ACUTE AND SUBACUTE METHYLMERCURY TOXICOSIS IN THE RAT

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ABSTRACT

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By

Sheldon S. Diamond

A series of experiments was performed to ascertain the acute and subacute effects of methylmercury dicyandiamide (MMD) in the rat. The LD_{50} dose of MMD was used for the acute study and was 1.335 mg/100 g of body weight. Weekly sublethal doses of 1.0 mg/100 g of body weight were used in the subacute study.

Clinical signs which could be attributed directly to methylmercury toxicosis were not observed in the acute study. Deaths occurred within 72 to 78 hours postinoculation (PI) in this phase. Only a small percentage of the rats in the subacute study had overt clinical signs of a nervous system disorder. Emaciation and/or loss of weight was a constant finding in those rats that died in both studies. No significant gross lesions were found at necropsy.

The primary microscopic lesions were found in the nervous system, spleen and liver. Two changes were observed in the cerebrum and spinal cord in both the acute and subacute studies. Patchy areas of neuronal degeneration occurred in all levels of the cerebrum, hippocampus and spinal cord. Areas of fibrinoid necrosis of the capillary walls, a heretofore unreported lesion in the rat, were also seen with a patchy

distribution. An apparent relationship existed between these lesions. Increased severity of the vascular lesions was always accompanied by severe neuronal changes. This relationship, plus the patchy distribution, suggested that the vascular lesions were responsible for the neuronal changes. The increased permeability of the capillaries may allow the methylmercury radical to escape from the blood stream which would lead to neuronal degeneration by direct contact. A progressive regression of these lesions in the acute study suggested that they were reversible.

Demyelination was observed in the sciatic nerve of the rats that were necropsied during the first week of the acute study. The nerves of the rats necropsied thereafter were normal. Severe demyelination was present in the nerves of the rats in the subacute study. Demyelination of peripheral nerves is probably an early change that either becomes more severe as methylmercury accumulates in the body, or reverts to normal if there is no more exposure to the compound.

Rats affected subacutely had a loss of cells in the granular layer of the cerebellum, which probably represents a late change.

Lymphoid depletion of the spleen was observed in both studies, but the cause could not be ascertained in these experiments. Atrophy of the hepatocytes was observed in the subacutely affected rats, and may have been due to the direct action of methylmercury on the liver cells.

A correlation between the concentration of mercury in the tissues and the presence and severity of lesions could not be made.

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Sheldon S. Diamond

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Organomercurial compounds have been widely used for some time. They have been used in agriculture as a fungicidal seed dressing; by the paper industry to prevent slime formation; in the textile industry for prevention of mildew; and as catalysts in chemical processes. The most commonly used organomercurial compounds today are those belonging to the alkyl group. This is unfortunate, since these compounds have also been found to be the most toxic of the organic mercury group. The toxicity of the organomercurials has been well documented in England, Sweden and Japan. Interest in organomercurial toxicosis in the United States was prompted by the discovery of high levels of mercury in fish caught in Lake Erie and Lake St. Clair in the spring of 1970, and was fortified by the accidental poisoning of a family in New Mexico.

The outbreak of the so-called Minamata disease in Japan in 1953 was the stimulus for extensive studies of alkyl mercury toxicosis. It is now believed that the toxicosis is primarily a neurological disease affecting many and varied species, including man. Most of the reports are principally concerned with the chronic or long-term intake of methylmercury compounds. There is a paucity of information on the acute effects of these compounds. Reports of as basic a study as LD_{50} determinations for the methylmercury compounds are also lacking.

This investigation was designed to fill this void as well as to try to correlate the findings in acute toxicosis with those of a more chronic nature. It was designed also to see what, if any, correlation can be

made between concentration of mercury in tissues and the histopathologic lesions.

The rat was selected as the test animal because it has been the animal used by most of the present day investigators. Methylmercury dicyandiamide (MMD), which is marketed under the trade name of Panogen, was chosen because this is one of the most widely used compounds in the United States.

REVIEW OF LITERATURE

History

King (1957) stated that there are indications that mercury was known in the 15th and 16th centuries B.C. Mercury compounds were thought to be used as drugs in India, Persia, and Greece in about 400 B.C. Goldwater (1957) reported that the ancient medical writers, such as Hippocrates, Pliny the Elder, and Galen mentioned mercury toxicosis. Arabian physicians used mercurial compounds to treat chronic skin diseases in about 1000 A.D. (Bidstrup, 1964). In 1495, when syphilis spread throughout Europe, mercury and its compounds were used for treatment.

Goldwater (1936) reported on four books dealing with industrial diseases that appeared in the 16th and 17th centuries. Ulrich Ellenbog's book in 1524 stated that mercury vapors were more deadly than the metal itself. Jean Fernel, in 1557, was the first to publish a detailed clinical description of occupational mercury toxicosis. Paracelsus, a physician who was also trained in metallurgy, published an exhaustive monograph on heavy metal poisonings in 1567. In 1656 Samuel Stachausen differentiated between the relative toxicities of many of the heavy metals. King (1957) stated that the toxic and cumulative effects of mercury vapor were well known in Spain where the red sulfide ore, cinnabar, had been mined for about 2,500 years. The 17th century miners of Almadén were granted the shortest working hours in the history of labor because of the toxic effects of the vapor.

Occupational mercury toxicosis by inorganic compounds was rampant in certain trades from the Middle Ages until the early 1900's. Bidstrup (1964) summarized many reports that showed that goldsmiths, gilders and mirror makers, as well as the early scientists Faraday and Pascal, succumbed to mercury's toxic effects. Early physicians, such as Frankland and Duppa in 1863, Edwards in 1865, Prumers in 1870, and Hepp in 1887, were aware of the central nervous system involvement (Hunter, Bomford and Russell, 1940; Swensson, 1952). Erethism and a marked tremor associated with mercury toxicosis were commonly seen in people manufacturing felt hats and gave rise to the terms "hatters' shakes" in England and "Danbury shakes" in the United States. The erethism probably was the reason for the phrase "mad as a hatter", and was immortalized in Lewis Carroll's character, "The Mad Hatter" in the story of "Alice in Wonderland" (Bidstrup, 1964). Bidstrup also added the interesting historical fact that, until the advent of the barbiturates, the drug of choice for suicide had been bichloride of mercury.

The organomercurial compounds have been used for some time. Hunter *et al.* (1940) stated that they have been used in chemical research as early as 1863, in therapeutics since 1887, and as a fungicidal seed dressing since 1914. These compounds are also used by the paper industry to stop slime formation, in the textile industry to prevent mildew, and as catalysts in chemical processes. Hunter and his group described organomercurial toxicosis in four men working in a seed treating factory in 1940. Despite their warnings of the toxicity of the methylmercury compounds, further deaths due to these compounds have occurred. Ahlmark (1948) reported on five cases of methylmercury toxicosis in Sweden. In a review of organomercurial toxicosis in 1952, Swensson tabulated 32 cases occurring between 1865 and 1951 in Canada, England, Germany, and Sweden. Over 100 people living in the Minamata Bay area of Japan were

poisoned by mercury between the years 1953 and 1966. A similar outbreak of mercury poisoning occurred in 1964 in the Niigata area of Japan (Nose, 1969; Löfroth, 1970). Deaths and poisonings from organomercurial compounds have also been reported by the press in Iraq, West Pakistan and Guatemala. In the United States, a family in New Mexico was poisoned late in 1969 because they ate meat from pigs that were fed grain which had been treated with organomercurial fungicides.

General Considerations

Swensson and Ulfvarson (1963) reported that the properties of the organomercurial compounds depend mainly on the organic radical. The anion usually has no effect on the metabolism of the compound within the body. They found that mercuric chloride was more toxic than the alkylmercury compounds when given in a single dose, regardless of the route of administration. Berlin (1967) stated that a variation in toxicity between different organomercurial compounds exists. Swensson (1952) confirmed the suspicions of Hunter *et al.* (1940) in his investigation of the relative toxicity of the different organomercurials. He found that the alkyl group was the most toxic.

Swensson (1967) reported that methylmercury compounds are rapidly absorbed through the lungs and gastrointestinal tract but slowly absorbed through the skin. Swensson and Ulfvarson (1963) postulated that most occupational poisonings were caused by inhalation. The accidental toxicoses occurring at Minamata and Niigata were caused by eating fish that had been contaminated by methylmercury in the effluent from a chemical plant (Irukayama, 1966; Nose, 1969). Poisonings have also been produced by the therapeutic use of methylmercury thioacetamide for fungal diseases of the skin (Suzuki and Yoshino, 1969). In a review of mercury toxicosis,

Brown, Jose and Kulkarni (1967) stated that the route of administration of mercury compounds was immaterial, since mercury which entered the body as a vapor behaved in the same way as did injected mercury.

Berlin (1967) stated that the alkylmercury compounds behaved differently from the other organomercurials. Gage (1964) found that methylmercury dicyandiamide (MMD) was rapidly absorbed from the site of inoculation and high levels were found in the erythrocytes. Aberg (1969) confirmed this finding, using ^{203}Hg-labeled methylmercury nitrate. Swensson and Ulfvarson (1963) observed that organomercurials are bound to the erythrocyte, whereas inorganic mercury salts are bound to the plasma proteins. Swensson (1952) observed that rats given repeated intraperitoneal injections had a peritonitis and adhesions developed within the peritoneal cavity.

In a three-part study on mice, Berlin and Ullberg (1963a,b,c) found that inorganic mercury and organomercurials, with the exception of the alkyl group, accumulated in the kidneys, liver and intestines. They observed that the arylmercury compounds also accumulated in the muscles and were retained there for a longer period of time. Alkylmercury compounds are uniformly distributed and taken up by all tissues. They do not accumulate in the kidneys in as high concentration as do the other mercury compounds. The highest levels are found in the liver, the next highest level in the kidney and less in the intestines (Berlin and Ullberg, 1963c; Swensson and Ulfvarson, 1963). Swensson (1952), using MMD in rabbits, found high concentrations in the brain, which remained high for at least 44 days. The content was about the same for the cerebrum and cerebellum, but was lower in the brain stem and spinal cord (Swensson and Ulfvarson, 1963). A similar pattern of distribution of methylmercury was reported in 1961 by Morikawa in cats, and by Hanko

et al. in 1970 in ferrets. Hanko *et al.* (1970) also found high levels of mercury in the skeletal muscles of ferrets. Morikawa (1961b) and Nonaka (1969) found that alkylmercury readily passes through the placenta in cats and rats, respectively. Löfroth (1970) concluded that the human fetus concentrates the mercury and may have a heavier accumulation than its mother.

Swensson and Ulfvarson (1963) found that the alkylmercury compounds are excreted more slowly than are the other mercurials. Excretion of the other compounds starts almost immediately, and one-third is excreted in the urine; the remainder is excreted in the feces. The alkylmercury compounds have only a small fraction excreted in the urine, 85 to 95% being eliminated in the feces. This is done slowly and, therefore, they concluded that the tendency for accumulation in the body was more pronounced with the alkyl compounds. Löfroth (1970), in quoting several investigators, reported that the biological half-life of methylmercury in man is 70 days. Swensson (1967) stated that the half-life in animals is approximately 15 days.

Löfroth (1970) concluded that methylmercury pollution of the environment is a hazard to all living systems. He also stated that the use of mercury compounds in agriculture, industry and community activities results in ever increasing mercury concentrations. He found that an additional source of these compounds has been found in nature, where inorganic mercury is methylated by anaerobic bacteria. Quoting from the work of Westöö, he reported that liver homogenates have been found to methylate inorganic mercury. Ui (1969) reported that soil bacteria are able to methylate inorganic mercury compounds. Hanko *et al.* (1970) and Ulfvarson (1969) observed that the alkylmercury compounds pass through the food chain without a change in their chemical characteristics. They

also found that animals fed contaminated meat seemed to concentrate the methylmercury, probably due to the slow excretion of the compounds. Berlin and Ullberg (1963c) concluded that the mercury was held intracellularly, and the methylmercury radical remained stable. Nonaka (1969), quoting Miyakawa (1968), and Brown and Yoshida (1965) pointed out that there is an interference with protein synthesis in the brain with organomercurials. Bidstrup (1964) stated that the organomercurials have an affinity for thiol groups and probably produce their toxic effects by interfering with enzyme systems.

Clinical Signs of Methylmercury Toxicosis

Most of the articles dealing with naturally occurring or experimentally induced mercurial toxicosis are concerned with the chronic manifestations of these toxicoses.

The dominating clinical features of the disease are the neurologic signs. They were first reported by Edwards in 1865. Hunter *et al.* (1940) published an extensive description of the clinical signs and symptoms that they found in four cases of methylmercury toxicosis that still stands as the classic description. They found that their patients lacked the signs and symptoms associated with inorganic mercury poisoning, with the exception of tremor. All of their patients showed severe generalized ataxia, dysarthria and abnormal plantar extensor response. One of the most striking findings was a gross constriction of the visual fields even though memory and intelligence were unaffected. Auditory and speech disorders were also observed. Hunter and Russell reiterated these findings in another report in 1954. Identical symptoms appeared in the patients suffering from the so-called Minamata disease, and this led the investigators in 1956 to look for a source of methylmercury (Takeuchi

et al. (1962). McAlpine and Araki (1958), working with Takeuchi and his associates, found that the disease affected the peripheral nervous system as well as the cerebellum, hearing and vision. It affected the pyramidal tracts less frequently. Tokuomi *et al.* (1961) found that one of the early symptoms of Minamata disease was a numbness of the distal extremities, tongue and lips. They observed two forms of nervous changes superimposed on those already reported in chronic cases. One led to psychic excitement or motor irritability, with generalized convulsions. In the other, the extremities gradually became fixed by contracture, which restricted active and passive movements. Miyakawa *et al.* (1970) reported that polyneuritis is a finding in this disorder. Quoting Tsubaki, they stated that sensory disturbances are among the first symptoms of organomercurial toxicosis.

Methylmercury toxicosis in animals follows a similar course. McIntee (1950) stated that the main signs seen in pigs that had been fed mercury-treated grain were glossopharyngeal paralysis, blindness and ataxia. Piper, Miller and Dickinson (1971) produced methylmercury toxicosis experimentally in pigs and they reported central nervous system disturbances. Morikawa (1961a) and Irukayama (1966), working with cats, observed essentially the same signs as found in man. Morikawa (1961b) was able to produce congenital cerebellar hypoplasia in kittens born from mothers given subsymptomatic doses of methylmercury. Nonaka (1969) stated that the cat is more susceptible to methylmercurialism than is the rat. Hanko *et al.* (1970) reported ataxia, paralysis and apathy in their experiments with ferrets. In 1966, Irukayama reported that fish-eating birds in the Minamata Bay area were also affected by methylmercury. A similar finding in the seed-eating birds in Sweden in the 1950's was summarized by Löfroth (1970).

Rats have been the most extensively used laboratory animal in the study of methylmercury toxicosis. Hunter *et al.* (1940) used rats and one monkey to confirm their diagnosis. These animals had similar signs to those seen in the human patients, such as severe ataxia, loss of sense of position and muscular incoordination. A short latent period was observed before signs developed. They found that the monkey had a more severe reaction to a lower dosage than did the rats, and they concluded that primates are more susceptible to methylmercury than are rats. Irukayama (1966), after feeding 6 to 13 mg of methylmercury to rats, observed weight loss, disabled gait and extended hind limbs. Swensson (1967) reported that animals given a lethal dose of alkylmercury showed no signs for a latent period of 1 to 2 weeks, and then had poor coordination. In chronic studies, the same central nervous system disturbances reported elsewhere were also found by Swensson (1967). Lehotzky and Bordás (1968) reported that rats given methoxyethylmercury chloride had tremor, ataxia and occasional palsy of the hind limbs. Steinwall and Olsson (1969), using MMD intravenously, reported the rats were drowsy but mentioned no other symptoms. Miyakawa *et al.* (1970), using methylmercury sulfide, observed that their rats began losing weight after the ninth day. The rats' hair tended to stand up, and they became inactive, irritable and anorectic. Ataxia developed slowly and was seen first in the hind limbs; sometimes it involved the forelimbs. Sera, Murakami and Sera (1961) and Suzuki (1969) found identical signs in mice.

Pathologic Changes Due to Methylmercury Toxicosis

The gross lesions seen at necropsy in man are not striking. Takeuchi *et al.* (1962), reporting on 18 cases, found emaciation and swelling of the brain in patients with chronic toxicosis. They found

no gross lesions in those people who died acutely. Hunter *et al.* (1940) reported emaciation in their experimental rats. Hanko *et al.* (1970), using ferrets, observed loss of weight with decrease in body fat. They also reported that the liver and kidneys were pale, and that muscular atrophy was present.

Histologically, the most striking findings in man were present in the nervous system and corresponded to the clinical signs of the patients. There was neuronal degeneration in the granular layer of the cerebellum and a selective destruction of neurons in the visual cortex, especially at the anterior end of the calcarine fissure (Hunter and Russell, 1954; McAlpine and Araki, 1958; Takeuchi *et al.*, 1962). Irukayama (1966) reported that there was a loss of Purkinje cells in the cerebellum in severe cases. Cavanagh (1969) stated that neuronal degeneration was seen elsewhere in the brain and changes were present in the long spinal tracts. He also reported that the changes in the peripheral nerves were quite striking, with degeneration occurring in sensory nerves only, ascending as far as the dorsal roots. Miyakawa *et al.* (1970) observed that organic mercury first affects the peripheral nervous system. The pathologic changes are selectively seen in sensory nerve fibers and consist of swelling and degeneration of Schwann cells and changes in both the myelin sheaths and axons, which tend to begin at the nodes of Ranvier. They speculated that sensory disturbances might be the only changes seen in some cases of organomercurial toxicosis.

The microscopic picture in animals, like that in man, is also dominated by changes in the nervous system. Hunter *et al.* (1940) reported that rats showed severe degeneration in the peripheral nerves, dorsal spinal roots and the trigeminal nerves. Early degeneration of the myelin sheaths consisted of fragmentation of the myelin into various sizes of

ovoid, globular masses. They also found that the nerves of the hind limbs were more severely affected than those of the forelimbs. Later changes observed were degeneration of the dorsal spinal tracts and patchy degeneration of the granular cells in the middle lobe of the cerebellum. They stated that no cerebral cortical changes were seen. Swensson (1952) reported degeneration of Purkinje cells, as well as of granular cells, in the cerebellum. He stated that no degeneration of myelin was present and concluded that this was due to the acute nature of the study. Morikawa (1961) observed changes in the cat that were similar to those described in man. He also reported that a slight thickening of the walls of blood vessels was present. He produced cerebellar atrophy in kittens borne of queens that had been fed methylmercury. This change in the kittens was restricted to the granular cell layer. Takeuchi *et al.* (1962), working with rats, mice and cats, found that the major changes were in the granular layer of the cerebellum and in neurons of the cerebral cortex. They stated that the neurons disappeared early in the disease following disintegration. They described vacuolar degeneration of neurons as being more frequent in the rat and mouse. Steinwall and Olsson (1969) demonstrated disturbed permeability of cerebral blood vessels in a study using rats. No lesions of the vessels were reported. Miyakawa *et al.* (1970) discussed the changes found in the peripheral nerves of rats. They observed hypertrophic, deeply staining Schwann cells after eight days. Later, the myelin sheaths became hypertrophic and irregular in shape. Proliferation of fibroblasts and macrophages was also observed. Hanko *et al.* (1970) found vacuolation and partial to complete disappearance of the myelin in the peripheral nerves of the ferret. They stated that slight gliosis and neuronal degeneration were present in the cerebral cortex. In 1968, Tryphonas and Nielsen reported that the vessels of the

cerebrum underwent endothelial proliferation, fibrinoid necrosis and scarring, with thickening of the walls in pigs given alkylmercury compounds.

Microscopic lesions of other organs were inconclusive. Cloudy swelling and fatty change of the epithelial cells of the proximal tubules in the kidney was one of the consistent findings (Hunter *et al.*, 1940; Morikawa, 1961; Hanko *et al.*, 1970). Hanko and his associates (1970) also found hypoplasia of lymphatic tissue of the spleen in their ferrets.

MATERIALS AND METHODS

A four-phase study was undertaken to investigate the acute and chronic effects of methylmercury dicyandiamide (MMD) administered intraperitoneally to rats. Male Sprague-Dawley rats (Spartan Research Animals, Inc., Haslett, Michigan, 48849) weighing approximately 100 g each were housed in standard metal cages, 7 to 8 rats per cage, except in Phase 2, in which the rats were penned individually. All the rats were given water and commercially pelleted feed *ad libitum*. Panagen^{R} 15 (Morton Chemical Company, 110 N. Wacker Drive, Chicago, Illinois) containing 2.2% MMD was used. The compound was diluted with triple-distilled water so that a dose of not more than 1 ml was injected. The intraperitoneal route was chosen so that a controlled dose could be delivered.

Phase 1. Determination of the LD_{50} for MMD

Eight groups of 7 or 8 rats each were given doses of 0.5, 1.0, 1.25, 1.5, 1.75, 2.5, 5.0 or 25.0 mg/100 g of body weight intraperitoneally. The injections were made halfway between the umbilicus and the prepuce and 5 mm to the right of the midline. The animals were observed for 1 week and the LD_{50} was calculated using the method of Reed and Muench (1938).

Phase 2. Study of the Acute Effects of MMD

Twenty rats were each given the calculated LD_{50} dose, using the same technique as in Phase 1. Six rats were injected with triple-distilled water in the same manner as the other rats, and they served as controls.

The test animals were examined at 2- to 3-hour intervals each day for clinical signs of methylmercury toxicosis and weighed once each day.

The general plan in this phase was to necropsy all rats that died acutely at or soon after the time of their death. One surviving test animal and 1 control animal were also killed at this time. One quarter of the remaining rats and 1 control rat were euthanatized with ether at weekly intervals. A system of random selection was used to decide the order in which the rats were to be euthanatized. Necropsies were performed on each animal, and tissues from the stomach, colon, kidneys, lungs, liver, spleen, right vastus medialis and rectus femoris muscles, adrenal gland, pancreas, submandibular salivary gland, lymph node, heart, thymus, testicle, brain, spinal cord and right sciatic nerve were saved in 10% buffered formalin for histopathologic examination.

Tissue preparation and staining were performed according to commonly accepted methods, as outlined in the Armed Forces Institute of Pathology Manual (1967). Hematoxylin and eosin were routinely used in the staining process. Sections of nerve, spinal cord and brain were also submitted to luxol fast blue stain, Gomori's trichrome stain and the periodic acid-Schiff (PAS) reaction. Several frozen sections of liver were stained with oil red O. The colon, kidney, liver, brain, left sciatic nerve and muscles of the hind limbs were frozen and saved for Phase 4.

Phase 3. Study of the Effects of Multiple Sublethal Doses of MMD

Fifteen rats were each given 1.0 mg/100 g of body weight intraperitoneally at weekly intervals using the same technique as in the above studies. These animals were examined twice daily for clinical signs of methylmercury toxicosis. Necropsies were performed on those animals that

died during the experiment in the same manner as in Phase 2 and tissues were saved. At the end of 8 weeks all surviving animals were euthanatized and necropsied.

Phase 4. Determination of Mercury Levels in Tissues

Frozen sections of colon, kidney, liver, brain, muscles and left sciatic nerve saved from Phases 2 and 3 were used. Mercury concentrations were determined by atomic absorption, following the mercury residue analysis procedure by M. Zabik, Pesticide Research Department, Michigan State University, East Lansing, Michigan.

RESULTS

Phase 1. Determination of the LD_{50} for MMD

The rats given doses of 25.0, 5.0 or 2.5 mg MMD/100 g of body weight all died within 12 hours of the intraperitoneal injections. Within minutes of the injection, all were reluctant to move, had ruffled hair, distended abdomens, closed eyes and rapid respirations.

The groups given 1.75, 1.5 or 1.25 mg MMD/100 g of body weight had mortality rates of 5/7, 6/7 and 4/8, respectively. These rats all had the same signs as seen in the other groups. The rats that survived these doses appeared to be normal within 24 hours. The rats that did not survive had the clinical signs until they died. The majority of the deaths occurred between 24 and 52 hours postinoculation (PI).

The rats given 0.5 or 1.0 mg MMD/100 g of body weight all survived. Some of these animals showed an unsteady gait and ruffled hair coat for the first 4 hours PI.

The LD_{50} dose of 1.335 mg MMD/100 g of body weight was calculated by the method of Reed and Muench (1938).

Phase 2. Acute Effects of MMD

Clinical Signs. Within one-half hour following injection of the LD_{50} dose, all of the rats receiving MMD were reluctant to move, arched their backs, had ruffled hair coats and distended abdomens. Their respirations were rapid and labored. The control animals behaved normally. By 3 hours PI, 14 of the 20 rats would move if they were disturbed, but the movement

was unsteady. By the third day PI, all but 5 rats appeared to be normal. The 5 abnormal appearing rats still had the same clinical signs that had been present since the beginning of the experiment, with the addition of having an ocular discharge and seeming to be thinner. Four rats died 72 hours PI and 1 died 78 hours PI. The other rats remained asymptomatic throughout the remainder of this phase.

Weight. The daily mean weights are presented in Figure 1. All but 2 rats lost weight within 24 hours PI. By 48 hours, 5 rats were gaining weight and, by 96 hours, all surviving rats were gaining weight. The daily mean weight gain of the control rats was greater than that of the test rats, with a mean difference of 47.8 g between the 2 groups at the end of the experiment.

Gross Necropsy Findings. The 5 rats that died presented similar gross findings. They all appeared thin, had ruffled hair coats and distended abdomens. Subcutaneous fat was absent. The peritoneum was grayish brown and thickened. The stomach contained a light brown fluid and the intestines had a greenish brown fluid in the lumen. The serosal and mesenteric vessels were prominent. Two rats had several reddish purple pin-point spots on the serosa of the intestines. In contrast to these 5 rats, the test rat that was euthanatized had a clear, transparent peritoneum and normal contents in the digestive tract. This rat had no subcutaneous fat and its liver was pale tan-brown. The control rat killed at the same time was well fleshed and had subcutaneous fat. No gross lesions were noted in this rat.

Three rats were killed 1 week PI. They all appeared to be well fleshed and had small amounts of subcutaneous fat. No recognizable gross lesions were present. The control rat was normal.

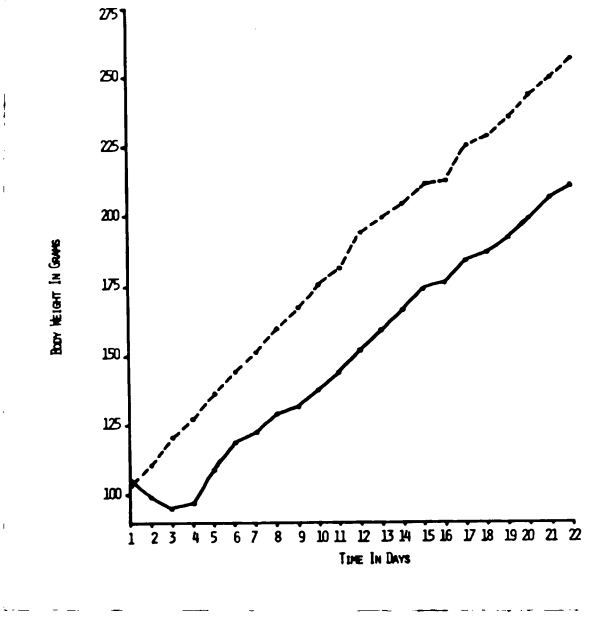

Figure 1. Comparison of the daily mean weights of the test rats (solid line) and control rats (broken line).

The 3 rats euthanatized 2 weeks PI were well fleshed, had normal subcutaneous fat and a small amount of visceral fat. The liver capsule was thickened and dull white, and was adherent to the diaphragm in 2 of the 3 rats. The lungs of 1 rat were congested. The control rat was normal.

Three of the 4 rats killed 3 weeks PI were well fleshed, and subcutaneous and visceral fat was present. The other rat was thin, and fat was absent from the carcass. The liver was tightly adherent to the diaphragm in all 4 rats. The control rat was normal.

The same findings were seen in the 4 rats euthanatized 4 weeks PI as in those euthanatized 3 weeks PI. The control rat was normal.

Microscopic Findings

Brain. Focal areas of neuronal degeneration and vascular changes were present. These changes were most pronounced in the rats that died and in the rat that was euthanatized at the same time.

Neurons in the cortex and hippocampus were undergoing degeneration and necrosis. No consistent pattern could be established. The degeneration occurred in small foci located in all lobes and at all levels throughout the cortex. The most prominent features were a deepening of the basophilia of the cells, and chromatolysis and pyknosis. Satellitosis and neuronophagia could be seen in these areas, along with occasional vacuoles in the cytoplasm of degenerating neurons. The nerve cell bodies in surrounding areas appeared to be unaffected. This change was also present in the rats killed 1 week PI. The extent of the degeneration was greatly diminished by 2 weeks PI, and was limited to a few foci of 4 to 6 neurons within the plane of the section. The lesion was not observed in test rats euthanatized thereafter, or in any of the control rats.

The vascular lesions also had a patchy distribution and appeared to affect capillaries and small arterioles. The severity of the vascular change followed the same grouping as seen in the neuronal degeneration. The perivascular space surrounding the affected vessels was slightly larger than the space around normal appearing vessels. The vessel walls were thickened, appeared smudged, and had roughened borders (Figure 2). The walls contained material that was PAS-positive, and took the collagen stain with Gomori's trichrome stain. In the first group, the nuclei were pyknotic. In the group killed 1 week PI, there were thickened walls as described previously, but the nuclei appeared normal. Only slight changes were seen in the group killed 2 weeks PI. Changes were not seen in the later groups or in the control rats.

Cervical spinal cord. Focal neuronal degeneration similar to that seen in the brain was present in 2 of the rats that died. Demyelination occurred in the ventral and lateral columns in both of these rats.

Sciatic nerve. Early demyelination was present in the sciatic nerves of the 5 rats that died, as well as in the test animal euthanatized at the same time. Rats killed thereafter had no change in the nerve. The affected nerves had swollen myelin sheaths, with small clear globular- to oval-shaped spaces, most noticeably at the nodes of Ranvier (Figures 3 and 4). These areas did not stain for myelin when a luxol fast blue stain was used. The integrity of the axon and Schwann cells appeared intact. Fibroblasts and macrophages were not evident.

Spleen. The normal architecture of the spleen was absent in the rats that died and in the test rat that was euthanatized at this time. The spleens were devoid of lymphoid follicles and an increase in the number

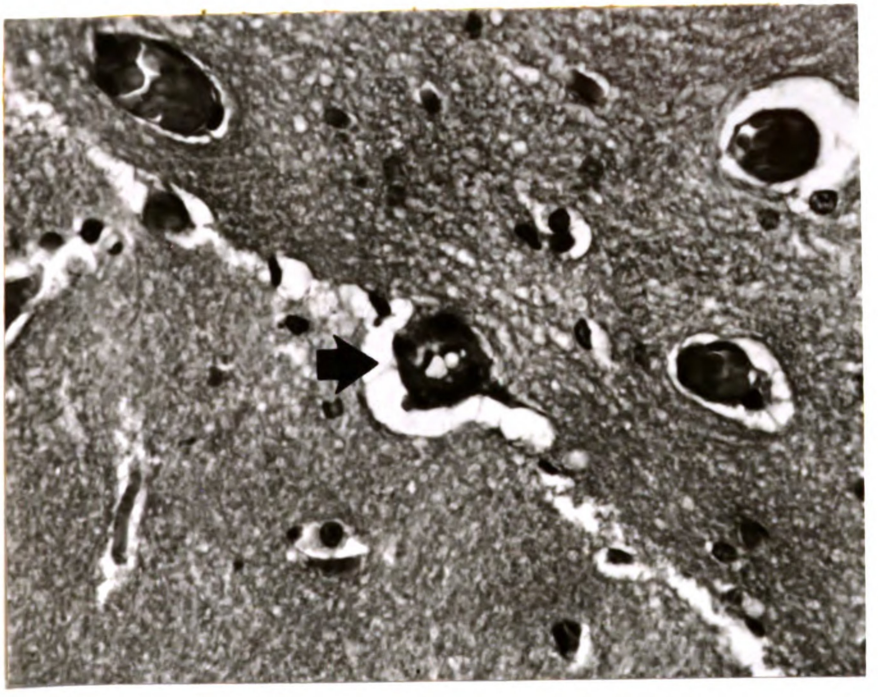

Figure 2. Fibrinoid necrosis of a capillary in the cerebrum (arrow). Normal capillaries are present above the affected vessel. H & E stain. x 560.

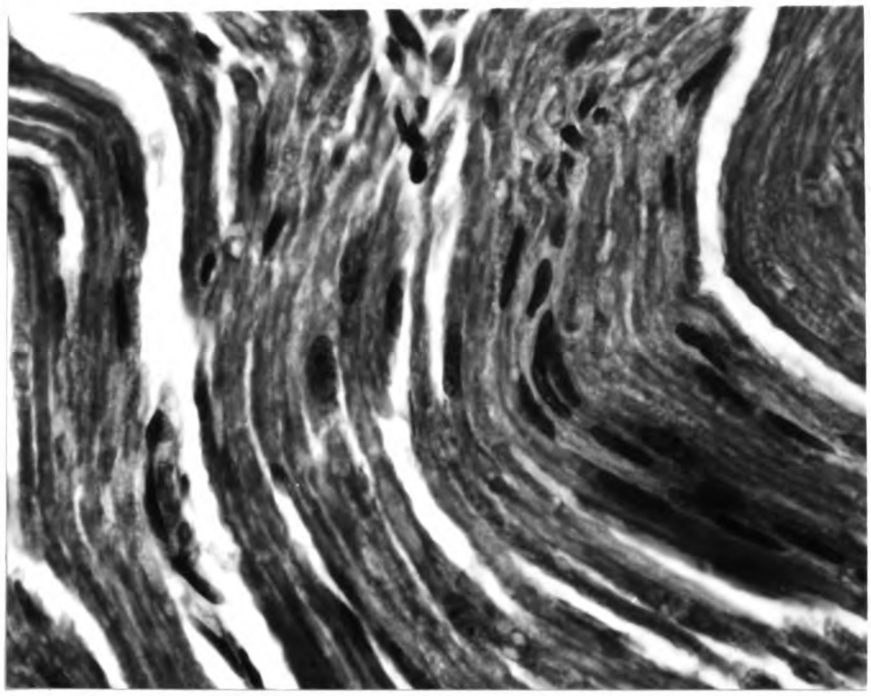

Figure 3. A normal sciatic nerve. H & E stain. x 600.

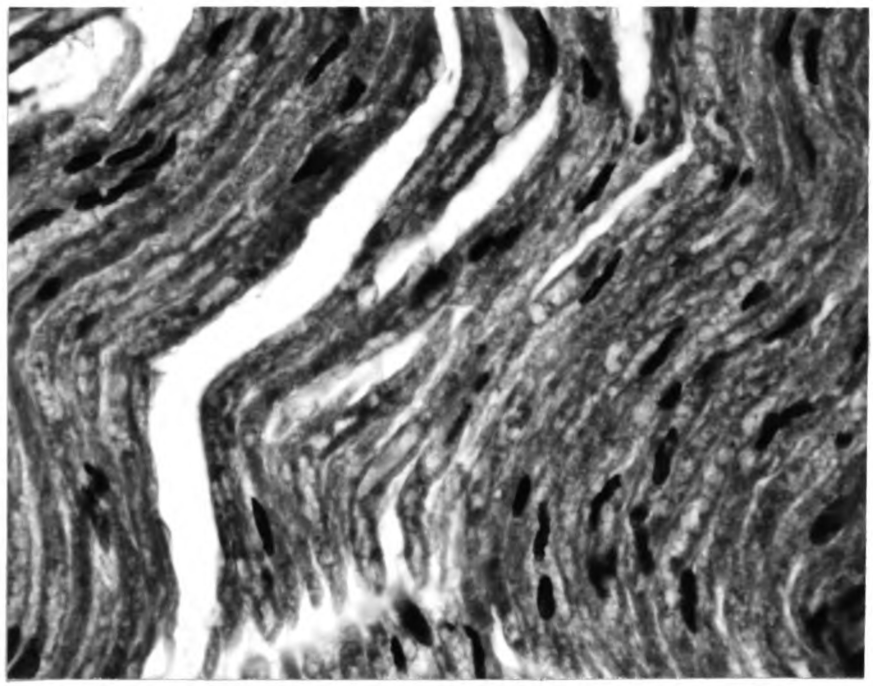

Figure 4. Sciatic nerve from a rat that died 72 hours PI to show early demyelination. H & E stain. x 600.

of reticuloendothelial (RE) cells was present. The 3 rats killed 1 week PI and 2 of the 3 rats killed 2 weeks PI also had lymphoid hypoplasia and RE cell hyperplasia, but to a lesser extent. A few small lymphoid follicles were present in these animals. The other test rats and all the control rats had normal appearing spleens. The lymph nodes and thymus glands in all but 1 rat appeared to be normal. The lymph nodes in this rat had normal follicles, but an increased number of RE cells was present in the medullary sinuses.

Kidneys. All the rats that died and the test rat euthanatized at the same time had a few small foci of vacuolar degeneration present in the epithelial cells of the proximal tubules. Four of the 5 rats that died had focal areas in which the tubular epithelial cells were swollen. Two rats killed 4 weeks PI had regenerating tubular cells.

Liver. All the rats euthanatized from 1 week PI through 4 weeks PI had thickened capsules and adhesions between the liver and diaphragm.

Other organs. Three of the rats that died had focal areas of acute, fibrinous serositis present in the intestinal area near the site of the injection. One rat had a mild, subacute, interstitial pneumonitis. One rat that was euthanatized 1 week PI had a decreased number of cells in the granular layer of the cerebellum in the middle folia.

Phase 3. Subacute Effects of MMD

Clinical Signs. After each of the first 5 weekly injections, most of the rats had ruffled hair coats, were reluctant to move and, when forced to move, had an unsteady gait. Some of the rats had rapid respirations, arched backs and closed eyes. These signs generally abated within 6 hours, and the rats appeared normal.

After the 6th weekly injection, all the rats behaved as described above, and all but 3 appeared normal after 6 hours. The 3 rats continued to show clinical signs, stopped eating and lost weight. Two of these rats continually bumped into objects when forced to move. These animals died 5 days following this inoculation. The third rat died the next day. The mean weight for these animals was 158.9 g, compared with 325 g for the survivors.

One rat had difficulty walking and appeared to bump into objects the day after the 7th weekly injection. On the third day, the rat held the hind legs in a flexed position when still, but could extend them when walking. The rat could not use the hind legs by the fifth day, and dragged itself around the cage by its front legs. The hind limbs were fixed in a flexed position. One other rat was having difficulty walking at this time. By the next day, its hind legs were also flexed. The first rat died in 7 days. The second rat could not extend the hind legs at all on this day, and died that night. The mean weight for these rats was 186.9 g compared with 346 g for the remaining rats.

One rat died 1 day after the eighth weekly injection. One other rat died 5 days PI. Neither of these rats had clinical signs after the 6-hour PI period. The 7 remaining rats were euthanatized 7 days PI. All appeared to be normal. The mean weight for this group was 335.2 g, with a range of 288.8 g to 380 g.

Gross Necropsy Findings. The first 5 rats that died appeared emaciated, and subcutaneous and visceral fat was absent. The remaining rats were well fleshed, and had subcutaneous and visceral fat. All but 2 of the animals had adhesions between the liver and the diaphragm, as well as between the peritoneum and intestines at the site of injection. In all

of the rats the liver appeared smaller than normal, friable, and ranged in color from mahogany to purplish-black. Three rats had large reddish-purple to greenish lesions in the intestinal wall. The peritoneum was thickened in 1 rat. This rat had a metallic blue spleen.

Microscopic Findings

Brain. Neuronal changes similar to those seen in Phase 2 were present in all these rats, but they were more severe. The focal areas of degeneration were larger and there were more areas involved. No pattern was evident. An increase in glial cells was seen and, in some areas, glial nodules occurred. Beside the neuronal degeneration in the hippocampus, a clear area was seen between the neurons and the surrounding neuropil (Figures 5 and 6). The rats had varying degrees of atrophy of the granular layer in the cerebellum. In most cases, this change was restricted to the middle folia but, in 4 rats, the atrophy was observed in all the folia.

The vascular changes were widespread and numerous in the cerebrum, but had a patchy distribution. The capillary walls were greatly thickened and the nuclei of the walls were pyknotic (Figure 7). The non-nuclear material in the walls stained the same as those in Phase 2. The pervivascular space was greatly enlarged.

Cervical spinal cord. Patchy areas of neuronal degeneration and demyelination were present. Some of the capillaries had changes in the walls that were similar to those described in the brain.

Sciatic nerve. Severe demyelination was present in all the rats (Figure 8). The nerves were disrupted, and macrophages, fibroblasts and collagen were present between the nerves. The loss of myelin was seen

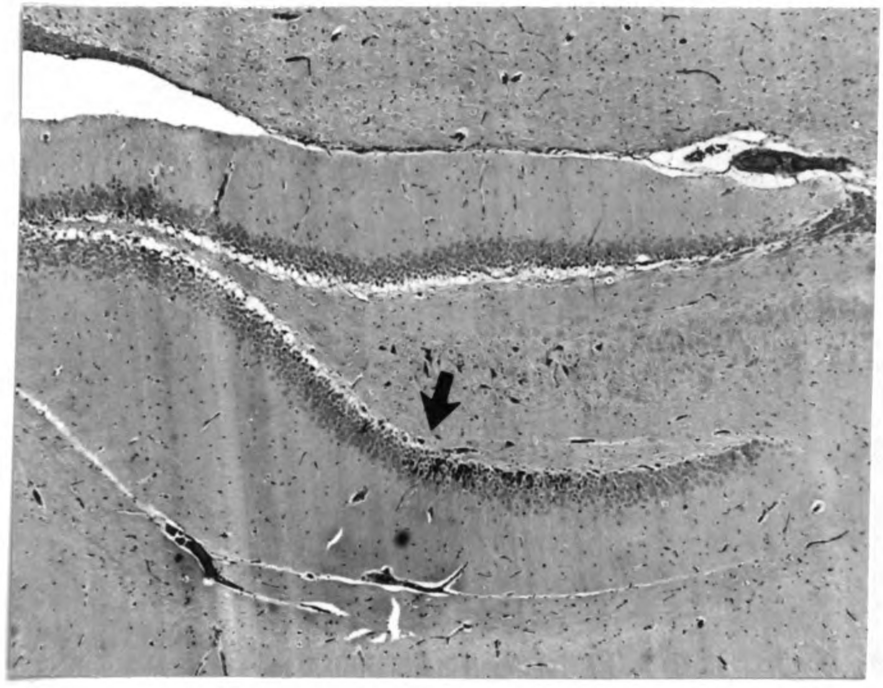

Figure 5. Hippocampus with area of neuronal degeneration and separation of neurons from the surrounding neuropil. H & E stain. x 60.

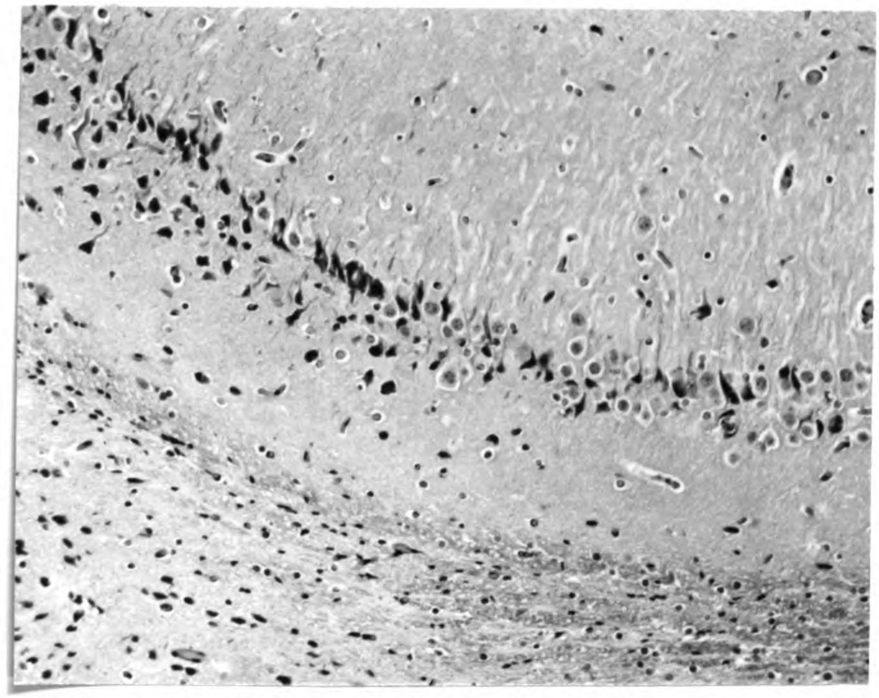

Figure 6. Higher magnification of hippocampus with area of degeneration next to area of normal neurons. H & E stain. x 150.

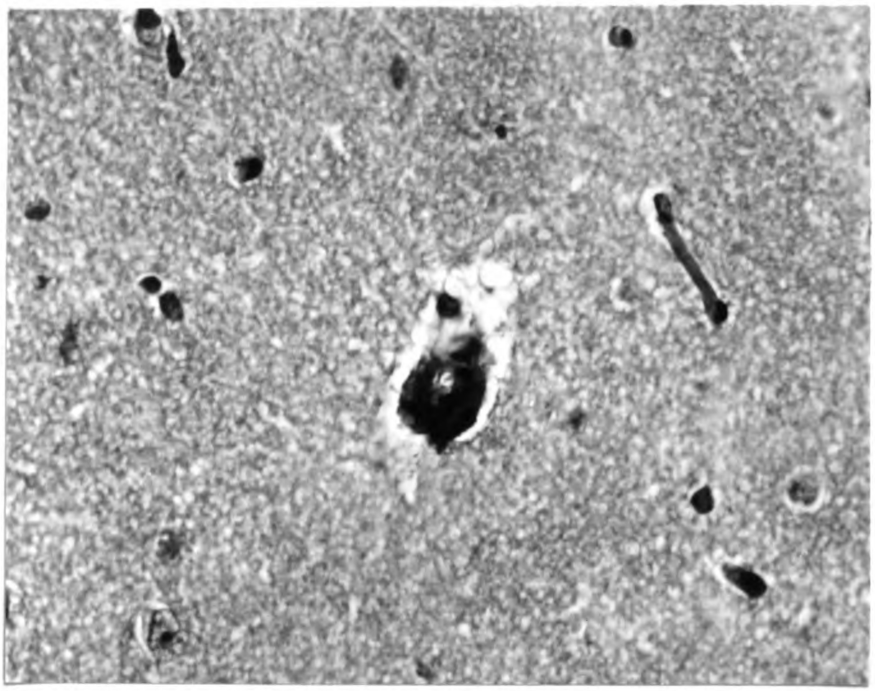

Figure 7. Capillary in cerebrum with fibrinoid necrosis of the wall, pyknotic nuclei and an increased perivascular space. H & E stain. x 560.

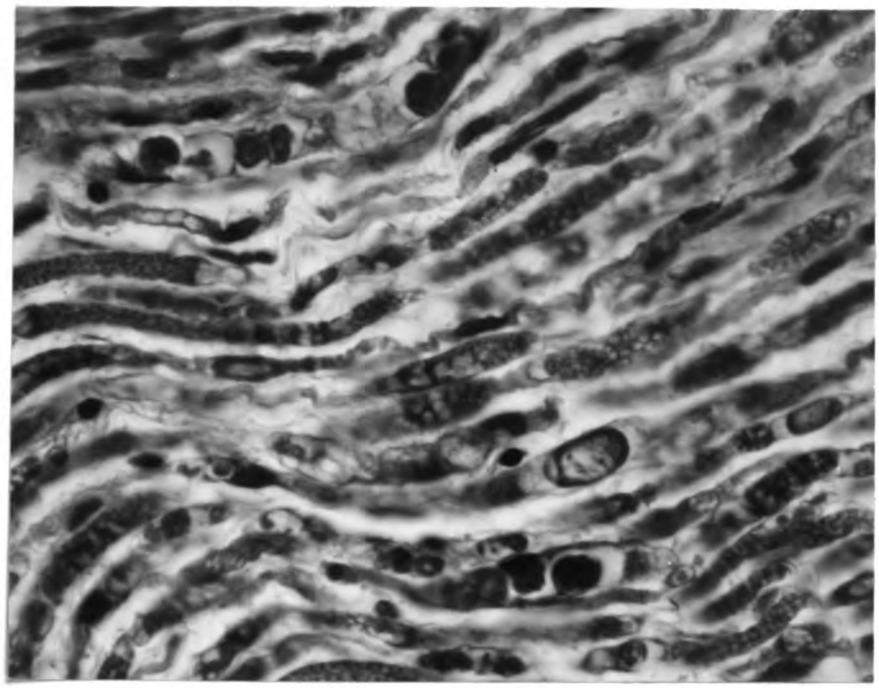

Figure 8. Severe demyelination and fibrous proliferation of a sciatic nerve. H & E stain. x 600.

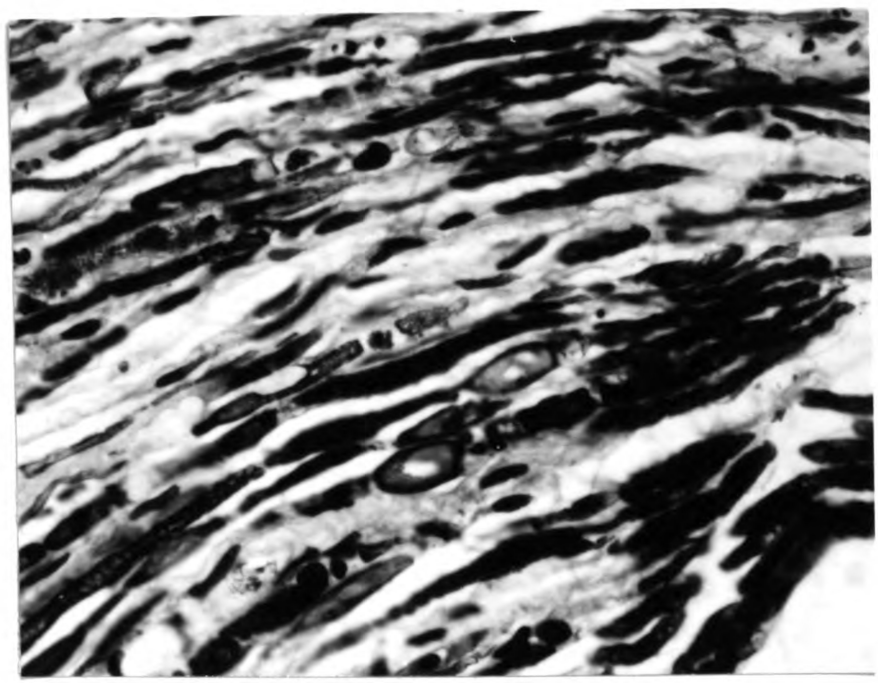

Figure 9. Same nerve stained with the luxol fast blue stain. x 600.

in sections stained with luxol fast blue (Figure 9). The extent of fibrosis was easily seen using Gomori's trichrome stain.

Spleen. A decreased number of lymphoid follicles was evident in the animals that died showing clinical signs. A similar change was present in the lymph nodes of these animals. The rats that died without clinical signs and the rats that were euthanatized had normal appearing spleens and lymph nodes.

Liver. Adhesions between the liver and diaphragm were seen in all but 2 rats. The hepatocytes appeared smaller than normal and more numerous than those of the control rats. An average of 271 hepatocytes was present per high power field in the affected rats, compared with 132 cells per high power field in the controls.

Peritoneum. A diffuse peritonitis was present in all the rats, characterized by macrophages, lymphocytes, plasma cells, fibroblasts and connective tissue formation. Some areas had neutrophils and hemorrhage present. These changes were most noticeable in the mesentery, serosal surface of the intestine, the perirenal area, and around the capsule of the spleen.

Kidney. Mild, patchy swelling and vacuolation of the tubular epithelial cells was present in the rats that died. The other rats had normal appearing tubular cells.

Small intestine. One rat had a large, penetrating, suppurative ulcer extending from the serosal surface through the mucosa.

Phase 4. Mercury Levels in Tissues

Mercury was not detected in the tissues of the rats used as controls. The concentrations of mercury in the tissues of the acutely affected rats and subacutely affected rats are summarized (Tables 1 and 2).

Table 1. Concentrations of mercury in tissues at terminal samplings from rats injected with a single LD_{50} dose of methylmercury

Acute Study	Concentration of Mercury (ppm)					
	Nerve	Brain	Kidney	Liver	Muscle	Colon
72-78 hrs. PI	53.77	10.00	36.12	8.46	12.00	6.77
		18.16	23.44	20.88	4.96	12.37
		---	113.23	56.17	12.03	8.32
	ND*	12.53	16.13	13.67	9.38	1.50
1 wk. PI	10.00	8.13	31.11	12.97	7.63	2.57
		6.88	28.95	6.15	2.12	0.73
2 wks. PI	2.66	2.88	4.23	3.57	2.89	2.38
		1.79	20.27	2.85	2.18	0.92
3 wks. PI	3.08	---	11.09	2.67	1.37	1.58
		3.62	15.65	3.01	0.81	2.51
4 wks. PI	4.17	0.78	8.40	0.87	0.81	0.38
		1.58	9.64	2.23	0.66	3.42
Mean	12.28	6.63	26.52	11.12	4.80	3.62
p value	.25	<.001	.01	.005	.005	.025

*ND = not detectable.

Table 2. Concentrations of mercury in tissues at terminal samplings from rats injected weekly with sublethal doses of methylmercury

Subacute Study	Concentration of Mercury (ppm)					
	Nerve	Brain	Kidney	Liver	Muscle	Colon
	105.00	31.92	170.40	82.32	19.88	40.74
	48.31	17.81	111.02	7.73	18.42	31.36
	53.44	21.17	147.85	80.00	5.41	32.93
Mean	68.92	23.63	143.09	56.68	14.57	35.01
p value	.05-.10	.025-.05	<.001	.10	.005-.01	.10-.20

DISCUSSION

These studies show that the acute and subacute effects of MMD in the rat parallel the findings in the chronic toxicoses documented in the literature review. The acute and subacute diseases primarily affect the nervous system.

The clinical signs observed in both Phase 2 and Phase 3 were of a nonspecific nature. Emaciation and/or loss of weight was a consistent finding in the rats, starting 3 to 4 days before death. The unsteady gait seen in most of the test animals was suggestive, but not diagnostic, of a central nervous system disturbance. In the subacute study (Phase 3), 4 rats showed definite signs of a nervous disorder. Two of the rats continually bumped into objects and 2 had severe flexion of the hind limbs. In the acute study (Phase 2), death occurred within 72 to 78 hours PI. The affected rats were never able to overcome the initial signs that had been present since the injection of MMD. Those animals that survived the LD_{50} dose had no signs of toxicosis except for the early changes which disappeared within 6 hours PI.

The major pathologic lesions in both the acute and subacute phases were seen in the nervous system. The severity of the lesions seen in the cerebrum and spinal cord followed a definite pattern. They were most severe in the subacute experiment. The remission of the lesions to a normal state in the acute study suggested that these changes were reversible. The neuronal and vascular lesions disappeared within 2 to

3 weeks. The changes in the sciatic nerve required only 1 week to revert to normal.

The pattern of severity of the vascular lesions and areas of neuronal degeneration, and their patchy distribution, suggest a relationship between these changes. Steinwall and Olsson (1969) showed an increased permeability of the cerebral blood vessels in their study in rats, but did not report on vascular lesions. The fibrinoid necrosis of the capillaries found in both groups of rats may well be the cause of the breach of the so-called blood-brain barrier. The increased capillary permeability of these vessels may allow the methylmercury radical to escape from the blood stream, leading to neuronal degeneration by direct contact. It becomes clear that this avenue should be explored further.

The lesions of the peripheral nerves and cerebellum in the subacute study were similar to those reported in chronic studies. The sciatic nerve in the acute studies had slight demyelination during the first week PI, confirming the supposition of Miyakawa *et al.* (1970) that the peripheral nerves are affected early in methylmercury toxicosis. Only 1 of the 20 rats in the acute study had a reduction in the number of cells in the granular layer of the cerebellum, which suggested that this lesion is one of the late changes and may appear only with multiple doses of MMD.

The lymphoid depletion and RE hyperplasia observed in the spleen also followed a pattern. Both were most severe in the acutely affected rats that were necropsied during the first week of the study. A gradual increase in lymphoid follicles and a decrease in RE cells occurred until 3 weeks PI, when the spleens appeared to be normal. All of the rats that died showing clinical signs in the subacute study had decreased lymphoid tissue in the spleen. The other rats had normal appearing spleens. This suggested that the change is reversible. Since the changes were most

severe in those rats that died, there may be a relationship between lymphoid depletion and lethality of the compound.

Another phenomenon observed in the subacute study was the atrophy of the hepatocytes. This change may be due to the direct action of the methylmercury radical on the cells as the compound accumulates in the liver.

The changes in the tubules of the kidney were minimal but may also be due to the direct action of methylmercury on the cells.

The peritonitis and adhesions were attributed to the irritating effect of the compound on the peritoneum. The early clinical signs seen in all the rats immediately following the injection of MMD were probably due to the acute peritonitis. The ulcerations present in some of the intestinal walls were attributed to the direct irritating effect of the compound on the tissues. These changes had no direct effect on the studies, and were considered to be a side effect due to the chosen route of administration.

Mercury residues in the tissues of acutely and subacutely affected rats followed the same distribution as reported in chronic studies. The highest concentrations of mercury were found in the kidney and liver. A correlation between the concentration of mercury in the tissues and the presence and severity of lesions could not be made. A progressive decrease in tissue concentration was evident in the acutely affected rats which indicated a slow excretion of mercury. The tissues from the subacutely affected rats had higher concentration of mercury which indicated a tendency for the compound to accumulate in the body with weekly sub-lethal injections. This was also shown in the work of Swensson and Ulfvarson (1963).

The variations of concentrations found within the groups indicate inherent problems in the analytical techniques. The technique calls for using 10 g of tissue for analysis. In the case of the nerves, brain

and kidney, only smaller amounts of tissue were available for analysis. A mathematical factor had to be used in the final calculations for mercury concentrations in order to correct for the lesser amounts of tissue. In those tissues that contained large concentrations of mercury, notably the kidney and liver, a dilution factor had to be used to obtain a reading on the photometer. These factors were in the range of 100 to 200 in some cases, which introduced a large mathematical error.

Weissler (1971) reported that the precision of atomic absorption is only moderate, being in the vicinity of $\pm$20%. In a review of analytical tests, he described a method used in Sweden that utilized 1 g of tissue. This technique would eliminate some of the mathematical errors found in this experiment. He also described a method of measuring methylmercury by gas chromatography. The reliability of this test, however, is also in the vicinity of $\pm$20%.

SUMMARY AND CONCLUSIONS

A series of experiments was performed to ascertain the acute and subacute effects of MMD in the rat. The LD_{50} dose of MMD was used for the acute study and was 1.335 mg/100 g of body weight. Weekly sublethal doses of 1.0 mg/100 g of body weight were used in the subacute study.

Clinical signs which could be attributed directly to methylmercury toxicosis were not observed in the acute study. Deaths occurred within 72 to 78 hours PI in this phase. Only a small percentage of the rats in the subacute study had overt clinical signs of a nervous system disorder. Emaciation and/or loss of weight was a consistent finding in those rats that died in both studies. No significant gross lesions were found at necropsy.

The primary microscopic lesions were found in the nervous system, spleen and liver. Two changes were observed in the cerebrum and spinal cord in both the acute and subacute studies. Patchy areas of neuronal degeneration occurred in all levels of the cerebrum, hippocampus and spinal cord. Areas of fibrinoid necrosis of the capillary walls, a heretofore unreported lesion in the rat, were also seen with a patchy distribution. An apparent relationship existed between these lesions. Increased severity of the vascular lesions was always accompanied by severe neuronal changes. This relationship, plus the patchy distribution, suggested that the vascular lesions were responsible for the neuronal changes. The increased permeability of the capillaries may allow the methylmercury radical to escape from the blood stream which would lead

to neuronal degeneration by direct contact. A progressive regression of these lesions in the acute study suggested that they were reversible.

Demyelination was observed in the sciatic nerve of the rats that were necropsied during the first week of the acute study. The nerves of the rats necropsied thereafter were normal. Severe demyelination was present in the nerves of the rats in the subacute study. Demyelination of peripheral nerves is probably an early change that either becomes more severe as methylmercury accumulates in the body, or reverts to normal if there is no more exposure to the compound.

Rats affected subacutely had a loss of cells in the granular layer of the cerebellum, which probably represents a late change.

Lymphoid depletion of the spleen was observed in both studies, but the cause could not be ascertained in these experiments. Atrophy of the hepatocytes was observed in the subacutely affected rats, and may have been due to the direct action of methylmercury on the liver cells.

A correlation between the concentration of mercury in the tissues and the presence and severity of lesions could not be made.

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VITA

Sheldon S. Diamond was born January 24, 1934, in Philadelphia, Pennsylvania.

He graduated from the School of Veterinary Medicine, University of Pennsylvania, in 1958. Following graduation, he entered the Air Force and served as Base Veterinarian at Griffiss Air Force Base in Rome, New York. He separated from the Air Force in 1960, and engaged in private practice for 3 years in Toms River, New Jersey.

He was recalled to active duty in the Air Force in 1963 and served as Assistant Base Veterinarian at Elmendorf Air Force Base in Alaska and as Chief, Veterinary Services, at Oxnard Air Force Base in California.

Prior to entering graduate training at Michigan State University, he completed a 2 year residency in Veterinary Pathology at the Armed Forces Institute of Pathology in Washington, D.C.

He has co-authored 2 papers on the treatment of *Dirofilaria immitis* in dogs.

He is married to the former Natalie Mitnik of Philadelphia, and is the father of 3 children, Cindy, Ellen and David.