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THE UNIVERSITY OF ARIZONA M.S. 1984

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THE ROLE OF NEURAL TISSUE CONCENTRATION IN ACRYLAMIDE NEUROTOXICITY

by

Leslie A. Rylander Yueh

A Thesis Submitted to the Faculty of the

DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

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WITH A MAJOR IN TOXICOLOGY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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STATEMENT BY AUTHOR

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Pharmacology and Toxicology

April 27, 1984
Date

DEDICATION

This thesis is dedicated to my family for their unflagging support of my scientific endeavors and my husband, Eddie, for his pride in my accomplishments and his appreciation of the difficulties of being a student.

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ABSTRACT

Age-related differences in tissue deposition of acrylamide (as ^{14}C, a cumulative neurotoxin, were found when 5 and 11 week old male Holtzman rats were singly - or multiply - dosed. In general, the concentration of acrylamide (as ^{14}C) was significantly greater in tissues from 11 week old rats than 5 week old rats. Together with a previous study by Kaplan and Murphy (1973) showing earlier onset of acrylamide - induced neurotoxic signs in 11 week old vs. 5 week old rats, this suggested a relationship between extent of tissue deposition and onset of neurotoxic signs. Phenobarbital treatment, previously shown by Kaplan et al. (1973) to delay the onset of acrylomide-induced neurotoxicity, also altered acrylamide deposition. Reduced tissue concentrations of acrylamide (as ^{14}C) were associated with phenobarbital treatment in male Fisher - 344 rats. However, attempts to relate this finding to delayed onset of neurotoxic signs were unsuccessful.

CHAPTER 1

INTRODUCTION

Purpose

Monomeric acrylamide (ACMD) is neurotoxic to humans and laboratory animals. Early studies characterizing ACMD toxicity related total cumulative dose to the production of neurotoxic signs (Kuperman, 1958; Fullerton and Barnes, 1966). That is, exposure to a threshold amount of toxic material was required to compromise nervous function to the point of visible neurological impairment. Biochemically, this property was attributed to the inactivation of a cofactor or enzyme necessary for maintenance of neuronal energy production (Schoental and Cavanaugh, 1977; Spencer and Schaumburg, 1978).

Hashimoto and Aldridge (1970) discovered small amounts of TCA-unextractable material in rat tissues after a single iv dose of (1-^{14}C) ACMD. This material was detectable in tissues 14 days after dosing. Pharmacokinetic studies confirmed the persistence of a slowly cleared tissue component following a single iv dose of (2,3-^{14}C) ACMD, with an estimated terminal elimination half life of approximately 8 days (Miller et al., 1982). Accumulation of this component in tissues during multiple dosing is therefore likely and could provide quantitative, though indirect, support for the

cumulative biochemical lesion theory of ACMD neurotoxicity. That is, changes in the distribution or amount of this component in the body, particularly the nervous tissue, could affect the onset of neurotoxic signs.

To test the hypothesis that tissue-associated material plays a critical role in ACMD-induced neurotoxicity, two experimental designs were used. The first was based on work published by Kaplan and Murphy (1972) demonstrating the effect of age on susceptibility to ACMD. These authors found that 11 and 14 week old rats became neurotoxic significantly sooner than 5 week old rats, when rats were dosed with 50 mg/kg/day ACMD (given ip) and balance on a rotating rod was used as a measure of neurotoxicity. In addition, both the 11 and 14 week old rats lost more weight than the 5 week old rats over the treatment period, another sign of greater susceptibility. Using this model, rats at different stages of ACMD-induced neurotoxicity could be produced and the relationship between onset of neurotoxicity and tissue level of ACMD could be examined. Therefore, 5 and 11 week old male Holtzman rats were singly- or multiply- dosed with ACMD (50 mg/kg/day, given ip) containing (2,3 - ^{14}C) ACMD as a tracer. Distribution and deposition of ^{14}C-label were measured to determine if changes in these parameters could be a factor in the age-related differences in onset of ACMD neurotoxicity. Elimination of a single ip dose of ACMD (50 mg/kg) was also examined for age-related differences, as another possible cause of differences in susceptibility.

A second set of experiments testing the critical tissue level hypothesis was based on work describing the effects of classical cytochrome P450 mixed function oxidase inducers and inhibiters on ACMD neurotoxicity. Kaplan et al. (1973) pretreated rats with phenobarbital or DDT to see if these microsomal enzyme inducers would modify the onset of ACMD neuropathy. They found that these pretreatments significantly delayed the onset of functional neurological impairment as measured by ability to balance on a rotating rod. Increased detoxification of parent ACMD was thought to be the reason for the delay in the onset of neurotoxic effects. This was supported by the increased disappearance of ACMD from incubations containing hepatic 9,000 x g supernatant from phenobarbital pretreated rats vs. control rats. These pretreatments could therefore affect the amount of ACMD available for distribution to and depostion in tissues, lending indirect support to the critical tissue level hypothesis. Phenobarbital was chosen as the model cytochrome P450 inducer, being the better characterized of the two used by Kaplan et al. (1973). Neurotoxicity tests were performed on the various treatment groups in an effort to duplicate and extend the published findings. Distribution and deposition of ^{14}C-label were measured to determine if changes in these parameters could be the cause of delays in ACMD-induced neurotoxic effects. In addition, the clearance of a single iv dose of (2,3-^{14}C) ACMD (50 mg/kg) was followed to determine the effects of phenobarbital pretreatment on ACMD excretion. Conversely,

the effect of ACMD on the inducing ability of phenobarbital was also studied.

Background

Chemistry of the Compound

Acrylamide (ACMD) is a highly reactive vinyl monomer that has been in industrial use since the early 1950's. A white crystalline solid, ACMD is highly water soluble (approx. 2 gm/ml). Although it is considered thermally stable, it does sublime at room temperature. Other physical and chemical properties are listed in Appendix A.

The majority of ACMD is used to produce a variety of homopolymers and copolymers. These polymers are used primarily as flocculants in water purification, strengtheners in the manufacture of cardboard and paper, chemical grouting agents, and in textile treatment (NIOSH, 1976). Approximately 50 million pounds of ACMD were produced in the United States in 1972, increasing to at least 70 million pounds by 1974 (US Department of Congress, 1976). Further growth in the market for ACMD-based polymers is expected due to expanded use by the petroleum industry and in water treatment applications.

With increased production, the potential for exposure to the toxic monomer also increases. Exposure of the general public could conceivably occur via leaching of monomer from ACMD-based polymers used to treat drinking water or from polymer-treated cardboard used to package food. To prevent this, residual monomer in polymer used

for these purposes is limited to 0.05%. In addition, ACMD is rapidly biodegraded, probably reducing monomer in effluent and disposal sites to very low levels (US Department of Commerce, 1976). Therefore widespread environmental contamination by ACMD monomer is not considered a serious threat.

The current time-weighted average for worker exposure to ACMD monomer is 0.3 mg/cu m of air (NIOSH, 1976). This theoretically limits absorption by the worker to no more than 0.05 mg/kg/day. Workers at highest risk are those involved in manufacture and polymerization of ACMD monomer, and workers exposed to large amounts of the polymer, including those in water treatment, in paper and cardboard manufacturing, or in the use of chemical grouting agents. (These latter groups would be exposed primarily to residual monomer contained in the various polymers used.) Approximately 20,000 workers are currently considered at risk in the country (NIOSH, 1976).

Toxicity in Humans

Human ACMD intoxication was first documented in the 1960's although it was probably occurring much earlier (Garland and Patterson, 1967). Early symptoms included sweating, reddening, and peeling of the skin on the hands. These were followed by neurological disturbances such as sensory loss in the hands and feet, fatigue, tingling sensations in the extremities, loss of balance, and incoordination, suggesting both central and peripheral components of toxicity. Neurological examination of the victims revealed small

losses of tendon reflexes. More severe cases also displayed muscle weakness in the arms and legs, wasting of the small muscles in the hands, and weight loss. These symptoms appeared gradually, becoming more severe as length of exposure increased: a latency period of 2 weeks was noted in one study of 6 patients (Kesson, Lawson, and Baird, 1977). Intoxication appeared to be limited to those working directly with the monomer. Dermal absorption was the primary route of exposure, although ingestion and inhalation were also possible (Garland and Patterson, 1967; Kesson, Lawson, and Baird, 1977). Cessation of exposure, via better protective clothing or time away from the work place, led to complete recovery in the less severe cases, although the required recovery period was sometimes as long as 12 months. The more severe cases had not fully recovered (ie., there was continued sensory loss and lack of tendon reflexes) even 15 months after exposure had ceased. (Kesson, Lawson, and Baird, 1977).

Electrophysiological recordings of motor nerve conduction velocities and motor and sensory nerve action potentials from three ACMD-intoxicated workers revealed considerably prolonged conduction times in the distal portions of nerves in the arms and legs (Fullerton, 1969). Histological examination (via nerve biopsy) of nerve fibers from sural nerves revealed some axonal degeneration and a decreased number of large (9-16 μm) diameter fibers compared to control nerves. Surprisingly, regenerating and degenerating axons were present within the same nerve trunks in one patient, implying that these processes were concurrent.

Animal Toxicology

Early studies in animals further established ACMD as a cumulative neurotoxin. Kuperman (1958) dosed cats with what are considered high doses of ACMD to study the apparent central component of ACMD toxicity. Daily doses of 1 - 50 mg/kg were given either intravenously or intraperitoneally. Large single doses (100 mg/kg) given iv caused convulsions and death. All doses eventually caused ataxia and postural and muscular incoordination, suggesting central involvement. More importantly, it was noted that time to onset of these signs decreased with increasing size of the daily dose. A cumulative dose of approximately 100 mg/kg was needed to produce overt toxicity regardless of dosing rate or route of administration. Death occurred if dosing continued long enough but cessation of dosing allowed complete clinical recovery provided intoxication was not too severe. Cats required a few weeks up to several months to recover, similar to ACMD-intoxicated humans.

A general toxicology study of ACMD was published in the 1960's. Rats, guinea pigs, rabbits, cats, and/or monkeys were used to determine the LD_{50}'s and study short and long term effects of orally-administered ACMD (McCollister et al., 1964). The oral LD_{50} for rats, guinea pigs, and rabbits was determined to range from 150 to 180 mg/kg. A threshold daily dose of 300 ppm ACMD in the diet was found to produce neurotoxic signs (ataxia, loss of proprioception) in rats after 21 days. By 42 days, all male rats and all but 4 female rats were dead. The survivors recovered completely after being off

ACMD treatment for 57 days. Again, it was found that a higher dose (400 ppm) produced toxicity in less time. Weight loss was a frequent finding in more severely intoxicated rats. Another symptom noted by these authors was bladder distension.

One year feeding studies in cats and monkeys also demonstrated a relationship between dosing rate and onset of toxic signs. A daily dose of at least 10 mg/kg was required to produce the characteristic hindquarter weakness and incoordination of ACMD intoxication. Microscopic examination of central nervous tissue from intoxicated animals revealed no pathology, although tendon reflexes were absent in the monkeys tested.

Pathology

The first electrophysiological and histological data from ACMD-intoxicated animals were published by Fullerton and Barnes (1966). Rats were given varying amounts (100 - 400 ppm) of ACMD in their food until signs of toxicity were noted. Again, onset of signs was more rapid at higher daily doses of ACMD. When examined electophysiologically, the maximal motor nerve conduction velocities in nerves supplying the small muscles of the foot progressively decreased, indicating a peripheral neuropathy. Histopathological changes were noted in peripheral nerves, consisting of fiber degeneration affecting first, and most severely, the distal ends of the longest nerve fibers. No changes were noted in the central nervous system.

An extensive histological study of central and peripheral nerve fibers and cell bodies from ACMD-intoxicated cats was conducted by Prineas (1969). Both peripheral axons and axons from the spinal cord exhibited what were labeled 'dying-back' changes. The earliest change was marked accumulation of neurofilaments, particularly at the nodes of Ranvier and nerve terminals. More severe degenerative changes were found primarily in the distal ends of fibers, although they also occurred in spinal nerve roots and within sensory fibers near their origin in dorsal root ganglion cells.

ACMD-induced degeneration of the peripheral nerves, particularly in the distal portions of the longest fibers, was also demonstrated in baboons (Hopkins and Gilliatt, 1967; Hopkins, 1970), cats, and monkeys (Leswing and Ribelin, 1969). Loss of large diameter fibers and swollen myelin sheaths and axons were characteristic histological findings in nerves from the hind limbs of intoxicated animals. Two years after severe neuropathy had been produced and dosing ended, total fiber content and fiber density of peripheral nerves had returned to normal, and the external diameter of remyelinated and regenerated fibers had reached two-thirds normal (Hopkins, 1970). Detectable decreases in nerve conduction velocities were present only after severe intoxication, but continued even after cessation of exposure and marked improvement in limb function.

Up to this point, it appeared that peripheral nerves were targeted in ACMD-induced neuropathy, particularly the long, large diameter fibers leading to the hind limbs. In addition, the overall

pathology resembled a classic 'dying-back' neuropathy, that is, a gradual degeneration of the axon progressing from distal regions of the axon to proximal regions.

Mechanisms of Toxicity

The early accumulation of neurofilaments and other organelles in peripheral nerve fibers led to the first proposed mechanism of ACMD toxicity. That is, distal degeneration of the axon involves the slowing of axoplasmic flow from the cell body down the axon (Prineas, 1969). Furthermore, energy-producing reactions in the axon, or supplies of substances from the cell body necessary to maintain axoplasmic flow, were suggested as primary targets for ACMD toxicity. It was argued that this hypothesis could adequately explain why the extremities of fibers, and particularly those of the longest, largest fibers, showed the first degenerative changes.

Study of the biochemical behavior of ACMD expanded as interest in the underlying mechanisms of its toxicity increased. Injury to the perikarya was one of the first areas explored. For example, incubation of brain cortex slices with 10 mM ACMD did not affect oxygen uptake or the final concentration of pyruvate or lactate in the media, apparently dismissing glycolysis as a specific target system for ACMD toxicity (Hashimoto and Aldridge, 1970). Nor was oxidative phosphorylation in respiring rat liver mitochondria affected.

Incorporation of ^{3}H-leucine into the cell bodies of anterior horn cells was studied *in vivo* in mice given 250 ppm ACMD in their

amino acid into perikaryal protein preceded any ultrastructural changes seen in the distal axons and preceded clinical signs, suggesting a possible causal relationship between this phenomenon and diminished axonal flow. Conflicting results were found when in vitro incorporation of L-lysine and L-methionine into brain, spinal cord, and sciatic nerve of rats fed 500 ppm ACMD in the diet for 4 weeks followed by 4 weeks without treatment (Hashimoto and Ando, 1973). In general, incorporation of these amino acids into spinal cord and sciatic nerve increased following ACMD treatment, while incorporation into brain slices was unaffected. However, this phenomenon was thought to be related to repair mechanisms turned on by toxic injury rather than itself a cause of the injury. The in vivo incorporation of ^{3}H-leucine into spinal cord and brain stem proteins was re-examined in rats by Schotman et al. (1977). In rats treated chronically with ACMD, the incorporation of ^{3}H into proteins was altered in a manner which seemed related to the phase of intoxication. That is, before toxic signs appeared, incorporation of radioactivity was reduced by 12%; when clinical signs were apparent, the reduction reached 20%. Incorporation increased by 11% when rats were allowed to recover such that clinical signs had disappeared. This apparent correlation between incorporation of an amino acid into neural protein and the stage of neuropathy, led the authors to suggest a relationship between a disturbance in protein metabolism and ACMD-induced neuropathy.

Impaired axonal transport of proteins to distal sections of nerve fibers was proposed as an alternative mechanism for ACMD neurotoxicity (Pleasure et al., 1969). Absence of the slow component of axonal flow in dorsal and ventral roots of cats intoxicated by ACMD was noted, and was implicated as a possible cause of distal axonal degeneration. This explanation was chosen rather than a defect in protein synthesis, because accumulation of radioactivity (from the incorporation of radiolabeled amino acids into proteins) near cell bodies was found, implying synthesis was indeed occurring.

Fast and slow transport were studied by Bradley and Williams (1973) in dorsal root ganglia along the proximal regions of sciatic nerves of mildly and moderately intoxicated cats. In contrast to the findings of Pleasure et al. (1969), ACMD treatment had no effect on the slow component of axonal transport, but was associated with a slight decrease in the velocity of fast transport. However, no decrease in the amount of labeled protein carried by either slow or fast transport was found, refuting the hypothesis that decreased supply of protein to the distal axon is the cause of axonal degeneration during ACMD intoxication. In addition, there was no apparent correlation between rates and amounts of axoplasmic flow and clinical or histological severity of the neuropathy. With these findings in mind, it was speculated that delivery of a specific protein to the distal axon may be involved in axonal degeneration, one that is only a minor fraction of the total protein carried.

Sidenius and Jakobsen (1983) used the protein incorporation and transport of ^{35}S-methionine, ^{3}H-fucose, and ^{3}H-proline to study fast and slow anterograde transport in sensory nerves of ACMD-intoxicated rats. No significant alterations in transport velocity were found, although a reduction in amount of protein carried in the slow axonal transport component was associated with severe neuropathy. This was thought to be a result of axonal injury rather than a cause. A second study, concentrating on retrograde transport, demonstrated a depression in the amount of material being transported, even though anterograde transport parameters were normal (Jakobsen and Sidenius, 1983). A close connection between degree of neuropathy and decrease in retrograde accumulation of labeled protein was noted. Retrograde transport is associated with large membranous bodies and reduction in the amount of material transported by this process may explain the accumulation of neurofilaments that occurs early in ACMD intoxication.

Retrograde transport was also examined by Miller et al. (1983a). In this study, the retrograde transport of iodinated nerve growth factor was altered by a single ip dose of ACMD in a dose-dependent manner, and by a subtoxic dose given repeatedly. These alterations preceded detectable alterations in peripheral nerve function and correlated with cumulative dose of ACMD. Thus, altered retrograde transport appears to be associated with the induction of ACMD neuropathy and may indeed reflect the primary biochemical lesion of ACMD toxicity, while not actually being that lesion.

Neither of the mechanisms proposed thus far adequately explained the exceptions to the 'dying-back' peripheral neuropathology associated with ACMD-induced neuropathy. For example, Hopkins (1970) noted, in one baboon in particular, that the extent of histological and electrophysiological changes in peripheral nerves was not in keeping with the severity of its clinical symptoms. A central component of ACMD toxicity, as was studied by Kuperman (1958) and suspected in cases of human intoxication, was suggested as the cause. Secondly, histological examination of fibers in cats after ACMD intoxication revealed multifocal degeneration of distal axons, not a gradual, proximal movement of degeneration (Schaumburg et al., 1974). This study also demonstrated preferential involvement of sensory, rather than motor, fibers. More importantly, it showed the simultaneous degeneration of Pacinian corpuscles (a sensory organ) in the forefeet and hindfeet, refuting the hypothesis that fiber length is the critical factor in determining fiber vulnerability. Another feature of ACMD-induced neuropathy left unexplained was the presence, both implied electrophysiologically and verified histologically, of regenerating nerve fibers in intoxicated humans and animals (Fullerton, 1969; Schaumburg et al., 1974). Finally, no explanation had been found for the characteristic weight loss noted during ACMD intoxication.

A third hypothesis, discussed by Prineas (1969), proposed direct axonal damage by ACMD as its toxic mechanism. Under this premise, ACMD penetrated the axon at vulnerable points (particularly

terminals and nodes of Ranvier, where protective myelin is lacking) and inactivated substances, such as metabolic cofactors (Schoental and Cavanaugh, 1977) or enzymes involved in energy production (Spencer and Schaumburg, 1978), necessary for the maintenance of axonal transport and impulse conduction. As more ACMD was introduced, re-supply of these substances, presumably from the perikaryon, could not satisfy the increased axonal demand. Distal portions of the axon suffered first, as limited supplies from the perikaryon were depleted in the proximal axon and transport down the axon was increasingly compromised. More severe intoxication led to involvement of the proximal axon. Eventually, a steady state, balancing the rate of re-supply and inactivation by succeeding doses of ACMD, was reached, allowing maintenance of a short section of axon and some regeneration.

This hypothesis was attractive for a number of reasons. As outlined above, it helped explain the pathology associated with ACMD intoxication. In addition, the cumulative nature of ACMD toxicity became more understandable - a certain degree of cofactor or enzyme inactivation was required before detrimental effects on the axon occurred. Thus, a cumulative threshold amount of toxin had to penetrate nerve fibers before signs of toxicity developed. The existence of an inverse relationship between dosing rate and onset of toxic signs, noted by several groups (Kuperman, 1958; McCollister et al., 1964; Fullerton and Barnes, 1966), also became clear. As larger doses of ACMD were given, more of the available target substance was

inactivated after each dose and the threshold level of inactivation was reached more quickly.

Interaction between neural tissue and ACMD had already been demonstrated when the direct axonal damage hypothesis was formally proposed. Rats were given a single iv dose of 100 mg/kg (1-^{14}C)/ACMD in an effort to characterize its distribution and retention in the body (Hashimoto and Aldridge, 1970). Tissues were extracted with 5% trichloroacetic acid to separate free ACMD (as ^{14}C) from protein-bound material. Protein-bound material persisted in all tissues examined, including brain, spinal cord, and sciatic nerve, at least 14 days after dosing. This finding lent credence to the proposal that ACMD reacts with some component of nervous tissue, the product of which persists long enough to allow its accumulation during multiple dosing. Furthermore, this reaction product was suspected as the source of the cumulative nature of ACMD neurotoxicity.

The nature of the target tissue component was as yet unknown. Subcellular distribution of radioactivity in the brain and liver (from ^{14}C-ACMD) 24 hours after a single iv dose was very uniform and tissue-independent (Hashimoto and Aldridge, 1970). The in vitro chemical reactivity of ACMD with glutathione and hemoglobin was very high (Hashimoto and Aldridge, 1970). Indeed, decreased non-protein sulfhydryl content in brain and spinal cord was found 4 hours after a single oral dose of ACMD, and levels of radioactivity (from ^{14}C-ACMD) were maintained at high levels in red blood cells long after plasma levels had dropped. The significance of these findings was

questioned by the authors, however, because a relatively non-neurotoxic analog, N-hydroxymethyl-ACMD, had similar properties.

Work by Edwards (1975) supported the distribution data, sulfhydryl reactivity data, and the effects on hepatic glutathione levels discussed above. In addition, glutathione conjugates of ACMD and its analog, N-hydroxymethyl-ACMD, were detected in bile from rats, as similar proportions of the total dose given indicating no preferential reactivity of glutathione toward ACMD *vs.* N-hydroxymethyl-ACMD. It was concluded by this author that either certain sulfhydryl groups show differential reactivity toward ACMD and N-hydroxymethyl-ACMD or the binding site related to the development of neuropathy is not a sulfhydryl group.

More recently, attention has been focused on enzymes of the glycolytic pathway as common targets for several chemically unrelated neurotoxins (Spencer et al., 1979). In particular, the inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by ACMD was studied since it is thought to be important in energy production required for axonal transport. Both crystalline GAPDH and enzyme present in brain and peripheral nerve tissue were inhibited by ACMD *in vitro*. So, too, was phosphofructokinase, another regulatory enzyme in the glycolytic pathway. In both cases, enzyme activity was protected by sulfhydryl-containing reagents.

Both central and peripheral nervous tissue contain a unique isozyme of enolase, a glycolytic enzyme (Howland et al, 1980a). This isozyme was found in all species investigated including the rat, cat,

and human, and is known to contain sufhydryl groups. These properties make the neuron specific enolase (NSE) an excellent candidate for involvement in ACMD neurotoxicity. When this was investigated, NSE from rat brain was completely inhibited after incubation with 10 mM ACMD. Rats chronically dosed with up to 550 mg/kg ACMD and displaying signs of neurotoxicity had only 60% of control NSE activity in brain and no detectable activity in sciatic nerve. Interestingly, the I_{50} for ACMD against NSE (3.3 mM) was not significantly different from that against total enolase activity (3.7 mM). In in vivo studies however, total enolase activity was unchanged from control, suggesting an in vivo selectivity for the neuron specific isozyme may be linked to the neurotoxic properties of ACMD.

Inhibition of enolase was reversed by dialysis, indicating little or no covalent binding of ACMD to the enzyme (Howland et al., 1980b). In vitro inhibition of this enzyme was potentiated by dithiothreitol and glutathione, two sulfhydryl group donators. It was thought that in vitro, the disulfide bonds of the enzyme were opened by these agents, exposing the resulting free sulfhydryls to attack and binding by ACMD.

A more extensive in vitro study considered inhibition of several glycolytic enzymes in rat brain homogenate by ACMD (Howland et al, 1980b). A differential sensitivity of the enzymes was revealed. Specifically, phosphofructokinase (PFK) was less sensitive than enolase, which was less sensitive than GAPDH. The biological significance of these findings was studied in ACMD-intoxicated cats

(Howland, 1981). NSE and GAPDH activities were decreased in both peripheral and central nervous system tissues, particularly in the distal portions of peripheral nerves. Two enzymatic targets were therefore suggested, with the tissue selectivity of ACMD residing in its ability to inhibit the neuron-specific isozyme of one target enzyme. In addition to changes in activities in nervous tissue, GAPDH activities in skeletal muscle and liver were also depressed, suggesting a possible cause of the general weight loss associated with ACMD intoxication.

Theoretically, if inactivation of certain glycolytic enzymes is the fundamental cause of ACMD-induced neuropathy, then providing the axon with an adequate supply of pyruvate, the end product of glycolysis, should prevent the onset of toxicity. Sterman et al., (1983) studied the onset of neurotoxic signs in rats treated with ACMD (50 mg/kg/day, given ip) with and without simultaneous feeding of sodium pyruvate (3.5 mg/kg/day). Using a battery of 6 neurobehavioral tests, they showed a very slight protective effect attributable to pyruvate treatment. However, there was no detectable effect on the measured rate of disease progression. Examination of peripheral nerve fibers and dorsal root ganglia by light microscopy revealed no differences between ACMD-only and ACMD + pyruvate groups. These findings are far from conclusive, however, since no effort was made to determine how much, if any, pyruvate actually reached peripheral nervous tissue. Alternatively, these authors speculate

that inactivation of glycolytic enzymes may not be the only requirement for the induction of ACMD neuropathy.

CHAPTER 2

METHODS

General Methods

Chemicals

Unlabeled acrylamide (ACMD) was purchased from Aldrich Chemical (Milwaukee, WI). Purity was greater than 99% by thin-layer chromatographic (TLC) analysis.

(2,3 - ^{14}C) ACMD was custom synthesized by New England Nuclear Co. (Boston, MA). Two different lots were used: #1202-167, specific activity = 0.512 μCi/mmole; #1533-153, specific activity = 0.702 μCi/mmole. Radiochemical purity was 97 - 98% by TLC and high pressure liquid chromatographic (HPLC) analysis.

All other chemicals and solvents were reagent grade quality unless otherwise specified.

Purification and Storage of ^{14}C-ACMD Monomer

(2,3 - ^{14}C) ACMD monomer was received in solid form in an amber glass bottle. Lot #1202-167 was initially stored in this form at -10°C. Aliquots of the solid were purified before use by mixing them with 100% ethanol then filtering the resulting solution to remove insoluble polymer. Eventually, polymerization became excessive (>30%) and a stock solution of monomer was made up in 100% ethanol. This solution was stored at -10°C. Upon arrival, lot

#1533-153 was immediately dissolved in 100% ethanol and filtered to remove polymer. The resulting stock solution was then stored at 4°C.

Analytical Methods for ACMD Monomer/Parent ACMD

^{14}C-labeled and unlabeled ACMD monomer were assayed for radiochemical and chemical purity, respectively using TLC and HPLC techniques.

Analysis of ACMD by TLC was performed using high efficiency Silica plates with fluorescent indicator from Analtech (Newark, DE). An ethanol/ammonium hydroxide (5:1) solvent system was used as described by Edwards (1975). ACMD monomer was identified as a UV visible spot (Rf = 0.70). In the case of (2,3-^{14}C) ACMD, plates were scraped in 1 cm sections, suspended in distilled water and scintillation fluid (Betaphase, Westchem, San Diego, CA), and analyzed sequentially for ^{14}C by liquid scintillation counting (LSC).

An HPLC analysis was performed as a supplemental purity assay for (2,3-^{14}C) ACMD and as a means of separating parent ACMD from metabolites in blood extracts and urine. A Partisil 10-PAC column (Chromanetics, Baltimore, MD) was used for the analysis, with a running solvent system of methylene chloride/methanol (80:20) and a flow rate of 2 ml/min. Eluate was collected at 1 minute intervals for 8 minutes and mixed with scintillation fluid for liquid scintillation counting. ACMD monomer/parent ACMD eluted at 3 minutes.

Dosing Solution Formulation

In all cases, dosing solutions were made up in distilled, deionized water. Ethanol from ^{14}C-ACMD stock solution was limited to less than 10% of the total volume of the final dosing solution.

For solutions containing (2,3-^{14}C) ACMD, stock solution was diluted with distilled, deionized water, then added to a solution of unlabeled ACMD in water. The resulting solution contained 50 mg/ml ACMD and radioactivity ranging from 50 µCi/ml to 19 µCi/ml. The solutions were formulated in this manner so that dosing volumes were kept at 1 ml/kg. All dosing solutions containing (2,3-^{14}C) ACMD were assayed for radioactivity in duplicate by LSC prior to use.

Analysis of Blood and Tissues for ACMD (as ^{14}C)

In all studies, rats were killed by ether overdose.

Blood and Plasma. Blood was drawn from the inferior vena cava and stored in heparinized tubes at 4°C prior to analysis. Whether sampled as whole blood only or as whole blood and plasma, aliquots for analysis were taken within 2 hours after samples were collected. Aliquot volumes remained constant for all studies (whole blood = 0.25 ml; plasma = 0.20 ml) unless the volume of sample limited the aliquot size. Aliquots were combusted to $^{14}CO_2$ as described by Miller et al., (1982) except that Betaphase (Westchem) was used as the scintillation cocktail. Total weight of blood was estimated as 9% of total body weight (Miller et al., 1982).

Tissues. Tissues to be analyzed for ^{14}C content were removed, rinsed with tap water, blotted dry, and frozen on dry ice.

Tissues were then stored at -10°C prior to analysis. Analyses were performed within 7 days of tissue collection. Total tissue weights were recorded, then duplicate aliquots (0.10 - 0.20 gm) were taken. Spinal cord aliquots were smaller due to lack of tissue. Each sciatic nerve (right and left) constituted one aliquot, the sum of which was used as total tissue weight. Total kidney weight included both kidneys; one aliquot was taken from each kidney. Tissues were dried overnight, then combusted to $^{14}CO_2$ in the same manner as blood and plasma. ^{14}C content of the tissue aliquots was then determined by LSC.

Analysis of Excreta for ACMD (as ^{14}C)

Rats were kept in individual metabolism cages to allow separate collection of urine and feces.

Urine. Urine was collected in plastic tubes. After collection, these tubes were removed and the cage bottoms were rinsed with soapy water to remove residual ^{14}C. This cage wash was collected, its total volume measured, and samples taken. Duplicate aliquots of urine and cage wash (urine = 0.1 - 0.2 ml; cage wash = 0.5 - 1.0 ml) were added to 7 ml of Betaphase (Westchem) and analyzed for ^{14}C content by direct LSC. Total urinary ^{14}C content (expressed as percent of dose) was calculated as the sum of urinary and cage wash ^{14}C contents.

Feces. Feces were collected on a wire mesh screen covering the bottom of the metabolism cage. After digestion in 0.5N NaOH, feces were weighed and duplicate aliquots (0.5 gm) were dried over-

night prior to combustion to $^{14}CO_2$. Combustion was as for blood and tissues. ^{14}C content was determined by LSC.

Analysis of Blood and Urine for Parent ACMD

Samples of whole blood and urine were prepared for analysis by the following methods, then analyzed by HPLC.

Whole Blood. Samples of whole blood (1 ml in most cases) were prepared for analysis within 2 hours of collection. Samples were extracted exhaustively with 3 volumes of 100% methanol containing 0.1% tris-(hydroxymethyl)-aminomethane (THAM) until resulting extracts had only background levels of radioactivity. The extracts were combined, concentrated, and centrifuged to remove particulates. Five to 10 µl aliquots were used for analysis. Percent of dose as parent was calculated according to equations presented in Appendix B.

Urine. Aliquots of urine were mixed with 2 volumes of 100% methanol, then centrifuged prior to HPLC analysis. Aliquots of the diluted urine were also analyzed by direct LSC for ^{14}C content. Percent of dose as parent was calculated according to equations presented in Appendix B.

Analysis for ^{14}C by Liquid Scintillation Counting

An LS-100C scintillation counter (Beckman Instruments, Fullerton, CA) was used for all scintillation counting. Samples were mixed with scintillation fluid (Betaphase) as described previously. Quench correction for the different types of samples was accomplished

using the external standard ratio for each sample and a series of quench curves. These curves were generated for samples corresponding to all the different handling conditions (combustion, HPLC, TLC, direct counting). Decay of ^{14}C was monitored by determining the amount of radioactivity present in all stock and dosing solutions prior to use.

Microsomal Preparation and Analysis

Preparation of Microsomes. Rats were anesthetized with ether, and kept unconscious during the procedure with ether-soaked gauze held over the nose.

The peritoneal cavity was opened and the liver perfused via the portal vein with ice-cold buffer until blanching of the tissue was extensive (approximately 25 ml of buffer). The aorta was then cut and the liver allowed to drain. The liver was removed, weighed, and minced. An aliquot (5 gm) of mince was homogenized in buffer, then centrifuged (Beckman J-21C centrifuge with a JA-20 rotor, Beckman Instruments, Fullerton, CA) successively at 1,100xg for 10 minutes, 12,000xg for 10 minutes, and 27,000xg for 10 minutes. Supernatants were poured through glass wool to remove lipid. Ultracentrifugation of supernatants employed an L8-55 ultracentrifuge (Beckman Instruments, Fullerton, CA) equipped with a TI-50 rotor. Supernatants were spun at 100,000xg for 40 minutes at 4°C. The resulting clear supernatants were discarded. Pellets were loosened by rinsing with cold buffer and gentle vortexing, then transfered to a Dounce homogenizer and resuspended in buffer. The ultracentri-

fugation procedure was then repeated. Final pellets were resuspended in a known volume of buffer and stored a -70°C. Washed microsomes were used within 1 week of preparation.

Protein Determination. Protein concentration in the microsomal suspension was determined by the Lowry method (Lowry et al., 1951) using bovine serum albumen (Sigma Chemicals, St. Louis, MO) as a standard. Microsomal suspensions were diluted for analysis with distilled, deionized water rather than buffer because of interference with color development.

Concentration of Cytochrome P450. The concentration of cytochrome P450 in microsomal suspensions was determined by carbon monoxide and dithionite difference spectra as described by Omura and Sato (1964). An Acta III UV-visible spectrophotometer equipped with recorder (Beckman Instruments, Fullerton, CA) was used for analysis. Concentrations were expressed as nmoles cytochrome P450 per mg microsomal protein.

Benzphetamine-N-demethylase Activity. The method of Mazel (1972) for aminopyrine-N-demethylase was followed except that benzphetamine (USP; Upjohn, Kalamazoo, MI) was substituted for aminopyrine at a final concentration of 1 mM. Incubation mixtures containing boiled microsomes were used as blanks. All incubations were run in duplicate. Enzyme activity was measured by formaldehyde formation over a 5 minute incubation period, and was expressed as nmoles formaldehyde formed per mg microsomal protein per minute.

Age Comparison Studies

Animals

Male Holtzman Sprague-Dawley (S/D) rats, aged 4 and 10 weeks, were purchased from Holtzman Co. (Madison, WI). Animals were allowed to acclimate for one week, thereby reaching 5 and 11 weeks of age prior to the start of each study. Rats were housed by treatment group (3 - 5 rats per cage) or in individual metabolism cages. Food and water were provided ad libitum throughout all studies.

^{14}C-ACMD in the Final Dose

Purpose. According to Kaplan and Murphy (1972), 11 week old male Holtzman rats are measurably neurotoxic after 5 days of ACMD treatment (50 mg/kg, given ip), while 5 week old rats are not comparably neurotoxic until 7 days of treatment. It was of interest to determine if tissue levels of ACMD (as ^{14}C) are higher in 11 week old rats than 5 week old rats after 5 days of treatment, and if these levels are comparable after 7 days of treatment.

Treatment Schedule. Rats in each age group were divided in 2 treatment groups (n = 5). One treatment group of each age was treated for 5 days (50 mg/kg/day ACMD, given ip). The other groups were treated for 7 days (50 mg/kg/day ACMD, given ip). In both cases, the final dose of ACMD given contained (2,3-^{14}C) ACMD (50 µCi/kg; specific activity of dosing solution = 7.61×10^{-5} µCi/nmole) as a tracer to follow the distribution and deposition of ACMD (as

^{14}C). Rats were killed 20 hours after receiving the last treatment of ACMD.

Tissue Sampling. Tissues (brain, spinal cord, sciatic nerve, lung, liver, and kidney) were removed and analyzed for ^{14}C. Distribution and deposition of ACMD (as ^{14}C; expressed as percent of dose and as concentration in nmoles per gm wet tissue, respectively) were calculated according to equations presented in Appendix B.

Statistics. Two-way analysis of variance (ANOVA) was used to determine if age or treatment period length were significant sources of between-group variation. Mean tissue levels (as percent of dose and concentration) were then examined for age-related differences by Newman-Keuls' Multiple Range test. A p value of 0.05 was chosen as the level of significance.

^{14}C-ACMD in All Doses

Purpose. Distribution and deposition of successive doses of ACMD (as ^{14}C) were studied in 11 and 5 week old male Holtzman rats to determine if age-related differences in susceptibility to ACMD toxicity were related to differences in handling individual doses of ACMD.

Treatment Schedule. Each age group was divided into 3 treatment groups (n = 3 each). One treatment group of each age was treated for 1, 3, or 5 days with ACMD (50 mg/kg/day, given ip) containing (2,3-^{14}C) ACMD (23 μCi/kg; specific activity of dosing solution = 3.28×10^{-5} μCi/nmole) as a tracer to follow distribution

and deposition of ACMD (as ^{14}C). Rats were killed 20 hours after the final treatment.

Tissue Sampling. Blood samples were drawn and tissues (brain, spinal cord, sciatic nerve, liver, and kidney) were removed and analyzed for ^{14}C. Distribution and deposition of ACMD (as ^{14}C; expressed as percent of dose and concentration in nmoles per gm wet tissue, respectively) were calculated according to equations presented in Appendix B. Tissue levels were corrected for ^{14}C contained in residual blood within the tissues by the method of Somjen et al., (1973).

Statistics. Two-way ANOVA was used to determine if age and/or treatment period length were significant sources of between-group variation. Mean tissue levels (as percent of dose and concentration) were then examined for age-related differences by Newman-Keuls' Multiple Range test. A p value of 0.05 was chosen as the level of significance.

Clearance of a Single ip Dose

Purpose. Excretion, distribution, and depostion of ACMD (as ^{14}C) after a single ip dose were studied in 11 and 5 week old male Holtzman rats to determine if age-related differences in susceptibility to ACMD toxicity were related to the ability of the rats to clear ACMD from the body.

Treatment Schedule. Five and 11 week old rats were given single ip doses of ACMD (50 mg/kg) containing (2,3-^{14}C) ACMD (22

μCi/kg; specific activity of dosing solution = 3.13×10^{-5} μCi/nmole). Three rats of each age were killed 4, 8, 12, and 24 hours after treatment.

Tissue Sampling. Blood (as whole blood and plasma) was collected, sampled, and analyzed for ^{14}C content. Percent of dose and concentration of ACMD (as ^{14}C) were calculated according to equations presented in Appendix B.

Urine and Feces Collection and Analysis. Urine and feces were collected separately for all time points and analyzed for ^{14}C content. Excretion of ^{14}C in urine and feces was expressed as percent of dose.

Statistics. Two-way ANOVA was used to determine if age and/or time after treatment were significant sources of variation in urinary, blood, or plasma levels of ACMD (as ^{14}C). Mean levels of ACMD (as ^{14}C) were then compared by Newman-Keuls' Multiple Range test. A p value of 0.05 was chosen as the level of significance.

ACMD-Phenobarbital Interaction Studies

Animals

Fisher-344 (F-344) rats were chosen for study because previous pharmacokinetic work had used this strain (Miller et al., 1982).

Male F-344 rats were purchased from M. A.Bioproducts (Walkersville, MD) or obtained from a breeding colony established in our Division of Animal Resources (Arizona Health Sciences Center,

University of Arizona, Tucson, AZ). Body weights ranged from 160 to 220 gm. Rats were housed by treatment group (3 - 5 rats per cage) or in individual metabolism cages. Food and water were provided ad libitum throughout all studies.

Male Sprague-Dawley (S/D) rats used in the induction study were obtained from our Division of Animal Resources (Arizona Health Sciences Center, University of Arizona, Tucson, AZ). Body weights ranged from 160 to 210 gm. Rats were housed by treatment groups (5 rats per cage). Food and water were provided ad libitum throughout the study.

Neurotoxicity Testing Methods Study

Purpose. Several easily administered, sensitive neurotoxicity tests were needed to distinguish ACMD-intoxicated rats from control rats, in order to determine tissue levels of ACMD present in a measurably neurotoxic animal. To minimize the amount of radiolabeled ACMD needed, procedures that had been shown to detect ACMD-induced neurotoxicity after a cumulative dose less than 500 mg/kg were chosen for study.

Treatment Schedule. Rats were divided into 2 groups (n = 5). One group (ACMD-treated) received daily ip injections of ACMD (50 mg/kg, given ip); the other group (Control) received injections of distilled water (1 ml/kg, given ip). Dosing was started after all rats in both groups could successfully complete 2 trials on the rotorod. Rats were dosed after completing the daily battery of neurotoxicity tests. Rats were dosed and tested until 3 out of 4

tests showed significant differences betweeen results from ACMD-treated and Control groups.

Body Weight. Weight loss is a characteristic sign of ACMD intoxication (McCollister et al., 1964). Daily body weights were recorded for all rats. Group means were compared daily by Student's one-tailed t-test.

Rotorod. The rotorod is a standard means of testing for neurotoxicity. The apparatus used consisted of a wooden rod, 3 inches in diameter, suspended approximately 1 foot above a wooden platform. The rod surface was covered with a textured plastic to provide better footing. The rod was divided into 4 sections by circular partitions which visually screened individual rats from one another if they were being tested simulataneously. These partitions also kept rats from moving laterally on the rod. The rpms of the apparatus were controlled by a variable speed motor and were set at 11-12 rpms.

Rats were trained by allowing them to walk onto the rotating rod then requiring them to balance on the rod for 2 120-second trials. Trials were separated by 20 minute rest periods. Attempts by rats to turn around or jump off the rod were discouraged by a tap on the nose with a blunt pencil. Rats were considered trained if they completed both training sessions successfully and required little or no prodding with the pencil.

After dosing was started, the amount of time a rat could stay on the rod (trial duration) was recorded for 2 daily trials. The

ACMD-treated rats were considered neurotoxic when their group mean trial duration was significantly different from that of the control group. Differences between means were tested statistically by Student's one-tailed t-test.

Ocular Zingerone. Application of a 1% solution of zingerone, a chemical irritant derived from ginger root (Pfaltz and Bauer, Inc., Stamford, CT), to the eye of a rat causes a rapid and vigorous response. This response consists of blinking, wiping of the eye and face with the forepaws, and scratching of the affected eye with the hind foot. The duration of the response is consistent in untreated rats, but treatment with ACMD has been shown to reproducibly lengthen response time (Miller et al., 1983).

Rats were restrained as zingerone was applied to the eye as a single drop from a Pasteur pipette. They were then released, and response time was recorded. Cessation of response was indicated when the rat stopped scratching and looked up. Two daily trials were performed on each rat, one trial per eye. The mean response time for each rat was determined; a group mean was then calculated. Daily group means from Control and ACMD-treated rats were compared by Student's one-tailed t-test.

Landing Foot Splay. The method of Edwards and Parker (1977) was used to measure the landing distance between the hind feet of rats dropped onto a horizontal surface. This distance (splay) was found to increase with continued exposure to ACMD.

For ease of recording, the hind feet of the rats were inked, and the landing surface was covered with white paper. Three daily trials were performed on each rat, with rest periods between trials. The mean splay for each rat was determined and used to calculate a daily group mean. Daily group means from Control and ACMD-treated rats were compared by Student's one-tailed t-test.

Choice of Tests. Tests were chosen for use in subsequent studies based on their ability to distinguish ACMD-intoxicated rats from Control rats after the lowest possible cumulative dose.

Neurotoxicity and Deposition of ^{14}C-ACMD

Purpose. Tissue levels of ACMD (as ^{14}C) have never been related to a stage of intoxication. Simple neurotoxicity tests were administered to ACMD-treated and untreated rats to determine when ACMD-treated rats were quantitatively neurotoxic. Tissue distribution and deposition of ACMD (as ^{14}C) in these rats were then calculated.

Treatment Schedule. Male F-344 rats were divided into 2 groups. One group (ACMD only; n = 5) received daily injections of ACMD (50 mg/kg, given ip) containing (2,3-^{14}C) ACMD (20 μCi/kg; specific activity of dosing solution = 2.84×10^{-5} μCi/nmole). The second group (Vehicle control; n = 5), received injections of distilled water (1 ml/kg, given ip).

Dosing was started after all rats in both groups could successfully complete 2 trials on the rotorod. Rats were dosed after completing the daily battery of neurotoxicity tests. Rats were dosed

and tested until all tests detected significant differences between ACMD only and vehicle control groups. Rats were killed 20 hours after the final dose.

A second group of Vehicle control rats (n = 5) was killed on the first day of ACMD dosing for determination of hepatic cytochrome P450 concentration.

Body Weight. Daily body weights were recorded. Group means were calculated and compared as described previously.

Rotorod. Two daily trials were performed. Successful trial duration was reduced to 90 seconds for this study. Group performances were compared as described previously.

Ocular Zingerone. Two daily tests were administered. Group mean response times were compared as described previously.

Tissue Sampling. Tissues (blood, plasma, brain, spinal cord, sciatic nerve, liver, and kidney) were collected, sampled, and analyzed for ^{14}C. Tissue levels were corrected for ^{14}C contained in residual blood in tissues by the method of Somjen et al. (1973).

Microsomes. Livers from Vehicle control rats were used to prepare microsomes on the first and last days of ACMD treatment. Cytochrome P450 concentrations were determined.

Induction of F-344 Rats with Phenobarbital

Purpose. A phenobarbital (PB) treatment regimen that could elevate hepatic P450 concentration and maintain that elevation up to one week (the approximate length of the ACMD treatment period) without incapacitating F-344 rats was required. A regimen used

successfully for S/D rats was tried.

Treatment Schedule. F-344 rats and S/D rats were divided into 2 groups (n = 5), one to be treated with PB (PB-treated) and one to remain untreated (PB control). PB-treated rats received a single injection of PB (100 mg/kg, given ip) followed by 0.1% PB in drinking water for 4 days. Control rats received a single injection of distilled water (1 ml/kg, given ip). A 24-hour washout period was allowed prior to preparation of microsomes to clear residual PB from the body. Washed microsomes from PB-treated and PB control rats were prepared. Cytochrome P450 concentrations were determined.

Neurotoxicity and Deposition of ^{14}C-ACMD after PB Treatment

Purpose. PB treatment has been shown to delay the onset of ACMD-induced neurotoxic signs (Kaplan et al., 1973). Therefore, tissue levels of ACMD (as ^{14}C) from PB-treated and non-treated rats given the same cumulative dose of ACMD were compared for PB-related differences. At the same time, the protective effect of PB treatment was re-examined to substantiate or refute previous claims.

PB Treatment Schedule. A PB treatment regimen shown to elevate and maintain hepatic cytochrome P450 levels in F-344 rats was used for all rats. In this case, however, 2 injections of PB (80 mg/kg, given ip) were given on successive days rather than a single injection of 100 mg/kg. Rats were maintained on 0.1% PB in drinking water throughout the rotorod training period and ACMD treatment period.

ACMD Treatment Schedule. Rats were divided into 2 groups (n = 5). One group (PB + ACMD) received daily injections of ACMD (50 mg/kg, given ip) containing (2,3-^{14}C) ACMD (20 μCi/kg, specific activity of dosing solution = 2.84×10^{-5} μCi/nmole). The other group (PB control) received daily injections of distilled water (1 ml/kg, given ip). Dosing was started when all rats completed training on the rotorod. Rats were dosed after completing the daily battery of neurtoxicity tests. Dosing and testing continued until a previously determined, neurotoxic cumulative dose of ACMD was reached. Rats were then killed 20 hours after the final dose.

A second group of PB control rats (n = 5) were killed on the first day of ACMD dosing for determination of hepatic cytochrome P450 concentrations.

Body Weight. Daily body weights were recorded. Daily group means were compared as described previously.

Rotorod. Two daily trials were performed. Successful trial duration was reduced to 60 seconds. Group performances were compared as described previously.

Ocular Zingerone. Two daily tests were administered. Mean group results were compared as described previously.

Tissue Sampling. Tissues (blood, plasma, brain, spinal cord, sciatic nerve, liver, and kidney) from PB + ACMD rats were collected, sampled, and analyzed for ^{14}C. Tissue levels were corrected for ^{14}C contained in residual blood in tissues by the method of Somjen et al., (1973). Mean tissue contents and concentrations from these rats

were compared to those from ACMD-only rats (Neurotoxicity and Deposition Study) by Student's two-tailed t-test. A p value of 0.05 was chosen as the level of significance.

Microsomes. Livers from PB control rats were used to prepare washed microsomes on the first and last days of ACMD treatment. Cytochrome P450 concentrations were determined.

Effect of ACMD Treatment on PB Induction

Purpose. The effect of ACMD on cytochrome P450 concentration and benzphetamine-N-demethylase activity were studied in PB-treated and non-treated F-344 rats.

Treatment Schedule. Male F-344 rats were divided into 4 treatment groups (n = 3). Vehicle controls received daily injections of distilled water (1 ml/kg, given ip). PB controls received 2 injections of PB (80 mg/kg, given ip) followed by 0.1% PB in drinking water until the end of the study. ACMD-only rats received daily injections of ACMD (50 mg/kg, given ip). PB + ACMD rats received 2 injections of PB (80 mg/kg, given ip) followed by 0.1% PB in drinking water and concurrent daily injections of ACMD (50 mg/kg, given ip). ACMD and/or PB treatment continued until a previously determined, neurotoxic cumulative dose was reached.

Body Weight. Daily body weights were recorded. Group means were calculated and compared as described previously.

Ocular Zingerone. Two daily tests were administered. Mean group results were compared as described previously.

Microsomes. Livers from all rats were used to prepare washed microsomes. Cytochrome P450 concentrations and the activity of an associated enzyme, benzphetamine-N-demethylase, were determined. Mean biochemical values were analyzed by two-way ANOVA and compared by the Bonferoni multiple comparison test.

PB Effects on Clearance of a Single iv Dose

Purpose. The protective effect of PB against ACMD neurotoxicity may be due to increased detoxification of ACMD. Detoxification can be estimated by the disappearance of parent ACMD from the blood, decline of ACMD (as ^{14}C) levels in blood and plasma, and appearance of parent ACMD and uncharacterized ^{14}C in urine and feces.

Treatment Schedule. Male F-344 rats were divided into 2 treatment groups (n = 15). One group (PB + ACMD) was pretreated with 2 injections of PB (80 mg/kg, given ip) followed by 0.1% PB in drinking water for 4 days. The other group (ACMD-only) received no pretreatment. Both groups then received ACMD (50 mg/kg, via tail vein) containing (2,3-^{14}C) ACMD (20 μCi/kg; specific activity of dosing solution = 2.84×10^{-5} μCi/nmole). Rats (n = 3 from each group) were killed 1, 2, 4, 6, and 8 hours after dosing.

Tissue Sampling. Blood samples were drawn and analyzed for ^{14}C as whole blood and plasma. Blood was also analyzed for parent ACMD. Mean ^{14}C content and amount of parent from PB + ACMD and ACMD-only rats were analyzed by two-way ANOVA and compared by Newman-Keuls' multiple range test. A p value of 0.05 was chosen as the level of significance.

Excreta. Urine and feces were collected for all time points and analyzed for ^{14}C. Urine was also analyzed for parent ACMD. Mean ^{14}C content and amount of parent from PB + ACMD and ACMD-only rats were analyzed by two-way ANOVA and compared by Newman-Keuls' multiple range test. A p value of 0.05 was chosen as the level of significance.

CHAPTER 3

RESULTS

Age Comparison Studies

^{14}C-ACMD in the Final Dose of a Multiple Dose Regimen

Five and 11 week old rats were treated with ACMD (50 mg/kg, given ip) either 5 or 7 days. The final dose was spiked with (2,3-^{14}C) ACMD (50 μCi/kg) as a tracer to follow distribution and deposition of ACMD (as ^{14}C) in the rats.

Daily body weights from control (n=2) and ACMD-treated groups (n=4 or 5) were recorded (Fig 1). Five week old treated rats showed an overall weight gain (+5 gm when treated for 5 days; +4 gm when treated 7 days). This gain was slightly less than that of control rats (+9 gm). In contrast, 11 week old rats in both treated groups experienced an overall weight loss (-4 gm for both groups). Eleven week old control rats gained about 4 gm over the same time period.

The general appearance of the various groups reflected the weight data. The 11 week old treated groups began to look thin, especially in the hindquarters, as treatment continued. In addition, their gait became stiff and their hindquarters were carried lower than controls or the 5 week old rats. One rat from the 11 week, 7 day treated group and one rat from the 5 week, 7 day treated group

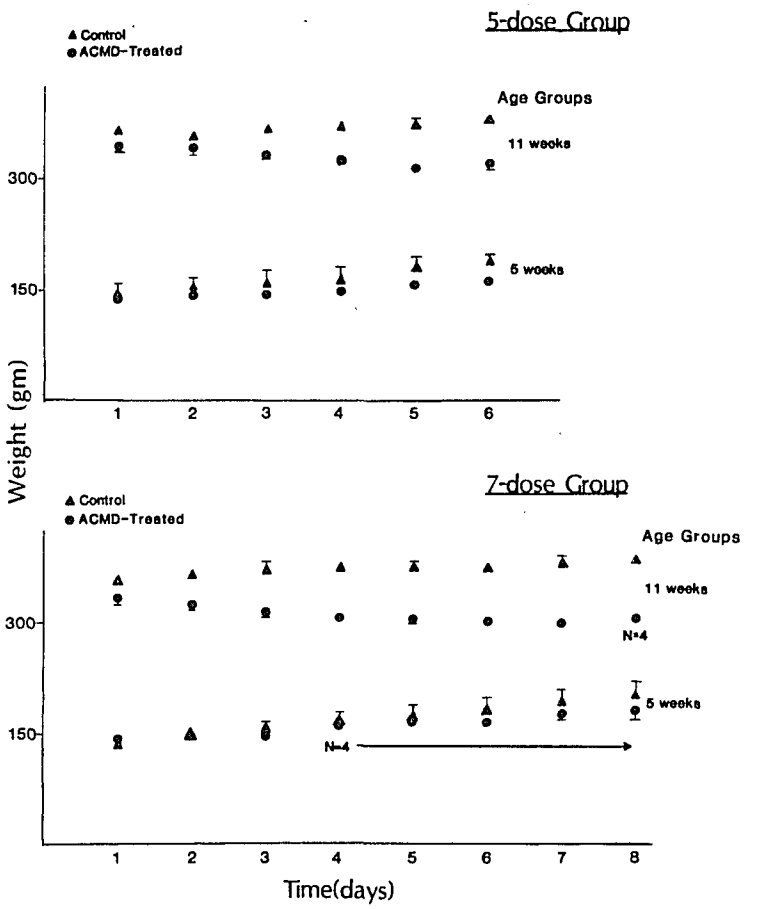

Figure 1. Body weights for 5 and 11 week old male Holtzman rats dosed ip 5 or 7 days with ACMD (50 mg/kg/day). The final dose contained (2,3-^{14}C) ACMD (50 μCi/kg). Data are expressed as mean ± SD (n=4 or 5). One rat in each of the 7 dose groups died before completion of dosing, reducing n to 4 in those groups.

died during the study. Both deaths were preceded by a substantial weight loss.

The distribution of ACMD (as ^{14}C) to tissues, expressed as percent of total dose, was similar in 5 and 11 week old rats after 5 days of treatment (Fig 2). Liver contained the largest portion of the dose and sciatic nerve the smallest. Lengthening the treatment period to 7 days did not affect the distribution pattern. Analysis of neural tissue content (as percent of dose) by two-way ANOVA showed that neither age nor treatment period length was a significant source of between group variation. Age-related comparisons of mean neural tissue content after the same treatment period length revealed no significant differences between 5 and 11 week old rats.

When the data were expressed as concentration of ACMD (as ^{14}C equivalents; nmoles/gm wet tissue), values from 11 week old rats were generally greater than 5 week old rats (Fig 3). Age was found to be a significant ($p<0.05$) source of between group variation. Comparison of means revealed significant differences ($p<0.05$) between brain ACMD concentrations from 5 and 11 week old rats after the same treatment period. Spinal cord ACMD concentration was significantly ($p<0.05$) less in 5 week old rats *vs.* 11 week old rats after 5 days but not after 7 days. No age-related differences in sciatic nerve concentrations were found after either treatment period.

Data from all ACMD-treated 5 week old and 11 week old rats were combined to calculate tissue:body weight ratios (Table 1). The proportion of total body weight made up by brain was significantly

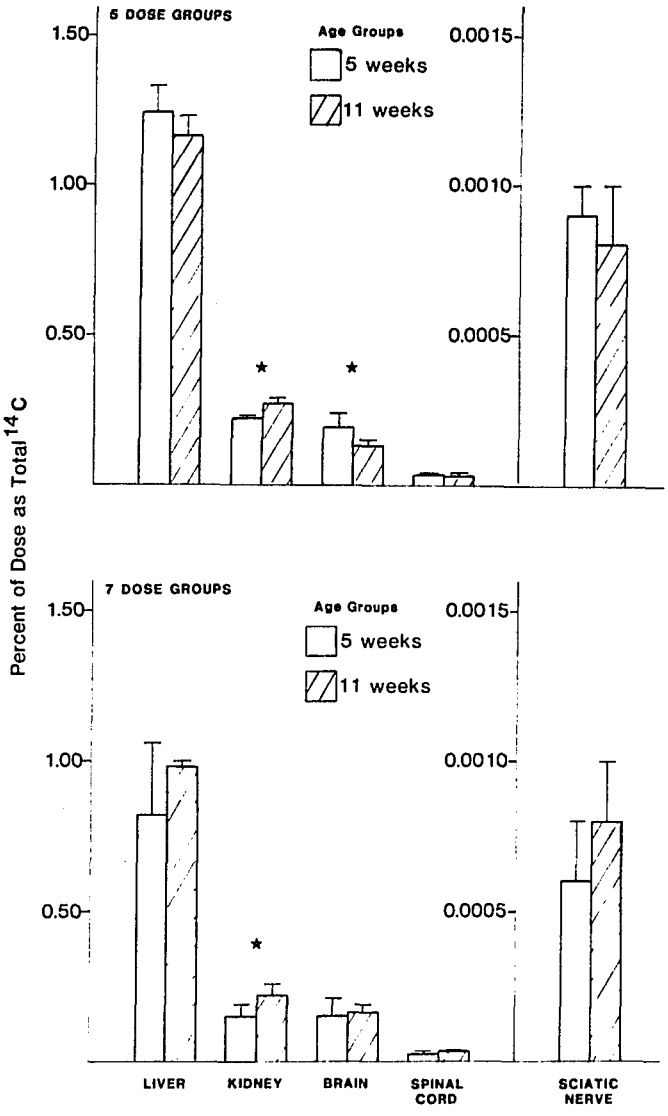

Figure 2. Distribution of ACMD (as ^{14}C) to tissues of 5 and 11 week old male Holtzman rats dosed ip 5 or 7 days with ACMD (50 mg/kg/day). The final dose contained (2,3-^{14}C) ACMD (50 μCi/kg). Rats were killed 20 hours after the final dose. Data are expressed as mean ± SD (n=4 or 5). (*) indicates a significant difference ($p<0.05$) between age groups.

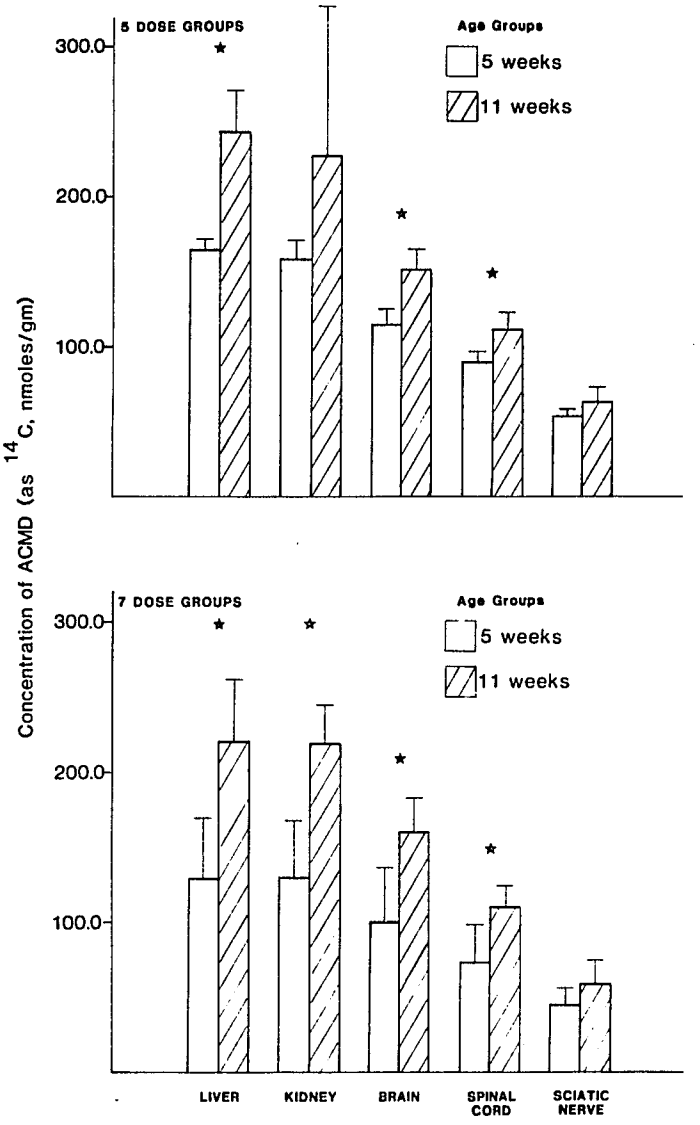

Figure 3. Concentration of ACMD (as ^{14}C) in tissues from 5 and 11 week old male Holtzman rats dosed ip 5 or 7 days with ACMD (50 mg/kg/day). The final dose contained (2,3-^{14}C) ACMD (50 μCi/kg). Rats were killed 20 hours after the final dose. Data are expressed as mean ± SD (n=4 or 5). (*) indicates a significant difference ($p<0.05$) between age groups.

Table 1

Tissue: Body Weight Ratios[a] From Age Comparison Study, ^{14}C-ACMD in Final Dose

	5 Week Old Rats	11 Week Old Rats
Brian	10.7 ± 0.7^{b}	6.4 ± 0.2^{b}
Spinal Cord	2.5 ± 0.2^{c}	2.0 ± 0.2^{c}
Sciatic Nerve	0.11 ± 0.02	0.10 ± 0.01
Lung	7.0 ± 0.7^{d}	5.2 ± 0.4^{d}
Liver	50 ± 5.0^{e}	33 ± 3.0^{e}
Kidney	8.8 ± 0.8^{f}	7.1 ± 0.6^{f}

[a]Calculated as gm tissue/kg body weight. Expressed as mean $\pm$ SD Values from all 5 week old male Holtzman rats and 11 week old male Holtzman rats in the study were combined; combined n=9 for each age group.

b,c,d,e,f - paired superscripts indicate means are significantly different ($p<0.05$).

($p<0.05$) smaller in 11 week old rats than 5 week old rats. Visceral tissues (lung, liver, and kidney) were also a smaller portion of the total body weight in 11 week old rats than 5 week old rats.

^{14}C-ACMD in All Doses of a Multiple Dose Regimen

Five and 11 week old rats were treated with ACMD (50mg/kg/day, given ip) for 1, 3, or 5 days. All doses were spiked with (2,3-^{14}C) ACMD (23 μCi/kg) as a tracer to measure the distribution and deposition of ACMD (as ^{14}C) in the rats.

Body weights were recorded daily and averaged for all animals involved in the study (Fig 4). On days 1 and 2, n=9 for the ACMD-treated groups and n=3 for control groups. These numbers were reduced by 3 and 1, respectively, on days 3 and 5, as successive groups were killed. As in the previous study, it appeared that the 11 week old rats showed a small weight loss compared to controls over the treatment period while the 5 week old rats gained slightly. These results could not be compared statistically due to n=1 in the control group after day 4.

Using a multiple dosing scheme, changes in tissue distribution and deposition with successive doses of ACMD could be followed. Variations in neural tissue concentration and percent of total dose contained in neural tissues were examined for age- and dose-related differences by two-way ANOVA.

Age and number of doses received were both significant sources ($p<0.001$) of between group variation in brain and spinal cord

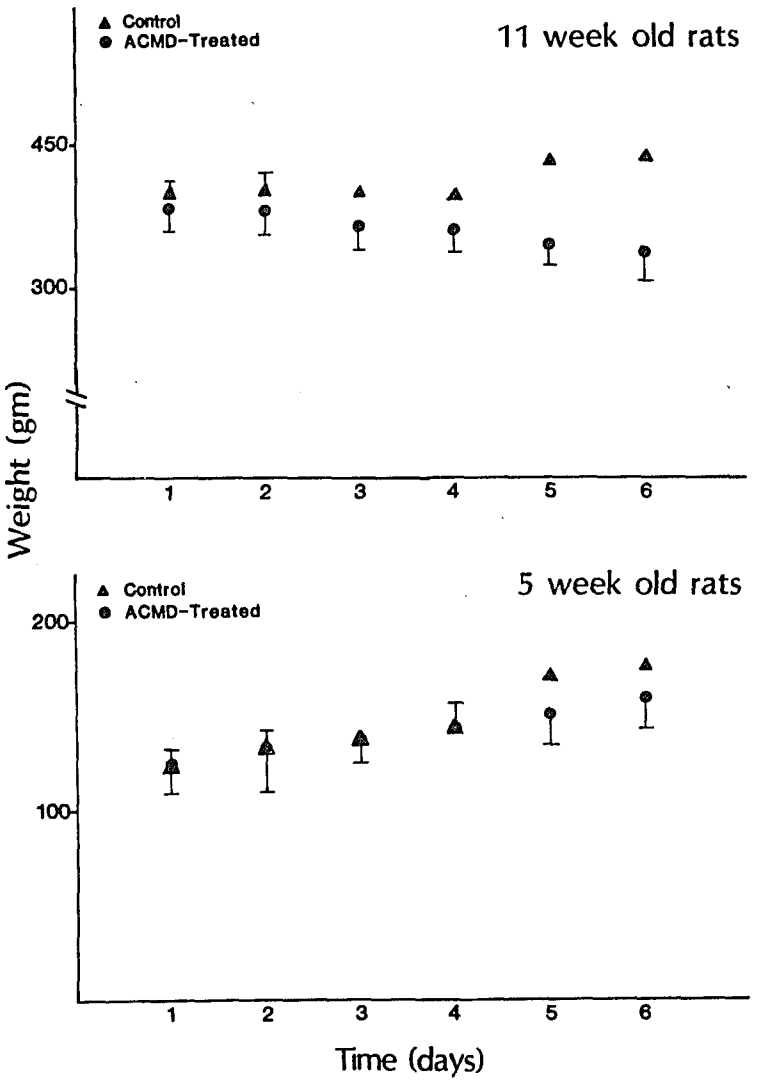

Figure 4. Pooled body weights of 5 and 11 week old male Holtzman rats given 1, 3, or 5 ip doses of ACMD (50 mg/kg) containing (2,3-^{14}C) ACMD (23 µCi/kg). On days 1 and 2, n=3 for controls and n=9 for ACMD treated rats. On days 3 and 4, n=2 for controls and n=6 for ACMD treated rats. On days 5 and 6, n=1 for controls and n=3 for ACMD treated rats.

concentration (Fig 5). Thus, concentration of ACMD (as ^{14}C equivalents) in these tissues was higher in 11 week old rats than 5 week old rats and increased with increasing number of doses. Indeed, when mean concentrations from 5 and 11 week old rats receiving the same number of doses were compared, all were significantly different ($p<0.05$) except values for spinal cord after a single dose.

Age was not a significant source of variation in sciatic nerve concentration. Number of doses received, however, proved to be a highly significant source ($p<0.001$). Comparison of means showed no significant differences between 5 and 11 week old rats receiving the same number of doses.

Age and number of doses received were significant sources of between-group variation in neural tissue content (expressed as percent of total dose; Fig 6). In this case, tissue content decreased with increasing number of doses. Also, 11 week old rats had lower percent of dose in neural tissues than 5 week old rats after the same number of doses. More specifically, 11 week old rats had significantly lower percent of dose in brain for all treatment period lengths, lower percent of dose in spinal cord after a single dose, and lower percent of dose in sciatic nerve after 1 and 3 doses.

Tissue: body weight ratios were calculated based on data from all treated or control rats in the same age group (Table 2). All values for tissues from 11 week old rats were significantly ($p<0.05$) lower than those from 5 week old rats except sciatic nerve, supporting the findings of our previous study. That is, tissue

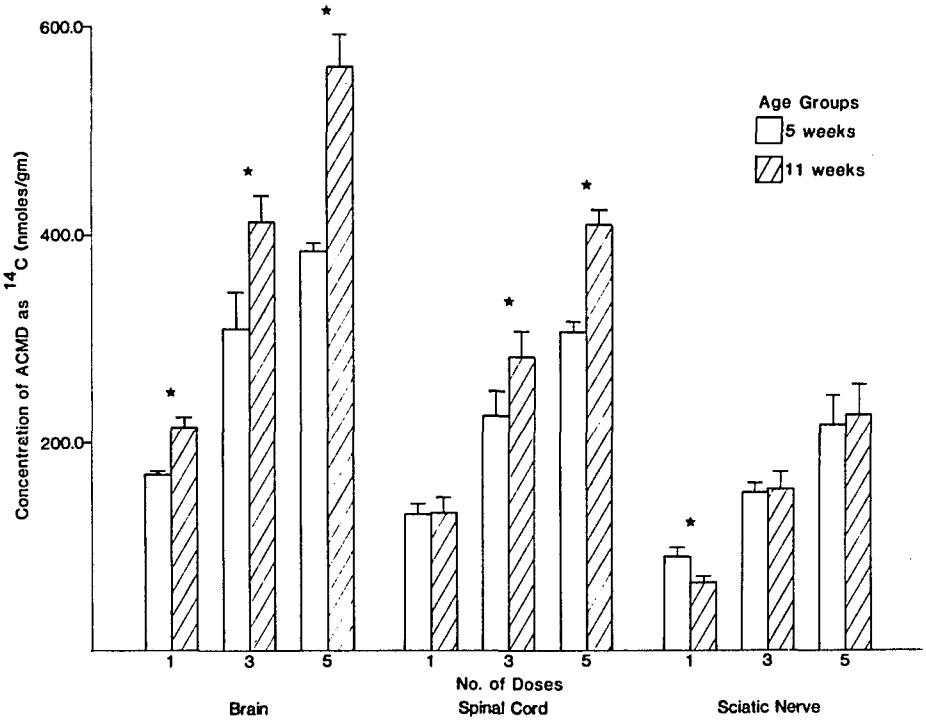

Figure 5. Concentration of ACMD (as ^{14}C) in tissues from 5 and 11 week old male Holtzman rats given 1,3, or 5 ip doses of ACMD 50 mg/kg) containing (2,3-^{14}C) ACMD (23 μCi/kg). Rats were killed 20 hours after final dose. Data are expressed as mean ± SD (n=3). (*) indicates a significant difference ($p<0.05$) between age groups.

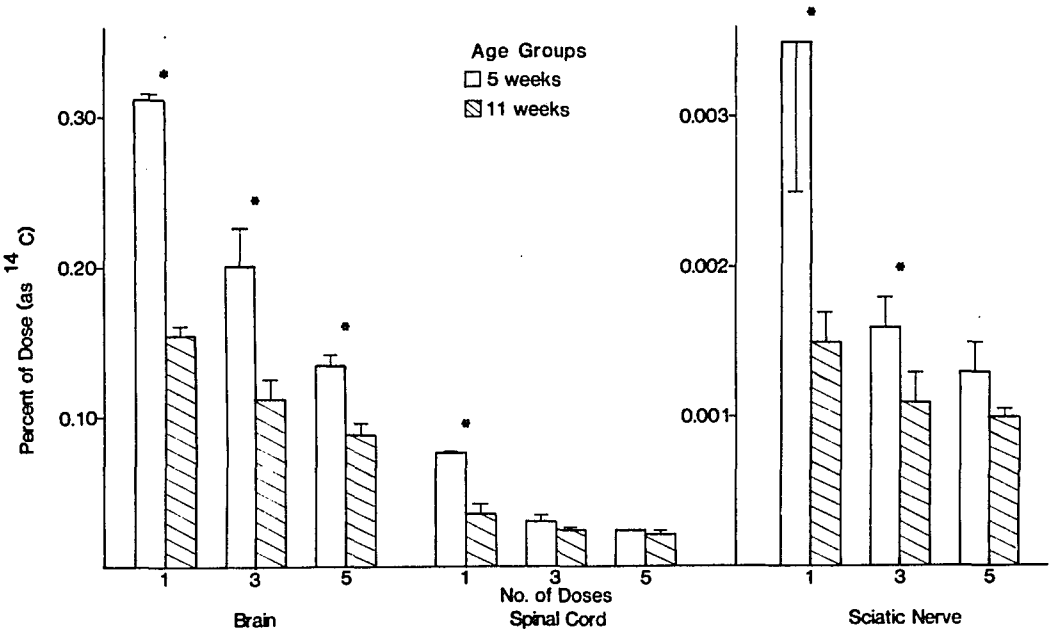

Figure 6. Distribution of ACMD (as ^{14}C) to tissues of 5 and 11 week old male Holtzman rats given 1, 3, or 5 ip doses of ACMD (50 mg/kg) containing (2,3-^{14}C) ACMD (23 μCi/kg). Rats were killed 20 hours after final dose. Data are expressed as mean $\pm$ SD (n=3). (*) indicates a significant difference ($p<0.05$) between age groups.

Table 2

Tissue: Body Weight Ratios[a] From Age Comparison Study, ^{14}C-ACMD in All Doses

	5 Week Old Rats	11 Week Old Rats
Brain	12.0 ± 1.0^{b}	5.7 ± 0.6^{b}
Spinal Cord	3.0 ± 0.8^{c}	1.8 ± 0.4^{c}
Sciatic Nerve	0.22 ± 0.06	0.16 ± 0.03
Lung	7.4 ± 1.0^{d}	4.7 ± 0.6^{d}
Liver	54 ± 3.4^{e}	36 ± 7^{e}
Kidney	9.8 ± 0.8^{f}	7.3 ± 0.5^{f}

[a]Calculated as gm tissue/kg body weight. Expressed as mean $\pm$ S.D. Values from all 5 week old male Holtzman rats and 11 week old male Holtzman rats in the study were combined combined n=9 for each age group.

b,c,d,e,f - paired superscripts indicate means are significantly different ($p<0.05$).

weights from the 11 week old rats did not increase in proportion to body weight increases.

Clearance of a Single ip Dose of ^{14}C-ACMD

Five and 11 week old rats were given a single ip dose of ACMD (50 mg/kg) containing (2,3-^{14}C) ACMD (22 μCi/kg). The appearance of ACMD (as ^{14}C) in urine and feces and its decline in blood and plasma were followed over a 24 hour period to determine if any age-related differences exist.

Age and time were significant ($p<0.05$) sources of variation in urinary excretion after a single ip dose of ^{14}C-ACMD (Table 3). Mean percent of dose in urine from 5 and 11 week old rats were compared by time point, but no significant age-related differences were found.

The decline of ACMD concentration (as ^{14}C equivalents) over time in blood and plasma was followed (Fig 7). Analysis of these data by two-way ANOVA showed age and time to be significant ($p<0.001$) sources of variation in both blood and plasma concentration. Mean concentrations were compared by time point for age-related differences. Blood concentrations in 11 week old rats were significantly greater than those in 5 week old rats at all time points. In contrast, no differences in plasma concentration means were detected except at the 24 hour time point.

Table 3

Excretion of ACMD (as ^{14}C) After a Single, ip Dose

(50 mg/kg; 22 Ci/kg)

A. 5 Week Old Male Holtzman Rats

Time Point	Percent of Dose[a] In Urine	Percent of Dose[a] In Feces	Percent of Dose[a] In Urine & Feces
4 hr	17.4 ± 2.5	0.38 ± 0.05	17.8 ± 2.5
8 hr	36.2 ± 3.1	0.68 ± 0.09	36.9 ± 3.0
12 hr	50.3 ± 0.5	1.30 ± 0.28	51.6 ± 0.8
24 hr	58.9 ± 3.4	1.97 ± 0.61	60.8 ± 3.1

B. 11 Week Old Male Holtzman Rats

Time Point	Percent of Dose[a] In Urine	Percent of Dose[a] In Feces	Percent of Dose[a] In Urine & Feces
4 hr	15.5 ± 1.8	0.22 ± 0.06	15.7 ± 1.8
8 hr	45.9 ± 2.2	0.52 ± 0.29	46.4 ± 2.3
12 hr	51.3 ± 5.1	1.17 ± 0.35	52.4 ± 5.2
24 hr	69.7 ± 1.8	1.24 ± 0.32	71.0 ± 1.7

[a]Mean ± SD, n=3.

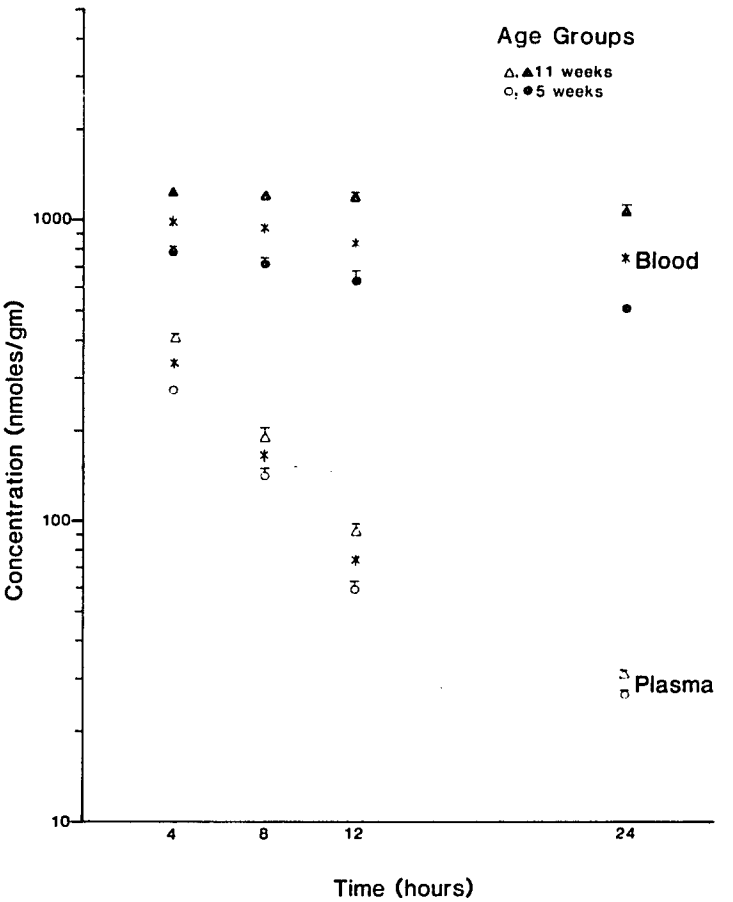

Figure 7. Concentration of ACMD (as ^{14}C) in blood and plasma from 5 and 11 week old male Holtzman rats after a single ip dose of ACMD (50 mg/kg) containing (2,3-^{14}C) ACMD (22 μCi/kg). Data are expressed as mean ± SD (n=3). (*) indicates significant differences ($p<0.05$) between age groups.

ACMD - Phenobarbital Interaction Studies

Neurotoxicity Testing in Methods Study

The ability to balance on a rotating rod, response to ocularly-applied zingerone, and landing foot splay were tested for their ability to reveal differences between ACMD-treated and control rats. Body weight changes were also monitored as a standard indicator of ACMD intoxication.

The body weights of ACMD-treated rats decreased steadily as treatment continued (Fig 8). A significant difference between group mean body weights was found after a cumulative dose of 100 mg/kg, and was maintained for the duration of the study. The ACMD-treated rats appeared to lose weight in their hindquarters - their haunches became extremely gaunt by the end of the study. This group also acquired a 'waddling' gait, as though the hind feet were being planted to the side of the body as a step was taken, rather than reaching forward. Overall, the treated rats maintained a well-groomed appearance and seemed as alert as control animals.

The rotorod test required the training of rats to maintain their balance on a rotating rod for a given length of time. This test proved difficult to administer and evaluate. Individual differences in the response of the rats to ACMD treatment caused wide variability in trial durations (Fig 9). However, a steadily decreasing ability to maintain balance was noted in the treatment group and a significant difference in mean trial duration (treated *vs.* control) was seen after a cumulative dose of 150 mg/kg ACMD.

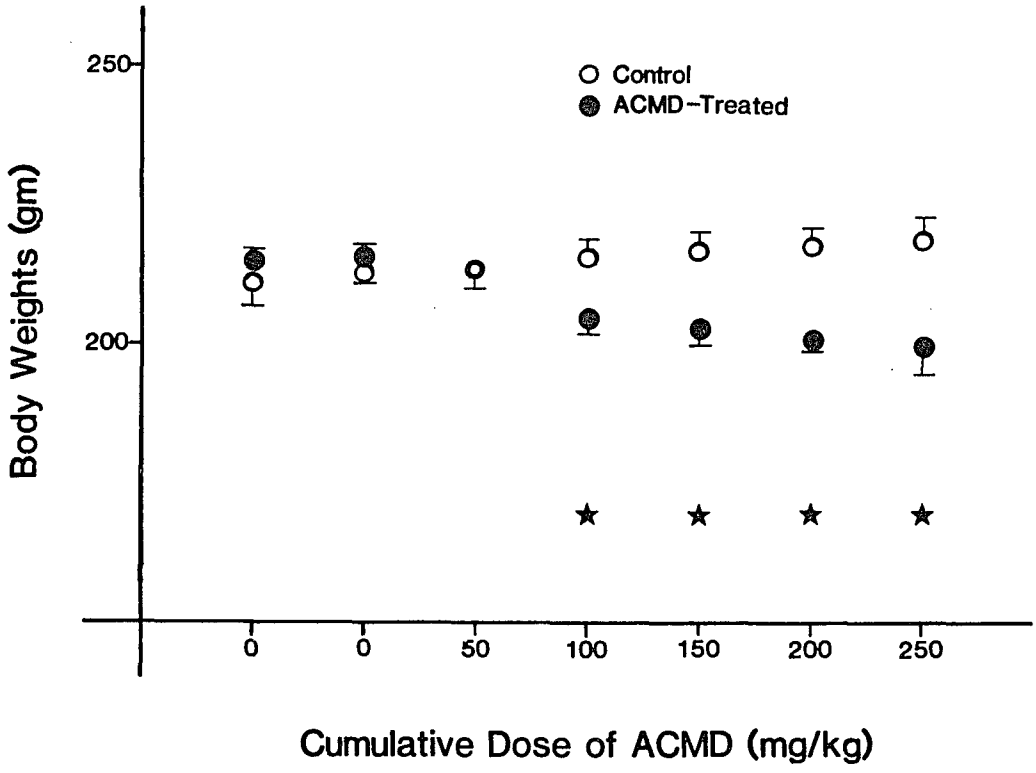

Figure 8. Daily body weights of male F-344 rats treated ip with either 50mg/kg/day ACMD or distilled water. Data are expressed as mean $\pm$ SD (n=5). (*) indicates significant difference ($p<0.05$) between control and treated rats.

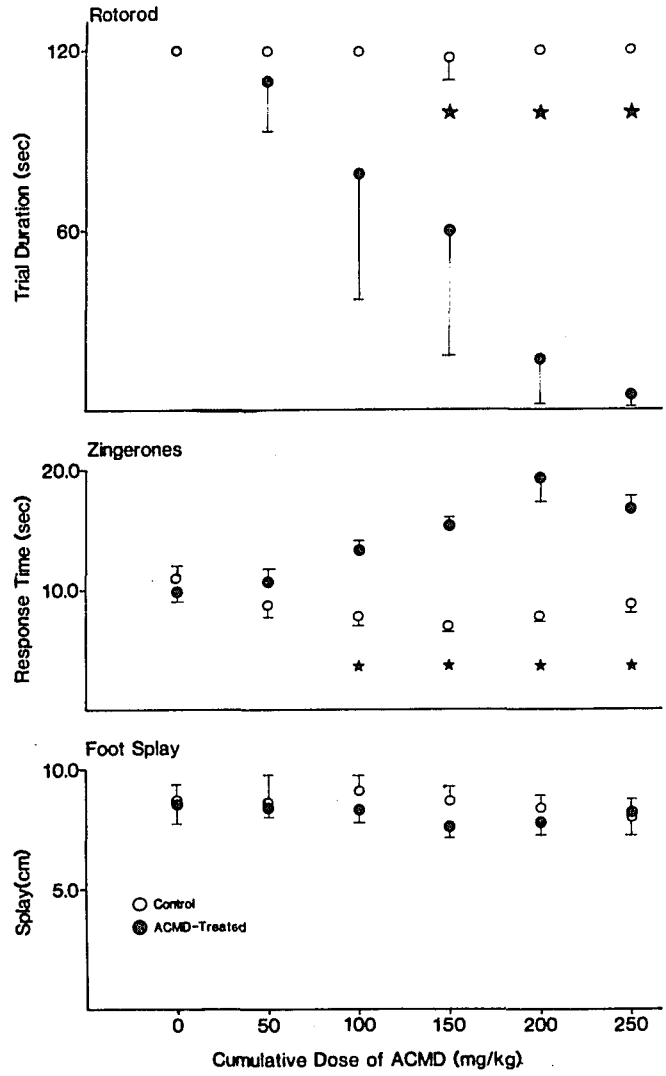

Figure 9. Results from three neurotoxicity tests administered to male F-344 rats receiving ACMD (50 mg/kg/day, given ip) or distilled water (1 ml/kg/day, given ip): rotorod balancing, response to ocular zingerone, and landing foot splay. (*) indicates significant difference ($p<0.05$) between ACMD-treated and control rats.

Rotorod - daily trial duration from ACMD-treated and control rats (mean $\pm$ SD; n=5; two trials per rat per day).

Zingerone - response time to application of zingerone to the eye of ACMD-treated and control rats (mean $\pm$ SD; n=5; two trials per rat per day).

Foot splay - distance between hind feet of ACMD-treated and control rats after dropping from a height of 32 cm (mean $\pm$SD; n=5; three trials per rat per day).

Failure during a trial was characterized by a gradual shift in the rat's center of gravity from directly over the peak of revolution toward the back of the rod. This forced the rat to push with its hind legs to lift its body back into position on top of the rod. Eventually, the rat's hindquarters could not provide the thrust needed to maintain its position on the rod and the rat fell off backwards. By a cumulative dose of 250 mg/kg ACMD, the mean trial duration of the treated rats had decreased to 5 sec from the original trail length of 120 sec.

Response time to ocularly-applied zingerone proved to be an easily administered test, with much lower variability than the rotorod test. However, it was discovered that the rats had to be tested in their home cages, as placement in a new cage for testing stimulated exploratory behavior which caused more variable results. That is, in the home cage, the rat remained stationary until response to the ocular irritant was complete. In a new cage, scratching was interrupted by efforts to explore the cage, then begun again, making detection of response cessation difficult. Response time increased with cumulative dose of ACMD and a significant difference between treated and control rats was detected after 100 mg/kg ACMD (Fig 9). Although maximum response time appeared to be reached after 200 mg/kg, testing after 300 mg/kg (not shown on graph) produced a mean response time of 25 $\pm$5 sec for treated rats compared to 12 $\pm$ 1 sec for controls.

The landing foot splay test, while easy to administer and evaluate, was never able to distinguish ACMD-treated rats from control rats (Fig 9). The rats tended to tuck their hind feet under their bodies before landing, resulting in narrow splay measurements. This behavior did not change even when other tests indicated intoxication.

Neurotoxicity and Neural Tissue ACMD Deposition

Rats were treated ip with distilled water or ACMD (50mg/kg/day) containing (2,3-^{14}C) ACMD (20 μCi/kg) as a tracer, until neurotoxicity tests detected a difference between the two groups. The rats were then killed, tissue samples taken, and distribution and deposition of ACMD (as ^{14}C) determined.

The ACMD-treated rats lost weight over the treatment period while controls showed a slight gain (Fig 10). However, body weight variability was greater in this study than previously seen due to lack of animals in the desired weight range. This variability masked the significance of any differences in mean body weight between treated and control groups.

Neurotoxicity tests were performed to determine the cumulative dose of ACMD that would produce overt signs of intoxication. Results from the rotorod test were somewhat unusual. After a steady decline in ability to balance on the rod, a temporary improvement in performance was noted in ACMD-treated rats (Fig 11). Performance during the succeeding test period (cumulative dose of 250 mg/kg), declined such that mean trial duration was significantly different

($p<0.05$) from controls. Response time after ocularly applied zingerone (Fig 12) showed changes similar to those observed during the Methods study (Fig 9). The scratching response became more vigorous and response time steadily increased up to a cumulative dose of 250 mg/kg. A significant difference ($p<0.05$) in mean response time (ACMD-treated vs. control rats) was detected after a cumulative dose of 200 mg/kg.

Based on the overall results from the neurotoxicity test, a cumulative dose of 250 mg/kg was considered neurotoxic. Percent of dose in tissues and tissue concentrations after this dose appear in Table 4. Relative tissue content and concentrations are similar to those seen in the age-related studies, with liver containing the greatest amount and highest concentration of ACMD (as ^{14}C equivalents) and sciatic nerve the least.

The concentration of cytochrome P450 in livers from control rats was determined on the first day of ACMD dosing and on the last day of the study (Table 6 - values corresponding to 'Control' group).

Induction Study

A phenobarbital treatment regimen shown to be successful in S/D rats was tested for its ability to elevate and maintain the hepatic cytochrome P450 concentration in F-344 rats. Maintenance of these levels for 5 days, the length of the ACMD treatment period sufficient to produce neurotoxic signs, was required.

Table 4

Tissue Levels[a] of ACMD in Neurotoxic Male F-344 Rats from Neurotoxicity and Deposition Study

Tissue	Percent of Total Dose[b]	Concentration (nmoles/gm)[c]
Whole Blood	9.68 ± 0.99	3890.0 ± 389
Plasma	0.11 ± 0.01	89.0 ± 6.82
Brain	0.10 ± 0.01	469.0 ± 50.5
Spinal cord	0.19 ± 0.004	340.0 ± 34.9
Sciatic Nerve	0.0012 ± 0.0002	279.0 ± 15.2
Liver	0.65 ± 0.05	630.0 ± 89.3
Kidney	0.14 ± 0.01	607.9 ± 55.9

[a]Rats were killed 20 hours after final dose.

[b]As ^{14}C; mean ± SD (n=5).

[c]As ^{14}C equivalents; mean ± SD (n=5).

A single ip dose of 100 mg/kg Phenobarbital (PB) followed by a maintenance regimen of 0.1% PB in the drinking water was sufficient to raise hepatic cytochrome P450 concentrations 150% in F-344 rats (Table 5). This was comparable in magnitude to the increase seen in S/D rats of similar weight. (Liver weights were not used as an index of induction because of the high variability introduced by perfusion.) Therefore, this regimen was considered appropriate for F-344 rats.

It was noted that PB-treated rats from both strains had slightly lower body weights after treatment than their respective controls even though starting weights were similar. These differences were not statistically significant, however.

Neurotoxicity and ACMD Deposition in Neural Tissues after PB Treatment

Phenobarbital pretreated rats were further treated with daily ip doses of distilled water or ACMD (50 mg/kg) containing (2,3-^{14}C) ACMD (20 μCi/kg) as a tracer. ACMD treatment was continued until a pre-determined, neurotoxic cumulative dose was reached. Neurotoxicity tests were performed during the ACMD treatment period. Rats were killed, tissue samples taken, and distribution and deposition of ACMD (as ^{14}C) determined. These values were then compared to

Lack of rats in the desired weight range increased the variability in mean body weights. Thus, only after a cumulative dose of 250 mg/kg ACMD did the mean weight of treated rats differ significantly from controls (Fig 10). Unlike the PB-treated rats in the

Table 5

Cytochrome P450 Concentrations in Livers of Sprague-Dawley and Fisher-344 Rats From the Induction Study

Sprague-Dawley Rats	Body Weight*	Dithionite Assay**	CO Assay**
Controls	241 ± 16	0.65 ± 0.08	0.59 ± 0.09
Phenobarb.	226 ± 9	1.48 ± 0.24	1.37 ± 0.33
Radio (Phenobarb/control)		2.23	2.32

Fischer-344 Rats	Body Weight*	Dithionite Assay**	CO Assay**
Controls	241 ± 5	0.58 ± 0.02	0.55 ± 0.04
Phenobarb.	227 ± 10	1.45 ± 0.06	1.40 ± 0.07
Radio (Phenobarb/Control)		2.50	2.55

*Body weight in grams on day killed, mean ± S.D. (n=5).

**Concentration of P450 in nmoles/mg microsomal protein; mean ± S.D. (n=5). Method of Omura and Sato, J. Biol. Chem. 239, 2370,1964.

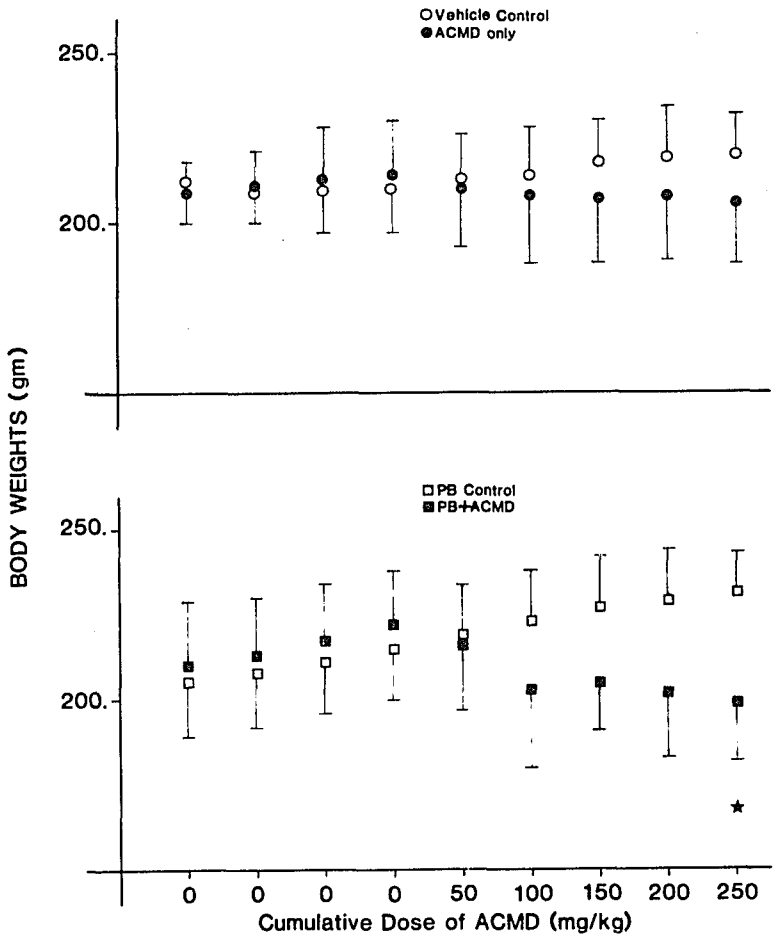

Figure 10. Daily body weights of control male F-344 rats or male F-344 rats treated with ACMD containing (2,3-14C) ACMD (20 μCi/kg) until a neurotoxic dose (250 mg/kg) was reached. PB control and PB + ACMD rats received phenobarbital before and during treatment with ACMD (50 mg/kg/day, given ip) or distilled water (1 ml/kg/day, given ip) as described in Methods. Data are expressed as mean ± SD (n=5). (*) indicates a significant difference ($p<0.05$) between the treated groups and their respective controls.

Induction study, that, as a group, failed to gain as much weight as their respective controls, the PB-treated controls gained weight steadily throughout the study. The final mean weight was greater for this group than for controls in the previous study (Fig 10), although starting weights were similar. Also, a larger weight loss was seen in the PB + ACMD group compared to the ACMD only group in the previous study.

It should be noted that for the rotorod test, the groups were only required to maintain their balance for a 60 second test period as opposed to 90 seconds for the groups in the previous Neurotoxicity study and 120 seconds for the groups in the Methods study. This change was necessary to guarantee that all rats could be trained to balance on the rod for the entire test period prior to the start of ACMD dosing.

A high degree of variability was seen in the results from the neurotoxicity tests performed. In the rotorod test, PB + ACMD-treated rats did not show the steady loss of ability (Fig 11) that the ACMD-only rats displayed in the previous studies (Fig 11 and Fig 9). In fact, a plateau was reached after an initial loss of ability; this level of performance was never significantly different from the control trial duration even after a neurotoxic cumulative dose. Results from the zingerone test were ambiguous. Up to a cumulative dose of 100 mg/kg, mean response times for PB-control vs. PB + ACMD-treated rats were comparable (Fig 12). The large increase in response time for the PB + ACMD-treated group seen after the next

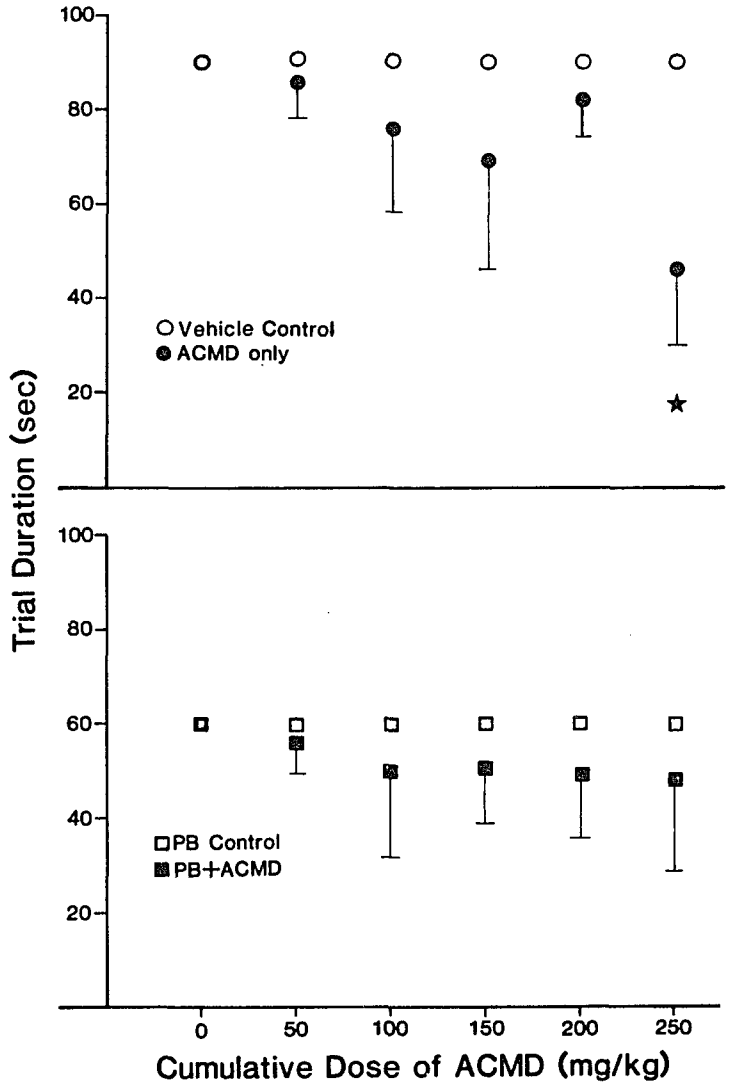

Figure 11. Rotorod trial duration results from control and ACMD-treated male F-344 rats receiving a neurotoxic cumulative dose (250 mg/kg). PB control and PB + ACMD rats received phenobarbital before and during treatment with ACMD (50 mg/kg/day, given ip) or distilled water (1 ml/kg/day, given ip) as described in Methods. Data are expressed as mean $\pm$ SD (n=5). (*) indicates a siginificant difference ($p<0.05$) between treated rats and their respective controls.

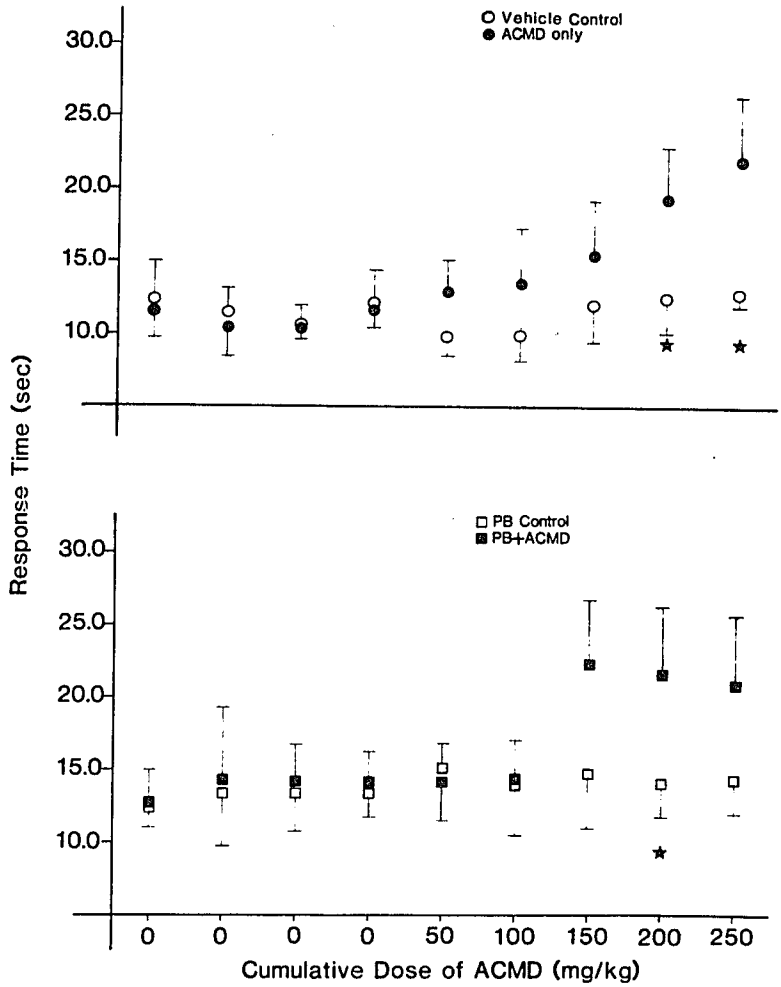

Figure 12. Response time after ocular application of zingerone for control and ACMD-treated male F-344 rats receiving a neurotoxic cumulative dose (250 mg/kg). PB control and PB + ACMD rats received phenobarbital before and during treatment with ACMD (50 mg/kg/day, given ip) or distilled water (1 ml/kg/day, given ip) as described in Methods. Data are expressed as mean $\pm$ SD (n=5). (*) indicates a significant ($p<0.05$) difference between treated rats and their respective controls.

dose was unlike the steady increase in response time seen in ACMD-only rats from the two previous studies (Fig 12 and Fig 9). A significant difference in mean response time was detected after a cumulative dose of 200 mg/kg but this difference disappeared, probably because of the large within-group variation in these results.

Tissue content and concentration of ACMD (as ^{14}C equivalents) from PB + ACMD-treated rats were compared to the values from ACMD-only rats in the previous study (Fig 13). Although relative tissue contents are similar to those seen in previous studies, a significantly smaller ($p<0.05$) percent of dose was found in the blood, plasma, liver, and kidney from PB-treated rats compared to ACMD-only rats. However, the percent of dose in neural tissues were not significantly different from one another. Concentration of ACMD (as ^{14}C equivalents) was significantly lower ($p<0.05$) in all tissues analyzed except sciatic nerve, which had higher within-group variation than the other tissues. The average decrease in concentration between tissues from PB + ACMD-treated and ACMD-only rats was 16%.

Microsomes prepared from livers of PB control rats on the first and last days of ACMD dosing had cytochrome P450 concentrations at least twice as great as those found in microsomes from control rats in the previous Neurotoxicity study (Table 6). These concentrations were comparable to those found in PB-treated rats in the Induc-

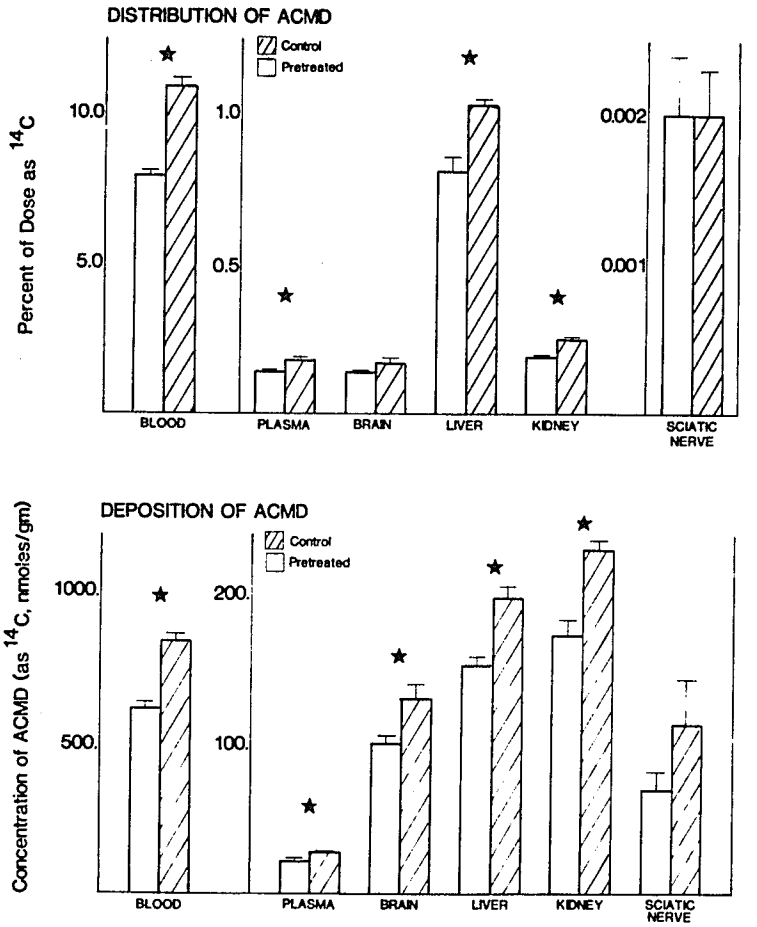

Figure 13. Tissue content and concentration of ACMD (as ^{14}C) in male F-344 rats receiving a neurotoxic cumulative dose (250 mg/kg) of ACMD containing (2,3-^{14}C) ACMD. Control rats received ACMD (50 mg/kg/day, given ip) only. Pretreated rats received phenobarbital before and during ACMD (50 mg/kg/day, given ip) treatment. Data are expressed as mean $\pm$SD (n=5). (*) indicates a significant difference ($p<0.05$) between pretreated and control rats.

Table 6

Hepatic Cytochrome P450 Concentrations in Control Male F-344 Rats From Neurotoxicity Study and Pretreatment/Neurotoxicity Study

First Dose Day (n=3)	Dithionite Assay**	CO Assay**
Control	0.51 ± 0.07	0.51 ± 0.13
Phenobarb.	1.27 ± 0.04	1.25 ± 0.07
Ratio	2.49	2.45

Final Dose Day (n=4)	Dithionite Assay**	CO Assay**
Control	0.46 ± 0.05	0.49 ± 0.05
Phenobarb.	1.09 ± 0.10	1.10 ± 0.11
Ratio	2.22	2.39

*Concentration of P450 in nmoles/mg microsomal protein; mean ± SD.

Treatment: Two ip injections, 80 mg/kg/day Phoenobarbital Sodium followed by 0.1% Phenobarbital in drinking water until the end of the study. Controls received injections of distilled water followed by tap water.

tion study (Table 5), reaffirming the adequacy of the PB-treatment regimen used.

Effect of ACMD on PB Induction of Hepatic Cytochrome P450

The effect of ACMD treatment on the normal and PB induced concentrations of cytochrome P450 in rat liver was studied. The effect of ACMD on the activity of a P450-associated enzyme, benzphetamine-N-demethylase, was also determined. Daily body weights were recorded and the ocular zingerone test was performed throughout the ACMD treatment period; results were compared to data from the two neurotoxicity studies.

Weight data resembled results from the Neurotoxicity studies; ACMD-only rats lost less weight than the PB + ACMD-treated rats, while both control groups gained a small amount over the treatment period (Fig 14). Within-group variation in weight was again a problem, particularly in the PB + ACMD group. In this study, however, weight losses were dramatic enough to counteract this problem. The results from the zingerone test were more ambiguous in this study than in the previous Neurotoxicity studies (Fig 15). The mean response time for the ACMD-only group began to increase as expected, but leveled off, and actually declined between a cumulative dose of 150 mg/kg and 250 mg/kg. Results from the PB + ACMD group did not display as dramatic an increase in mean response time as in the previous PB treatment study (Fig 12). Variability was again higher in this group, increasing as cumulative dose increased.

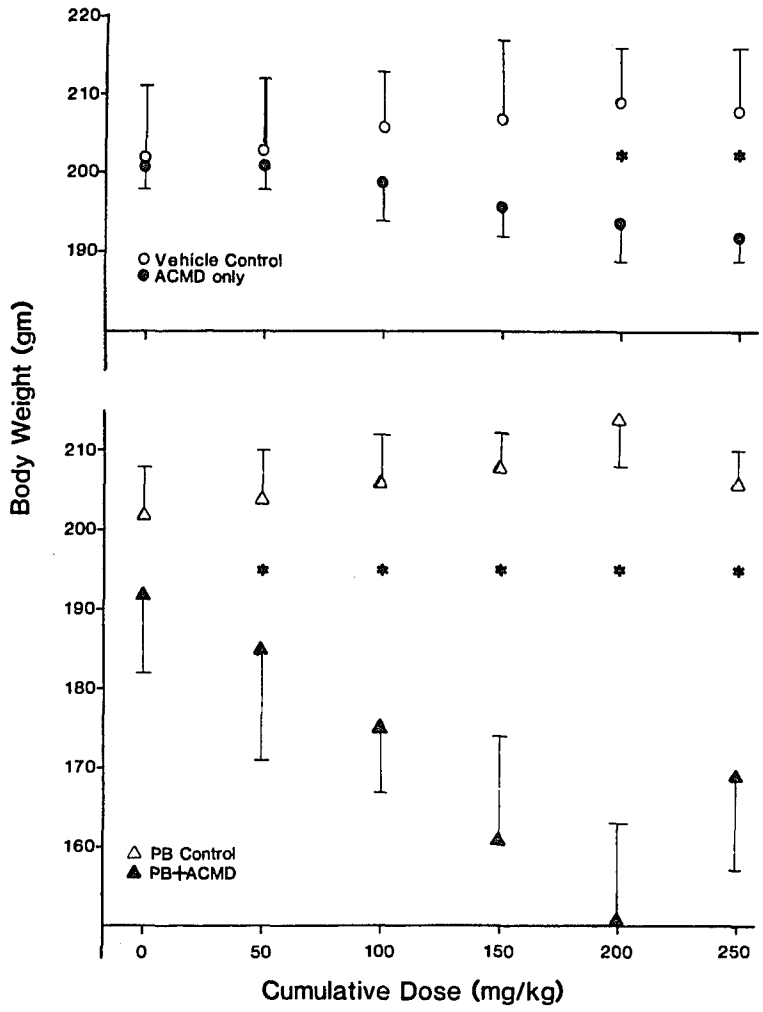

Figure 14. Daily body weights from control male F-344 rats and male F-344 rats treated with a neurotoxic cumulative dose of ACMD (250 mg/kg). PB and PB + ACMD rats received phenobarbital before and during treatment with ACMD (50 mg/kg/day, given ip) or distilled water (1 ml/kg/day, given ip) as described in Methods. Data are expressed as mean $\pm$ SD (n=3). (*) indicates a significant difference ($p<0.05$) between control and treated rats.

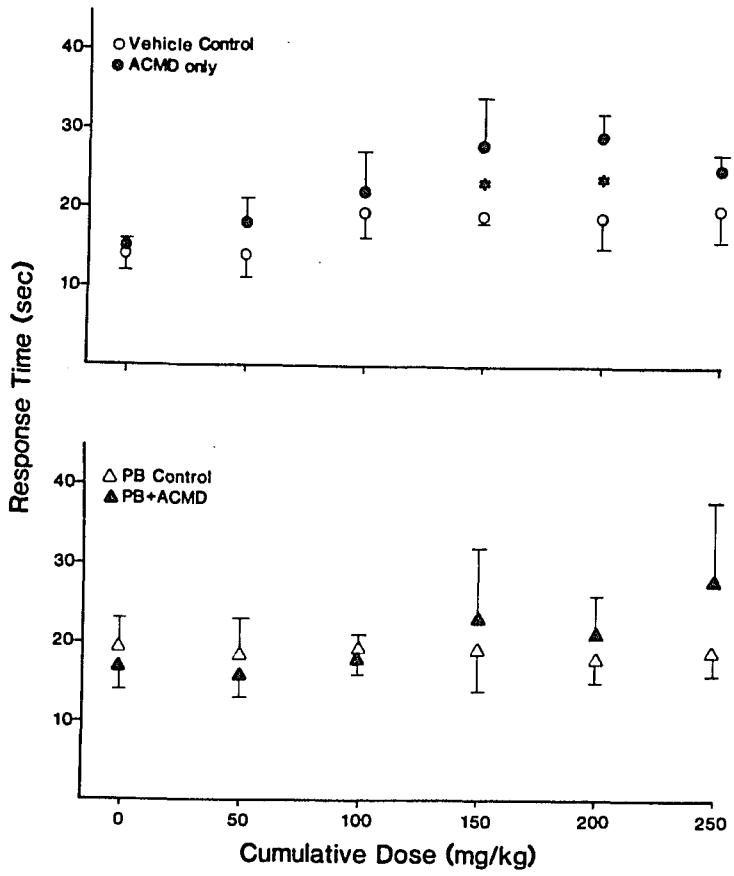

Figure 15. Response times after application of zingerone to the eyes of control male F-344 rats and male F-344 rats treated with a neurotoxic cumulative dose (250 mg/kg) of ACMD. PB + ACMD rats received phenobarbital before and during treatment with ACMD (50 mg/kg/day, given ip) or distilled water (1 ml/kg/day, given ip) as described in Methods. Data are expressed as mean $\pm$ SD (n=3). (*) indicates a significant difference ($p<0.05$) between control and treated rats.

ACMD alone did not alter P450 concentration from control values (Fig 16). However, ACMD treatment did prevent the expected elevation of P450 concentration by PB. Thus, hepatic P450 concentrations in PB + ACMD-treated rats were not statistically different from control levels, while PB-treated control levels were at least twice those in livers from vehicle control rats. Similar results were seen in the activity of a P450-associated enzyme, benzphetamine-N-demethylase (Fig 16). ACMD alone did not alter activity levels from those found in control rats, while simultaneous exposure to ACMD prevented the PB-induced increase in enzyme activity. PB-treatment alone caused an increase in activity to approximately 3 times control levels. Liver weights were not compared because of the high variability introduced by perfusion.

Effect of PB Treatment on Clearance of a Single iv Dose of ACMD

PB pretreated and non-pretreated rats were given a single iv dose of ACMD (50 mg/kg) containing (2,3-^{14}C) ACMD (20 µCi/kg) as a tracer. The appearance of ACMD as ^{14}C in urine and feces and as parent ACMD in urine was followed for 8 hrs. Also, the decline of parent ACMD in blood and ^{14}C in blood and plasma was monitored over the same time period.

The percent of total dose (as ^{14}C) remaining in whole blood did not decrease with time in ACMD-only rats (Fig 17). This agreed with results from a previous kinetics study (Fig 7). Treatment with PB before and during ACMD treatment had a significant effect on loss of total ^{14}C from the blood, although differences in mean percent of

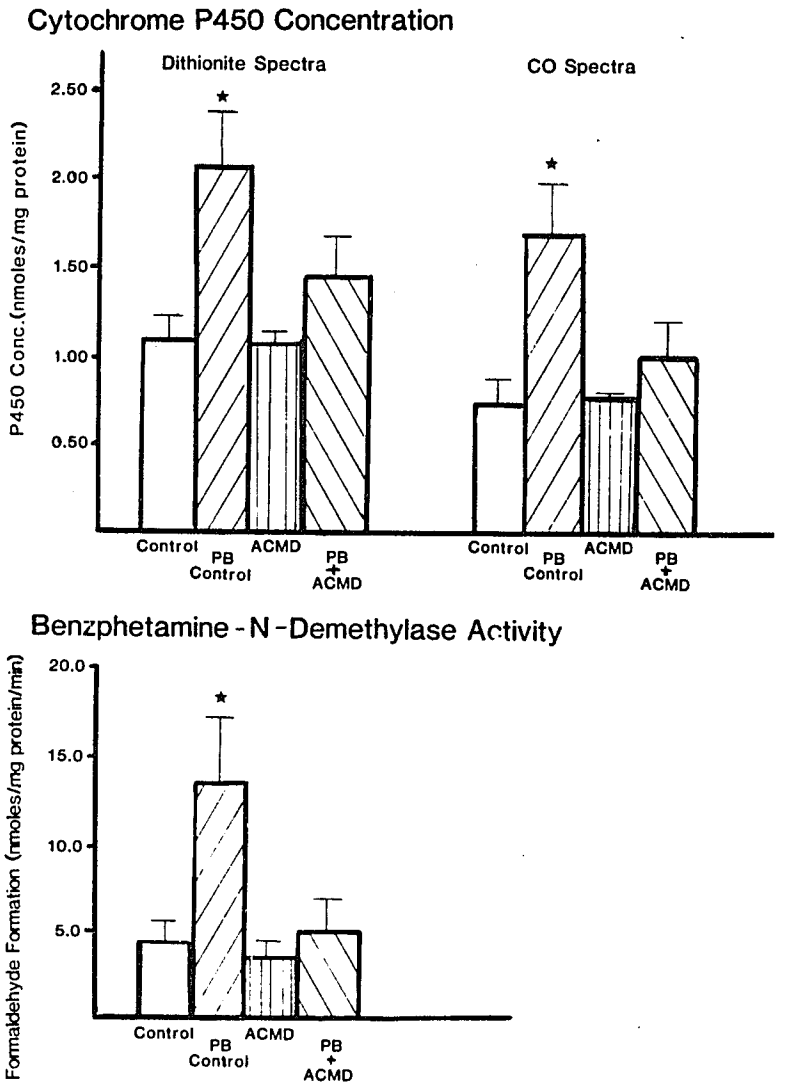

Figure 16. Hepatic Cytochrome P450 concentrations and benzphetamine-N-demethylase activities from control and PB-treated male F-344 rats with and without simultaneous exposure to a cumulative neurotoxic dose of ACMD (250 mg/kg, given ip at a rate of 50 mg/kg/day). Data are expressed as mean $\pm$ SD (n=3). (*) indicates significantly different from all other groups.

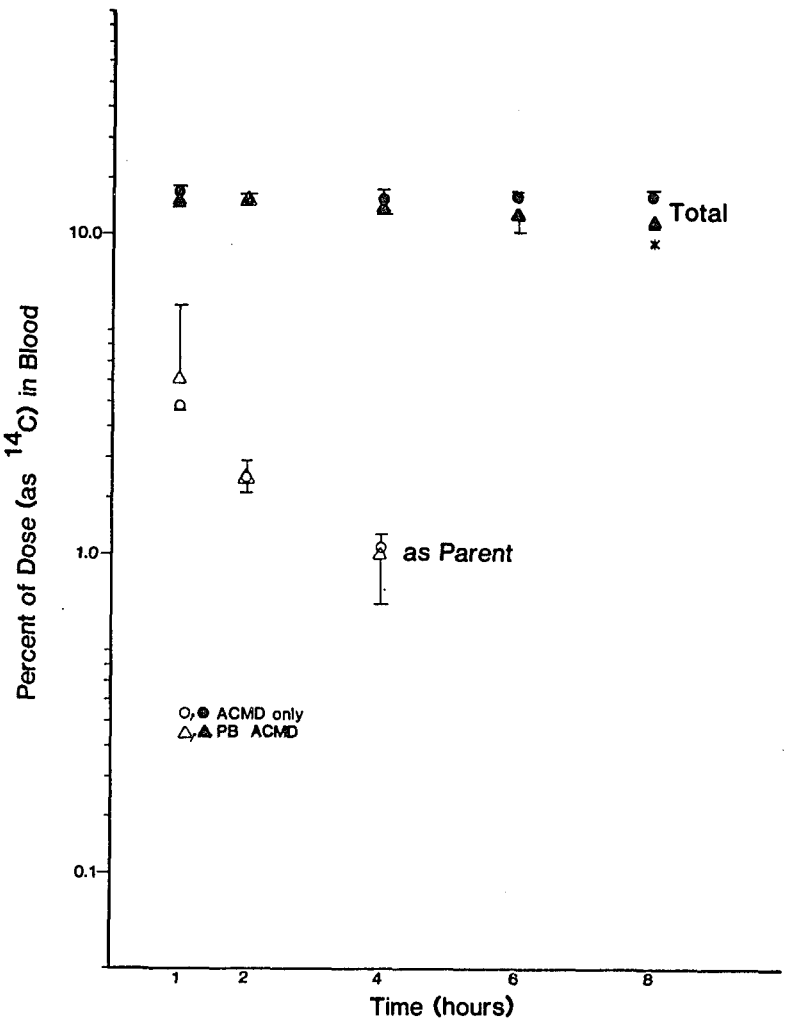

Figure 17. ACMD content of blood from male F-344 rats as uncharacterized ^{14}C and as parent ACMD after a single iv dose (50 mg/kg, 20 µCi/kg). Rats were treated with ACMD only or ACMD after pretreatment with PB. Data are expressed as mean ± SD (n=3). (*) indicates significant differences ($p<0.05$) between pretreated and non-pretreated rats.

dose were significant only at the 8 hour time point. The loss of ^{14}C corresponding to parent ACMD was not affected by PB treatment at the time points observed. Loss of parent ACMD from blood was rapid; analysis of later time points was not possible due to insufficient radioactivity in extracts of blood.

Plasma levels of ^{14}C (expressed as percent of dose) decreased rapidly over the observed time period to less than 1% of the total dose given (Fig 18). Pretreatment did not have a significant effect on loss of radioactivity from the plasma, although the mean percents of dose at the 6 hour time point were significantly different. The relatively large mean of the 6 hour ACMD-only group was primarily due to one animal, making the biological significance of this difference questionable.

Concentration of ACMD (as ^{14}C) in whole blood and plasma mirrored the percent of dose results (Fig 19). PB treatment significantly altered the disappearance of ^{14}C from whole blood such that 8 hours after dosing, mean blood concentration in PB-treated rats was significantly different from controls. Plasma concentration, in contrast, was not affected by pretreatment, and decreased rapidly over time. Again, the ACMD-only 6 hour time point does not follow the smooth decline in concentration illustrated by the other points on the curve because one ACMD-only rat had a plasma concentration nearly double that of the other rats in the group.

Excretion of ^{14}C in the urine was rapid (Fig 20). Approximately 50% of the total dose (as ^{14}C) was excreted by 8 hours after

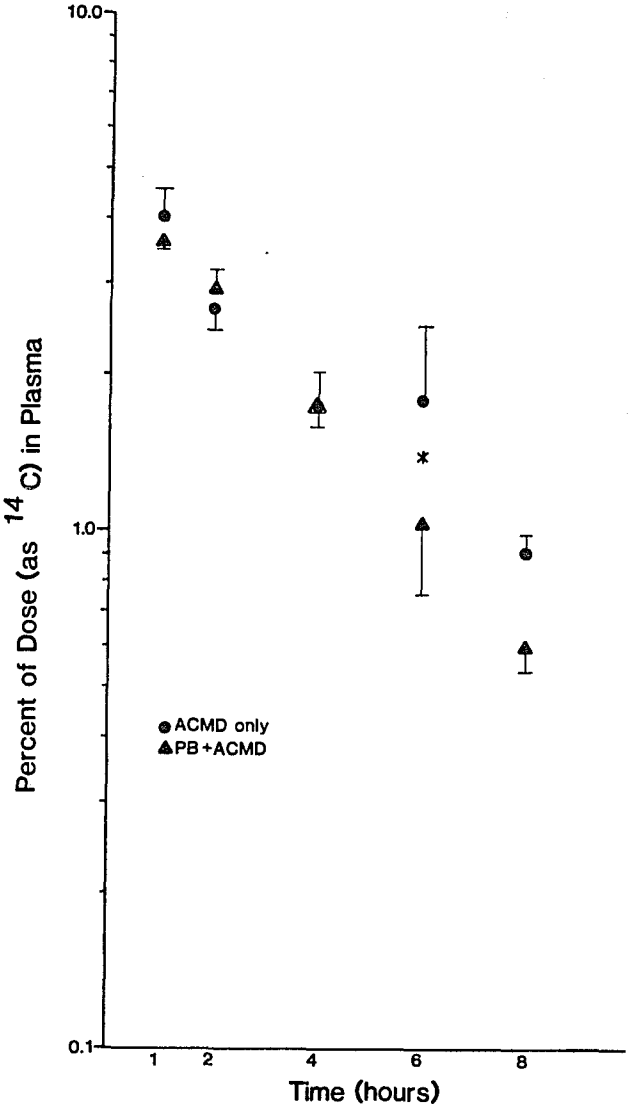

Figure 18. ACMD content of plasma from male F-344 rats as total ^{14}C after a single iv dose (50 mg/kg, 20 μCi/kg). Rats were treated with ACMD only or ACMD after pretreatment with PB. Data are expressed as mean ± SD (n=3). (*) indicates significant differences ($p<0.05$) between pretreated and non-pretreated rats.

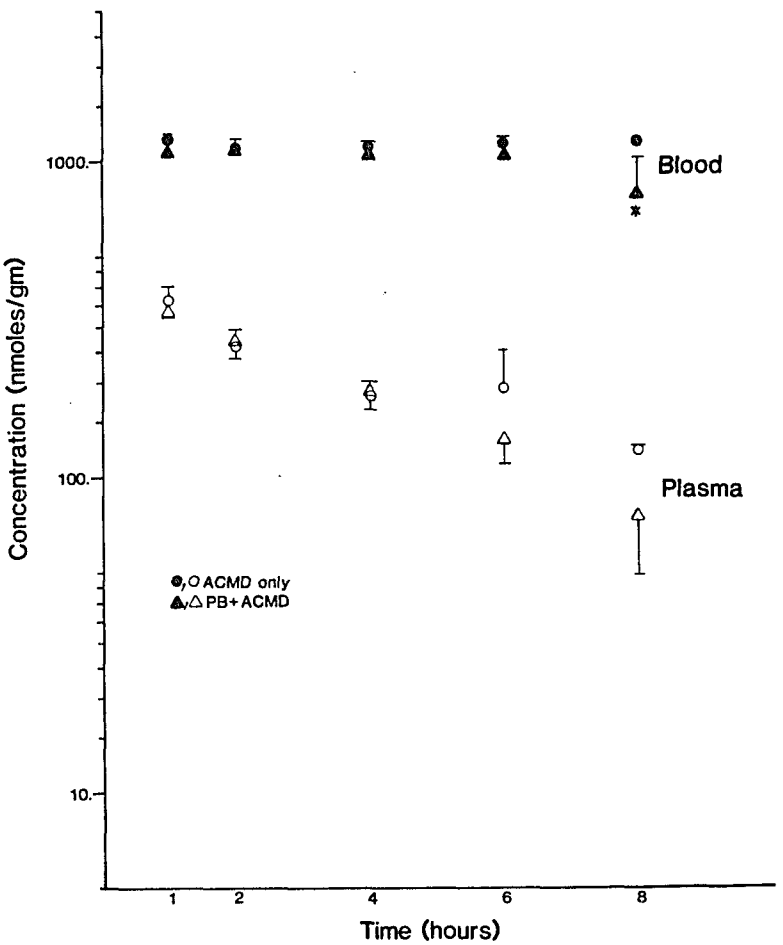

Figure 19. ACMD concentration (as ^{14}C equivalents) in blood and plasma from male F-344 rats after a single iv dose (50 mg/kg, 20 μCi/kg). Rats were treated with ACMD only or after pretreatment with PB. Data are expressed as mean ± SD (n=3). (*) indicates siginificant differences ($p<0.05$) between pretreated and non-pretreated rats.

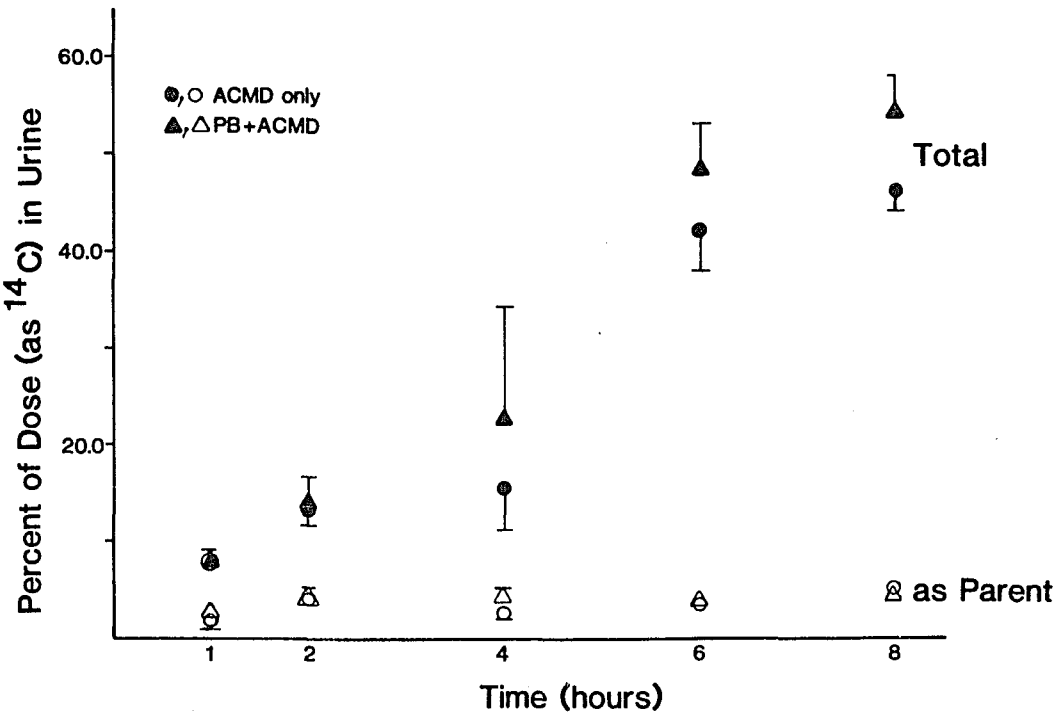

Figure 20. Percent of total dose excreted in the urine of male F-344 rats as uncharacterized ^{14}C and as parent ACMD after a single iv dose (50 mg/kg, 20 μCi/kg). Rats were treated with ACMD only or ACMD after pretreatment with PB. Data are expressed as mean ±SD (n=3). (*) indicates significant differences ($p<0.05$) between pretreated and non-pretreated rats.

dosing. Both PB treatment and time were significant ($p<0.025$) sources of variation in the urinary excretion of radioactivity. However, no significant differences were found between means from control and treated rats. Excretion of parent ACMD via the urine was unaffected by pretreatment;roughly 4% of the dose excreted in the urine was parent.

Very little radioactivity (<1%) appeared in the feces (Table 7) which agreed with our previous kinetics study (Table 3). Therefore, total excretion of ACMD (as ^{14}C) could be represented by urinary excretion, or approximately 50% of the total dose given.

Table 7

Excretion of ACMD (as ^{14}C) by Male F-344 Rats After a Single iv Dose (50 mg/kg, 19 Ci/kg)

A. Percent of Dose in Feces[a]

Time Point	Control	PB-Treated
1 Hr	0.03 ± 0.03	0.16 ± 0.13
2 Hr	0.01 ± 0.01	0.36 ± 0.30
4 Hr	1.70 ± 0.61	1.86 ± 1.21
6 Hr	0.21 ± 0.09	0.25 ± 0.13
8 Hr	0.42 ± 0.27	0.14 ± 0.05

B. Percent of Dose in Urine + Feces

Time Point	Control	PB-Treated
1 Hr	7.87 ± 1.11	8.24 ± 0.83
2 Hr	13.24 ± 1.80	14.17 ± 3.04
4 Hr	16.97 ± 3.83	24.70 ± 12.37
6 Hr	42.03 ± 4.09	48.83 ± 4.28
8 Hr	46.69 ± 1.85	53.80 ± 4.09

[a]Mean ± SD; n=3.

CHAPTER 4

DISCUSSION

Age Comparison Studies

Age-related differences in the concentration of ACMD (as ^{14}C) were found when 5 and 11 week old male Holtzman rats were singly- or multiply-dosed. In general, the concentration of ACMD (as ^{14}C) was significantly greater in tissues from 11 week old rats than 5 week old rats. In contrast, the distribution of ACMD, expressed as percent of dose in tissues, did not change with age; the relative distribution patterns in these two age groups were similar.

The elimination of ACMD (parent + metabolites) has been shown to be biphasic, involving the rapid clearance of parent and excretable metabolites followed by the slow loss of tissue-associated material (Hashimoto and Aldridge, 1970; Edwards, 1975; Miller et al., 1982). Total ^{14}C from (2,3-^{14}C) ACMD is cleared from the blood with an initial elimination half-life of 5 hours. In a multiple dosing regimen with a dosing interval of 24 hours, it was assumed that all rapidly excreted species from one dose would be cleared prior to the administration of the next dose, leaving only tissue-associated material. In addition, it was assumed that the same proportion of each dose becomes tissue-associated. Under these assumptions, the distribution and deposition of the last dose would

mimic all preceeding doses. For this reason, and to conserve radiolabeled material, only the final dose of ACMD contained (2,3-^{14}C) ACMD, in the initial study.

Using this dosing scheme, no significant differences were found in distribution or deposition of ACMD (as ^{14}C) that could be related to treatment period length. However, values for both parameters tended to be lower for tissues from groups treated 7 days compared to those from the same age group treated 5 days. This raised doubts about the validity of the assumption that the same proportion of each dose in a multi-dose regimen remains in the tissues.

To test this assumption, radiolabeled ACMD was included in all doses of a multi-dose study. Rats were killed after 1, 3, or 5 doses to determine if the same proportion of each daily dose becomes tissue-associated. Again, significant age-related differences in tissue deposition of ACMD (expressed as concentration in nmoles ACMD/gm wet tissue), but not distribution (as percent of total dose), were found. In addition, significant between-group variation in both concentration and percent of dose could be related to the number of doses received.

The percent of total dose remaining in the tissues decreased with increasing number of doses. This implies that a smaller proportion of each successive dose remains in the tissues, disproving the assumption that the same proportion of each dose remains in the tissues. Several explanations for this phenomenon are possible.

First, as dosing continues, the clearance of the tissue component from one dose could begin to overlap the introduction of new material. Since ACMD is only identified as ^{14}C, one cannot distinguish the material from any particular dose. As more ^{14}C is cleared, the decrease is reflected in the percent of total dose remaining. It is also possible that a finite number of sites capable of reacting with ACMD exist in tissues, such that during multiple dosing, these sites become saturated. A decreased retention of successive doses would result, eventually reaching a steady state of exchange of new material for material already associated with tissue sites.

In contrast, tissue concentration of ACMD (as ^{14}C equivalents) increased with increasing number of doses received, lending support to the hypothesis that a tissue-associated component accumulates in neural tissues during multiple dosing.

Early studies of ACMD neurotoxicity related dosing rate and total cumulative dose to time of onset of neurotoxic signs (Kuperman, 1958; Fullerton and Barnes, 1966). One mechanism of ACMD neurotoxicity proposes the binding of the parent compound (or a metabolite) to critical sites in neural tissues (Hashimoto and Aldridge, 1970). By this mechanism, toxicity occurs after a threshold number of these sites are chemically modified. According to Kaplan and Murphy (1972), 11 week old rats are measurably neurotoxic after 5 days of ACMD treatment (50 mg/kg/day, given ip). If the hypothesis that a critical cumulative tissue level of an ACMD specie(s) be reached prior to the onset of neurotoxic signs is valid, then tissue

levels in these rats should represent a neurotoxic threshold. In contrast, 5 week old rats are not measurably neurotoxic after 5 days of treatment and one would therefore expect cumulative tissue levels in these rats to be lower than those in the 11 week old rats.

The data support this hypothesis both by showing that accumulation of an ACMD specie(s) does occur and by revealing age-related differences in tissue concentration of an ACMD specie(s). In addition, this quantitative evidence tends to rule out suggestions that age-related differences in neural physiology are responsible for the differences in susceptability to ACMD neurotoxicity.

A more reasonable cause of the observed differential susceptability appears to be the method of dose calculation. It is clear that by dosing on a body weight basis, 11 week old rats (350 - 400 gm) received a larger absolute amount of ACMD than 5 week old rats (130 - 175 gm). Assuming the rate and extent of ACMD detoxiciation and excretion are similar for 5 and 11 week old rats, tissues in 11 week old rats would be exposed to higher levels of the neurotoxic ACMD specie(s). The threshold level of modified tissue sites necessary to impair neural function would be reached more quickly in this age group, ultimately leading to the earlier onset of neurotoxic signs. However, other factors that could influence tissue deposition of ACMD were studied for their contributions to this phenomenon.

The influence of age on elimination of total ^{14}C (parent + metabolites) from a single ip dose of (2,3-^{14}C) ACMD was examined over a 24 hour period. Although they received a larger absolute

ACMD-Phenobarbital Interaction Studies

Phenobarbital (PB), given before and during ACMD treatment, caused significant decreases in the deposition of ACMD (as ^{14}C) in tissues of male F-344 rats. The average decrease in concentration of ACMD (as ^{14}C) was 16%. PB also affected the distribution of ACMD to tissues; significantly lower percents of dose were found in non-neural tissues from PB-treated rats than non-treated rats.

It is possible that the decreased deposition of ACMD (as ^{14}C) after PB treatment is caused by PB effects on the detoxification of ACMD. Decreased levels of parent ACMD were found in urine from rats that had been pretreated with PB. However, it is doubtful that PB acts via cytochrome P450 induction. Previous work has shown that incubation of ACMD with hepatic microsomes from PB-treated rats caused a loss of cytochrome P450, cytochrome b_5, and NADPH-cytochrome c reductase activity (Ivanetich et al., 1978). Loss of cytochrome P450 from rat hepatic 9,000xg supernatant was also reported by Ortiz et al. (1981), accompanied by loss of aniline hydroxylase activity. Although ACMD alone did not affect hepatic cytochrome P450 concentration or the activity of benzphetamine-N-demethylase, an associated enzyme, it did prevent the expected PB-induced elevation of both these parameters. Thus, if PB does influence the detoxification of ACMD via the cytochrome P450 mixed function oxidase system, the effect would be temporary at best, due to the inhibitory action of ACMD.

PB may also influence ACMD detoxification through its stimulatory effects on glutathione-S-transferase activity. This enzyme group is thought to play a major role in ACMD metabolism (Miller et al., 1982) by catalyzing a reaction between ACMD and intracellular glutathione. This reaction is known to occur *in vitro* (Dixit et al., 1981), and is implied *in vivo* by the appearance of glutathione-ACMD conjugates and mercapturic acids of ACMD in the bile (Edwards, 1975), and urine (Miller et al., 1982) of rats. However, ACMD has also been shown to inhibit the activities of glutathione-S-transferases in rat brain and liver (Dixit et al., 1982), and one wonders if this is the reason PB treatment only delays rather than prevents ACMD-induced neuropathy. That is, during multiple dosing, ACMD may be able to inhibit its own detoxification, even though its detoxification pathway has been enhanced by chemical treatment.

The interaction of PB and ACMD may explain the somewhat ambiguous protective effect attributed to PB against ACMD-induced neurotoxicity. Specifically, results from only one neurotoxicity test, the rotorod, demonstrated a clear PB-related delay in the neurotoxic effects of ACMD. Neither the ocular zingerone test nor body weight changes showed definitive improvement when results from the PB+ACMD group and the ACMD-only group were compared to their respective controls. In fact, weight loss was more severe in PB+ACMD rats. If multiple doses of ACMD were able to minimize PB induction of cytochrome P450 and glutathione-S-transferase activity, then any protection afforded by the induction would also be

diminished. It should be noted that the PB treatment regimen employed in these studies differed from that used by Kaplan et al. (1973). In that study, injections of PB (50 mg/kg/day) were given throughout ACMD treatment. It is possible that unlike high dose injections, the maintenance program of 0.1% PB in drinking water was not able to counteract the inhibitory effects of ACMD.

Another source of ambiguity concerning the protective effect of PB against ACMD neurotoxicity may be the lack of a definitive decrease in ACMD concentration in the tissue of PB-treated rats compared to non-treated rats. The average decrease in tissue concentration was only 16%. It is possible that the biochemical protection afforded by this decrease was not sufficient to prevent the functional deficits caused by the biochemical injury that did occur.

Alternatively, PB may have had an adverse effect on the ability of rats to respond and/or perform during the chosen neurotoxicity tests. For example, the rotorod trial duration considered successful in the PB-treated groups was reduced to 60 seconds compared to 90 seconds for the non-treated groups. This reduction in trial duration was necessary because PB-treated rats as a group had difficulty balancing on the rod for more than 60 seconds. Changes in proprioception or the perception of pain (from falling off the rod) stemming from PB treatment may have caused this difficulty, and may have inadvertantly affected testing results. However, it should be noted here that Kaplan and Murphy (1973) placed an electric grid

underneath the rotorod used in their experiments. This strong negative feedback may have been a more effective deterrent than presented here against rats jumping and/or falling from the rod for reasons other than physical inability. While relying on more complex behaviors ('learning', 'memory'), use of this technique might have helped determine physical differences between PB-treated and non-treated groups. Both the unusual results from the ocular zingerone test and the dramatic changes in body weight seen in PB+ACMD rats may also have arisen from PB interaction with the normal functioning of the animals. If PB altered the perception of pain, changes in the response to zingerone application could have been produced. Additionally, PB-induced lethargy may have led to a decrease in food consumption, compounding whatever effect ACMD itself has on body weight.

The tests used for detection of ACMD-induced neural deficits must also be examined. Both rotorod balancing and response to ocular zingerone are complex combinations of behaviors. While simple muscle strength is involved, balance, pain perception, proprioception, and more subtle factors such as fatigue and wellness of the animal are also a part of the response/performance measured by these tests. It would be difficult to say that either of these tests exclusively detects the classic signs of ACMD-induced peripheral neuropathy i.e., ataxia and hind limb weakness. The level of neuropathy detected by these tests probably includes many of the central components of ACMD

neural effects as well as interactions of peripheral and central components.

Finally, the number of animals per group used in the neurotoxicity studies was small (n=5). In contrast, Kaplan and Murphy (1972) used 12 rats per group when studying age - related differences in the ouset of ACMD - induced neurotoxicity. When studying the effects of hepatic microsomal mixed function oxidase inducers on ACMD neurotoxicity, Kaplan et al. (1973) used 9 rats per group. Small numbers of animals per test group may account for the high degree of variability in the neurotoxicity test results presented here, which, in turn, may have masked differences between treatment groups and their respective controls.

Unfortunately, the hypothesis that onset of neurotoxic signs is associated with threshold tissue levels of ACMD could not be substantiated by these experiments. Nor was the protective effect of PB treatment against ACMD-induced neurotoxic effects evident, even when using a battery of neurotoxicity tests shown to be sensitive to the effects of ACMD. However, the hypothesis was not disproven by this body of work, either. A small decrease in neural tissue ACMD (as ^{14}C) concentration did appear to be associated with PB treatment and, under different experimental conditions, this association could prove useful in testing the hypothesis.

General Discussion

A disturbing finding in both groups of experiments was the lack of a significant difference between ACMD concentrations in sciatic nerve. Neither age nor PB treatment had a significant effect on deposition of ACMD in this tissue. This is surprising in light of the fact that ACMD is characterized as a peripheral neurotoxin. One explanation could be that at high subacute exposure levels, central effects (incoordination, behavioral changes) have been shown to precede peripheral involvement (Kuperman, 1958; McCollister et al., 1964). The dosing regimen used in both the age comparison studies and the PB-ACMD interaction studies called for a relatively high dose (50 mg/kg) given over a short period of time (5-7 days), and could have produced central neural deficits rather than peripheral ones. If this were true, significantly different ACMD concentration in the brain and possibly the spinal cord might be expected within the treatment period. The data from both sets of experiments support this proposal, suggesting that some sort of relationship exists between neural tissue ACMD deposition and the development of neurotoxicity.

At this point, however, one must question the use of tissue concentration of ACMD (as ^{14}C) as a measure of tissue levels of the ACMD special(s) involved in neurotoxicity. Since ACMD is capable of reacting with a variety of chemical groups present in tissue macromolecules (-SH, $-NH_2$, -OH), it is likely that binding of ACMD to many tissue components unrelated to toxicity (non-specific binding)

occurs concurrently with modification of critical tissue components (specific binding). Therefore, the total amount of ACMD (as ^{14}C) deposited in neural tissues represents both specific and non-specific binding. As a result, any changes in specific binding of ACMD (caused by chemical treatment or other factor) are measured against a background of non-specific binding. This need not be of great concern as long as the level of specific binding is of the same or greater magnitude as non-specific binding. That is, changes in total tissue levels of ACMD (measured as tissue concentration) should adequately represent changes in specific binding and one should be able to see a defined relationship between these changes and development of neurotoxicity. If, on the other hand, specific binding represents only a small portion of the total tissue ACMD burden, changes in total tissue concentration of ACMD (as ^{14}C) may not represent changes in specific binding. That is, statistically significant changes in total tissue concentrations may not reflect biologically significant changes in specific binding. Thus, a lack of correlation between statistically significant changes in total tissue levels of ACMD (as ^{14}C) and development of neurotoxicity might be observed.

Data on the composition of the brain lend support to the second scenario discussed above. Protein represents about 8% of the total brain weight of rate (Waehneldt and Shooter, 1973). Of this, 25-29% is composed of water soluble proteins, including the glycolytic enzymes. Neuron specific enolase (NSE), one of the

glycolytic enzymes purported to be a target in ACMD - induced toxicity (Howland, 1981), accounts for 1.5% of the mass of these soluble proteins in the brain (Marangos, Polak, and Pearse, 1982). Though NSE is considered a major protein constituent of the brain by these authors, an estimate of its content in an average rat brain weighing 1.6 grams (personal observation) is only 5.2×10^{-4} grams, out of an estimated total of 0.13 grams protein, a 250 - fold difference, or 0.4% of the total. Add to this the liklihood of binding to some lipid and/or sugar moieties, and the magnitude of non-specific binding relative to specific binding increases further. Thus, to draw conclusions about changes in specific binding and their impact on onset of neurotoxicity based on data that may reflect such a large amount of non-specific binding is risky at best. Indeed, this may explain the lack of a clear relationship between statistically significant changes in tissue concentration of ACMD (as ^{14}C) and the onset of neurotoxic signs observed in our experiments with phenobarbital.

Along these same lines, although differences in toxicity related to age were not re-examined, differences in neural tissue concentrations in those studies, though significant, were not dramatic. Since rotorod, with its inherent difficulties, was the only test used to determine neurotoxicity in the original study, the age-related differences in onset of ACMD-induced neural deficits reported by Kaplan and Murphy (1972) may not be conclusive evidence that this is a reproducible phenomenon. Indeed, the large size of the 11 week

old rats alone may have had a detrimental effect on the rats' ability to maintain their balance, creating a significant difference where physiologically, one did not exist.

Several of the difficulties encountered in the studies described might be overcome by reducing the size of the daily dose administered and extending treatment over a longer period of time. In this way, any suspected central effects would be minimized, simplifying the neurotoxic signs produced. Also, changes due to any additional treatments could be observed over a longer period of time, permitting detection of more subtle differences in development of neuropathy stemming from the additional treatment. For example, the suspected inhibitory effect of ACMD on its own metabolism may have been reduced with lower doses, allowing the protective effects of PB to become more apparent and, perhaps, more clear-cut. The relationship between neural deficit and peripheral nervous tissue levels of ACMD could be more easily examined with this dosing scheme as well. Neurotoxic signs developed over a longer period of time, at a lower dosing rate, are more likely to be due to the effects of ACMD on peripheral nerves. Correlation between these signs and peripheral nerve ACMD concetration would lend more support to the existence of such a relationship.

Conclusions

The effects of age and phenobarbital treatment on the distribution and deposition of ACMD were studied in an effort to

determine the role of tissue concentration in the onset of ACMD neurotoxicity. Although the experiments described previously failed to establish a clear relationship between concentration and neurotoxicity, a number of conclusions were drawn from the results. These are summarized below:

1. Age-related differences in tissue deposition of ACMD (expressed as concentration in moles ACMD/gm wet tissue) but not distribution (as percent of total dose) were found where 5 and 11 week old male Holtzman S/D rats were given single or multiple ip injections of ACMD (50 mg/kg, 20 μCi/kg). Neural tissues from 11 week old rats had significantly higher concentrations of ACMD (as ^{14}C) than those from 5 week old rats. This finding, together with a study by Kaplan and Murphy (1972) demonstrating earlier onset of ACMD neurotoxicity in 11 week old rats than 5 week old rats, indicates that a relationship between tissue concentration and onset of neurotoxicity exists.
2. Tissue concentrations of ACMD (as ^{14}C) were found to increase in neural tissues with increasing number of doses, indicating the accumulation of some ACMD specie(s) in these tissues. This supports the hypothesis that neurotoxic signs appear only after a threshold number of critical tissue sites are chemically modified by ACMD.
3. The percent of total dose remaining in the tissues decreased with increasing number of doses, implying that a smaller proportion of each successive dose remained in the tissues. This may have

resulted from an overlap of clearance and introduction of new material or from saturation of sites capable of reacting with ACMD.

4. Excretion of ACMD by 11 week old rats was significantly greater than by 5 week old rats, negating the possibility that age-related differences in excretion as a cause of the observed tissue concentration differences.
5. Tissue: body weights were significantly less in 11 week old rats than 5 week old rats, indicating that the larger doses of ACMD given to the 11 week old rats (based on body weight) were distributed to less than proportionally larger tissues. Together, these factors may explain the observed age-related differences in tissue concentration.
6. Decreased body weight, increased response time after ocularly applied zingerones, and inability to maintain balance on a rotating rod were all found to distinguish ACMD-intoxicated male F-344 rats from control rats. Significant differences between intoxicated and control rats were observed after a cumulative dose of 150 mg/kg ACMD, therefore these tests were considered adequate for evaluating rats for neurotoxicity.
7. Phenobarbital (PB), given before and during ACMD treatment, caused significant decreases in the deposition of ACMD (as ^{14}C) in tissues of male F-344 rats. It is possible that this decrease was caused by a PB-induced stimulation of ACMD detoxification pathway(s). Support for this hypothesis came from decreased

levels of parent ACMD in the urine, the primary route of excretion. However, ACMD was found to block the cytochrome P450 induction associated with PB treatment, making this pathway an unlikely candidate for a role in the observed tissue concentration decrease.

8. Phenobarbital treatment did not produce the definitive delay in the onset of ACMD-induced neurotoxic signs reported by Kaplan et al (1973). The possible influence of PB on neurotoxicity test results, the lack of dramatic decreases in neural tissue concentrations of ACMD (as ^{14}C), modification of the rotorod test system described by Kaplan and Murphy (1972), and the use of small numbers of animals (n=5) per test group, may explain our inability to reproduce the results of the study cited above.

While these findings do not provide strong support for the hypothesis that tissue concentration of ACMD plays a role in the onset of neurotoxicity, they do not disprove it, leaving the area open for investigation in other experimental systems.

APPENDIX A

Physical and Chemical Properties of Acrylamide

Molecular formula	$CH_2 = CHCONH_2$
Formula weight	71.08
Melting point	84.5 $\pm$0.3°C
Vapor pressure	0.007 mmHg at 25°C
Appearance	White crystalline solid
Boiling point (at 25 mmHg)	125°C
Density	1.122 g/ml at 30°C
Solubility in g/100 ml of solvent at 30°C:	
acetone	63.1
chloroform	2.66
ethanol	86.2
methanol	155
water	215.5

Copied from USDHEW, 1974.

APPENDIX B

Calculations

Percent of Dose in Blood, Plasma, Excreta, and Tissues

$$\text{Percent of Dose} = \frac{\dfrac{^{14}C \text{ in aliquot (dpm)}}{\text{weight of aliquot (gm)}} \times \text{total tissue weight}}{\text{Total } ^{14}C \text{ in dose(s) given (dpm)}} \times 100$$

Concentration of ACMD (as ^{14}C) in Blood, Plasma, and Tissues

$$\text{Concentration of ACMD (nmoles/gm)} = \frac{^{14}C \text{ in aliquot (dpm)}}{\text{weight of aliquot (gm)}} \times \text{Specific activity of the dose (nmoles/dpm)}$$

Percent of Dose as Parent in Blood

$$\text{Extraction Efficiency} = \frac{^{14}C \text{ content (dpm) per ml of blood} - ^{14}C \text{ content (dpm) of extract from 1 ml of blood}}{^{14}C \text{ content (dpm) per ml of blood}} \times 100$$

$$\text{Percent of Dose as Parent} = \text{Extraction Efficiency} \times \frac{^{14}C \text{ in extract (dpm)} - ^{14}C \text{ as parent (dpm)}}{^{14}C \text{ in extract (dpm)}} \times 100$$

Percent of Dose as Parent in Urine

$$\text{Percent of Dose as Parent} = \frac{^{14}C \text{ in diluted urine (dpm)} - ^{14}C \text{ as parent (dpm)}}{^{14}C \text{ in diluted urine (dpm)}} \times 100$$

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