Ultrastructural Hepatotoxicity Induced by an Organotin Ester

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Adult mice and rats were intubated with daily one-fourth LD_{50} amounts of the organotin dibutyltin diacetate. Histopathology by light microscopy, and cellular ultrastructural changes by electron microscopy to hepatic tissue was determined at 24-hr. intervals to elucidate the mode of hepatotoxic action of the compound. It was found that early mitochondrial damage with cristae swelling and subsequent hydropic degeneration occurred. Swelling of granular and agranular endoplasmic reticulum followed soon after mitochondrial changes were seen, and progressed to hydropic vesicular formation. There was a progressive congestion of bile canaliculi due to cellular and microvilli swelling. The proposed mechanism of action of the organotin was dithiol inhibition of ATP production in the mitochondria, with consequent interference of ATP-dependent membrane activity in other parts of the cell. Rats maintained greater cellular integrity possibly due to early inhibition of phagocytosis or phagolysosomal activity.

A SERIES OF organotin esters has previously been studied in this laboratory for their toxic effects to biological systems *in vivo* and *in vitro*. It was found that certain of these compounds even in low concentrations, and particularly those possessing the acetate ester moiety, were highly toxic. In investigations by Guess *et al.* (1) and Calley *et al.* (2), these compounds have been shown to rapidly cause death of cell cultures *in vitro;* have an initial CNS depressant effect when administered orally or parenterally to rabbits, rats, or mice; are highly irritating to dermal, muscle, and ocular issue; and bring about hepatic disturbances resulting in biliary obstruction, jaundice, and liver necrosis in mice, and altered liver function in rats and rabbits.

No study reported to date has discussed the mechanism of toxicity to liver tissue by organotin at the subcellular level. According to Rees (3), exogenous toxic agents may cause biochemical changes in liver tissue which lead either to fat accumulation or to necrosis with resultant autolysis. One of these mechanisms of toxic action may be evidenced without the other, or both may occur simultaneously. A reduction or blockage of protein synthesis is the key event in fat accumulation (4–8). The changes in protein synthesis are detectable at a time when intracellular fat accumulations become visible by light microscopy. Harris, Robinson, and Seakins (4–6) have proposed that lack of protein, normally constituting part of the lipoprotein molecule, causes the formation and deposition of triglycerides intracellularly. According to Rees and Shotlander (7, 8), this process is coincidental with a fall in serum lipoprotein levels.

It is generally agreed that intracellular changes which lead to tissue necrosis involve at least two events. These are cell permeability changes and mitochondrial injury. In addition, lysosomal damage may sometimes occur. The latter is thought by de Duve and co-workers (9) to be a terminal stage of liver cell injury and may be the event precipitating necrosis. Disagreement on this point is based upon the late occurrence of above normal amounts of unbound serum lysosomal enzymes, signifying lysosomal damage. Investigations by Slater, Greenbaum, and Wang (10) at this level of liver cell injury have shown a preponderance of unbound enzymes only after necrosis has occurred. Mitochondrial injury is also thought to be a relatively late event in hepatic poisoning, although preceding lysosomal damage. The manner in which mitochondrial change occurs is uncertain, but according to Rees *et al.* (11) may be secondary to other changes. Rees (3) suggests that the initial point of attack of all liver poisons is the endoplasmic reticulum and that this sets into motion a series of events leading to necrosis or fatty degeneration, depending upon whether smooth or rough endoplasmic reticulum is affected, *i.e.*, whether cell permeability and ionic changes occur, or alternatively, protein synthesis is inhibited.

Status of knowledge concerning organotin toxicity through 1958 has been well documented in a monograph by Barnes and Stoner (12). Inhibition of oxidative phosphorylation has been demonstrated by Sone (13) in triethyltin poisoning. Tetraethyltin was found by Cremer (14) to be converted in small amounts to triethyltin, primarily by liver cells, when administered intraven-

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ously in rats. Barnes and McGee (15) found that dibutyltin salts produced lesions of the bile duct in rats and mice, and was found in highest concentration in liver and kidney tissue and was excreted unchanged in bile. They also found that species difference was noted as to bile duct damage, with rabbits, guinea pigs, and cats showing no effect, rats and mice showing lesions.

A serious lack of knowledge exists as to the mechanisms involved in toxicity induced to cellular material by the organotin esters. Changes which may be brought about to the cytoplasmic and nuclear membranes, to cell organelles such as mitochondria, lysosomes, Golgi apparatus, chromosomes, endoplasmic reticulum, *etc.*, and how these changes are related to enzyme levels and normal body function is virtually unknown. The electron microscope is, of course, the most useful tool in determining structural changes at the subcellular level. It was decided that a study combining the capabilities of this instrument with customary light microscope pathological determinations of liver tissue from dosed animals would be a rational approach to delineate some of the more subtle aspects of toxicity.

MATERIALS AND METHODS

Young adult female Holtzman rats, weighing between 275 and 300 Gm., were intubated daily with one-fourth the LD_{50} concentration of dibutyltin diacetate (LD_{50} 109 mg./Kg.). Cottonseed oil was employed as the vehicle and control. One rat was sacrificed at each 24-hr. interval, with the dosage and sacrifice schedule covering a 10-day period. A similar schedule was followed in which young adult female, Swiss-Webster white mice weighing between 17 and 20 Gm. were dosed and sacrificed daily. Liver tissue from both rats and mice was taken immediately upon sacrifice and prepared for histological examination by light microscopy and for ultrastructural interpretation by electron microscopy.

Liver tissue selected for light microscopy was fixed in 10% phosphate buffered formalin, paraffin embedded, and the mounted sections were hematoxylin-eosin stained for examination.

A cross-section of tissue from the center portion of the left lobule of the liver was prepared for electron microscopy. Liver slices approximately 2 mm. in thickness were placed in 2.5% gluteraldehyde buffered with 0.2 *M* collidine. All tissue was placed in preservative within 1 min. after sacrifice to prevent autolysis. After 10 days, the tissue was removed from the buffered gluteraldehyde, cut into 2 mm. by 2 mm. cubes, washed twice for 30 min. in collidine buffer, and post-fixed for 1.5 hr. in cold (4°) 1% osmium tetroxide buffered with collidine. After removal from the osmium fixative, the tissues were dehydrated in a graded series of ethanol solutions, with final dehydration of the tissue in acetone. The material was then embedded in a resin[1] and sectioned on a Porter-Blum Servall MT 1 microtome fitted with a diamond knife. Sections were post-stained with 0.5% uranyl acetate and lead citrate, examined, and photographed on an RCA model 3G electron microscope. Negative prints were taken at ×8,330 magnification and developed to obtain ×21,000 photomicrographs. In the liver tissue, parenchymal, Kupffer, and endothelial lining cells, as well as bile ductules were observed for ultrastructural changes from normal condition represented by the control tissues. Comparisons were made of the size, configuration, spatial relationship, and relative numbers of all cell organelles, including mitochondria, rough and smooth endoplasmic reticulum, lysosomes, phagosomes, Golgi bodies, lipid droplets, or other storage particles. In addition, changes in nuclear size, shape, content, and nuclear and cytoplasmic membranes were also observed.

Data from all procedures were analyzed and compared to determine whether changes had been induced in rats or mice as the result of oral administration of the organotin.

[1] An Epon resin, Shell Chemical Co., New York, N. Y.

RESULTS

Histopathological Determination by Light Microscopy

After about a 2 to 3-day lag period in the development of visible liver damage, both mice and rats began to exhibit progressive liver involvement that reached a peak of pathological change on the sixth and seventh days of the 24-hr. repeating dosage schedule (Fig. 1). In the rat the severity lessened after this critical point, whereas in the mouse the degree of histopathology continued to be manifest.

At the peak of hepatocellular response in the mouse on the seventh through ninth doses, loss of hepatic architecture was severe, often involving the histologic organization of the entire lobule. Cells showed a spectrum of responses including cloudy swelling, opacification, cytoplasmic granulation, some fatty degeneration and infiltration, hyaline change, rupture of cell wall, cellular debris, changes in nuclear size, nuclear dust, and chromatin condensation. Vascular channels lost their continuity, protein was precipitated, and necrosis was common. No repair processes were evident, and the injury to these liver cells was deemed irreversible.

In the rat all changes which were seen in mice were visible except those which might be considered irreversible and lethal to the hepatic cell. There was

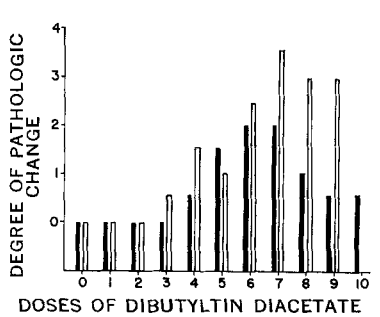

Fig. 1—Histopathological response to daily intubation with one-fourth LD_{50} concentration of dibutyltin diacetate in mice and rats. Degree of pathologic change was rated as: 0, normal; 1, minimal; 2, moderate; 3, moderately severe; 4, severe. Key: ■, rat; □, mouse.

minimal necrosis, rupture of cell walls, profound nuclear change, or vascular discontinuity even at the height of liver damage on the sixth and seventh day of dosing. In other words, the injury to the rat cells was not so extensive, and rats exhibited a terminal recovery pattern.

Ultrastructural Histopathology

Mitochondria—Normal mouse liver mitochondrial profiles (Fig. 2) exhibit a dense, nonvacuolated matrix with a few electron dense narrow lines representing the cristae, the structure bounded by a double membrane. Both mouse and rat hepatic parenchyma cells underwent vacuolar degeneration of the mitochondria as a direct consequence of poisoning with dibutyltin diacetate. These changes were visible 24 hr. after the first dose and were progressive in nature. Mice were more drastically affected than rats.

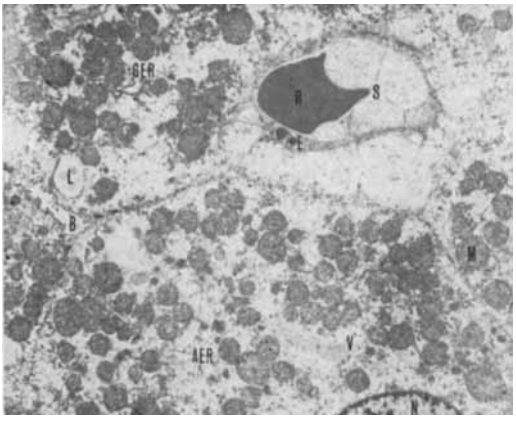

Fig. 2—Micrograph of control mouse hepatic cells showing normal parenchyma cell and organelle architecture. Key: AER, *agranular endoplasmic reticulum;* B, *bile canaliculus;* E, *endothelial lining cell;* GER, *granular endoplasmic reticulum;* L, *lysosome;* M, *mitochondria;* N, *nucleus;* R, *red blood cell;* S, *sinusoid;* V, *Golgi vesicle.*

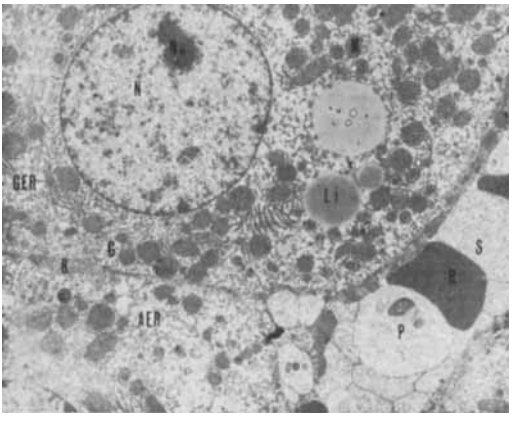

Fig. 3—Micrograph of mouse hepatic cells following the third successive dose of dibutyltin diacetate showing accumulation of lipid (Li), *condensation of chromatin in nucleolus* (Nu) *inside the nucleus, extrusion of phagolysosomes* (P) *into sinusoid* (S). *Several mitochondria* (M) *contain swollen cristae membranes. Lumen of bile canaliculi* (B) *is reduced and contains swollen microvilli. Also appearing unchanged are granular* (GER) *and agranular* (AER) *endoplasmic reticulum, a Golgi complex* (G), *red blood cell* (R), *and sinusoid* (S).

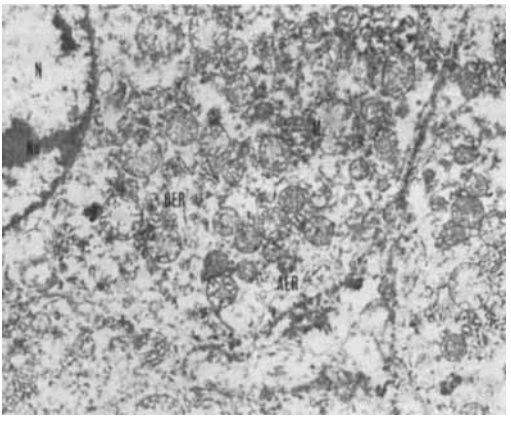

Fig. 4—Micrograph at higher magnification of mouse hepatic parenchyma cell following the sixth successive dose of dibutyltin diacetate showing hydropic degeneration and the formation of myelin figures in mitochondria (M), *swelling and vacuolization of granular* (GER), *and agranular* (AER) *endoplasmic reticulum.*

Initially, the cristae in a few mitochondria of both species became slightly distended (Fig. 3). After six successive doses, the mouse showed a pattern as illustrated in Fig. 4, in which the cristae were swollen and disrupted, the matrix of the mitochondria became less dense, and membranous whorls developed inside. It is obvious at this point that the mitochondrial damage was extensive and probably irreversible. With this degree of organelle injury, it is doubtful that a functional unit existed. Rat mitochondria did not degenerate to the extent described for mice, although they did develop numerous swollen cristae. A recovery pattern in rats was observed to take place beginning with dose seven. Here the cristae began to return to a more normal condition, although swelling of cristae, sometimes developing small vacuoles, could still be seen in some cells. Many cristae appeared normal, indicating a good recovery pattern or lack of original injury by the organotin.

Granular Endoplasmic Reticulum (GER)—In normal parenchyma cells, the double layered membranes of this organelle are often found in close proximity to mitochondria (Fig. 2). Their planar surfaces are covered with small electron dense particles called ribosomes. The cisternae between the membranes have a narrow, undulating appearance. Very little if any change in this pattern was seen after initial organotin dosing (Fig. 3). After repeated doses of organotin, both mice and rat GER showed a progressive swelling but with no noticeable loss of ribosomes. It can be seen in Fig. 4 that after six successive doses in mice, no normal GER can be found. Most cisternae have undergone gross swelling or enlargement and much GER appears to have dispersed as small vesicles, ribosomes intact, into the cytoplasm. Rats showed a similar pattern but to a lesser degree. Considerably greater amounts of GER either remained intact or were more rapidly regenerated in the rat cells (Fig. 5).

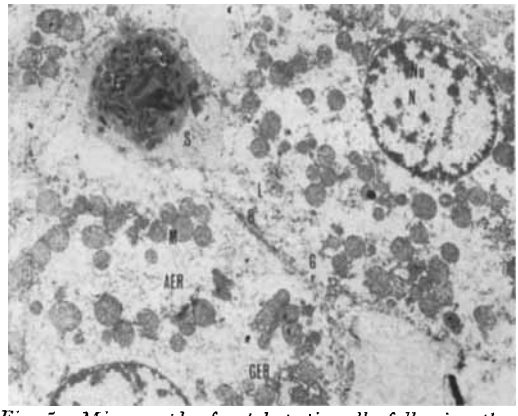

Fig. 5—Micrograph of rat hepatic cells following the tenth successive dose of dibutyltin diacetate in which organelles have been preserved or regenerated, in contrast to the condition found in the mouse at this dose interval. Agranular (AER) *and granular* (GER) *endoplasmic reticulum have near normal structure, bile canaliculus* (B) *remains obstructed, eosinophils* (Eo) *are present in sinusoid* (S), *Golgi* (G) *and lysosome* (L) *activity is present, mitochondria* (M) *appear normal except for a few vacuolated forms, chromatin in nucleolus* (Nu) *remains condensed.*

Agranular Endoplasmic Reticulum (AER)—In normal hepatic cells the AER were visualized as minute channels, often appearing in cross section as tiny vesicles (Fig. 2). These were most often irregularly dispersed in areas within the cell which were lacking in other structures, and having a somewhat transparent cytoplasmic matrix. The complexity of these structures greatly increased after the first three doses in both species, depicted in the mouse in Fig. 3. Further dosing led to irregular dispersion of these tubules, with some disruption into discrete vesicles appearing. Random invasion by cellular components of the areas formerly occupied only by the AER was evident (Fig. 4) creating a disorganized assemblage of vesicles from both AER and GER plus abnormal mitochondria and other vacuolar material. This pattern was seen in certain hepatic cells of both species, although much more prevalent in the mouse. Many of the rat cells appeared to regain or retain much of the original internal architecture (Fig. 5) and the AER was again found in normal amounts and sites inside the cells.

Bile Canaliculi—The normal condition of this structure in both rats and mice, as shown in Fig. 2, was an uncongested lumen into which projected a few small microvilli. Membranous whorls or myelin figures were commonly found unattached in the lumen, which was otherwise free of particles. In both study species, beginning with the second dose of organotin, these bile capillaries were rapidly closed (Fig. 3) so that after the third dose, no lumen was visible. The closing was attributed to two primary factors, one of these being the swelling of the parenchyma cell. The microvilli also appeared to have become swollen, further contributing to the congestion. No relief from this pattern was noted through the dose series in either mice or rats.

Lysosomes—Normal lysosomal activity involves discharge of contents from this aging organelle into the bile canaliculi as depicted in Fig. 2. After dosing with organotin, the bile capillaries became swollen and obstructed and lysosomes were seen to empty into the sinusoid instead, as shown in Fig. 3. As dosing frequency increased beyond the sixth in the series, an inhibitory effect was seen on parenchyma cell lysosomal activity, especially in mice. This lack of lysosomal activity was associated in both species with extreme disorganization and random dispersion of numerous vesicles and particles inside many cells (Fig. 4). In mouse cells, lysosomal activity was never reactivated to any appreciable extent. In rat cells, however, there was an apparent regeneration of lysosomal activity as shown in Fig. 5, even though the structures seen were much smaller and less numerous than in controls. Phagocytic activity, often associated with lysosome activity, was initially stimulated by dosing in mice but appeared to be inhibited in rats. This could have accounted in part for the damage seen in mouse cells as compared to rats.

Golgi Complex—The Golgi complex in normal cells is seen to arise from normal AER and is easily recognized by its structure (Fig. 2). Since the Golgi complex may be one of the sources for lysosomal production, it would be expected that damage to the AER would damage the Golgi and hence the lysosomal enzyme production. It was apparent that lysosomal activity showed a decrease at about the same time as the disappearance of the Golgi was noted. After the sixth dose of organotin in mice, the Golgi were not seen (Fig. 4) but are readily distinguishable in rat cells even after dose ten (Fig. 5).

Nucleus—The primary effect of organotin on the nucleus was a condensation of the nucleolus in both species to create a more dense often somewhat reticulated pattern shown in Fig. 3. This effect was noted after dose three and remained unchanged throughout the remainder of the series in mice and rats. Another effect was the accumulation of lipid and other small droplets in the nucleus seen during the later phases of dosing, primarily in mice.

Lipid Droplets—A noticeable increase in the number of lipid droplets began to be apparent from the first dose of organotin and were evident through the third dose (Fig. 3) in mice. These droplets were seen only rarely thereafter, indicating little or no fat accumulation in the cell or, alternatively, an increased mobilization from the cell, which was doubtful. In rats, small amounts of lipid were found after dose four and little or none at successive doses. This observation confirms the general observation that rat cells are still functioning.

Kupffer and Endothelial Lining Cells—Endothelial lining cells of both species showed increased thickness and granularity after dosing. Excessive swelling in some cells developed later, with sparse matrix containing small amounts of tiny vesicles. Kupffer cells increased in matrix density and showed greater numbers of dark staining granules in the cytoplasm.

DISCUSSION

The earliest noted ultrastructural change attributable to the dibutyltin diacetate dosing schedule was in the parenchymal cell mitochondria of both rats and mice. In this organelle, the cristae membranes responded initially by swelling and this became more

pronounced through time as doses of the organotin were repeated daily. This mitochondrial reaction was much more advanced in mice at any given dose than in the corresponding rat hepatic cells. As dosing continued in the mouse, the swollen cristae developed into the formation of large vacuoles and finally into a complete disorganization of the mitochondrial structures. At the latter stages of dosing, myelin figures were noted in grossly distorted mitochondria.

The observed fact that the mitochondria were the earlier organelles to be visually injured by the organotin appears to be in some disagreement with the views proposed by Rees (3). According to the scheme which he proposed, the granular endoplasmic reticulum is the first subcellular organelle to be injured by a liver poison. This injury to the GER would result in cellular permeability changes and ionic imbalance, resulting in a later mitochondrial injury.

Based on the over-all morphological changes in the mitochondria as observed in this study, one could propose that the first alteration of consequence would be the failure of the mitochondria to produce adequate ATP for the cell to maintain normal energy requiring functions. It is known that the cristae of the mitochondria are involved with electron transport enzymes and are more susceptible to swelling than the outer limiting membrane (16). Christie and Judah (17) concluded from their studies on vacuoles of mitochondrial origin obtained from poisoned hepatic cells that such poisons could induce hydropic changes to mitochondria and this in turn interferred with the tricarboxylic acid cycle at the citrate and maleate level, thus preventing final metabolism of pyruvate and acetate necessary for eventual ATP formation. The possible mechanism of interference with the tricarboxylic acid cycle has been proposed by Peters (18) and Williams (19) as interfering with the activity of α-keto acid oxidases. A molecule of the divalent toxic substance, in this case, dibutyltin diacetate, will combine with two SH groups in the enzyme, which is thereby inhibited. The enzymes inhibited by this mechanism would be pyruvic and α-keto glutaric dehydrogenases, enzymes which are essential for the formation of ATP *via* these cycles. Robinson (20) found that 2,4-dinitrophenol, which makes energy released by oxidation unavailable for ATP formation, causes tissues to take up water, implying that energy is normally expended by cells to prevent hydropic degeneration. Price *et al.* (21) found that isolated liver mitochondria maintained normal water content when incubated under conditions favorable to oxidative phosphorylation. Other investigators (22–24) have also found that failure of ATP synthesis appears to be a critical factor in determining whether cell degeneration will proceed to autolysis. Mouse liver mitochondria showed a progressive degeneration and this was reflected in areas of eventual liver necrosis in these animals, but was not as extensively shown in rats. However, as pointed out previously, the rat mitochondria were never injured to the same extent as the mouse tissue, thereby explaining the apparent partial cellular recovery in rats.

It can be postulated, then, that early mitochondrial injury in mouse and rat hepatic parenchyma cell poisoning is probably one of the underlying causes for toxic manifestations to other cellular organelles having a dependency on ATP for normal function.

Under the conditions of this study the granular or rough endoplasmic reticulum (GER) was the second cell organelle to manifest degenerative change. A progressive hydropic change with gross swelling and vacuolization of the cisternae of this structure is considered to be a contributing factor to the cloudy swelling as seen through the light microscope. Confirming studies by Molbert (25) and Oberling (26) also have shown that vacuolization of the endoplasmic reticulum and dilation of its cisternae were contributing factors to hydropic degeneration and cloudy swelling. It was apparent from the series of micrographs of normal cells that a very close spatial relationship existed in these cells between mitochondria and the GER. There is, no doubt, a direct functional dependency. The GER, for example, depends upon material elaborated by the mitochondria for membrane transport. In pathological cells, particularly those showing greatest degenerative change, the close relationship between these two organelles was lost. In the rat, the ability to maintain to a greater degree the integrity of both structures in certain areas of the cell was evident. It is probable that in such cases the GER maintains its membrane activity by ATP supply from mitochondria, and in the absence of this supply, cannot adjust to ionic and/or water imbalance. When the latter exists, swelling of the cisternae develops.

Porter and Bruni (27) postulate that the agranular or smooth endoplasmic reticulum (AER) portion of the vacuolar system is formed from the terminals of the GER and has a function in carbohydrate metabolism. In addition Essner and Novikoff (28) proposed that secretory and hydrolytic Golgi complexes were formed from the AER. Shnikta (29) indicated that microbodies may also arise from the AER, but in a different manner than do the secretory or hydrolytic bodies. As a result of the administration of the organotin compound, there was an obvious disruption of the AER as well as the GER, followed by a disappearance of the above-mentioned elaborated structures. If these structures are no longer elaborated by the AER, there would follow a cessation of various enzyme formation and activity, an accumulation of precursors and raw materials usually subject to metabolism by enzyme action.

Of particular importance, perhaps, was the loss of ysosomal action which maintains intracellular digestion and either recycles or extrudes from the cell the breakdown products. According to de Duve and Wattiaux (30), lysosomes are almost always involved in the response of the cell to challenging agents and by failing to perform their lytic actions adequately or performing them in injurious fashion may contribute to the cell pathology. Their malfunction may be due to uptake of inhibitors by a derangement of enzyme biosynthesis, or as Triederici and Pirani (31) suggest, by the production of abnormal proteins of low molecular weight which contribute to edema of the cell. It is not thought, however, that compressive or congestive lysosomal damage was a major factor in poisoning by dibutylin diacetate in rats, although in mice there was an obvious initial lysosomal or phagolysosome stimulation which declined with successive dosing.

One of the prime responses of the hepatic cells of both mice and rats was the rapid congestion of the bile capillaries upon dosing with the test compound.

This feature was not observed after the first dose, but some congestion was observed after the second dose, and almost complete reduction of the lumen of the canal was observed after the third repeated dose. This injury was of great significance to the welfare of the hepatic cells, since it obstructs a primary route of discharge of unwanted waste products, causing them to pile up inside the cell or, when possible, to be discharged into the sinusoid or extracellular spaces. In rats, lysosomal enzymes are all present in the bile, according to de Duve and Wattiaux (30), at a concentration indicating a daily unloading of about 5% of the total hepatic lysosomes. Confer and Stenger (32) and Bruni and Porter (33) suggest that the peribiliary location of many liver lysosomes and the presence of amembranous dense bodies and myelin figures in the lumen of bile capillaries suggest elimination by exocytosis into this structure. This suggests that the bile may be the major pathway for unloading lysosomes or their contents by hepatic parenchyma cells. The release of lysosomal enzymes into body fluids or into extracellular fluids has been observed by several researchers (34–37) to be associated with pathological phenomena. This congestion then left the parenchyma cell without a normal route of elimination of these cellular waste products, possibly resulting in an intracellular accumulation and subsequent necrosis.

SUMMARY AND CONCLUSIONS

Dibutyltin diacetate was administered daily in one-fourth LD_{50} doses for a period of 10 days. Ultrastructural damage to liver parenchyma cells after each 24 hr. of dosing was evaluated by the electron microscope and by the light microscope. Chronologically, the order of injury to the subcellular organelles and structures were as follows.

1. Mitochondrial damage with early cristae swelling followed by vacuolization and hydropic swelling.

2. Swelling and vacuolization of the granular endoplasmic reticulum.

3. Progressive congestion of the bile canaliculi due to cellular and microvilli swelling.

4. Formation of a dense population of small vacuoles resulting from the disruption of the rough and smooth endoplasmic reticulum.

It is felt then that the initial and progressive mitochondrial lesions interfered with normal biochemical and synthetic activity through a reduction and/or blockage of ATP production by dithiol inhibition. As a consequence, other cell functions involving active transport across membranes as well as certain synthetic and metabolic functions were inhibited. The net result of these inhibitory processes was cell necrosis in both mice and rats, although moderated in the latter. The ability of the rat hepatic cells to maintain better internal organization throughout the dosage schedule seemed to be related in part to an early cessation of phagocytosis or phagolysosomal activity, thus preventing intake of lethal (cellular) doses of the organotin by these routes.

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