Pathogenesis of Trimethyltin Neuronal Toxicity

Ultrastructural and Cytochemical Observations

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The ultrastructural cytopathologic and cytochemical effects of trimethyltin (TMT) neurotoxicity were delineated in hippocampal and pyriform neurons of acutely intoxicated adult rats. TMT produced neuronal necrosis that preferentially involved hippocampal formation and pyriform cortex. The first subcellular alterations were multifocal collections of dense-cored vesicles and tubules and membrane-delimited vacuoles in the cytoplasm of the perikaryon and proximal dendrite. Ultrastructural cytochemical examination revealed that the vesicles and tubules had acid phosphatase activity analagous to Golgi-associated endoplasmic reticulum (GERL). Shortly after the appearance of the GERL-like vesicles and tubules, autophagic vacuoles and polymorphic dense bodies accumulated in the neuronal cytoplasm. Some dense bodies appeared to arise from the dense-cored tubules. Neuronal necrosis was characterized by increased electron density of the cytoplasm and large, electron-dense intranuclear masses. Alterations of mitochondria and other organelles were not observed in the early stages of cell injury. No light- or electron-microscopic alterations were found in liver or kidney. Comparable subcellular alterations were observed in adult and neonatal rats chronically intoxicated with TMT. A series of other trialkyl and tricyclic tins and dimethyltin did not produce similar pathologic findings.

The GERL-like accumulations are unique in neuronal cytopathology. These findings suggest that GERL and autophagy play an important role in the pathogenesis of TMT-induced neuronal injury. (Am J Pathol 1981, 104:237–249)

THE NEUROTOXICITY OF certain organotin compounds is well recognized. Among these toxicants, the neuropathologic characteristics of triethyltin (TET) have been the most extensively studied. This compound produces marked intramyelinic edema in the nervous system, and particularly in the central nervous system (CNS).[1] Although ultrastructural alterations in neurons are reported by some investigators,[2] there is no discernible neuronal necrosis *in vivo* with TET.[1]

Recently, there has been a renewal of interest in the neuropathology of the organotins and, in particular, trimethyltin (TMT). Unlike TET, TMT produces neuronal necrosis rather than intramyelinic edema.[3] Of particular interest is the accentuation of the TMT-induced neuronal necrosis in the hippocampal formation and pyriform cortex. The mechanism by which TMT produces neuronal necrosis is unknown, as is the reason for the preferential localization of the necrosis in the hippocampal formation and pyriform cortex.

The present study extends the original light-microscopic descriptions[3] of the pathologic features of TMT neurotoxicity by defining the ultrasructural basis of the pathologic changes that occur within hippocampal and pyriform neurons prior to their death. The sequence of subcellular pathologic changes, which was analyzed in acutely intoxicated adult rats, began with the multifocal accumulation of smooth membranes in the cytoplasm of the neuronal perikaryon and proximal dendrite. Ultrastructural cytochemical study of the hippocampal formation revealed that these abnormal collections of smooth membranes exhibited activity for acid phosphatase such as is found in Golgi-associated endoplasmic reticulum (GERL).[4] These multifocal collections of GERL-like

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membranes were followed by the massive accumulation of cytoplasmic dense bodies and autophagic vacuoles, and finally by neuronal necrosis. Similar subcellular pathologic changes were found in chronically intoxicated adult and suckling rats.

Materials and Methods

Dosage

The Long-Evans rat, originally obtained from Charles River, was used in all of the studies. Adult and neonatal rats of both sexes were used, and all were obtained from within our own animal breeding colony. The rats were treated with a single daily oral dose of the toxicant via gastric intubation. Chronic neonatal exposure was based upon dosing with trimethyltin hydroxide (TMT), 1 mg/kg, on alternate days from the 3rd through the 29th day of life. The suckling rats were killed when they were 30 days old. Chronic adult exposure consisted of treating 45- to 60-day-old rats with TMT (1 mg/kg) daily for 14–16 days. The adult rats were sacrificed 1 day after the last dose.

Acute TMT exposure consisted of intoxicating adult rats with 5 mg/kg/day. Rats were killed 24 hours after one, two, three, four, or five daily doses (rats rarely survived more than four doses).

Structure-related neurotoxic activity was also investigated in developing rats. Neonatal rats were dosed from the 3rd through the 30th day of life with a series of trialkyltin compounds. The maximal daily oral dose of organotin that allowed the rats to survive the 30-day period was used and was as follows: TMT, 1 mg/kg/alternate days; triethyltin sulfate (TET), 1 mg/kg/day; tri-n-propyltin chloride, 3 mg/kg/day; tri-n-butyltin acetate, 10 mg/kg/day; tricyclohexyltin bromide, 30 mg/kg/day; and triphenyltin acetate, 30 mg/kg/day. The neonatal rats were killed 1 day after the last dose.

The relation between the number of alkyl groups and neurotoxicity was also investigated. Neonatal rats and adult rats were exposed to dimethyltin dibromide (DMT), 35 mg/kg/day, or diethyltin dichloride, 10 mg/kg/day, for 27 days in the suckling rat and 20 days in the adult rat. The animals were killed 24 hours after the last dose.

All of the organotins were obtained from Alfa Products (Danvers, Mass), except TET, which was obtained from Organisch Chem Institute, I.N.O., Utrecht, Holland.

Preparation of Tissues

For conventional light-microscopic examination, the animals were anesthetized with pentobarbital, the left ventricle of the heart was canulated, and the animal was perfusion-fixed for 10–15 minutes with phosphate-buffered 4% formaldehyde at room temperature. After storage overnight in a refrigerator at 4 C, the CNS, liver, and kidney were removed and portions taken for embedding in paraffin. The brain was sectioned in the coronal plane, and sections for light microscopy were taken from the cerebrum, cerebellum, brain stem, spinal cord, and dorsal root ganglia. Sections of the nervous system, liver, and kidney were cut at 6–8 μ and stained by the periodic acid–Schiff (PAS) technique, with solochrome and eosin, or with cresyl violet (Nissl stain).

Rats selected for electron-microscopic examination were anesthetized and perfusion-fixed for 10–15 minutes with 2% glutaraldehyde and 2% paraformaldehyde in a 0.06M phosphate buffer, pH 7.2, at room temperature. The final 15 ml of the perfusate contained 4% glutaraldehyde and 2% paraformaldehyde in phosphate buffer. After continued fixation overnight at 4 C, the brain, liver, and kidney were removed, and small portions were selected for embedding in araldite, after postfixation in buffered 1% osmium tetroxide and dehydration in ascending alcohols and propylene oxide. The brain was coronally sectioned, and blocks of tissue were taken from the hippocampal formation and pyriform cortex. One-micron survey sections were stained with toluidine blue; thin sections were collected on Formvar-coated slot grids and stained with uranyl acetate and lead citrate.

Histochemistry

All animals were fixed via cardiac perfusion at room temperature with 1% glutaraldehyde and 4% paraformaldehyde in 0.05M cacodylate buffer, pH 7.2, with 0.025% $CaCl_2$ added. Perfusion was for 15 minutes, and fixation was continued for an additional 2–3 hours at 4 C. The brains were removed, and a block of the ventral hippocampal formation was isolated, buffer washed three times, and kept overnight in 0.1 M cacodylate buffer containing 5% sucrose. An 80-μ horizontal section of hippocampal formation was cut with a Sorval TC-2 Tissue Sectioner and collected in the cacodylate-sucrose buffer. Acid phosphatase (AcPase) activity was demonstrated using sodium β-glycerophosphate as the substrate by the Gomori method, as modified by Barka and Anderson,[5] or with cytidine-5′-monophosphate (CMP) as the substrate by the method of Novikoff.[6] Dinucleosidase (TPPase) activity was demonstrated with the thiamine pyrophosphate method, described by Novikoff and Goldfischer.[7] All substrates were obtained from Calbiochem, La Jolla, California.

In preparing the histochemical media, glass-distilled, preboiled water was used, and the reactions were carried out in acid-cleaned glassware. Five percent sucrose was added to each histochemical medium. Incubations were carried out at 37 C with agitation for 60–90 minutes; the medium was replaced with freshly filtered medium every 30 minutes. Controls consisted of substrate blanks and, for AcPase activity, preincubation in 5mM NaF. The reactions were stopped with three washes in the appropriate incubating medium buffer and a final wash in veronal buffer, pH 7.2, with 5% sucrose. The tissues were postfixed in a veronal-buffered solution, pH 7.2, containing 1% osmium tetroxide, 1.5% potassium ferricyanide,[8] and 5% sucrose; some tissues were also in-blocked stained with aqueous uranyl acetate. The tissues were rapidly dehydrated in ascending concentrations of ethanol, placed in propylene oxide, and embedded in araldite. After identifying the hippocampal formation in 1-μ sections, the blocks were trimmed so that the fascia dentata and area CA4 of the cornu ammonis were included in the thin sections. Thin sections were collected on Formvar-coated slot grids and viewed in the election microscope either unstained or after lead citrate staining. Unincubated tissue was also prepared for electron microscopy.

Designations for the subdivisions of the cornu ammonis were taken from Lorente de Nó (Figure 1).[9]

Results

General Observations in Organotin Intoxication

Neonatal rats chronically exposed to TMT (1 mg/kg on alternate days) had up to a 20–30% decrease in weight. The pups exhibited spontaneous tremors and seizures; they were reactive to noise but were not aggressive. Adult rats chronically exposed to TMT (1 mg/kg/day) lost weight after 10–12 days and then became self-mutilating, highly aggressive, and required separate cages. Brown et al have reported similar behavioral changes.[3]

Adult rats acutely intoxicated with TMT (5 mg/kg/day) exhibited weight loss after two doses, self-mutilation and aggressiveness after three doses, and generally fatal seizures after four doses. If the toxicant was stopped after three doses, the rats became progressively less aggressive.

The DMT intoxication in both the developing and adult rat models produced weight loss but did not produce paresis, tremors, convulsions, or fatalities.

Neonatal rats chronically exposed to TET at a dose of 1 mg/kg/day demonstrated neurologic abnormalities and alterations in myelin.[10] No neurologic or pathologic abnormalities were noted when the neonatal rats were chronically given 0.3 mg/kg/day of TET.[10]

Chronic intoxication of neonatal rats with tri-n-propyltin and tri-n-butyltin produced "ill-kept" small rats but no discernible neurologic abnormalities. Decreased weight gain was the only effect noted with a maximal nonlethal dose of the tricyclohexyltin and triphenyltin.

Light-Microscopic Examination in TMT Intoxication

The degree and distribution of pathologic changes that we found in the paraffin-embedded tissues of rats intoxicated with TMT agreed with the detailed observations of Brown et al.[3] In brief, the pathologic changes were limited to the CNS. The prominent and

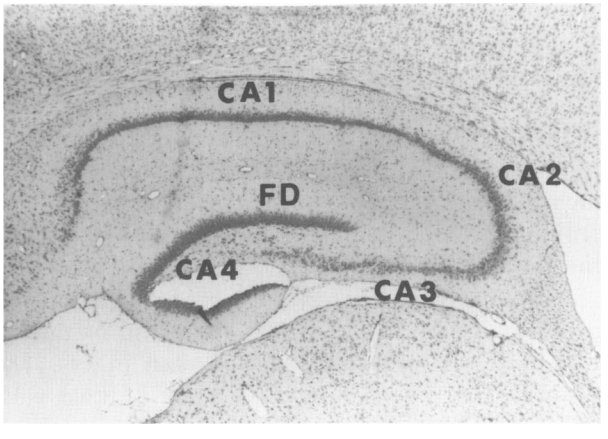


Figure 1—Coronal section through hippocampal formation of untreated 30-day-old rat. Fascia dentata (*FD*) and areas CA1-CA4 of cornu ammonis are denoted. (Cresyl violet, × 20) (With a photographic reduction of 5%)

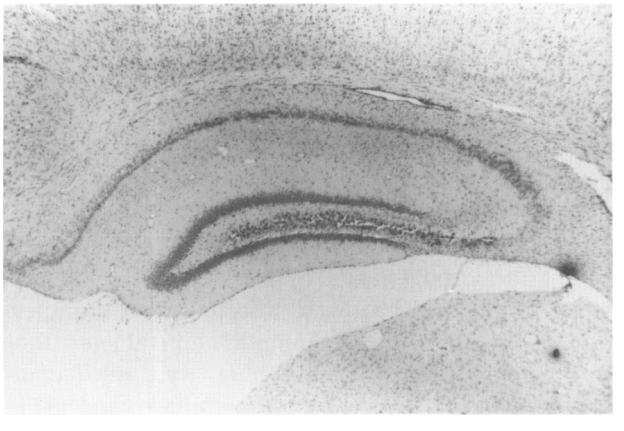


Figure 2—Coronal section through hippocampal formation of 30-day-old rat after fourteen alternate-day doses of TMT (1 mg/kg). There is a loss of neurons in areas CA3 and CA4. Many of the dead neurons in area CA3 remain as dark mineralized cells. (Cresyl violet, × 20) (With a photographic reduction of 5%)

major pathologic finding in TMT neurotoxicity was neuronal necrosis, as evidenced by shrunken neurons with dense, eosinophilic cytoplasm and pyknotic nuclei. The necrotic neurons were found in the neocortex, pyriform cortex, hippocampal formation, basal ganglia, brain stem, spinal cord, and dorsal root ganglia. Although quantitative studies were not done, it was very apparent that the necrotic neurons were most numerous in the hippocampal formation and pyriform cortex. The neuronal necrosis was followed over the next several days by an influx of microglial cells, neuronophagia, astrocytic proliferation, and gliosis. Mineralization of degenerated hippocampal neurons was sometimes striking, especially in the chronically intoxicated suckling rats (Figure 2). Nissl-stained sections revealed occasional neurons with central chromatolysis; in PAS-stained sections, the chromatolytic area was sometimes PAS positive. Edematous white matter, such as is found in TET intoxication, was not observed.

The use of 1-μ plastic-embedded sections of the hippocampal formation from acutely intoxicated adult rats permitted a much more detailed cytologic study of the sequential pathologic changes in hippocampal neurons prior to their undergoing necrosis. The first discernible light-microscopic changes in hippocampal neurons were found 24 hours after the third daily dose (5 mg/kg) of TMT to adult rats. At this time, dead neurons were occasionally present in the fascia dentata; much less frequently, dead neurons could also be found in the cornu ammonis. Neuronal death was evidenced in the toluidine-blue-stained plastic sections by shrunken neurons with dense dark-blue cytoplasm and nuclear pyknosis. By 24 hours after the fourth daily dose (5 mg/kg) of TMT, there were frequent dead neurons in the fascia dentata and occasional dead neurons in the cornu ammonis (Figure 3).

Another pathologic change, which has not been previously described in TMT neurotoxicity, was a progressive granulation of the neuronal cytoplasm. Whereas normally the hippocampal neurons have a very fine basophilic dust in their perikaryal cytoplasm, after 3 doses of TMT an occasional hippocampal neuron contained numerous dense granules. The cytoplasmic granulation became much more evident after 4 daily doses of TMT, when numerous hippocampal neurons were affected. The granules, which were about 1 μ in diameter and stained orthochromatically with toluidine blue, were most commonly found in the perikaryal cytoplasm and proximal dendritic cytoplasm of pyramidal cells of the entire cornu ammonis. Neurons with granular cytoplasm were also present, but much less conspicuous,

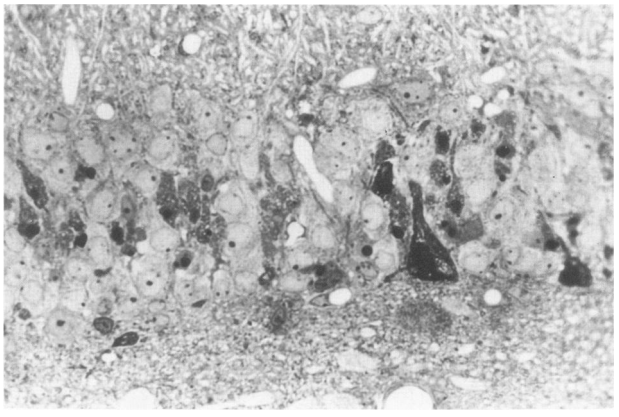


Figure 3—Fascia dentata (1-μ section) from adult rat 24 hours after four daily doses of TMT (5 mg/kg). Numerous necrotic neurons are present within the granule-cell layer. The necrotic neurons have pyknotic nuclei, darkly staining cytoplasm, and small cytoplasmic vacuoles. (Toluidine blue, ×350) (With a photographic reduction of 5%)

in the granule cell layer of the fascia dentata. The granules so completely filled the cytoplasm of some neurons that their nuclei became eccentrically located. Occasionally, granulated neurons also displayed vacuolation of their perikaryal cytoplasm. Granulated neurons were also noted in the pyriform cortex.

The pathologic findings described above were not limited to the acutely intoxicated adult rats. Similar pathologic changes, including granulated neurons and neuronal necrosis, were observed in chronically intoxicated suckling rats and chronically intoxicated adult rats. However, these dose-related studies did reveal one peculiar topographic feature of TMT neurotoxicity not previously emphasized. Whereas in the acutely intoxicated (high-dose, short-term) adult rats the neuronal necrosis in the hippocampal formation almost exclusively involved the neurons of the fascia dentata, in the chronically intoxicated (low-dose, long-term) neonatal and adult rats the neuronal loss was much more marked in the pyramidal neurons of the cornu ammonis than in the granule cells of the fascia dentata (Figure 2). It is interesting that granulation of the neuronal cytoplasm did not follow such a relationship; granulated neurons were more prominent in cornu ammonis in both the acutely and chronically intoxicated rats.

A second topographic feature of chronic TMT neurotoxicity was that, within the cornu ammonis, certain areas were more affected than others. The neuronal loss was always most severe in areas CA3 and CA4, generally less severe in area CA1, and often least severe in area CA2 (Figure 2). Cytoplasmic granulation was, however, frequently present in

pyramidal neurons of area CA2. The relative sparing of area CA2 was not evident in the acutely intoxicated rats; neuronal necrosis was rare throughout the entire cornu ammonis in these animals.

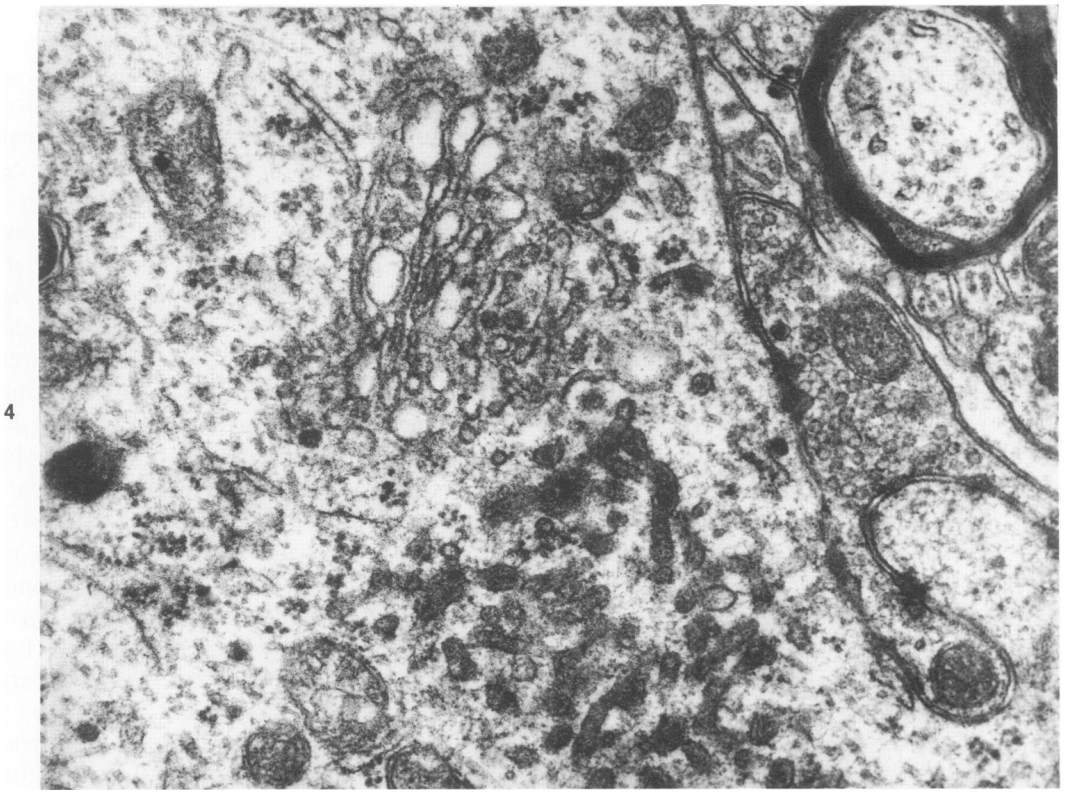
Electron-Microscopic Examination in TMT Intoxication

The hippocampal formation of adult rats acutely intoxicated with TMT was examined in the electron microscope to characterize the evolution of the subcellular pathologic changes leading to neuronal necrosis. The hippocampal formation was chosen for detailed study not only because of its predilection for TMT-induced neuronal necrosis, but also because the organization of this structure is relatively well characterized. The acutely intoxicated adult rat model was chosen because the evolution of the pathologic changes occurred in a highly predictable manner over a relatively short period (5 days).

Twenty-four hours after one dose of TMT (5 mg/kg), no ultrastructural alterations were present in the hippocampal formation. By 24 hours after the second daily dose, however, distinctive cytoplasmic alterations could be found in neurons of both the fascia dentata and cornu ammonis. These initial subcellular changes consisted of multifocal accumulations of smooth unit membranes in the cytoplasm of the perikaryon and proximal dendrite (Figure 4). These smooth membranes, which formed vesicles and tubules, contained electron-dense, amorphous material. The plane of section was occasionally such that a few of these dense-cored tubules could be seen to branch, and, thus, they resembled smooth endoplasmic reticulum (SER) (Figure 5). The frequent discovery of transitional profiles between the dense-cored tubules and vesicles suggested that many of the vesicles were likely to represent transverse sections through the tubules. Although many of the cytoplasmic accumulations consisted only of vesicles and tubules, some of the accumulations also contained scattered dense bodies, multivesicular bodies, and autophagic vacuoles (Figure 5). Occasionally, dense bodies appeared to arise directly from the dense-cored tubules (Figure 5, inset). Collections were only infrequently adjacent to stacks of Golgi (Figure 4).

Another neuronal alteration in the 2-dose animals was the occasional discovery of cytoplasmic vacuoles (Figure 5). The vacuoles were membrane-bound and were generally the size of mitochondria or slightly larger. The vacuoles were found only in neurons that also contained cytoplasmic accumulations of dense-cored vesicles and tubules; however, not all neurons with vesiculo-tubular accumulations contained vacuoles. At this stage in the evolution of TMT-induced neuronal necrosis, no alterations could be discerned in the mitochondria, polysomes, or rough endoplasmic reticulum (RER). There was no evidence of neuronal necrosis, and no pathologic changes were found in glial or endothelial cells.

Twenty-four hours after the third daily dose of TMT, the first signs of neuronal necrosis were evident. The necrotic neurons, which were very infrequent, had condensed electron-dense cytoplasm and pyknotic electron-dense nuclei. Electron-dense necrotic dendrites were also noted. Of the remaining viable hippocampal neurons, many contained cytoplasmic accumulations of dense-cored vesicles and tubules in their perikarya and proximal dendrites; occasional neurons had cytoplasmic vacuoles. At this stage of the intoxication, however, the most striking finding was that neuronal perikarya and proximal dendrites contained numerous cytoplasmic dense bodies, which were the ultrastructural correlate of the granules noted by light microscopy (Figure 6). Many, but not all, of the neurons in the fascia dentata and cornu ammonis were affected by this accumulation of dense bodies. The internal structure of the dense bodies varied, but all had a limiting membrane. The internal structure of some dense bodies was that of extremely electron-dense amorphous material, while other dense bodies had one or more membranous fragments embedded in an electron-dense matrix. Multivesicular bodies were also occasionally present. Many of the dense bodies had a double limiting membrane and strongly resembled autophagic vacuoles (Figure 6). Occasionally, we found profiles illustrating the various stages in the entire sequence of autophagocytosis. A few nascent autophagic vacuoles contained structures suggestive of mitochondria, while other vacuoles contained normal appearing cytoplasm or moderately electron-dense cytoplasm (Figure 6). However, the majority of the autophagic vacuoles contained amorphous electron-dense granular material or fragments of membranes. The dense bodies and autophagic vacuoles were sometimes adjacent to the cytoplasmic accumulations of dense-cored vesicles and tubules; at other times, the dense bodies and autophagic vacuoles were scattered throughout the cytoplasm. Although the cytoplasmic dense bodies occupied a considerable amount of the perikaryal cytoplasm at this stage, there was still no evidence of abnormal mitochondria, dispersion of polysomes, or degranulation of RER. The collections of dense bodies in the dendrites often had a peripheral subplasmalemmal distribution, with the center of the dendrite occupied by numerous parallel neurotubules (Figure 7). No



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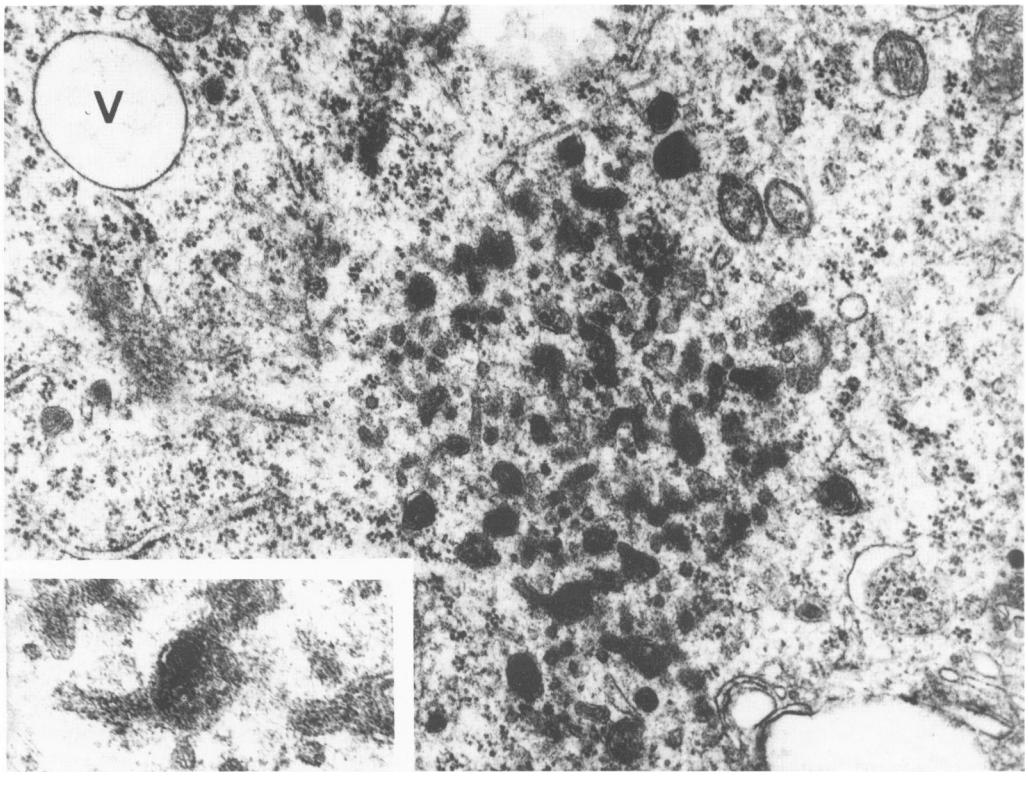

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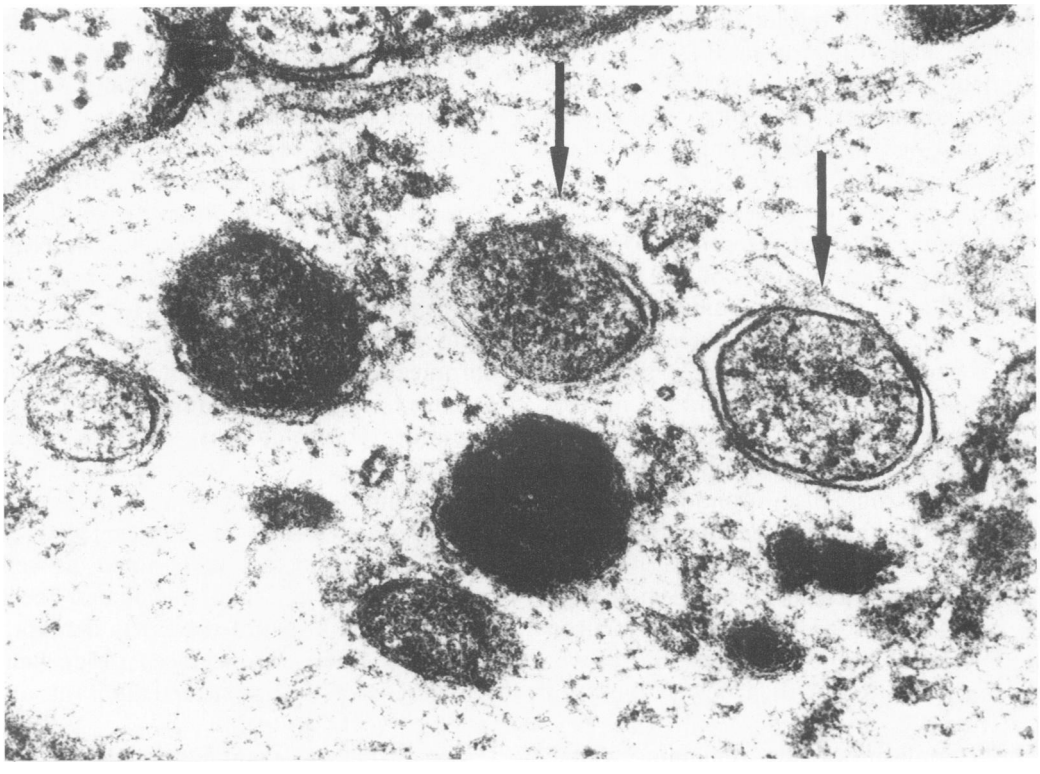
Figure 4—Electron micrograph of adult rat 24 hours after two daily doses of TMT (5 mg/kg). The cytoplasm of a dentate granule cell contains an abnormal collection of dense-cored vesicles and tubules (*bottom center*). A Golgi apparatus (*top center*) is adjacent to the abnormal collection. (Uranyl acetate and lead citrate, ×51,000) **Figure 5**—Electron micrograph of adult rat 24 hours after two daily doses of TMT (5 mg/kg). A collection of dense-cored vesicles, tubules, and dense bodies is present within a dentate granule cell. A membrane-delimited vacuole (*V*) is also shown. (Uranyl acetate and lead citrate, ×31,000). **Inset**—At higher magnification, one of the dense bodies appears to arise from a dense-cored tubule. (×68,000)



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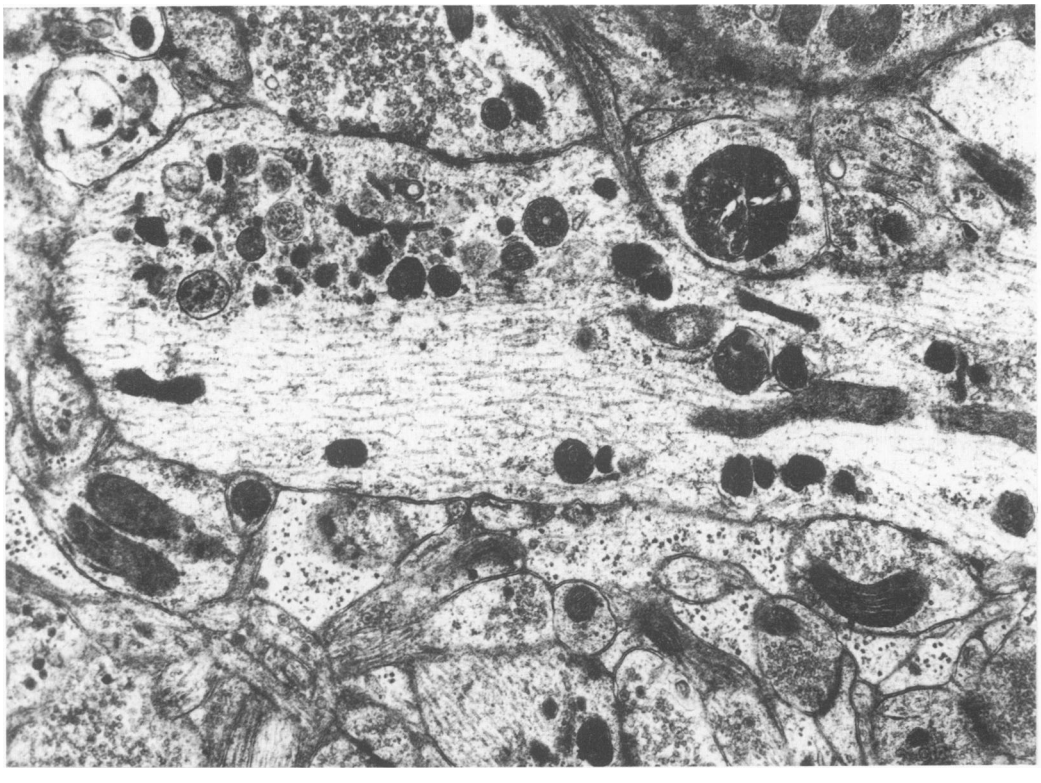

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Figure 6—Electron micrograph of adult rat 24 hours after three daily doses of TMT (5 mg/kg). The dendritic cytoplasm of a hippocampal neuron contains polymorphic dense bodies and autophagic vacuoles (*arrows*). (Uranyl acetate and lead citrate, ×71,000) **Figure 7**—Electron micrograph of adult rat 24 hours after three daily doses of TMT (5 mg/kg). Dense-cored vesicles and tubules and polymorphic dense bodies are in the dendrite of a hippocampal neuron. As was frequently the case, the dense bodies have collected beneath the plasma membrane. (Uranyl acetate and lead citrate, ×21,000)

pathologic changes were found in glial cells or endothelial cells at this stage of intoxication.

Twenty-four hours after the fourth daily dose of TMT, necrotic neurons were frequently found in the fascia dentata and occasionally found in the cornu ammonis (Figure 8). The necrotic neurons were characterized by an electron-dense granular cytoplasm in which were embedded the outlines of mitochondria and polymorphic dense bodies. Necrotic electron-dense dendrites were also numerous at this time. Scattered cytoplasmic vacuoles were present in many of the necrotic cells. The nuclei of the dead neurons were moderately shrunken, and the nucleoplasm had become more electron dense. Many of the necrotic neurons had nuclei that contained an amorphous, extremely electron-dense, round mass, which was several times larger than a nucleolus; nucleoli, as such, were not found in necrotic neurons. The formation of these electron-dense intranuclear masses sometimes preceded the necrosis of the neuron (Figure 8). The cytoplasmic processes of macrophages were beginning to engulf a few of the dead neurons.

Within the cytoplasm of the remaining viable neurons of the 4-dose animals, dense bodies were now more numerous, more polymorphic internally, and more variable in size (Figure 9). Much larger autophagic vacuoles were present and contained cytoplasm and numerous dense bodies. The neuronal mitochondria remained normal in appearance, but there were now fewer profiles of RER and fewer polysomes in neurons which had numerous cytoplasmic dense bodies. Some of the remaining viable neurons of the cornu ammonis had eccentric nuclei (Figure 9). In these neurons, the cytoplasm was filled with numerous dense bodies and autophagic vacuoles; the mitochondria tended to group in the center of the perikaryon, with the nucleus and numerous dense bodies occupying the periphery of the neuronal soma.

Another pathologic finding, first noted in the four-dose animals, was accumulation of dense bodies in occasional myelinated axons within the hippocampal formation. More rarely, these abnormal myelinated axons also showed early axonal degeneration, as evinced by granular transformation of the axoplasm.

The hippocampal formation in adult and suckling rats, chronically intoxicated with TMT, was examined to determine 1) if the sequence of ultrastructural changes leading to neuronal necrosis in acute TMT toxicity was also present in chronic TMT toxicity, and 2) if the sequence of ultrastructural changes leading to neuronal necrosis in the mature rodent nervous system was also present in the immature nervous system of the suckling rat. Ultrastructural changes similar to those described in the acutely intoxicated adult rats were found in the chronically intoxicated adult and suckling rats. Specifically, accumulations of dense-cored vesicles and tubules, polymorphic dense bodies, and autophagic vacuoles were found in the cytoplasm of neurons in both the fascia dentata and cornu ammonis. Necrotic cells, similar to those found in the acutely intoxicated animals, were also present. However, as noted by light microscopy, the neuronal dropout in the chronically intoxicated animals was principally localized in the cornu ammonis, rather than in the fascia dentata. In contrast to the acutely intoxicated rats, the chronically intoxicated rats had numerous reactive fibrous astrocytes and macrophages in the hippocampal formation. Degenerating myelinated fibers were also occasionally noted, as well as a conspicuous loss of the small myelinated fibers normally present in the hilum of the fascia dentata. No ultrastructural differences in degenerating neurons were found between the intoxicated adult rats and suckling rats.

Ultrastructural examination of neurons from the pyriform cortex of adult rats acutely intoxicated with TMT revealed a sequence of subcellular pathologic changes identical to that found in the hippocampal formation.

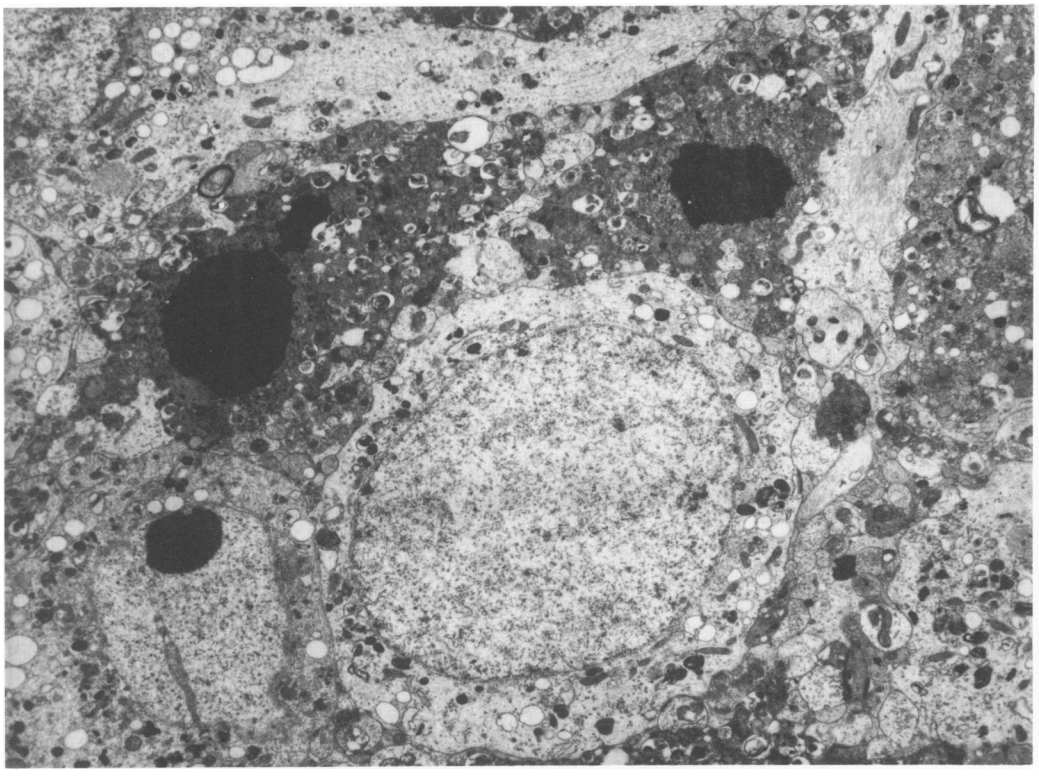
The liver and kidney in adult rats acutely and chronically intoxicated with TMT were examined by electron microscopy. There were no cytoplasmic accumulations of dense-cored vesicles and tubules, no dense bodies, and no mitochondrial abnormalities.

Light- and Electron-Microscopic Examination in Other Organotin Intoxications

No light- or electron-microscopic evidence of neuronal necrosis was found in the hippocampal formation or pyriform cortex of neonatal and adult rats intoxicated with dimethyltin, diethyltin, tri-n-propyltin, tributyltin, tricyclohexyltin, or triphenyltin. The TET produced, as reported by others, widespread severe intramyelinic edema[1]; the tributyltin produced bile duct ectasia and portal tract fibrosis in the liver.[11]

Ultrastructural Cytochemistry

Electron-microscopic examination of hippocampal neurons from control rats revealed that TPPase activity was essentially limited to the *trans* aspect of the Golgi apparatus; AcPase activity was limited to GERL and the occasional dense body. These observations are in agreement with those of Novikoff, who studied



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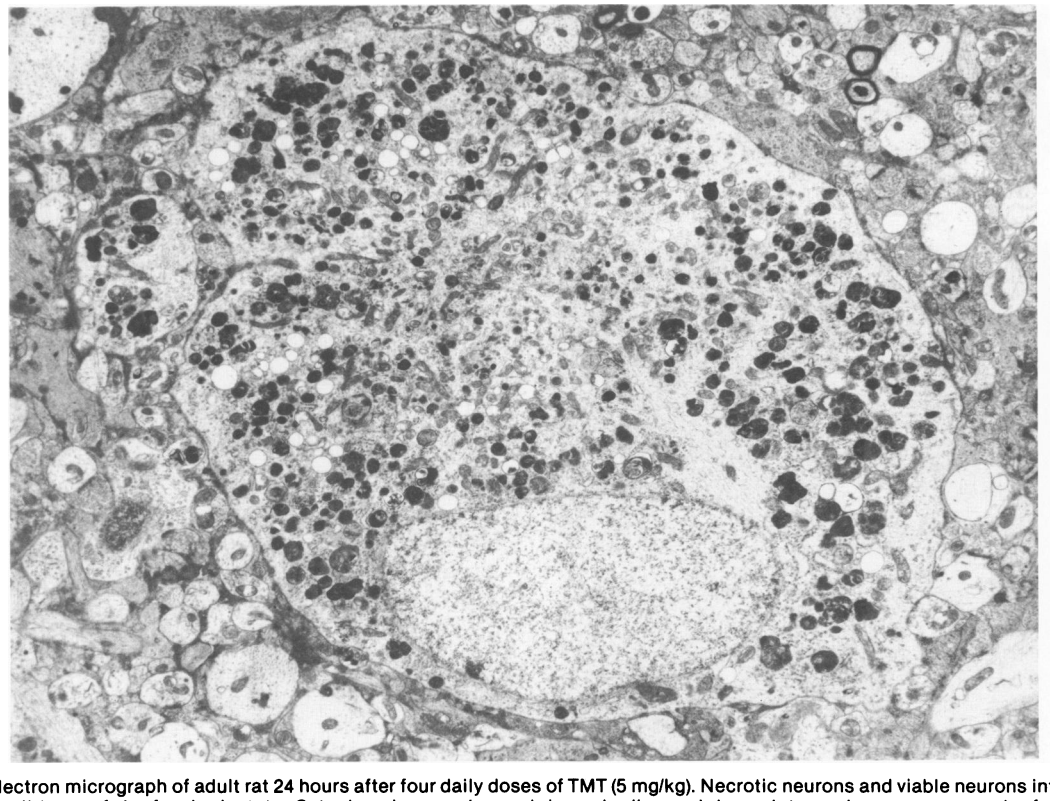

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Figure 8—Electron micrograph of adult rat 24 hours after four daily doses of TMT (5 mg/kg). Necrotic neurons and viable neurons intermingle in the granule-cell layer of the fascia dentata. Cytoplasmic vacuoles and dense bodies and dense intranuclear masses can be found in both necrotic and viable neurons. (Uranyl acetate and lead citrate, ×5500) **Figure 9**—Electron micrograph of adult rat 24 hours after four daily doses of TMT (5 mg/kg). A neuron from area CA4 demonstrates the ultrastructural correlates of the central chromatolysis noted by light microscopy. The nucleus is eccentric and there are many cytoplasmic dense bodies. The cell's normal organelles tend to be grouped in the center of the perikaryon. (Uranyl acetate and lead citrate, ×5000)

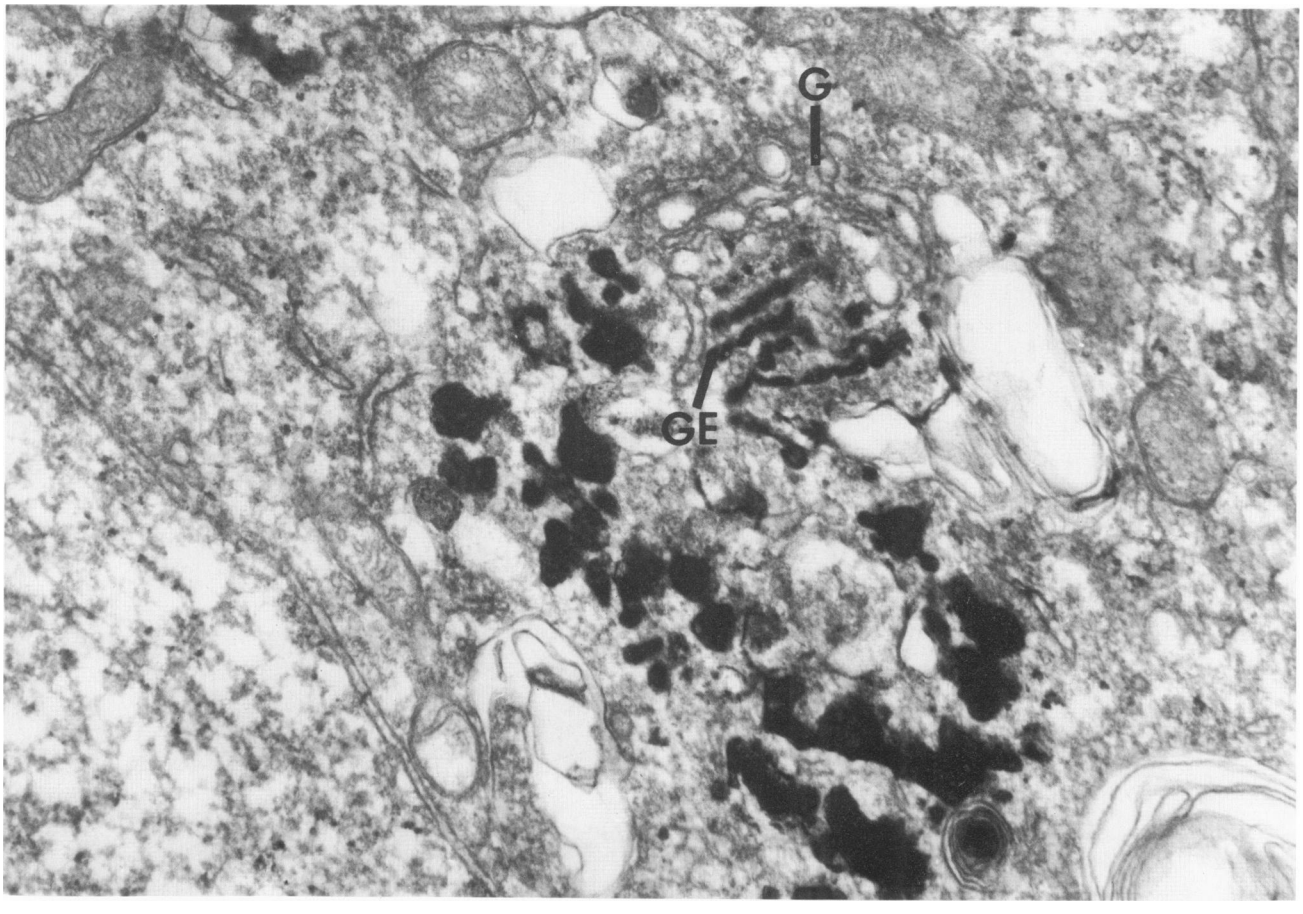

Figure 10—Electron micrograph of adult rat 24 hours after three daily doses of TMT (5 mg/kg); tissue incubated in CMP medium for AcPase. Reaction product is present within several GERL cisterns (*GE*), numerous vesicles and tubules, and several dense bodies. No reaction product is found within the adjacent Golgi complex (*G*). (Uranyl acetate, in-block, and lead citrate, × 54,000)

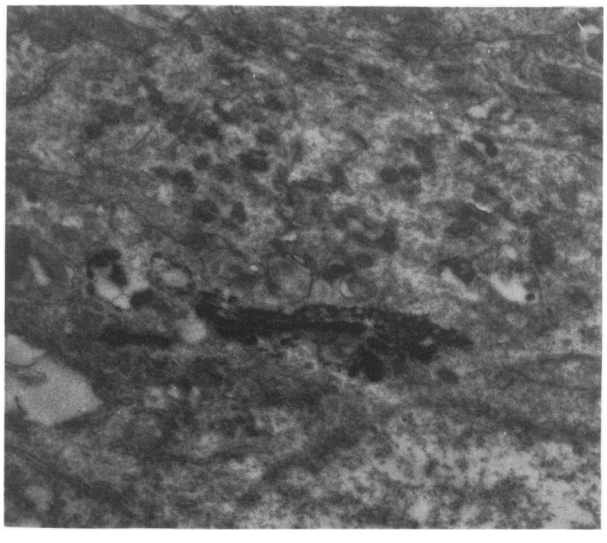

Figure 11—Electron micrograph of adult rat 24 hours after three daily doses of TMT (5 mg/kg); tissue incubated for TPPase. Reaction product is present within Golgi cisterns, but none is found in the adjacent collection of dense-cored vesicles and tubules. (× 19,100) (With a photographic reduction of 5%)

the cytochemistry of neurons in the dorsal root ganglia of rats.[4]

Hippocampal tissue from adult rats, killed 24 hours after three daily doses of TMT (5 mg/kg), was incubated for TPPase and AcPase activity. By electron microscopy, the intraneuronal accumulations of dense-cored vesicles and tubules and the polymorphic dense bodies showed strong AcPase activity, but no TPPase activity (Figures 10 and 11). Some of the membrane-delimited cytoplasmic vacuoles demonstrated TPPase activity, but none of the vacuoles had AcPase activity. As in control animals, AcPase activity was also present in membranous cisterns of GERL, and TPPase activity was present in the *trans* aspect of the Golgi apparatus.

Discussion

Our light-microscopic studies of TMT poisoning in rats indicated that the pathologic findings were limited to neuronal necrosis within the CNS, and that,

whereas neurons of many different regions of the CNS were affected to some degree, the neurons of the hippocampal formation and pyriform cortex were most vulnerable to the toxic effect of TMT. Similar light-microscopic findings have been reported by Brown et al.[3] One topographic feature, which was prominent in our studies and not emphasized by Brown et al, was that the neuronal necrosis in the hippocampal formation preferentially involved the neurons of the fascia dentata in the acutely intoxicated rats (short-term, high-dose) and the neurons of the cornu ammonis in the chronically intoxicated rats (long-term, low dose). The reasons for such a hierarchy of neuronal vulnerability in TMT neurotoxicity are not known.

Our electron microscopic studies of the hippocampus and pyriform cortex in TMT-intoxicated rats revealed that specific subcellular pathological changes occurred in neurons prior to their death. The subcellular pathologic changes, which were characterized by cytoplasmic accumulations of dense-cored vesicles and tubules, autophagic vacuoles, and polymorphic dense bodies, appeared characteristic of TMT intoxication, regardless of whether the animal was acutely or chronically intoxicated, or whether the intoxicated animal had a mature or immature CNS.

The earliest signs of neuronal injury in TMT poisoning were cytoplasmic collections of smooth unit membranes in the form of vesicles and tubules; these vesicles and tubules, which contained an electron-dense core, were limited to the neuronal perikarya and proximal dendrites. The ultrastructural appearance of the vesicles and branching tubules suggested that they arose from the neuronal SER. Furthermore, the cytochemical characteristics of the vesicles and tubules suggested that they were derived from a specialized region of the neuronal endoplasmic reticulum (ER) known as GERL.[4]

GERL, as defined by the Novikoffs, is a "hydrolase-rich region of ER, spatially related to the Golgi apparatus (but not part of it), from which lysosomes appear to form."[12] It is from the neuron's GERL that primary lysosomes, dense bodies, and autophagic vacuoles are thought to arise.[13,14] Histochemically, the collections of vesicles and tubules found in TMT neurotoxicity would qualify as GERL, as the vesicles and tubules demonstrated considerable activity for AcPase and no activity for TPPase, a marker for the *trans* aspect of the Golgi apparatus. Ultrastructurally, the vesicles and tubules were similar to GERL, in that they were formed of unit membrane and contained an electron-dense material.[12] The intimate association of autophagic vacuoles and dense bodies with dense-cored vesicles and tubules, and the occasional profiles suggesting the derivation of dense bodies from dense-cored tubules, further suggested that the dense-cored vesicles and tubules were functioning as GERL. The discovery of dense-cored vesicles and tubules in proximal dendrites does not exclude their close association with the Golgi apparatus, as Golgi stacks are present in the proximal dendrites of hippocampal neurons.[15] However, it should be noted that many of the abnormal collections of vesicles and tubules that we observed were not spatially related to the Golgi apparatus (at least in the plane of section, since serial sections were not done).

Whether the dense-cored vesicles and tubules that we have observed should be considered as hypertrophied GERL, as abnormal accumulations of SER, or as hypertrophied Golgi elements, is a moot point. As pointed out by Hand,[16] there are several studies that demonstrate how developmental, functional, or pathologic changes in cellular activity may produce marked changes in the morphologic and cytochemical properties of GERL and the Golgi apparatus. One must, therefore, exercise caution in differentiating between GERL and Golgi apparatus by means of limited cytochemical studies.[16] Furthermore, the Novikoffs' suggestion that the presence of AcPase activity indicates that the GERL is a specialized region of SER distinct from the Golgi apparatus has been questioned by several contemporary authors.[17,18] Activity for AcPase has been demonstrated in the Golgi apparatus of several cell types,[16,17,18] and Quatacker[17] and Mayahara et al[18] have reported cytochemical and ultrastructural findings which link the GERL much more closely to the Golgi apparatus.

Neuronal autophagic vacuoles and dense bodies are considered to be derived from GERL,[13,14] and, thus, the early presence of accumulations of GERL-like membranes in TMT-intoxicated neurons may simply reflect a reactive change of the neuron in preparation for the markedly increased synthesis of lysosomes and autophagic vacuoles. Supporting this interpretation of the pathologic findings are ultrastructural and cytochemical studies of guinea pig luteal cells during normal postpartum involution. Regressing luteal cells are characterized by both a marked increase in the number of autophagic vacuoles and lysosomes and a striking hypertrophy of GERL.[19] In this model, the hypertrophy of GERL precedes the accumulation of dense bodies and autophagic vacuoles, which appear to arise from the hypertrophied GERL. A similar subcellular sequence of events is reported to occur in anuran lateral motor column neurons that degenerate after failing to make connections with the periphery.[20]

Increased autophagic activity was a prominent and

early response of the neuron to TMT intoxication. Autophagy is a normally occurring cell function that permits the cell to dispose of effete organelles. Increased autophagy, as evidenced by markedly increased numbers of autophagic vacuoles, can be a pathologic cellular response to a wide variety of sublethal cellular injuries, including starvation, exposure to numerous toxins, and irradiation.[21] As noted previously, neuronal autophagic vacuoles may arise from GERL.

The basis for the increased autophagy in the TMT-intoxicated neurons is not apparent from our ultrastructural studies, as no stimulus for increased autophagocytosis, such as altered cytoplasmic organelles, was identified. The mitochondria, RER, and polysomes were decreased only in the late stages of neuronal toxicity, when autophagocytosis and dense-body formation were far advanced. It is possible, of course, that transient morphologic alterations in organelles did occur, and that examination of hippocampal neurons at more frequent intervals during the early stages of intoxication would reveal such alterations.

The accumulation of intracytoplasmic dense bodies was a striking light- and electron-microscopic feature of TMT neuronal toxicity. The dense bodies, which included primary and secondary lysosomes and occasional multivesicular bodies, showed prominent AcPase activity. The accumulations of dense bodies occurred *para passu* with the increase in autophagic activity, and undoubtedly a number of the dense bodies were derived from autophagic vacuoles. Many of the dense bodies, however, gave no indication of having arisen from autophagic vacuoles, and at least some of the dense bodies appeared to arise directly from the dense-cored tubules. That a portion of the dense bodies did not arise from the process of autophagy suggests that other processes, such as increased formation of lysosomes and possibly heterophagy, also play a role in the pathogenesis of the cytopathology of TMT neuronal toxicity. The induction of massive accumulations of cytoplasmic dense bodies within neurons is not unique to TMT, as a wide variety of drugs have been shown to induce similar accumulations.[22] To our knowledge, however, these other experimental models of drug-induced dense-body accumulation have not shown the prominent collections of dense-cored vesicles and tubules that characterize the earliest stage of TMT neuronal toxicity.

Previous biochemical studies reveal that trialkyltins are potent inhibitors of mitochondrial function.[23] The lack of early ultrastructural lesions in the neuronal mitochondria, RER, and polysomes in TMT neurotoxicity suggests, however, that the pathogenesis of TMT neurotoxicity does not involve an initial defect in mitochondrial or ribosomal function. The lack of mitochondrial changes also argues against the observed pathologic changes being related to TMT-induced seizures, as mitochondrial swelling is one of the earliest neuronal alterations associated with generalized seizures.[24]

Recently, Ito et al reported that in the gerbil, a "reactive change" can occur in hippocampal neurons after a slight ischemic insult.[25] By light microscopy, this "reactive change" in the neuron is evidenced by central chromatolysis, a shift of the nucleus to the periphery of the soma, and cytoplasmic granules that stain positively with toluidine blue. Ultrastructurally, "reactive" neurons have an accumulation of organelles in the center of their soma; these central accumulations include altered mitochondria, vesicles, vacuoles, large dense bodies, and clumps of electron-dense material. Also prominent in such "reactive" neurons are a disintegration of the Golgi complexes, a loss of RER, and a disruption of polysomal clusters.[26,27] Brown et al report that "selective chromatolysis," a light-microscopic alteration that they equate with Ito's "reactive change," is present in neurons from animals poisoned with TMT.[3] They did not, however, examine the neurons showing such changes by electron microscopy to determine the ultrastructural correlate of their light-microscopic observation. We, too, found in TMT poisoning that occasional neurons in the hippocampus and elsewhere showed either focal chromatolysis or a more conspicuous central chromatolysis with nuclear eccentricity in Nissl-stained sections. Electron microscopy, however, revealed that neurons showing eccentric nuclei had multifocal collections of dense-cored vesicles and tubules and a marked accumulation of dense bodies and autophagic vacuoles, rather than the conspicuous central accumulation of organelles, disintegration of Golgi, and loss of RER and polysomes that characterize "reactive change."

The genesis of the cytoplasmic vacuoles, which were another early ultrastructural feature of TMT neuronal toxicity, is not clear from our ultrastructural studies, as transitional forms between vacuoles and cytoplasmic organelles were not identified. The cytochemical studies, however, revealed that some of the vacuoles contained TPPase activity. This finding suggests that at least a portion of the vacuoles are derived from the *trans* aspect of the Golgi apparatus. There was no evidence of AcPase activity in the vacuoles, and thus no histochemical evidence to suggest an origin from SER or GERL.

In the course of studying the biochemical mechanisms of organometal toxicity, Cremer has shown

that the neurotoxic effects of tetraethyltin and tetraethyl lead reside not in the parent compounds, but in one of their biotransformed products, a trialkyl metal.[28,29] Because a similar situation might also be operative in TMT neurotoxicity, we investigated the neurotoxicity of DMT. We found that in our animal models, DMT did not produce neuropathologic changes such as were associated with TMT neurotoxicity. From these findings we conclude that the neurotoxic effects of TMT are not mediated by DMT, a metabolite in the course of organotin dealkylation.[30] This suggests that the neurotoxic effects of TMT reside in the parent molecule or in the initial hydroxylated form, dimethylmethoxytin. The molecular mechanism of TMT neurotoxicity, however, remains to be defined.

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