicate the presence of two transport processes, $K_m$ and $V_{max}$ values obtained for both processes are presented in Table 2. Each point is the mean of 2-4 determinations involving 2-4 separate MCM preparations. Assay performed in duplicate at 37°C for 3.0 min with simultaneous 0°C controls.
Figure 5. Lineweaver-Burk plot of the uptake data for ^3^H-5HT presented in Figure 4. The obtained $K_m$ and $V_{max}$ values are presented in Table 2. Crossbars designate the S.E.M. of the data collected from 2-4 MCM preparations. Circles without bars represent individual observations.
below 0.25 μM and the low affinity uptake operating above 5-HT concentrations of 0.5 μM. Using a Lineweaver-Burk reciprocal plot of 1/v versus 1/S (Figure 5) a more accurate estimation of the \( K_m \) and \( V_{\text{max}} \) was obtained. With this approach the \( K_m \) and \( V_{\text{max}} \) for the high affinity uptake were calculated as 0.45 μM and 2.86 pmoles/mg protein/min, respectively. The low affinity uptake was determined to have a \( K_m \) of 3.64 μM and a \( V_{\text{max}} \) of 10 pmoles/mg protein/min. The distinction between these two processes is evident with the intersection of the two lines in Figure 5.

NE concentrations ranging from 0.05-10 μM were used to obtain the information that is graphically presented in Figures 6 and 7. A smooth perpendicular right-handed hyperola is obtained in Figure 6 in a plot of substrate concentration versus velocity and suggests that first-order kinetics are appropriate at low substrate (NE) concentrations. By replotting this data on a reciprocal plot of 1/v versus 1/S (Figure 7) the \( K_m \) and \( V_{\text{max}} \) were identified as 1.11 μM and 4.65 pmoles NE/mg protein/min, respectively. A single set of kinetic constants were obtained indicating the existence of only one uptake process for NE in synaptosomes.

The concentrations of 5-HT and NE used for the majority of this study were based on the \( K_m \) values described above. For 5-HT 0.1 μM was the concentration chosen while 0.5 μM was used for all experiments involving NE uptake.

**Localization of NE after incubation in MCM preparations**

MCM preparations were enriched in synaptosomes but contained, to a lesser extent, mitochondria and various types of membrane
Figure 6. Standard substrate vs velocity plot for accumulation of $^3$H-NE at various concentrations into MCM fractions. Each point represents the mean of 2-4 determinations involving 2-4 different MCM preparations. Assays performed in duplicate at 37°C for 5.0 min with simultaneous 0°C controls.
Figure 7. Lineweaver-Burk plot of the uptake of $^3$H-NE by the MCM fraction. The data presented is a re-plot of the information presented in Figure 6. The obtained $K_m$ and $V_{max}$ values are presented in Table 2. Crossbars designate the S.E.M.
fragments (Cahill and Medzihradsky, 1976). Several experiments were performed to determine if the non-synaptosomal components were competing with synaptosomes for the NE or 5-HT used in the uptake studies. In Table 3 the results of purifying the MCM preparation after exposure to NE indicate that greater than 95 percent of the NE retained in the preparation after assay was found in the synaptosome fraction (B). Less than 5 percent was found in the mitochondrial fraction (C) while the myelin fraction (A) had no measurable retention of the NE. In this study the assay buffer was without pargyline, a monoamine oxidase inhibitor, which would have prevented mitochondrial metabolism of the NE. However, in Table 4 the NE and 5-HT uptake was measured in the presence of pargyline. In these experiments pargyline did not increase the biogenic amine uptake into synaptosomes by inhibiting mitochondrial metabolism of these compounds and thus increase amine concentrations available to synaptosomes. The information presented in Tables 3 and 4 indicate that the contaminating elements of the MCM preparations did not interfere with synaptosomal uptake of NE or 5-HT.

Lactate Dehydrogenase Activity as a Normalization Factor Between MCM Preparations

Since many MCM preparations were used for this study it was necessary to determine the degree of membrane intactness between preparations in order to assess the feasibility of directly comparing data from each preparation. To accomplish this, lactate dehydrogenase activity (LDH) was monitored in several preparations. LDH measurements
Table 3. Localization of $^3$H-NE after uptake into a synapotosomal preparation.

<table>
<thead>
<tr>
<th>Gradient fraction</th>
<th>pmoles NE $^b$</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0°C</td>
<td>37°C</td>
</tr>
<tr>
<td>A</td>
<td>0.02</td>
<td>ND</td>
</tr>
<tr>
<td>B</td>
<td>0.68</td>
<td>8.50</td>
</tr>
<tr>
<td>C</td>
<td>ND</td>
<td>0.34</td>
</tr>
</tbody>
</table>

$^a$Uptake assay performed as described in methods after which MCM pellet resuspended and layered on a 3 - 13% discontinuous ficoll-sucrose gradient.

$^b$Values obtained were assayed at the indicated temperature and are expressed as pmoles NE/ml.

$^c$Composition determined by Kurokawa et al. (1965).

$^d$ND indicates radiolabel was not detected.

$^e$4.5% of fraction B.
Table 4. Effect of pargyline on NE and 5HT uptake into MCM fraction.

<table>
<thead>
<tr>
<th>Pargyline (M)</th>
<th>5HT or NE</th>
<th>Amine Uptake$^a$ pmoles/mg protein/min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5HT (0.1 x 10^{-6} M)</td>
<td>0.48</td>
</tr>
<tr>
<td>0</td>
<td>''</td>
<td>0.38</td>
</tr>
<tr>
<td>10^{-5}</td>
<td>''</td>
<td>0.46</td>
</tr>
<tr>
<td>10^{-4}</td>
<td>''</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>NE (0.5 x 10^{-6} M)</td>
<td>3.08</td>
</tr>
<tr>
<td>10^{-4}</td>
<td>''</td>
<td>2.38</td>
</tr>
<tr>
<td>5 x 10^{-4}</td>
<td>''</td>
<td>0.58</td>
</tr>
</tbody>
</table>

$^a$Uptake determined in one MCM preparation.
were determined for the extra- and intra-synaptosomal compartments. Since synaptosomal concentrations varied between preparations the monitoring of LDH activity in both compartments enabled batch comparisons to be made by using the extra- to intra-synaptosomal ratio (extra/intra) as an indicator of synaptosomal membrane integrity. In Table 5 the synaptosomal LDH ratio is less than 0.15 in eight of the nine preparations indicating a very intact and non-permeable membrane. The 5-HT uptake was determined in each of these preparations and the information in Table 5 shows that this activity is very similar in all preparations. The correlation coefficient obtained when comparing the LDH ratio to the 5-HT uptake is 0.043 and indicates that such a correlation does not exist.

Chemical Influence on NE and 5-HT Uptake

Chlopromazine and ouabain were used as positive controls for validating the functionality of the synaptosomal preparations. Data in Figure 8 indicates that chlorpromazine inhibited the synaptosomal uptake of NE and 5-HT. The IC$_{50}$ (the concentration of test chemical that inhibited the uptake by 50 percent) for both processes were similar with 50 percent inhibition of NE uptake occurring at $1.1 \times 10^{-5}$ M while 5-HT uptake was inhibited by 50 percent at $3.2 \times 10^{-5}$ M. Although the IC$_{50}$'s were nearly identical the shape of the two curves were dissimilar. In Figure 8 the NE process is below control levels between $10^{-6}$ - $10^{-4}$ M chlorpromazine. With 5-HT uptake chlorpromazine was effective in a much narrower range $10^{-6}$-$10^{-4}$ M and the slope of the line is much steeper than that seen with the NE process.
Table 5. Correlation of 5HT uptake with the MCM Lactate Dehydrogenase activity.

<table>
<thead>
<tr>
<th>MCM Preparation</th>
<th>LDH ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>5 HT uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.285</td>
<td>0.69</td>
</tr>
<tr>
<td>2</td>
<td>0.132</td>
<td>0.72</td>
</tr>
<tr>
<td>3</td>
<td>0.146</td>
<td>0.91</td>
</tr>
<tr>
<td>4</td>
<td>0.124</td>
<td>0.69</td>
</tr>
<tr>
<td>5</td>
<td>0.101</td>
<td>0.70</td>
</tr>
<tr>
<td>6</td>
<td>0.059</td>
<td>0.69</td>
</tr>
<tr>
<td>7</td>
<td>0.092</td>
<td>0.72</td>
</tr>
<tr>
<td>8</td>
<td>0.117</td>
<td>0.75</td>
</tr>
<tr>
<td>9</td>
<td>0.123</td>
<td>0.059</td>
</tr>
</tbody>
</table>

Mean<sup>c</sup>: 0.131 ± 0.063
5 HT uptake: 0.72 ± 0.08

Correlation coefficient: 0.043

<sup>a</sup>Ratio of extra-synaptosomal/intra-synaptosomal LDH activity, determined spectrophotometrically by the measurement of the conversion of NADH → NAD.

<sup>b</sup>5HT uptake determined using 0.1 μM 5HT for 3 min at 37°C, activity expressed as pmoles 5HT/mg prot./min.

<sup>c</sup>Mean ± s.d.
Figure 8. Chlorpromazine inhibition of the NE (0—0) and 5-HT (●—●) uptake into the synaptosomal preparations. Concentrations of the biogenic amines were NE, 0.5 μM and 5-HT, 0.1 μM. Crossbars designate S.E.M. for data from 3 MCM preparations, circles without bars represent individual observations. Horizontal dashed lines indicate confidence limits for control values.
Ouabain inhibition of the synaptosomal uptake processes was positive only for NE uptake, the 5-HT uptake was not affected. The IC$_{50}$ value for the NE transport was $1.1 \times 10^{-4}$ M with an effective range occurring between $0.5 \times 10^{-4}$-1.0 $\times 10^{-3}$ M (Figure 9). The slope obtained for NE uptake is a linear function of the ouabain concentration over the range tested. With the 5-HT uptake system control values were obtained between $10^{-6}$-$10^{-3}$ m ouabain.

In testing the effects several heavy metals have on the synaptosomal transport process information presented in Figures 10 and 11 and in Table 6 indicate that the two mercurials, CH$_3$HgCl and Hg(NO$_3$)$_2$, inhibited both the NE and 5-HT process while CdCl$_2$ had no significant effect on either process. The alkyl mercury compound, CH$_3$HgCl, appeared to inhibit both NE and 5-HT uptake in a similar manner, as well as to a similar degree. IC$_{50}$'s for the uptake processes were NE, $1.1 \times 10^{-4}$ M and 5-HT, $2.5 \times 10^{-4}$ M. The actual effective range is quite small. Hg(NO$_3$)$_2$, an inorganic mercurial, was not as effective an inhibitor of the transport systems. Although the IC$_{50}$ obtained with Hg(NO$_3$)$_2$ was within the same magnitude as the CH$_3$HgCl, Hg(NO$_3$)$_2$ did not completely inhibit synaptosomal transport of NE or 5-HT. At $10^{-3}$ M, CH$_3$HgCl inhibition of the NE or 5-HT uptake was complete while the Hg(NO$_3$)$_2$ inhibition at the same concentration was only 50-65 percent complete.

Kepone was capable of completely inhibiting both NE and 5-HT uptake as is indicated in Figure 12. IC$_{50}$ values were estimated to be $9.4 \times 10^{-5}$ M for NE and $3.3 \times 10^{-4}$ M for 5-HT. The effective inhibition range was small for both processes, NE inhibition occurred between $0.5 \times 10^{-4}$-$0.5 \times 10^{-3}$ M Kepone and 5-HT inhibition was less
Figure 9. Contrasting effects of ouabain on the biogenic amine uptake into synaptosomes. NE (0--0) uptake was assayed at 0.5 μM NE while 5HT (∗∗) uptake was determined at 0.1 μM 5HT. Crossbars designate the S.E.M. for experiments from 2-3 MCM preparations, circles without bar represent individual observations. Horizontal dashed lines indicate confidence limits for control uptake.
Figure 10. CH$_3$HgCl inhibition of synaptosomal biogenic amine uptake. NE (0--0) and 5-HT (●●●) uptake were followed using 0.5 μM and 0.1 μM 5-HT, respectively. Crossbars are the S.E.M. for 2 MCM preparations while horizontal dashed lines designate the confidence limits for the control uptake. Circles without bars represent individual observations.
Figure 11. Hg(NO$_3$)$_2$ influence on MCM NE (-----0) and 5HT (•—•) uptake. NE uptake was measured in the presence of 0.5 μM NE while 0.1 μM 5HT was used for the 5HT assay. Crossbars designate S.E.M. for data collected from 2-3 MCM preparations, horizontal dashed lines indicate confidence limits for the control uptake. Circles without bars represent individual observations.
Table 6. Inhibitory influence of various environmental pollutants on synaptosomal uptake of NE and 5-HT.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration (M)</th>
<th>% Control Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NE</td>
</tr>
<tr>
<td>CH₃HgCl</td>
<td>10⁻³</td>
<td>0*</td>
</tr>
<tr>
<td></td>
<td>10⁻⁴</td>
<td>26*</td>
</tr>
<tr>
<td></td>
<td>10⁻⁵</td>
<td>115</td>
</tr>
<tr>
<td>Hg(NO₃)₂</td>
<td>10⁻³</td>
<td>35*</td>
</tr>
<tr>
<td></td>
<td>10⁻⁴</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>10⁻⁵</td>
<td>94</td>
</tr>
<tr>
<td>CdCl₂</td>
<td>10⁻³</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>10⁻⁴</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>10⁻⁵</td>
<td>94</td>
</tr>
<tr>
<td>Kepone</td>
<td>10⁻³</td>
<td>0*</td>
</tr>
<tr>
<td></td>
<td>10⁻⁴</td>
<td>49*</td>
</tr>
<tr>
<td></td>
<td>10⁻⁵</td>
<td>95</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>10⁻³</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>10⁻⁴</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>10⁻⁵</td>
<td>88</td>
</tr>
<tr>
<td>DFP</td>
<td>10⁻³</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>10⁻⁴</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>10⁻⁵</td>
<td>94</td>
</tr>
<tr>
<td>Paraoxan</td>
<td>10⁻³</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>10⁻⁴</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>10⁻⁵</td>
<td>84</td>
</tr>
</tbody>
</table>

*p<0.05

Non-detectable
Figure 12. Kepone inhibition of NE (O---O) and 5HT (●---●) synaptosomal uptake. 0.5 μM NE and 0.1 μM 5HT were used for the assay. S.E.M. for the data collected in 2-3 MCM preparations is indicated by the crossbars while the confidence limits for the control uptake is designated by the horizontal dashed lines. Circles without bars represent individual observations.
than control levels in the $10^{-4}$-$10^{-3}$ M range. As was already shown in Figures 10 and 11 for CH$_3$HgCl and Hg(NO$_3$)$_2$, respectively, the slope within the effective inhibitory range for Kepone was also quite steep.

Table 6 lists several other chemicals that were tested for their ability to inhibit the synaptosomal transport processes. Acrylamide, DFP, and paraoxon were unable to produce a significant inhibition of either the NE or 5-HT uptake. Although acrylamide and DFP did inhibit the process 30-40 percent at the highest concentrations tested, $10^{-3}$ M, this was not interpreted as being significantly different from the control values.

**Chemical Influence on Synaptosomal K$^+$-Dependent Phosphatase Activity**

In the assay for the K$^+$-phosphatase activity of the synaptosomal membrane chlorpromazine was found to be an effective inhibitor of this enzymatic process (Figure 13). The degree of inhibition was seen to be directly proportional to the chlorpromazine concentration of the assay. The graphical presentation of this effect shows a linear decrease in enzyme activity over the concentration range tested, $10^{-5}$-$10^{-3}$ M. The IC$_{50}$ for this inhibition was observed to occur at $8.4 \times 10^{-5}$ M chlorpromazine. Although the enzyme inhibition was dependent on the concentration of the drug it could not be determined from the data whether chlorpromazine was capable of producing a complete enzyme inhibition.
Figure 13. Measurement of synaptosomal $K^+$-dependent phosphatase in the presence of various concentrations of chlorpromazine. Enzyme activity determined at 37°C with 3-O-methylfluorescein phosphate (1.0 μM) as substrate. S.E.M. for data collected with 2-3 MCM preparations indicated by crossbars, circles without bars represent individual observations.
CH₃HgCl was found to be a more potent inhibitor of the phosphatase activity than chlorpromazine with an IC₅₀ of 1.5 x 10⁻⁶ M (Figure 14). The effective concentration range for inhibiting the phosphatase activity was 5.0 x 10⁻⁵-1.0 x 10⁻⁷ M CH₃HgCl. The concentrations used for this determination provide a well balanced group of values that indicated the synaptosomal enzyme activity to be directly proportional to the CH₃HgCl concentration of the assay.

Hg(NO₃)₂ was determined to be the most potent inhibitor of the phosphatase. As can be seen in Table 7 it was 50 times more potent than either CdCl₂ or CH₃HgCl, and 400 and 2600 times as effective as Kepone or chlorpromazine, respectively. Figure 15 reveals the initial inhibition to be related to the concentration of the compound in the assay while the lowest concentration of Hg(NO₃)₂ tested, 3.0 x 10⁻⁸ M, sharply departed from the linear relationship.

CdCl₂ was also a potent inhibitor of the K⁺-dependent phosphatase with an IC₅₀ of 1.5 x 10⁻⁶ M (Table 7). Figure 16 expresses the inhibitory relationship as being linear throughout the range of concentrations tested (10⁻⁸-10⁻⁴ M). In comparing the CdCl₂ and CH₃HgCl inhibitions it was evident that their effects on the synaptosomal phosphatase activity was identical with the IC₅₀ of 1.5 x 10⁻⁶ M for each and the shape of the line connecting the points in Figures 14 and 16 very similar.

Kepone, at 3.33 x 10⁻⁴ M, completely inhibited the phosphatase enzyme (Figure 17). The IC₅₀ for this inhibition was 1.3 x 10⁻⁵ M and Kepone, like chlorpromazine, CH₃HgCl and CdCl₂, inhibited the enzymatic cleavage of phosphate from the substrate in a manner that
Figure 14. Measurement of synaptosomal K\textsuperscript{+}-dependent phosphatase activity in the presence of CH\textsubscript{3}HgCl. Phosphatase activity determined at 37°C with 1.0 \( \mu \text{M} \) 3-0-methyl-fluorescein phosphate as substrate. S.E.M. for data collected from 2-3 MCM preparations indicated by crossbars. Circles without bars represent individual observations.
Table 7. Concentration of test compound producing 50 percent inhibition (IC$_{50}$) of synaptosomal NE or 5HT uptake or K$^+$-dependent phosphatase activity.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>IC$_{50}$ (uM)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NE</td>
<td>5HT</td>
<td>K$^+$-dep. phosphatase</td>
<td></td>
</tr>
<tr>
<td>CH$_3$HgCl</td>
<td>110</td>
<td>250</td>
<td>1.50</td>
<td></td>
</tr>
<tr>
<td>Hg(NO$_3$)$_2$</td>
<td>400</td>
<td>600</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>CdCl$_2$</td>
<td>--</td>
<td>--</td>
<td>1.50</td>
<td></td>
</tr>
<tr>
<td>Kepone</td>
<td>94</td>
<td>330</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Acrylamide</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>DFP</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>Paraoxon</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>11</td>
<td>32</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>Ouabain</td>
<td>110</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Insufficient inhibition for detection of IC$_{50}$.
Figure 15. Hg(NO$_3$)$_2$ inhibition of synaptosomal K$^+$-dependent phosphatase. Enzyme activity determined at 37$^\circ$C with 1.0 μM 3-0-methylfluorescein phosphate as substrate. S.E.M. for data collected from 2-3 MCM preparations indicated by crossbars. Circles without bars represent individual observations.
Figure 16. Measurement of synaptosomal K⁺-dependent phosphatase in the presence of various concentrations of CdCl₂.
Phosphatase activity determined at 37°C with 1.0 μM 3-O-methylfluorescein phosphate as substrate. Crossbars designate the S.E.M. for data collected from 2-3 MCM preparations.
Figure 17. Kepone inhibition of synaptosomal \( K^+ \)-dependent phosphatase as measured in the presence of 1.0 \( \mu M \) 3-0-methylfluorescein phosphate at 37\( ^\circ \)C. S.E.M. for data collected from 2-3 MCM preparations designated by crossbars, circles without bars represent individual observations.
was proportional to the concentration of Kepone in the assay. Kepone exerted its inhibitory influence over a wide concentration range, $10^{-7}$-$10^{-3}$ M, which was also true for the other compounds that strongly inhibited this enzyme.

The information presented in Table 8 shows acrylamide, paraoxon, DFP and ouabain to be ineffective in depressing the synaptosomal phosphatase activity. Although acrylamide shows a 15-20 percent inhibition of this activity between $10^{-6}$-$10^{-4}$ M and ouabain produces a 24 percent inhibition of $3.0 \times 10^{-4}$ M these decreases were not found to be of statistical significance.

Table 7 compares the IC$_{50}$'s for NE and 5-HT uptake and K$^+$-dependent phosphatase activity. From this information chlorpromazine, used as a positive control for the uptake inhibition, had the lowest IC$_{50}$ for both the NE and 5-HT transport processes while it was the least effective phosphatase inhibitor of the compounds that were able to reduce this activity more than 50% of the control. Hg(NO$_3$)$_2$, on the other hand, was the least effective inhibitor of the uptake processes, of those inhibiting this function by more than 50 percent, yet it was the most effective inhibitory agent on the K$^+$-dependent phosphatase by several orders of magnitude. Ouabain, another positive control, inhibited NE uptake but surprisingly was not effective against the 5-HT uptake mechanism or the phosphatase enzyme of the membrane.
Table 8. Test compounds producing only mild inhibition of synaptosomal $K^+$-dependent phosphatase.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration (M)</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>$3.33 \times 10^{-5}$</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>$3.33 \times 10^{-4}$</td>
<td>83</td>
</tr>
<tr>
<td>Paraoxon</td>
<td>$3.33 \times 10^{-5}$</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>$3.33 \times 10^{-4}$</td>
<td>96</td>
</tr>
<tr>
<td>DFP</td>
<td>$3.33 \times 10^{-4}$</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>$6.67 \times 10^{-4}$</td>
<td>96</td>
</tr>
<tr>
<td>Ouabain</td>
<td>$3.33 \times 10^{-5}$</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>$3.33 \times 10^{-4}$</td>
<td>76</td>
</tr>
</tbody>
</table>
DISCUSSION

This study was undertaken to determine what influence a select group of environmental pollutants exerts on synaptosomal inactivation of norepinephrine (NE) and 5-hydroxytryptamine (5-HT). The test chemicals used in this study were CH$_3$HgCl, Hg(NO$_3$)$_2$, CdCl$_2$, diisopropylfluorophosphate (DFP), paraoxon, acrylamide, and Kepone. In addition to the examination of the uptake process after in vitro exposure to the test compounds the Na, K$^+$ gradient was indirectly monitored in the synaptosomal membranes by determining the K$^+$-dependent phosphatase activity in the presence and absence of the test chemicals. Since the uptake process is dependent on the transmembrane Na$^+$ gradient the monitoring of this gradient was able to provide information to help assess whether the chemical influence on the membrane transport process is specific in nature or the result of non-specific membrane interactions.

Prior to the actual examination of the uptake activity in the presence of the test chemicals it was necessary to determine the suitability of the synaptosomal preparations for the study. This was accomplished by first determining the optimum conditions for the uptake assay (time, synaptosome concentration and neurotransmitter concentration) then by obtaining the $K_m$ and $V_{max}$ for each uptake process. Once these $K_m$ and $V_{max}$ values were obtained they were compared to the previously reported values of Cahill and Medzihradksy (1976). After this comparison chlorpromazine and ouabain were used as positive inhibitors of the membrane uptake to further
determine the suitability of the synaptosomal preparations for use in this study.

Suitability of the Synaptosomal Preparations for Uptake Studies

Since the first isolation and characterization of synaptosomes, their usefulness for increasing the understanding of synaptic transmission has been widely accepted. Most procedures for their isolation incorporate differential and density-gradient centrifugation as a necessary step in their purification. However, Cahill and Medzihradsky (1976) devised a method which only requires differential centrifugation to isolate what they refer to as the "modified crude mitochondrial" fraction (MCM). They have shown this MCM fraction to be capable of carrying out the same functions as the more purified synaptosomal preparations. Not only does this procedure shorten the preparation time but it also reduces the handling of the synaptosome fraction and the possibility of membrane lysis that might occur under a more extensive isolation procedure.

In Table 2 the $K_m$ and $V_{max}$ for the transport of NE and 5-HT are listed. NE has a single high affinity process with a $K_m$ of 1.1 μM and a $V_{max}$ of 4.65 pmoles/mg prot./min. 5-HT uptake occurs via two separate transport phenomena with the high affinity process being the most active at low substrate concentrations with a $K_m$ and $V_{max}$ of 0.40 μM and 2.54 pmoles/mg prot./min, respectively. The low affinity uptake is characterized by a $K_m$ and $V_{max}$ of 3.64 μM and 10.0 pmoles/mg prot./min, respectively. Cahill and Medzihradsky (1976) listed the $K_m$ for the NE uptake as 0.39 μM while the 5-HT
Kᵣ is 0.1 M for the high affinity uptake, $V_{\text{max}}$'s are 2.9 and 3.0 pmoles/mg prot./min for NE and 5-HT, respectively. The quantitative difference between this study and the Cahill study may well be due to the manner in which the uptake assay's were terminated. This study employed a centrifugation step, after transferring the 37°C samples to an ice-bath, to pellet the synaptosomes while the Cahill procedure called for vacuum filtration as the terminating step in the assay. It is possible that the filtration technique allows for a more complete removal of any radio-labelled amine that is non-specifically adhering to the synaptosomes than is possible by a similar clean-up procedure utilizing centrifugation.

In assessing the subcellular distribution of NE after uptake into the MCM it was determined that greater than 95 percent of the amine was located in the synaptosomal fraction (Table 3). In conjunction with this finding is the demonstration that the addition of pargyline to the incubation media had no enhancing effect on NE or 5-HT uptake (Table 4). Since pargyline is a monamine oxidase inhibitor and as such would prevent the metabolism of NE or 5-HT by mitochondria, consequently increasing the amine concentration available for uptake, this result is evidence that mitochondrial contamination of the MCM does not interfere to a significant extent with the synaptosomal uptake.

The determination of the synaptosomal LDH ratio (intra-/extra-synaptosomal) did not prove to be useful as a normalization factor for comparison of data from different sample preparations (Table 5), however, this LDH ratio is useful if used as an indicator of membrane
intactness. The ratios presented in Table 5 suggest that very intact synaptosomal membranes were present. Greater than 80 percent of the LDH activity occurred in the intra-synaptosomal compartment in almost all preparations. The data presented here indicate that the synaptosomes isolated for this study had a greater degree of membrane intactness than the synaptosomes used in the Cahill and Medzihradsky (1976) study where 30 percent of the LDH activity was present in the extra-synaptosomal medium. Although the LDH ratio is a good indicator of membrane integrity it unfortunately does not appear to be useful in assessing the functionality of the isolated synaptosomes. Because of this all uptake data is reported as percent of control uptake rather than a direct comparison of the actual uptake values.

Chemical Influence on Synaptosomal Uptake

Modification of synaptic inactivation of NE and 5-HT uptake is one means of producing behavioral changes in both animals and man. Chlorpromazine, a commonly used anti-psychotic drug, is believed to work through such a mechanism (Brucke et al., 1969; Koelle, 1975). In this study a variety of environmental pollutants were tested for this activity and the results obtained indicate that several have such a capability. Table 7 lists the concentration of test chemical producing 50 percent inhibition of NE or 5-HT uptake for all the compounds investigated. The two mercurials, methylmercuric chloride and mercuric nitrate, and the cyclodiene insecticide Kepone were the only compounds tested, other than the positive controls, that were able to produce greater than 50 percent inhibition of this process. Acrylamide, DFP and paraoxon induce a slight but
statistically insignificant inhibition (Table 6). The IC\textsubscript{50} values for the mercurials and Kepone were greater than 10\textsuperscript{-5} M indicating that this inhibition is not specific for the carrier-mediated transport of the biogenic amines.

With respect to Kepone, End et al. (1979b) demonstrated a synaptosomal membrane disruption at 10\textsuperscript{-5} M that was characterized by a Ca\textsuperscript{++} efflux and leakage of LDH into the assay media. With electron microscopy they also noted that Kepone produced a direct membrane lysis that apparently led to the LDH and Ca\textsuperscript{++} liberation. In another study Ho et al. (1980) noted that Kepone decreased catecholamine and 5-HT uptake, after both in vitro and in vivo exposure, into mouse synaptosomes. Using a Scatchard analysis they revealed that Kepone reduced the affinity of the transmitter towards the ligand as well as the maximum amount of binding. These studies suggest that Kepone was able to alter the uptake of the biogenic amine in two ways, the first is membrane perturbation, resulting in a loss of the ionic gradient that is required for membrane transport thus reducing the effectiveness of the carrier-mediated process. The second mechanism is possible through a direct interaction of Kepone with the neurotransmitters membrane receptor inhibiting the transmitter binding to the uptake site.

Mercurials are also known to specifically bind to membranes (Chang and Hartman, 1972c), in part this may be a function of their high affinity for sulfhydryl groups as well as a lesser affinity for amino, phosphate, carboxylate and hydroxyl radicals (Oehme, 1978). Mercury compounds accumulating in the region of the synapse would be
expected to readily associate with membrane components. Such an interaction could ultimately result in the loss of membrane function as is evidenced in this study of biogenic amine transport. Since the neurotransmitters have functional groups that are capable of complexing the mercury, direct interaction with the transmitter may enhance the inhibition of the uptake process. If such a direct interaction with transmitters was to occur physiologically in exposed animals the transmitter-metal complex would not only affect the inactivation process of the amines but would also have a specific and possibly more dramatic effect on post-synaptic interaction of the neurotransmitters and their membrane receptors.

In examining the information contained in Figures 10, 11, and 12 it is noted that the mercurials and Kepone have a very narrow effective range \((10^{-4} - 10^{-3} \text{ M})\) where significant uptake inhibition is detected. Such a narrow concentration range is suggestive of an all-or-none mechanism where uptake inhibition occurs only after high enough concentrations are present to produce membrane perturbations. If complexing of neurotransmitter and toxicant were occurring it would be expected that a much greater dose-response effect would be seen. Extending the relationship further it might be argued that these chemicals do not produce a chronic toxicity by acting at the synapse because the high concentrations required to disrupt the system are higher than one might expect to accumulate during chronic low-level exposures.

With the other compounds, DFP, paraoxon, acrylamide and CdCl\(_2\), a direct effect to influence synaptosomal uptake of the transmitters does not appear to occur. A significant modification of the
uptake process was not seen when tested for in the presence of these compounds. For DFP and acrylamide this is somewhat unexpected considering they both appear to act, at least partially, at the membrane level in eliciting a toxic response. DFP is known to readily phosphorylate membrane components (Johnson and Lauwerys, 1969) and it might have been anticipated that such phosphorylations would affect the uptake process, if not directly then indirectly by influencing the synaptosomal energy generation that is apparently required for membrane transport to occur. Although acrylamide initiates axonal degeneration it is still undecided whether this is a direct membrane effect or an indirect result of inhibition of axonal respiratory enzymes as recently suggested by Spencer et al. (1979).

**K⁺-Dependent Phosphatase Modification by Environmental Pollutants**

K⁺-dependent hydrolysis of organic phosphate is catalyzed by membrane preparations highly enriched in (Na⁺,K⁺)-activated adenosinetriphosphatase (Na⁺, K⁺-ATPase) (Bader and Sen, 1966; Judah et al., 1962). More recently it has been found that in the purification of the Na⁺, K⁺-ATPase enzyme, from a variety of different sources, the K⁺-dependent phosphatase activity remains associated with the enzyme and is actually purified along with it (Jorgensen et al., 1971; Pitts et al., 1973; Uesugi et al., 1971). In fact, it has been accepted that the K⁺-dependent phosphatase activity is a property of the Na⁺, K⁺-ATPase (Hokin et al., 1973; Kyte, 1971;
Lane et al., 1973). Monitoring of this enzyme activity in synaptosomal preparations, and in the presence of the environmental pollutants investigated in this study, gives an indication of the influence that these chemicals have on the Na\(^+\) gradient required for synaptosomal membrane transport.

Table 7 indicates that the heavy metal CH\(_3\)HgCl, Hg(NO\(_3\))\(_2\) and CdCl\(_2\) as well as Kepone and chlorpromazine produced greater than 50 percent inhibition of this enzyme. Hg(NO\(_3\))\(_2\) is considerably more potent in this respect than the others, but CH\(_3\)HgCl and CdCl\(_2\) also strongly inhibit the phosphatase at very low concentrations. This is in general agreement with other workers who indicate that heavy metals are very effective in modulating Na\(^+\), K\(^+\)-ATPase activity (Bond and Hudgins, 1979; Henderson et al., 1971; Karlish et al., 1979; Kinter and Pritchard, 1977). Kepone inhibition of the phosphatase activity is also in agreement with the finding that, as a class, organochlorines are effective inhibitors of ATPase (Glick, 1973). Chlorpromazine was the weakest of the inhibitors producing greater than 50 percent inhibition, requiring almost a 1.0 mM concentration to reduce the phosphatase to a half maximal rate. Ouabain, which is generally considered a highly specific inhibitor of Na\(^+\), K\(^+\)-ATPase, was found to be a poor inhibitor in this system. At 0.33 mM less than 25 percent inhibition of this activity occurred. This is possibly due to the lack of Na\(^+\) ions in the incubation medium and the high K\(^+\) concentrations present (10 mM). It is also possible that the enzyme substrate used, 3-0-methylfluorescein phosphate, does not support ouabain binding to the enzyme because of a higher
affinity for the binding site. Acrylamide, DFP and paraoxon did not demonstrate any inhibitory activity in this system. Although DFP, like other organophosphates, is considered to be a good enzyme inhibitor (Lehninger, 1975), it is apparent that the $K^+$-dependent phosphatase activity, as examined in this study, is not adversely affected by any protein phosphorylation initiated by DFP.

Comparison of the Biogenic Amine Uptake and $K^+$-Dependent Phosphatase Inhibition by Environmental Pollutants

Examining Table 7 closely it is evident that a correlation between uptake inhibition and $K^+$-dependent phosphatase activity does not exist. The inorganic mercurial, Hg(NO$_3$)$_2$, was the strongest inhibitor of the phosphatase activity but the weakest inhibitor of the uptake process of the chemicals capable of producing greater than 50 percent inhibition. Chlorpromazine had just the opposite properties, being the most effective uptake inhibitor but the weakest of the positive phosphatase inhibitors. CdCl$_2$, on the other hand, was very effective against the phosphatase enzyme but totally ineffective in inhibiting either uptake process and DFP which would be expected to exert a strong influence on any enzymatic process has no influence, even at a $10^{-3}$ M concentration, on either the synaptosomal uptake or phosphatase properties of the membranes. Although a correlation does not occur between the phosphatase and uptake function of the synaptosomal membrane, this data indicated that several of these environmental pollutants were highly effective inhibitors of enzymatic processes.
(as evidenced by the low IC\textsubscript{50} 's for the phosphatase). This would be of critical importance to synaptic, as well as neuronal function, if the test compounds were able to permeate the plasma membranes and gain access to the intracellular compartments.

It should be noted that the lack of an inhibitory influence for DFP was unexpected. As previously mentioned DFP is considered to be a good inhibitor of many enzymatic processes and Saunders (1957) estimated the IC\textsubscript{50} for horse-serum cholinesterase at $10^{-9}$ M DFP. Contrasting results have been reported for DFP effects on Na\textsuperscript{+}, K\textsuperscript{+}-ATPase isolated from rat brain. Glow et al. (1972) reported that exposure to sublethal doses of DFP in vivo increased the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase specific activity of whole brain homogenates while decreasing activities were found in Na\textsuperscript{+}, K\textsuperscript{+}-ATPase of rat cerebral cortices (Jovic et al., 1971). Whatever the reasons for the qualitative differences in these two studies may be, the important point is that DFP did alter the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase. Since K\textsuperscript{+}-dependent phosphatase is considered to be a property of the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase, the lack of effect exhibited by DFP on this enzyme was unexpected. A more extensive examination of the kinetics of the phosphatase would be useful in elucidating the extent of this ineffectiveness.

**The Significance of the Effects Environmental Pollutants Have on Rat Synaptosomal Membrane Function and Suggestions for Future Studies**

In examining the inhibition of the uptake process at various concentrations of toxicant it was concluded that at relatively high concentrations, CH\textsubscript{3}HgCl, Hg(NO\textsubscript{3})\textsubscript{2} and Kepone were effective inhibitors.
This low affinity inhibition suggests that a specific inhibition of the carrier-mediated transport may not exist. Rather, a non-specific membrane interaction may be the mechanism by which such inhibition occurs. The lipophilic nature of these compounds may allow for their insertion within the membrane, with subsequent interaction with membrane phospholipid or protein, leading to a disruption of the inherent order and functionality of the membrane.

The information collected indicates that a correlation between inhibition of rat brain synaptosomal uptake and K⁺-dependent membrane phosphatase does not exist. To account for this lack of correlation it is possible that the rat is not a sensitive enough species for such studies. It is widely accepted that the chicken is the species of choice for studies involving neurotoxic pathology because of its sensitivity to chemicals inducing such effects. Although it can be shown that rats and other rodents are susceptible to chemically induced neuropathies it is generally accepted that they are less sensitive than other species (i.e., chicken). Therefore, it is possible that a study of this nature performed with chicken synaptosome preparations may yield a more positive correlation.

The results of this study also suggest that inhibition of synaptosomal neurotransmitter uptake is not a key factor in the neurotoxicity of the compounds tested. However, in vivo exposure of animals to these compounds followed by analysis of the uptake functions would be a much stronger indicator of the existence as well as the significance of such an effect. It would also be of relevance to examine the nature of the interaction between these compounds and the
biogenic amines to determine whether a toxicant-amine complex formation occurs. Such a complex would be capable of directly influencing the information transfer that occurs across the synapse. Other in vitro studies that may provide significant information in assessing the potential toxicity of these compounds would include an examination of the actual membrane interaction that was postulated to occur. This could be accomplished by monitoring membrane fluidity as well as passive diffusional transport for changes in membrane permeability. It would also be of interest to determine whether these compounds actually permeate the membrane and accumulate within the intra-synaptosomal compartment. If this were to occur it may ultimately prove to be more critical for synaptic function since these neuortoxic compounds would then have access to the metabolic and synthetic machinery of the synaptic terminal and the concentration necessary to disrupt this activity would likely be much less than that necessary for membrane disruption.
SUMMARY AND CONCLUSION

This study was undertaken to determine if the neurotoxicity produced on exposure to several environmental pollutants could be attributed to membrane changes occurring at the synaptic processes of the central nervous system. An in vitro model system was used to determine if membrane changes were capable of being produced on in vitro exposure to CH$_3$HgCl, Hg(NO$_3$)$_2$, CdCl$_2$, DFP, paraoxon, acrylamide, and Kepone. Synaptosomal uptake of norepinephrine and serotonin, as well as the membrane K$^+$-dependent phosphatase activity, were monitored as indicators of membrane function.

The data presented indicates that the two mercurials, CH$_3$HgCl and Hg(NO$_3$)$_2$, and the cyclodiene insecticide Kepone, were effective inhibitors of both the NE and 5HT carrier-mediated uptake with more than 50 percent inhibition occurring at concentrations greater than 10$^{-4}$ M. The remaining test compounds were unable to induce a significant inhibition of this process at concentrations ranging up to 10$^{-3}$ M. Although CH$_3$HgCl, Hg(NO$_3$)$_2$ and Kepone produced a significant inhibition it should be noted that the K$_m$'s for the high affinity NE and 5-HT transport was established to be less than 10$^{-6}$ M. The high IC$_{50}$ values presented for these compounds as well as the demonstration that their effectiveness occurred over a narrow concentration range suggests a non-specific membrane perturbation as the initiating factor eliciting the inhibitory response.

The K$^+$-dependent phosphatase was strongly inhibited by Kepone, CH$_3$HgCl, Hg(NO$_3$)$_2$, and CdCl$_2$. The IC$_{50}$ values were determined to be
less than $10^{-5}$ M with Hg(NO$_3$)$_2$ having the strongest inhibitory influence. DFP, paraoxon and acrylamide at concentrations up to $10^{-3}$ M were unable to produce a significant inhibition of the phosphatase.

Since the NE and 5HT uptake is at least partially dependent on the energy generated from the Na$^+$, K$^+$ transmembrane gradient it was felt that the low affinity inhibition of the uptake processes might be a result of a direct effect on the cation gradient. Although the inhibition of the membrane phosphatase did occur at very low concentrations of the test chemicals a correlation between this inhibition and the monoamine uptake inhibition could not be demonstrated.

The data generated from this investigation indicates that inhibition of the synaptosomal NE and 5HT uptake on in vitro exposure to CH$_3$HgCl, Hg(NO$_3$)$_2$ and Kepone, is not a critical factor in the neurotoxicity produced by these compounds. The high concentrations and narrow dose-response curves suggest a direct membrane effect as the causative factor in the inhibitory response. Although the uptake inhibition was not demonstrated to be a highly specific process, inhibition of the K$^+$-dependent phosphatase was found to occur at sub-micromolar concentrations. This suggests that these compounds are able to influence synaptic membrane function and the possibility of other synaptic effects can not be ruled out.
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