A STUDY OF THE INTERACTION OF METHYLMERCURIC CHLORIDE AND GAMMA IRRADIATION ON PEROXISOMES AND LYSOSOMES

A DISSERTATION

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FAYEZ S. SALAITA, B.S., M.S.

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INTRODUCTION

The unlimited use of various mercury compounds as fungicides and for other purposes during the last thirty years has resulted in widespread contamination of the environment. Toxic trace metals such as mercury, cadmium, and arsenic are present in some fossil fuels and may be emitted in substantial quantities during the course of energy production (Fowler et al., 1974). Release of these elements into the environment from power plants may lead to their accumulation in soil, water and edible biota at appreciable distances from the original source. Ingestion of water or food organisms, such as fish, contaminated with these elements represents a potential human health hazard of unknown magnitude.

Several episodes of methylmercury poisoning in man have occurred in Japan, Iraq, Pakistan, and Mexico (Miyakawa et al., 1970). The implications of inorganic mercury contamination of the milieu are less clear. In spite of the recent advances in studies of the biotransformation of inorganic mercury by microorganisms, the exact mechanism is not very clear (Brun et al., 1976).

The problem of assessing biological effects in humans after prolonged low-level exposure to mercury, is complicated by a basic lack of understanding the *in vivo*

mechanism of metal toxicity, as well as the impact of trace element exposure on cellular responses due to other environmental toxicants such as carcinogens. It is presently clear, however, that many cellular systems affected by trace elements are also sensitive to other classes of toxicants.

Reports of mercury studies have appeared in great numbers in the literature (Hunter, 1943; D'Itri, 1972); however, despite this, there appears to be an absence of extensive research on interactions of methylmercury and other insults, such as gamma irradiation, which will be undertaken in this study.

The proposed study is an extension of the work of Earhart (1975) who demonstrated that hydrogen peroxide (H_2O_2) pretreatment protected against methylmercury induced mortality. He also showed that hydrogen peroxide pretreatment increased the number of peroxisome-like organelles in the nucleus arcuatus of the rat brain. He hypothesized that proliferation of peroxisome-like organelles protected against the effect of methylmercury on the nervous system. The results of the proposed study will show whether hydrogen peroxide pretreatment induces peroxisome proliferation in other tissues, for example, liver and kidney, and whether ionizing radiation with methylmercury pretreatment also elicits peroxisome proliferation. Whether H_2O_2

pretreatment also protects against radiation induced mortality will be investigated in the second phase of the proposed study. Specific objectives of the study are:

1) to investigate histochemically the effect of gamma-irradiation, methylmercury, the combination of methylmercury and gamma-irradiation, and hydrogen peroxide upon the proliferative activity of peroxisomes and lysosomes in the brain, liver, and kidney in male rats.
2) to study the effect of hydrogen peroxide pretreatment as a protective agent against lethality due to irradiation of male rats.

REVIEW OF LITERATURE

Mercury as a toxic agent has been known for many centuries. Since the outbreak of mercury poisoning in Minamata Bay and Niigata District of Japan in the 1950's, organic mercury, particularly methylmercury, has been recognized as a hazardous environmental pollutant (Katsuki et al., 1957). Mercury is found in the environment in its elemental form and in organic and inorganic compounds. Mercurial poisoning has occurred following treatment with diuretics, through careless industrial use of mercury, after consumption of food contaminated by fungicides, and as a result of other contamination. Intoxication by these mercurials is characterized by neurological symptoms such as sensory disturbances, ataxia, concentric constriction of visual fields, and loss of hearing (Friberg and Vostal, 1972).

Kidney, liver, and brain are the organs most vulnerable to intoxication by mercury compounds. Morphological changes in these organs after mercury intoxication have been studied extensively by numerous investigators (Miyakawa and Deshimaru, 1969; Sahaphong and Trump, 1971; Chang and Hartmann, 1972).

Since the advent of the "atomic age," numerous studies have been made concerning the biological effects of various types of ionizing radiation, as evidenced by the

large body of scientific literature (See reviews by Haley and Snider, 1962; Altman et al., 1970).

Both peroxisomes and lysosomes have features which indicate their involvement in a cellular response to toxic agents such as heavy metals and ionizing radiation. A peroxisome is a cytoplasmic organelle bounded by a single, well formed membrane and contains a finely granular amorphous matrix (Rouiller and Bernard, 1956). The matrix contains catalase and various H_2O_2 producing oxidases (De Duve and Baudhuin, 1966). In the liver and kidney, the presence of a crystalline nucleoid aids in its morphological identification (Hruban and Rechicigl, 1969). Since the development of the alkaline diaminobenzine (DAB) technique for visualization of the peroxidative activity of catalase (Novikoff and Goldfischer, 1969), 0.2 - 0.5 micrometer (μm) catalase positive particles have been identified in many tissues. In fact, it has been proposed by Hruban et al. (1972) and Novikoff and Novikoff (1973), who coined the term "microperoxisomes" for these particles, that they are ubiquitous in mammalian cells.

The peroxisomes of rat kidney were first described by Gansler et al. (1956). They are usually larger in the proximal convoluted tubules than the peroxisomes of rat liver and mouse kidney (Ericsson, 1964; Ericsson and Trump, 1966). They show a close spatial relationship to the

smooth endoplasmic reticulum and to the system of paramembranous cisternae (Ericsson, 1964; Ericsson and Trump, 1966). These peroxisomes are enclosed by a single membrane approximately 60 - 65 angstrom (A) thick (Ericsson and Trump, 1965) which often shows a scalloped or wavy outline.

Recently, Herzog and Fahimi (1974, 1976) and Hand (1974) independently described the presence of peroxisomes in the ventricular myocardium of the adult mouse and rat, respectively. These particles were described as measuring 0.2 - 0.5 μm in diameter and being in close association with mitochondria and the sarcoplasmic reticulum. Herzog and Fahimi (1976) also confirmed biochemically the presence of catalase and small amounts of two flavin oxidases in microsomal fractions of heart tissue and suggested that the particles in the mouse myocardium morphologically and biochemically resembled the peroxisomes of other tissues.

Hicks and Fahimi (1977) investigated the occurrence of peroxisomes, their cytochemical characteristics and their ultrastructural relationship to the neighboring organelles in the ventricular myocardium of four rodents (rats, rabbit, gerbil, and guinea pig) and two primates (Macaca java and Tupaya). The electron-dense reaction product of catalase was found in the myocardium of these species and was localized in 0.2 - 0.5 μm oval particles, surrounded by a single limiting membrane and located usually at the

junction of I and A bands. A close spatial association was found between the myocardial peroxisomes and mitochondria, lipid droplets, and the membranes of sarcoplasmic reticulum, especially the so-called junctional sarcoplasmic reticulum. These observations demonstrate the consistent occurrence of peroxisomes in the heart of various mammalian species and suggest that peroxisomes have important metabolic and physiological functions in the myocardium.

In a histochemical investigation, Itabashi et al. (1977) used the 3,3'-diaminobenzidine reaction to demonstrate the catalase activity, and thus variations in number of peroxisomes. Electron microscopic examinations were made of hyperplastic liver lesions in rats fed 0.06% 3-methyl-4-dimethylaminoazobenzene. At the 10th week of carcinogen feeding, hyperplastic lesions (hyperplastic foci, areas, and nodules) appeared and advanced to further stages. Most of the foci and some of the lesion areas and nodules showed very low catalase activity and, correspondingly, a small number of peroxisomes. When these workers administered ethyl- α -p-chlorophenoxyisobutyrate to rats, there was an increase in catalase activity and in peroxisome number.

Hand (1973) identified peroxisomes in three parenchymal cell types of the normal parotid gland of the rat. They average 0.33 μm in diameter in the acinar and intercalated duct cells, and 0.22 μm in the striated duct cells.

They were closely related to the endoplasmic reticulum, occasionally in continuity with smooth surfaced cisternae and often embraced by ribosome-free portions of endoplasmic reticulum which paralled their membrane. Glutaraldehyde fixation inhibited the endogenous peroxidase of the parotoid gland and allowed visualization of the peroxisomes following incubation in alkaline diaminobenzidine medium. Peroxisomal staining was unaffected by varying H_2O_2 concentrations or concentrations of potassium cyanide (KCN) below 0.1 M, but was prevented by aminotrazole and dichlorophenolindophenol. Examination of other exocrine glands, after incubation in diaminobenzidine medium, revealed the presence of peroxisomes in the pancreas, and in the submandibular, lacrimal, nasal mucosal and Von Ebner's glands. These studies indicated that peroxisomes are of widespread occurrence in exocrine tissues of the rat.

Studies by Staubli et al. (1977) of repeated administration of the hypolipidemic agent Su-13-437 (Nafenopin) to neonatal rats roughly doubled the number of peroxisomes in the liver tissue and caused a six-fold volumetric expansion of the peroxisomal compartment. During the proliferative response, the size-distribution of the peroxisomes was reversibly altered, enlarged particles appearing in numbers varying according to the dose given. By means of a new method of quantitative autoradiography, Staubli et al.

(1977) showed that a) the concentration of silver grains over the peroxisomes was comparable to that found over the endoplasmic reticulum (ER); b) the peak incorporation of tritiated arginine into the peroxisomes was delayed in comparison to that of the ER; and c) the label, once incorporated into the expanding peroxisomal compartment, displayed the same shift to large particles as did the whole population of peroxisomes. These results are compatible with the biosynthetic pathway for peroximal catalase proposed by De Duve (1973), and with the notion that the drug induced size-shift might have resulted from progressive growth of a particular class of peroxisomes formed in the presence of the agent. During the recovery period the larger peroxisomes were removed preferentially.

Staubli et al. (1977) administered BR-931, and ethanolamine derivative of [4-chloro-G-(2,3-xylidino)-2-pyrimidinythio] acetic acid, known as Wy-14,643, at a dietary concentration of 0.125% for 3 weeks to male F-344 rats. This resulted in a significant enlargement of the liver. The hepatomegaly appeared to be due to liver cell hyperplasia and hypertrophy resulting, in part, from peroxisome and smooth endoplasmic reticulum proliferation. The hepatic catalase and carnitine acetyltransferase activities increased significantly in association with peroxisome proliferation. All these hepatic effects were reversible when

the drugs were withdrawn from the diet. Reddy and Krishnakantha (1975) demonstrated that Wy-14,643 caused a significant increase in the liver weight and hepatic peroxisome population in rats and mice.

In an ultrastructural study of human liver biopsy specimens, Sternlieb and Quintana (1977) found that peroxisomes are regularly present in normal human hepatocytes. Some normal peroxisomes were found to display marginal plates or peripheral crystalline inclusions which were present in pathologic specimens as well. They also reported that in certain inherited metabolic disorders (Menkes' steely hair disease, analbuminemia) the volume of the individual peroxisomes appeared to be considerably reduced. Most pathologic processes affecting hepatocytes seem to produce one or several of the following: increased volume or numbers per cell, changes of shapes, alterations of the consistency of the matrix, appearance of dense inclusions, or clustering of peroxisomes in some portions of the cytoplasm. Sternleib and Quintana (1977) concluded that various pathologic processes involving the hepatocytic cytoplasm exert different effects on peroxisomes.

Srebro and Cichocki (1971) proposed that the glial "Gomori-positive" granulations are large peroxisome-like cytoplasmic organelles. It has also been reported that the number of the "Gomori-positive" or cysteine-rich

periventricular glial cells considerably increased following whole-body-X-irradiation (Srebro, 1969, 1970). An increase was observed in the number of "Gomori-positive" glial cells in rats irradiated with high doses of X-rays to the head region and in mice exposed to protracted total body UV-irradiations. This increase was considered as a compensatory formation of the peroxisome-like organelles in response to circulating radiotoxins of an organic peroxide type which occurs during the post irradiation period.

The peroxisome-like system of organelles demonstrated in the periventricular glia by Srebro et al. (1970) becomes extremely attractive as a possible response mechanism for the protection against methylmercuric chloride (MMC) toxicity. The presence of such a system has been described and shown to be rich in thiol and disulfide groups (Noda, 1959; Srebro, 1970; Srebro and Cichocki, 1971).

Lysosomes are also organelles that may play an important role in cellular response to radiation and other toxic agents. The prime criteria which appear necessary for the demonstration of lysosomes are 1) the presence of one or more acid hydrolases, known to be associated with lysosomes at particulate sites; 2) such sites should possess a single limiting membrane; and 3) the reactions of the particles for the acid hematein test, the periodic acid-Schiff reaction, or with the vital dyes (Gahan, 1965).

De Duve (1963) considered that the lysosomal hydrolases have one function, namely, that of acid digestion; he excluded any possible role in biosyntheses. De Duve further considered these enzymes to act in one of two ways, the one involving heterolysis or true intracellular digestion and the other involving autolysis or self-digestion of the cell. Novikoff and Shin (1964) suggested as a working hypothesis that a membrane-delimited cytoplasmic particle showing acid phosphatase activity is likely to contain other hydrolases and so may be considered as a lysosome.

Whole-body exposure of mice to ionizing radiation can result in marked changes in the activities and/or distribution of lysosomal hydrolases found in lymphoid tissue (Altman et al., 1970). There was an increase in the fragility of lysosomes following radiation exposure to 700 R gamma irradiation, and redistribution of enzymatic activity from the particulate to the supernatant fractions, indicating increased permeability of the lysosomal membrane in vivo (Roth and Hilton 1968; Rene et al., 1971).

Aikman and Wills (1974b) studied lysosomal membrane permeability and acid phosphatase activity histochemically in sections of tissues from mice which had been subjected to 50 - 1,000 rads whole-body irradiations. After a whole-body dose of 850 rads, they reported no change in lysosomal

membrane permeability or in enzyme activity of liver, kidney, heart, or adrenal; but, substantial increase of membrane permeability and acid phosphatase activity were observed in spleen and thymus. A dose of 100 rads caused an increase in the permeability of lysosomal membranes in the spleen; whereas, after 300 rads, the membranes were fully permeable. Enzyme activity was progressively increased by doses up to 850 rads and greater activation of the enzyme was observed in the thymus than in the spleen.

Several mechanisms for the release of hydrolases from lysosomes have been proposed. Some have suggested that labialization of the lysosomal membrane may result from direct attack of lipid peroxides on the membrane (Kocmiersha and Rodzka, 1972; Wills and Wilkinson, 1966). Lipoperoxides generally are present in tissues at a low level because water soluble antioxidants react with them (Wills, 1966).

Formation of lipoperoxides is related to the effects of radiation on membranes of subcellular particles, especially lysosomes (Wills and Wilkinson, 1966). Liberation of lysosomal hydrolases, as well as disruption of lysosomes, take place after relatively low doses of in vivo irradiation (100 - 700 R) (Gouiter and Gouiter, 1962) but requires much higher doses of 5 - 20 kR in vitro (Wills and Wilkinson, 1966).

The peroxidative process antecedes the release of enzymes from in vitro irradiated lysosomes (Wills and Wilkinson, 1966) and may be an obligatory step leading to the disintegration of the membrane. Others postulate that the permeability of the lysosomal membrane following radiation may be regulated by hormones (Rahman, 1963) or by mediators released from radiosensitive lymphocytes (Aikman and Wills, 1974a).

Chang and Reuhl (1977), by means of electron microscopy, reported an increase in lysosomal activities, formation of giant sized lysosomes and disintegration of the rough endoplasmic reticulum suggesting an early sign of cellular degeneration of the neurons when female rats were administered methylmercuric dicyandiamide. Thus, it is possible that lysosomes may be involved in the cell protective mechanism, based on the assumption that lysosomes engulf methylmercury particles thereby inactivating them.

Lysosomal enzymes are suggested to be involved in senescence of mammalian tissues (Finch, 1972; Wilson, 1973). Most previous studies have been concerned with change in total activities of enzymes while few investigations have focused on differences in the intracellular location of enzymes (Wilson, 1973). Goto et al. (1969) demonstrated that the ratio of acid ribonuclease activities in the soluble fractions to that in the particulate fractions of

liver increased during aging of animals and their results suggest an enhancement of lysosome fragility with a possible liberation of the enzyme into the cytoplasm with age. Cristofalo and Kabakjian (1975) reported an increase of acid phosphatase and β-glucuronidase activities in the lysosomal and supernatant fractions of a human lung cell line (WI - 38) during in vitro again, and the stabilization of lysosomes and life span extension of cells in hydrocortisone-treated culture. Shingo et al. (1979) found when the "senile" (34-37 months) rats and "old" (24-27 months) rats were compared, it was evident that the total activity of liver β-N-acetylglucosaminidase and brain acid deoxyribonuclease in the supernatant and the specific activity of brain β-N-acetylglucosaminidase in the microsomal fraction of senile rats were significantly greater. In most cases the ratios of activities of other enzymes in either or both the microsomal and supernatant fractions were very similar to those of liver and brain β-N-acetylglucosaminidase. These changes of the intracellular location of the lysosomal enzymes suggest two possibilities. First, the smaller lysosomal vesicles may accumulate during late senility. Second, the fragility of lysosomes may be enhanced with senescence (Goto et al., 1969; Cristofalo and Kabakjian, 1975) and the disruption of the particles during homogenization may be induced, resulting in the liberation of

fragments of the lysosomal membrane containing the enzymes into the supernatant and/or the microsomal fraction.

Mercury and other heavy metals accumulate in lysosomes of different tissues under normal conditions and during various diseases and intoxications (Brendeford, 1971; Sternlieb and Goldfischer, 1976). During exposure of animals to mercury, the kidney is the organ with the highest deposition (Clarkson, 1972) and mercurials have been widely used as diuretics acting primarily on renal sodium transport (Cafruny, 1968). In the kidney, mercury accumulates in cortical lysosomes during chronic intoxication with mercuric chloride (Madsen, et al., 1976) and accumulation has also been reported during intoxication with methylmercury hydroxide (Fowler et al., 1974), but whether mercury has any influence on normal lysosomal functions is unknown. It is well established that lysosomes in the proximal tubule cells play an important role in the degradation of proteins taken up from the glomerular filtrate (Christensen, 1976; Christensen and Maunsbach, 1972; Maunsbach, 1973).

Madsen et al. (1976) demonstrated that low concentrations of mercury interfere with normal lysosomal functions in the renal proximal tubule by decreasing lysosomal proteolytic activity. In vitro addition of mercurials to lysosomal enzymes induced a concentration dependent decrease in the degradation of both lysozyme and cytochrome c. It is

known that proteins absorbed in the kidney are transferred to lysosomes that contain a group of proteolytic enzymes, cathepsins (Straus, 1964; Maunsbach, 1973), including the thio-dependent cathepsin B (Shibko and Tappel, 1965). In as much as mercurials are potent enzyme inhibitors, primarily reacting with SH-groups (Webb, 1966), it seems likely that the demonstrated effect of mercury on lysosomal protein digestion is due to inhibition of some of the cathepsins (Madsen and Christensen, 1978).

Madsen and Christensen (1978) also showed no difference in total uptake and intercellular transport of protein to lysosomes of the proximal tubule in normal and mercury-intoxicated rats in contrast to results obtained in experiments with mice, in which it was suggested that mercury inhibits heterolysosome formation (Mego and Barnes, 1973).

Barratt and Wills (1979) reported that the activity of lysosomal acid phosphatase and β-glucuronidase was increased in both spontaneous and transplanted C_3H mouse tumors immediately after they had received hyperthermic treatment *in vivo*. The activation was not confined to neoplastic cells, as the lysosomal enzymes in the spleen were activated after lower-body hyperthermia. The latter activation occurred at a lower temperature than that used with tumors, since the water temperature was only 42°C and the spleen, being a deep tissue, was probably several degrees

cooler. They suspected that part of this activation was due to indirect stimulation by hormones. The immediate response of transplanted tumor lysosomes to hyperthermic treatment was not potentiated by tumor irradiation with 3,500 R of ^{60}Co gamma radiation prior to heating. The lysosomal activation 24 hours after irradiation increased if the radiation was followed by 1 hour at 43°C. The treatment used was one that has produced a large percentage of cures in the C_3H tumor system (Overgaard and Overgaard, 1972). The individual heat and radiation regimes alone would not cause a high percentage of cures so some interaction between the modalities must have occurred. In contrast, hyperthermia and irradiation did not appear to be synergistic in their effects on lysosomes. Lysosomal activation may not, therefore, be a primary event in tumor regression which results from combination therapy. Nevertheless, lysosomes are probably very important in the hyperthermic response and are involved in all types of tumor cell destruction, which involves autolysis, but as a secondary agent are triggered by some other biochemical event.

Klein et al. (1973) noted by light microscopic autoradiography a uniform distribution of lysosomes throughout all segments of the proximal convoluted tubule of the kidney following a single injection of labeled methylmercuric hydroxide into male rats.

In a study on rats Fowler et al. (1974) demonstrated that mercury was sequestered in lysosomes of male rat kidney and stored as discrete particles in lysosomes that differ morphologically from the characteristic phagosome or autophagosome. The mercury-containing lysosomes have a granular matrix, while phagosomes contain remnants of cell organelles and membrane profiles. Mercury-containing lysosomes of renal cells also differ from the normal renal lysosomes with respect to their localization in the second and third segments of the proximal convoluted tubule rather than in the first and second segments.

It has been suggested by Brun and Brunk (1970) that metals at low concentrations may be normal constituents of lysosomes perhaps existing as ligands to sulfhydryl groups. Lysosomal binding of metals, dyes, and cationic drugs has been associated with an acidic lipoprotein, probably of the lysosomal matrix, (Dingle and Barrett, 1968; and Goldstone et al., 1970). The coupling of mercury to such a component could explain the homogeneous, granular appearance of the lysosomes. Chronic low level mercury administration may simply stimulate a normal lysosomal mechanism for binding metals.

The mode of mercury entry into lysosomes is unclear. Other heavy metals, such as lead and gold, appear to gain entry into lysosomes through autophagy of poisoned

organelles, such as mitochondria, (Goyer, 1968; Stuve and Galle, 1970).

Hupp et al. (1977) reported that generally the effect of the co-insult treatment with ionizing radiation and methylmercuric chloride was less than that of either agent alone. Hupp et al. (1974) in assaying for brain serotonin and norepinephrine after single and co-insult treatment with X-irradiation and MMC, found indications that the effect of single insults upon neurotransmitter levels tended to be neutralized in the co-insult treatment. The mortality response of the rats to the co-insult treatment was intermediate to their responses exhibited after the single insult treatments, indicating a neutralization pattern in which the X-irradiation effect appeared to override the mortality effect of MMC (Hupp et al., 1977).

Earhart (1975) proposed that the neutralization effect, inferred from the mortality response of rats to the co-insult treatment with MMC and X-irradiation, indicated that X-irradiation affords a measure of protection against MMC toxicity. He proposed that the effect might be due to proliferation of peroxisome-like organelles in the brain elicited by the effects of the radiation, which in turn protected against methylmercuric chloride toxicity. This lead to his studies with hydrogen peroxide described in the introduction. However, the literature reviewed earlier

suggests that peroxisome proliferations in other tissues and also the possible role of lysosomes in the protective response should be investigated.

Rationale for Choice of Experiments

The histological and histochemical experiments, designed to determine any changes in the number of peroxisomes and lysosomes, were based upon results of Earhart (1975), who reported an increase in the number of peroxisome-like (P-L) organelles in the nucleus arcuatus of the rat brain, after treatments with hydrogen peroxide and a single insult of 800 R ^{60}Co whole body gamma irradiation. Peroxide injection may produce many effects, in addition to P-L organelles proliferation, capable of protecting against mercury-induced mortality, directly or indirectly making -SH groups more available in all tissues. Thus, methylmercury may have been trapped before reaching the brain.

The choice of liver and kidney as additional tissues to examine was based on the following:

a) Since intraperitoneally (I.P.) administration of the agents leads to liver accumulation, it seems that liver sequestration of mercury would most likely be a factor to consider.

b) Based upon the fact that the kidney is the primary excretory organ, it is possible that some of the mercury particles have been trapped by the proximal and/or distal tubules.

Earhart (1975) proposed that both hydrogen peroxide and gamma irradiation protected against the effect of MMC intoxication by causing a proliferation of P-L organelles. If this is a general phenomena, hydrogen peroxide should also protect against the indirect effects of radiation and, therefore, provide protection against a mid-lethal dose of gamma radiation. Thus the lethality experiment was designed to test this hypothesis.

MATERIAL AND METHODS

Animals

A total of 30 large mature male Sprague-Dawley-derived rats, approximately 170 days old and weighing 415 - 505 g, were used in a preliminary lethality study, and 110 mature male Sprague-Dawley-derived rats, approximately 90 days old and weighing 250 - 300 g, from the Texas Woman's University colony were used for the main studies. Rats were maintained on Purina Laboratory Chow and water and housed five to a cage. They were on a 12:12 hour light-dark cycle in a room approximately 21°C during the test period.

Treatment Procedures

Hydrogen Peroxide Treatment

Earhart (1975) showed that 1.8 ml of 1.5% hydrogen peroxide could be administered intraperitoneally without causing any observable detrimental effects. The hydrogen peroxide treated groups received five doses of 1.5% hydrogen peroxide at 24 hour intervals for five days. Control animals were injected with a corresponding volume of distilled water.

Gamma Radiation Treatment

Whole body ^{60}Co gamma radiation was delivered to unanesthetized experimental animals by a U. S. Nuclear

Corporation GR-9 gamma irradiator. Rats were placed in a cylindrical cardboard container 120 mm long and 80 mm wide. Sham irradiated animals were placed in the same type of container for a comparable period of time.

Methylmercuric Chloride (MMC) Treatment

From a stock of saline solution containing 3 mg of MMC/ml, injections were given at 8 mg/kg of body weight. All MMC doses were administered by intraperitoneal injection.

Co-Insult Treatment of Methylmercuric Chloride and Gamma Radiation

Acute doses of 8 mg/kg body weight of MMC were administered and five days later the animals were exposed to 800 R gamma irradiation.

Experimental Plan for Determining Changes in the Number of Peroxisomes and Lysosomes

Assignment of Animals to Treatment

A total of 50 rats were divided randomly into five groups of 10 animals each with these treatments: Group A, five daily doses of 1.8 ml of 1.5% hydrogen peroxide pretreatment; Group B, 800 R whole body gamma irradiation at the rate of 285.7 R per minute; Group C, one dose of 8 mg MMC/kg body weight; Group D, a dose of 8 mg MMC/kg body weight and five days later 800 R gamma irradiation; Group E, five doses of 1.8 ml H_2O and sham irradiation (Table I).

TABLE I

Experimental protocol using hydrogen peroxide, and single and co-insults of ^{60}Co gamma radiation and methylmercuric chloride to investigate changes in number of peroxisomes and lysosomes in brain, liver, and kidney of mature male albino rats. Intraperitoneal injections were used.

Group	No. of Animals	Treatment	Day from First Treatment to Kill
A	10	1.8 ml of 1.5% hydrogen peroxide daily for five days	7
B	10	800 R whole body gamma irradiation	7
C	10	8 mg MMC/kg body weight	2
D	10	Co-insult of 8 mg MMC/kg body weight; 800 R gamma irradiation 5 days later	7
E	10	Distilled water (1.8 ml) for 5 days, followed by sham gamma irradiation	7

Processing of Animal Tissues

Animals were killed after treatments by pentobarbital anesthesia. Tissues were fixed in 10% formaldehyde for 18 hours and prepared for light microscopy by accepted methods of dehydration and embedded in paraffin (Humanson, 1972). Serial sections seven micrometers (μm) thick were cut on an A. O. Spencer "820" microtome. The nucleus arcuatus region of the brain was located by using a stereotaxic atlas (Konig and Klippel, 1970).

The brain tissues were stained by Bargmann's modification of the chrom-alum haematoxylin-phloxin technique of Gomori (Pearse and Everson, 1960), to show peroxisome-like organelles. Other serial sections of the same brain area, liver, and kidneys were processed by a modification of the histochemical method of Novikoff and Goldfischer (1969), to show peroxisome and lysosome organelles. The modification were as follows:

a) The tissues were fixed in 10% formaldehyde instead of 3% glutaraldehyde.
b) The tissues were embedded in paraffin for sectioning.
c) The slide-mounted serial sections were incubated and stained separately to identify lysosomes from peroxisomes.
d) Potassium cyanide (0.1 M) was added to the incubation medium of lysosomes.

e) The tissue sections were deparaffinized and hydrated before being transferred to incubation media as follows:

1) The slides were deparaffinized in 2 changes of 100% xylene: 3 minutes in each;

2) Then they were transferred to 2 changes of 100% absolute alcohol: 1.5 minutes in each;

3) The tissues were hydrated in 2 changes of 95% ethyl alcohol: 1.5 minutes in each;

4) Then hydration was continued in 2 changes of 70% ethyl alcohol: 1.5 minutes in each;

5) The tissue sections were washed in running tap water for 5 minutes before being transferred to their specific incubation media.

Procedures to Demonstrate Peroxisomes

a) The incubation medium consisted of:

1) 20 mg DAB tetra-HCl

2) 9.8 ml 0.05 M 2-amino-2-methyl-1,3-propandiol buffer, pH 10

3) 0.2 ml freshly prepared 1% hydrogen peroxide

4) 65 mg potassium cyanide (KCN) to prevent the background staining of mitochondria. This action of KCN was noted earlier by Novikoff and Goldfischer (1969).

b) The pH was adjusted to 9.0 using concentrated HCl and the precipitate that formed was removed by filtration.

c) Incubation of tissue sections was performed at 37°C for 30 minutes.

d) Tissue sections were transferred to the lead citrate staining solution.

Procedures to Demonstrate Lysosomes

a) The incubation medium consisted of:

1) 20 mg cytidine 5'-monophosphoric acid (CMP)

2) 9.8 ml 0.05 M 2-amino-2-methyl-1,3-propandiol buffer pH 10

3) 0.2 ml freshly prepared 1% hydrogen peroxide

4) 65 mg (0.1 M) potassium cyanide, described earlier in the peroxisomes preparation procedure.

b) The pH was adjusted to 5.0 using 1.0 N NaOH.

c) The incubation of tissue sections was performed at 37°C, for 25 minutes.

d) The tissues were transferred to the lead citrate staining solution.

Procedures to Demonstrate Lysosomes and Peroxisomes in the Same Sections

a) The tissues were incubated first to show lysosomes with CMP, (pH 5, 25 minutes, 37°C).

b) Then they were transferred to DAB incubation medium to show peroxisomes (pH 9, 30 minutes, 37°C).

c) The tissue sections were transferred to the lead citrate staining solution.

Procedures for Staining with Lead Citrate

a) The lead citrate staining solution was prepared according to the procedure of Reynold (1962).

b) All previously incubated serial sections were stained in the lead citrate solution for 10 minutes.

c) The sections were washed in 0.02 N NaOH for 3 minutes.

d) The sections were washed with distilled water.

e) The sections were allowed to drain dry.

f) Cover slips were attached with Permount.

Procedure to Counterstain Against Lead Citrate

Tissues of brain, liver, and kidney were processed as described earlier. The haematoxylin counterstain was applied as follows:

a) After sections were stained in lead citrate, the slides were:

   1) Washed in 0.02 N NaOH for 5 minutes,

   2) Washed in distilled water for 5 minutes,

   3) Scott's solution: for 3 minutes,

   4) Running water: for 5 minutes,

   5) Haematoxylin counterstain: for 1 minute,

   6) Washed in running water: 5 minutes.

b) The slides were dehydrated in the following series:

   1) 70% ethyl alcohol: 2 dips,

   2) 95% ethyl alcohol: 3 dips,

3) Two changes of 100% absolute alcohol: 3 minutes in each,

4) Two changes of 100% xylene: 3 minutes in each.

c) The slides were air dried.

d) Cover slips were attached with Permount.

Identifiable Features of Peroxisomes and Lysosomes

Peroxisomes are a group of intracytoplasmic particles limited by a single membrane. The matrix is finely granular. Their presence has been demonstrated histochemically in this study in the hepatocytes, renal tubular cells and the nucleus arcuatus of rat brain. After serial sections of these tissues have been incubated in DAB medium (pH 9; 30 min. 37 °C), they show homogenous DAB reaction products. Peroxisomes measure 0.2 - 0.5 μm in diameter (Novikoff and Goldfischer, 1969). With the exception of fetal tissues, peroxisomes always maintain a close spatial relationships to the smooth endoplasmic reticulum or to the smooth portion of rough cisternae (Ericsson and Trump, 1966).

Peroxisomes were recognized as specific cytoplasmic particles characterized biochemically by their content of catalase and one or several oxidative enzymes with slightly alkaline pH optima (De Duve and Baudhuin, 1966).

To prove conclusively that particular types of granules observed in tissue sections correspond to lysosomes,

Bitensky (1962) considered that ideally, they should be shown to be: (a) inert when intact; (b) activated by treatments that will damage membranes; and (c) any diffuse reaction for acid phosphatase should be shown to have emanated from the particular sites. A feature of lysosomes is their limiting membrane, which appears to be primarily lipid-protein in nature.

Lysosomes were recognized in the present study, under light microscopy, after tissue sections incubated in cytidylic acid (pH 5, 25 min., 37°C), and stained with lead citrate. They are characterized by a dense precipitate of lead in the membrane and a nearly opaque matrix. The use of haematoxylin as a counterstain to lead citrate, again clearly demonstrated the presence of both peroxisomes and lysosomes in this study.

In the present work P-L organelles in the nucleus arcuatus of rat brain were visualized after tissues were stained in Bargmann's modification of the chrom-alum haematoxylin-phloxin technique of Gomori (Pearse and Everson, 1960). The P-L organelles appeared as dark pink particles, with a homogenous matrix and usually round, ellipsoid, or angular in shape.

Quantitation of the Cellular Organelles

Peroxisome-Like Organelles

Peroxisome-like organelles positive for the chrom-alum

haematoxylin-phloxin in the nucleus arcuatus were counted in a 0.322 mm^2 area of each of the 10 serial sections from each animal, using the 10X objective of the Reichert Vis-O-Pan projection microscope, giving a total magnification of 122 X.

Peroxisomes and Lysosomes

The lead citrate stained peroxisomes and lysosomes in the liver, kidney and the nucleus arcuatus of the rat brain were counted in a 0.020 mm^2 area of each of 10 serial sections from each animal, using the 40X objective of the Reichert Vis-O-Pan projection microscope, giving a total magnification of 545 X for the area examined. Differences between groups were tested by analysis of the variance.

EXPERIMENTAL PLAN FOR DETERMINING THE EFFECTS OF HYDROGEN PEROXIDE PRETREATMENT ON GAMMA RADIATION INDUCED LETHALITY

Experiment 1

Thirty mature male rats, approximately 170 days old and weighing 415 - 505 g, were used in this preliminary study (Table II). The experimental Groups, I, II, and III, received five doses of 1.0, 1.4, and 1.8 ml of 1.5% H_2O_2 respectively, and each received 775 R gamma irradiation delivered at 291 R per minute; Group IV, five doses of 1.8 ml of distilled water and 775 R gamma irradiation, delivered at 291 R per minute; Group V, sham radiation and five doses of 1.8 ml of 1.5% H_2O_2; Group VI, sham radiation and five doses of 1.8 ml distilled water (Table II).

Injections were given intraperitoneally for five days at 24 hour intervals, and rats were irradiated or sham irradiated 48 hours after the last injection. Animals were observed for 30 days after irradiation or sham irradiation and deaths were recorded daily.

TABLE II

Experimental protocol of preliminary lethality study of mature male rats injected intraperitoneally with 1.5% hydrogen peroxide (H_2O_2) and subsequently exposed to ^{60}Co gamma irradiation delivered at 290 R/min.

Group	No. of Animals	Injection*	Treatment Gamma-irradiation
I.	5	1.0 ml H_2O_2	775 R
II.	5	1.4 ml H_2O_2	775 R
III.	5	1.8 ml H_2O_2	775 R
IV.	5	1.8 ml H_2O	775 R
V.	5	1.8 ml H_2O_2	0
VI.	5	1.8 ml H_2O	0

*One injection a day per animal for 5 days

Experiment 2

Sixty male rats, approximately 90 days old and weighing 250 - 300 g, were used. The experiment was conducted in two replicates, with 5 animals in each treatment group in each replicate (Table III). The experimental treatments were the same as in the previous experiment. The gamma radiation dose rate was 287 R per minute in the first replicate, and 283 R per minute in the second. As in the previous experiment, animals were observed for 30 days after irradiation or sham irradiation and deaths recorded daily.

TABLE III

Experimental protocol of lethality study of mature male rats injected intraperitoneally with 1.5% hydrogen peroxide (H_2O_2) and subsequently exposed to ^{60}Co gamma irradiation, delivered at 287 or 283 R/min.

Group	No. of Animals	Injection*	Treatment Gamma-irradiation
I.	10	1.0 ml H_2O_2	775 R
II.	10	1.4 ml H_2O_2	775 R
III.	10	1.8 ml H_2O_2	775 R
IV.	10	1.8 ml H_2O	775 R
V.	10	1.8 ml H_2O_2	0
VI.	10	1.8 ml H_2O	0

*One injection a day per animal for 5 days

RESULTS

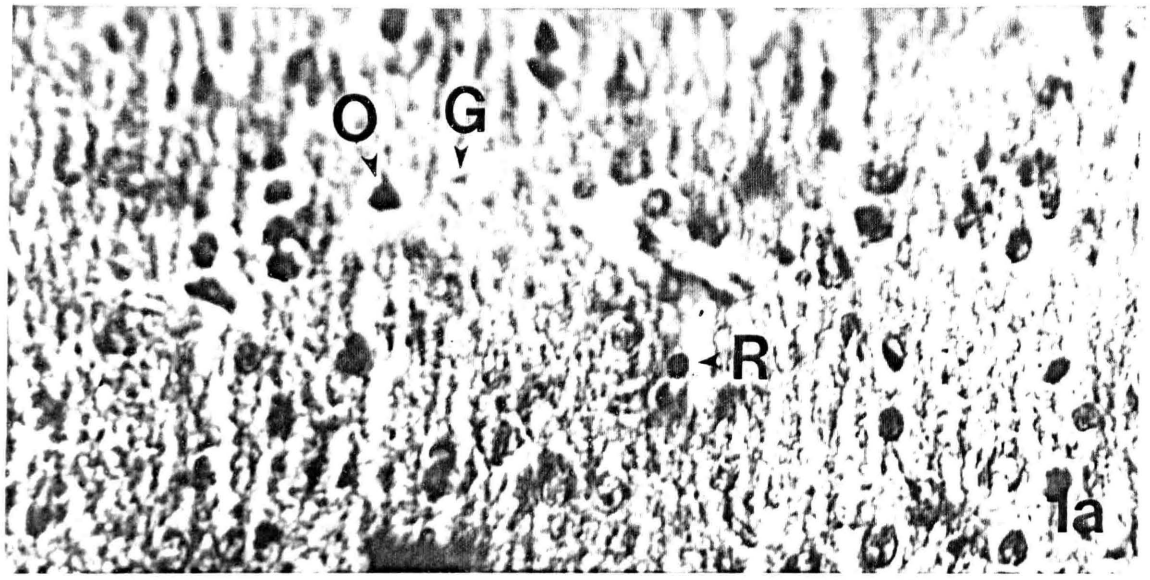
Light Microscopy of the Brain, Liver and Kidney

Light Microscopy of Peroxisome-Like (P-L) Organelles

Examination of the nucleus arcuatus of the rat brain for the presence of P-L organelles revealed these organelles as round, ellipsoid, or angular in shape (Fig. 1). Rats treated with distilled water showed P-L organelles as round structures with a homogenous matrix (Fig. 1a). The P-L organelles of the hydrogen peroxide and radiation groups did not appear to differ morphologically from those of the distilled water group. In MMC treated rats, P-L organelles appeared as round, ellipsoid, or angular in shape with a homogenous matrix (Fig. 1b). Rupture of the membrane of the organelles as indicated by decreased density of the matrix, irregular shape, lack of clear demarcation between the organelle and surrounding cytoplasm and evidence of extrusion of the contents of the organelle was observed in all sections examined. Occasionally, what appeared to be budding organelles were observed in close association with the rough endoplasmic reticulum. This process could lead to a proliferation of organelles. Rats treated with the MMC and gamma irradiation (Fig. 1c), also revealed the P-L organelles as round, ellipsoid, or angular structures with

Figure 1

Light micrographs illustrating peroxisome-like organelles of the nucleus arcuatus of the brain stained with Bargmann's modification of "Gomori-positive." (1a) Most of the organelles (O) of control rats appear as rounded (R) structures with a homogenous matrix. G, Glycogen granules. X 2000. (1b) Peroxisome-like organelles from animals treated with MMC, showing organelles with a homogenous matrix. Note membrane damage (D) to many of these organelles. They appeared as round (R), ellipsoid (E), or angular (AN) in shape. B, Budding P-L organelles; G, Glycogen granules; ER, Endoplasmic reticulum. X 2000. (1c) Co-insult treatment of MMC and 800 R gamma irradiation showing D, damaged peroxisomes. The organelles differ in matrix density and are round (R), ellipsoid (E), or angular (AN) in shape. B, Budding P-L organelles. X 2000.


O
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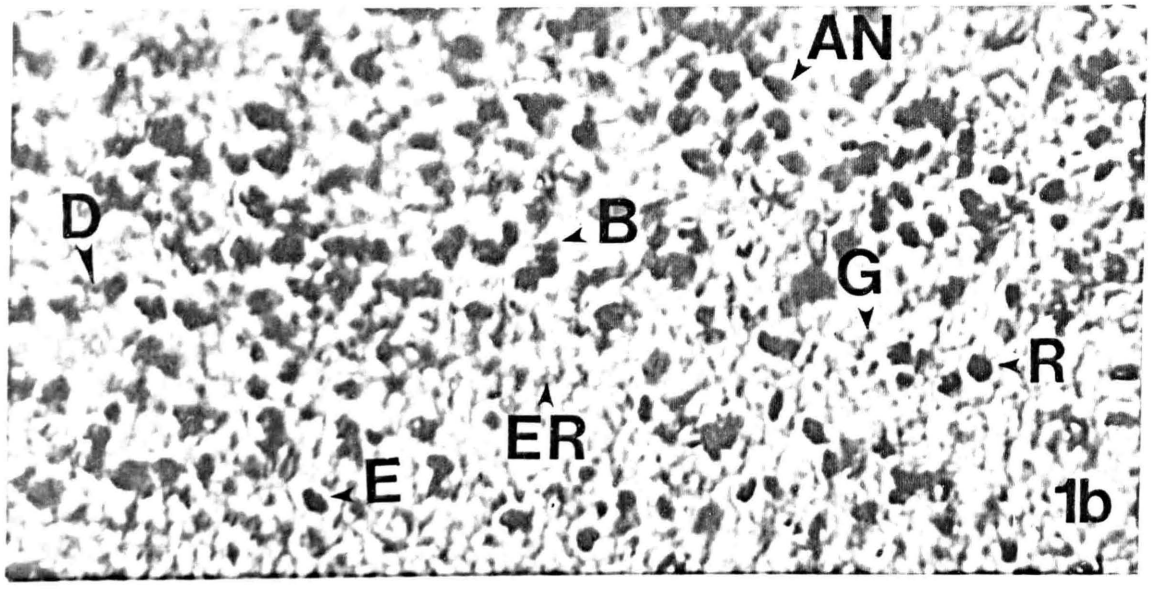
AN
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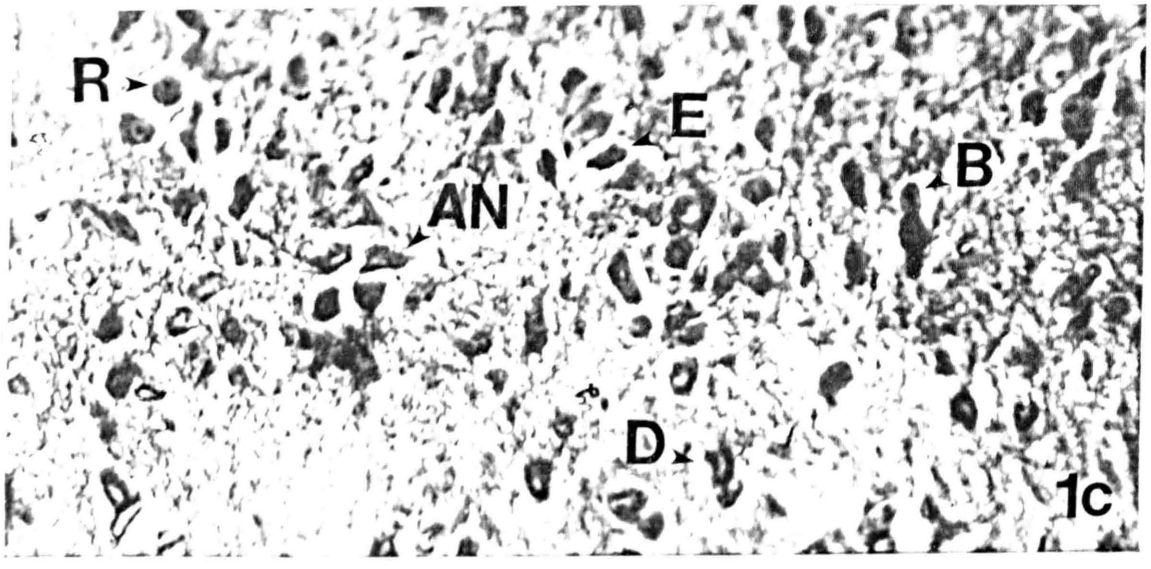

R
E
B
AN
D
1c

a variation in organelles' density. Some also showed membrane rupture.

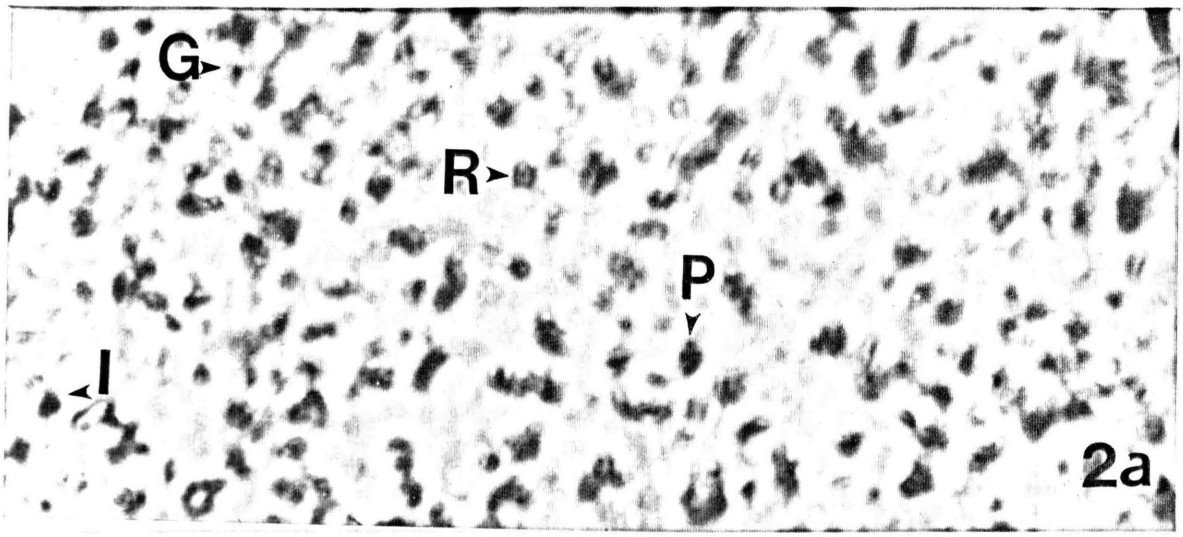
Light Microscopy of Peroxisomes

Serial sections of rat brain, liver and kidney were incubated in DAB medium and stained with lead citrate. Peroxisomes in the nucleus arcuatus of distilled water treated animals (Fig. 2a) exhibited normal peroxisomes of round structure with a homogenous matrix. Methylmercuric chloride treated animals (Fig. 2b) showed membrane rupture, this damage was illustrated in peroxisomes that exhibited precipitate of lead citrate in part of the membrane, and none in other areas, and by the presence of gaps in some membranes. Co-insult treated rats (Fig. 2c) showed similar types of damage to peroxisomes, however, most of these organelles were normal round structures with a homogenous matrix.

Peroxisomes of liver tissues (Fig. 3) showed homogenous round or ellipsoid organelles in control rats (Fig. 3a). Fusion of some organelles was observed. Infrequently, the peroxisomes exhibit budding where a smaller organelle seems to be developing from a larger one, a phenomenon observed that may act in the regeneration process. Hydrogen peroxide treated rats (Fig. 3b) had fewer fusing peroxisomes than control rats but the number of peroxisomes which

Figure 2

Light micrographs of peroxisomes of the nucleus arcuatus, sections incubated in DAB and stained with lead citrate. (2a) Control rats reveal peroxisomes (P) as intact (I), round (R) organelles, with a homogenous matrix. G, Glycogen granules. X 1260. (2b) Peroxisomes of MMC treated animals. Note damage to peroxisomal membrane (D). Organelles rounded (R) or ellipsoid (E). G, Glycogen granules; C, Cisterna of the endoplasmic reticulum; GP, gap. X 1260. (2c) Co-insult of MMC and gamma irradiation showing D, damaged peroxisomes. Peroxisomes appear as round (R) or ellipsoid (E) organelles. C, Cisterna of endoplasmic reticulum; G, Glycogen granules. X 1260.


G
R
P
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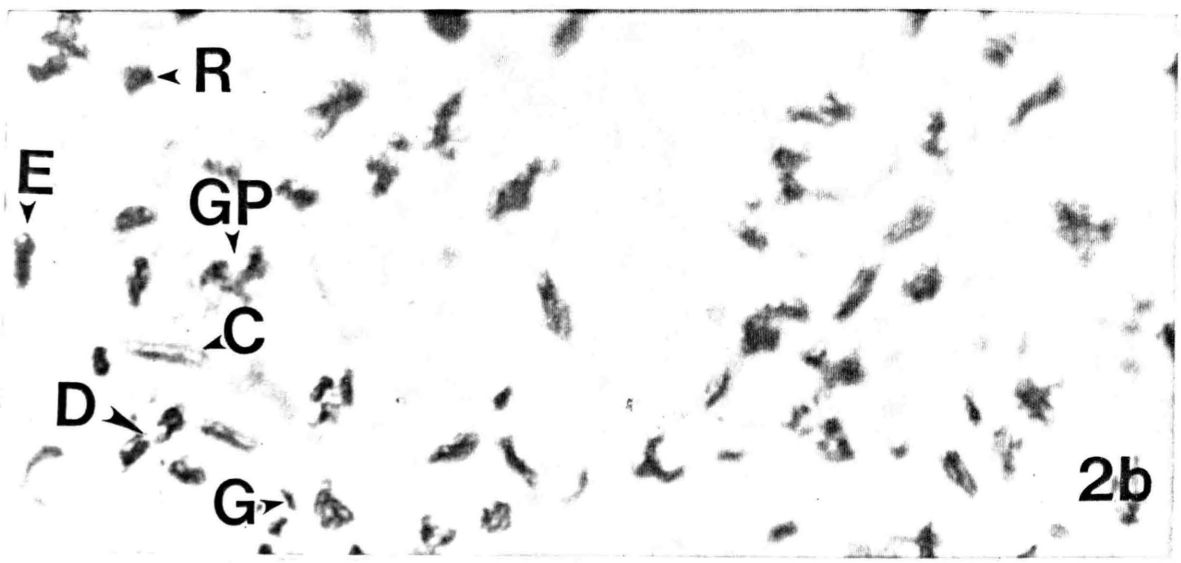
2a


R
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GP
C
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G
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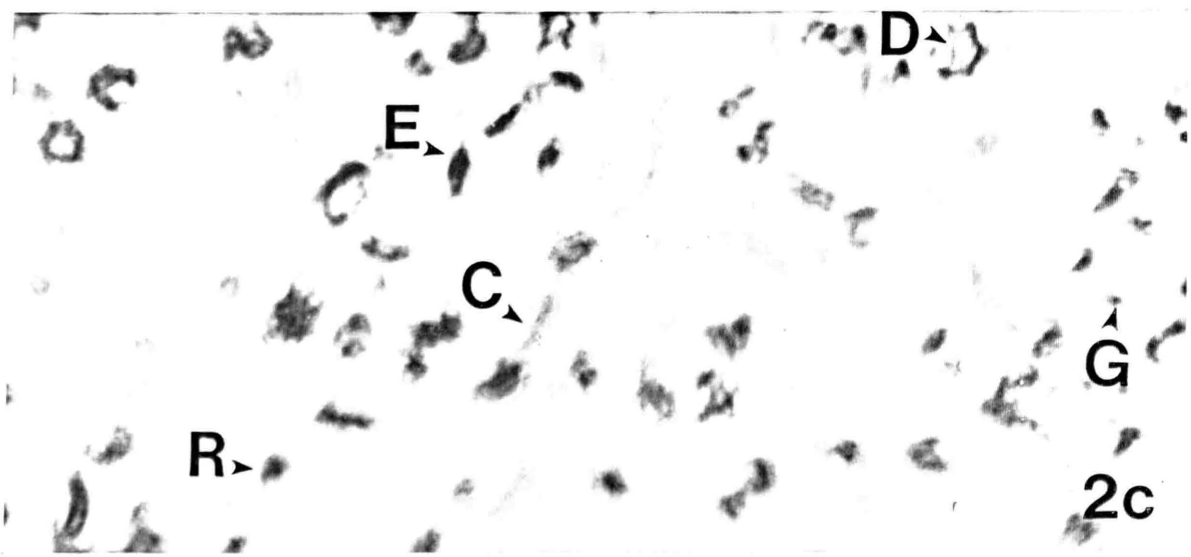
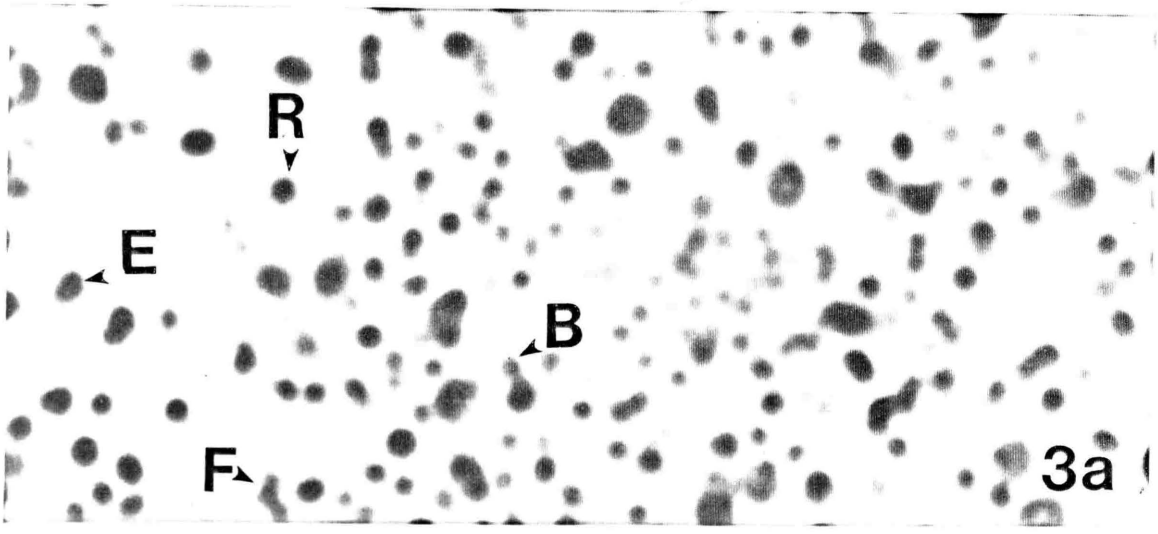

D
E
C
G
R
2c

Figure 3

Light micrographs of rat liver tissue incubated in DAB and stained with lead citrate. (3a) Control rats showing homogenous peroxisomal organelles of the liver, round (R) or ellipsoid (E) in shape. B, Budding peroxisomes; F, Fusion of organelles. X 1260. (3b) Hydrogen peroxide treated animals, illustrating peroxisomes of liver tissue, round (R) or ellipsoid (E). F. Fusions of two or more peroxisomes; B, Budding peroxisomes. (3c) Peroxisomes of 800 R gamma irradiated rats revealed enlargement in size of some peroxisomes (S1, S2). Fewer fusions of two or more peroxisomes (F) were observed. Budding (B) of peroxisomes was observed. X 1260.


R
E
B
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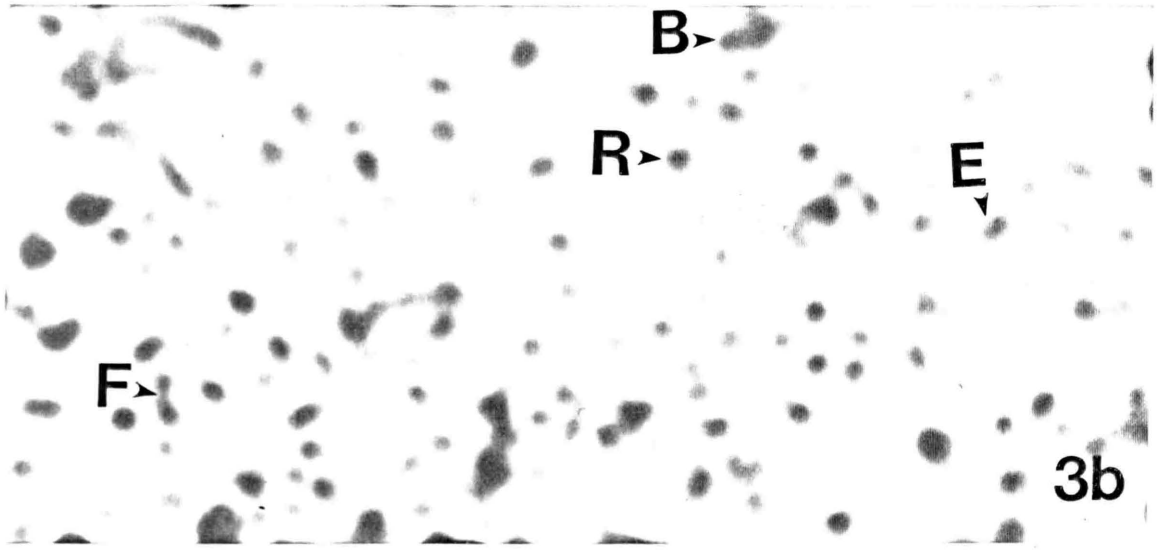
3a


B
R
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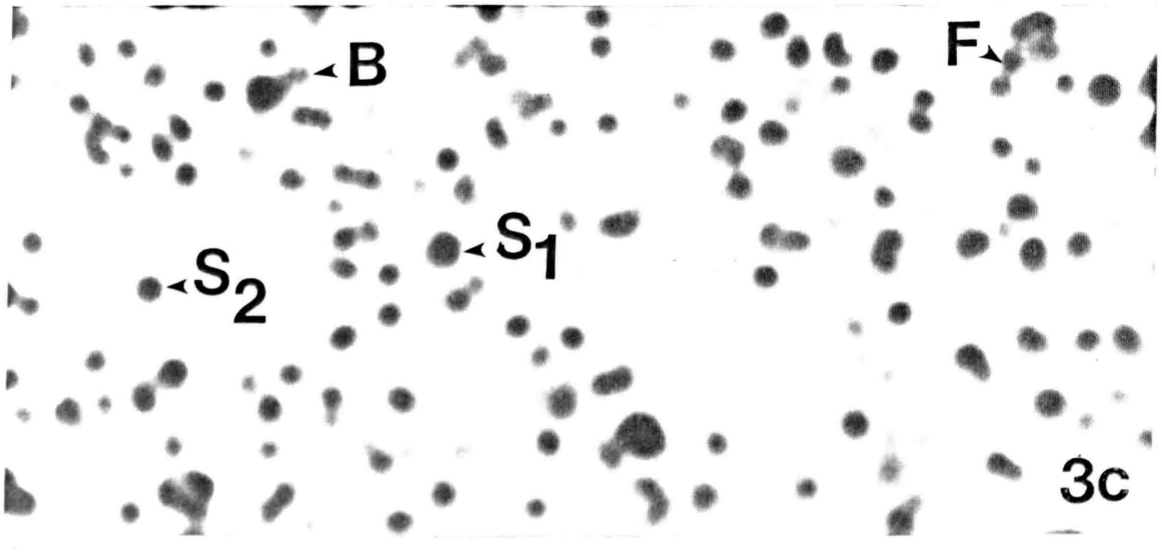

B
F
S1
S2
3c

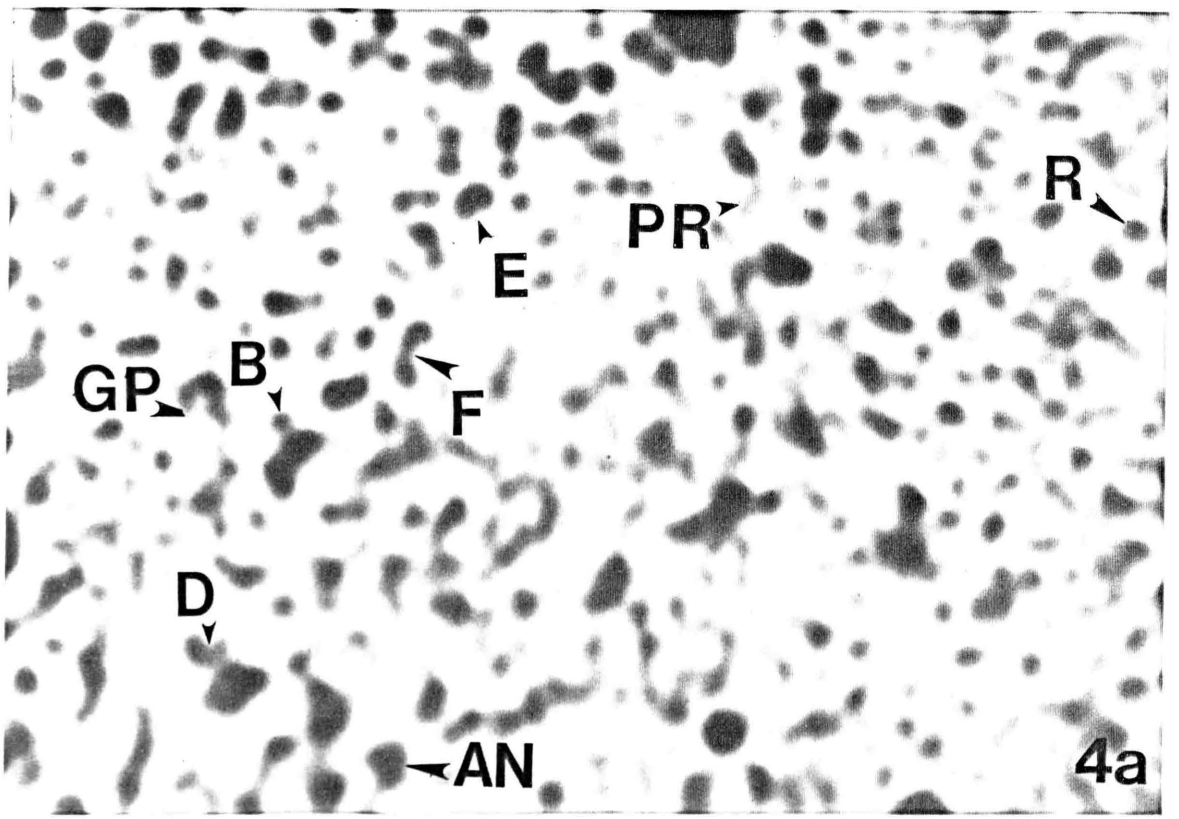
exhibited the budding process was comparable to control rats. Normal round or ellipsoid structures were observed. Photomicrographs of peroxisomes in whole body gamma irradiated rats (Fig. 3c) revealed a greater differential increase in their size.

Liver peroxisomes from animals treated with MMC (Fig. 4a) displayed membrane damage in the form of gap formations and less darkly stained protrusions from the peroxisomal membrane. Figure 4b (co-insult treated animals) illustrates again the damage inflicted by MMC toxicity on peroxisomes, and the antagonistic action of whole body gamma irradiation, in causing a possible neutralization of MMC toxicity. The morphological observations revealed that less peroxisomes were damaged than in those treated with MMC alone.

Peroxisomal organelles from kidney tissue of control rats, those pretreated with hydrogen peroxide, and gamma irradiation (Fig. 5a, 5b, 5c), respectively were normal in appearance. Many organelles that were less darkly stained than in brain and liver tissue were observed in all 3 groups. An increase in size of some of the organelles was observed in peroxide treated and irradiated animals. Peroxisomes from animals treated with MMC (Fig. 6a) and from co-insult treated animals (Fig. 6b) revealed a large number of

Figure 4

Light micrographs of rat liver tissue sections. (4a) MMC treated rats revealed characteristics of MMC action on peroxisomes. Note damage (D) to peroxisomes membrane, with possible release of enzymes and enzymes products into the cytoplasm. Organelles are round (R), ellipsoid (E) or angular (AN) in shape. B, Budding peroxisomes; F, Fusion of peroxisomes; GP, Gap; PR, protrusion. X 2000. (4b) Illustrates peroxisomes from rats after co-insult treatment of MMC and 800 R gamma irradiation. Note membrane rupture (D), clustering of more than one peroxisome organelle together (CL). Also many organelles are still intact (I), apparently undamaged by MMC. Organelles are round (R) or ellipsoid (E). F, Fusion of peroxisomes; B, Budding peroxisomes. X 2000.


R
PR
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D
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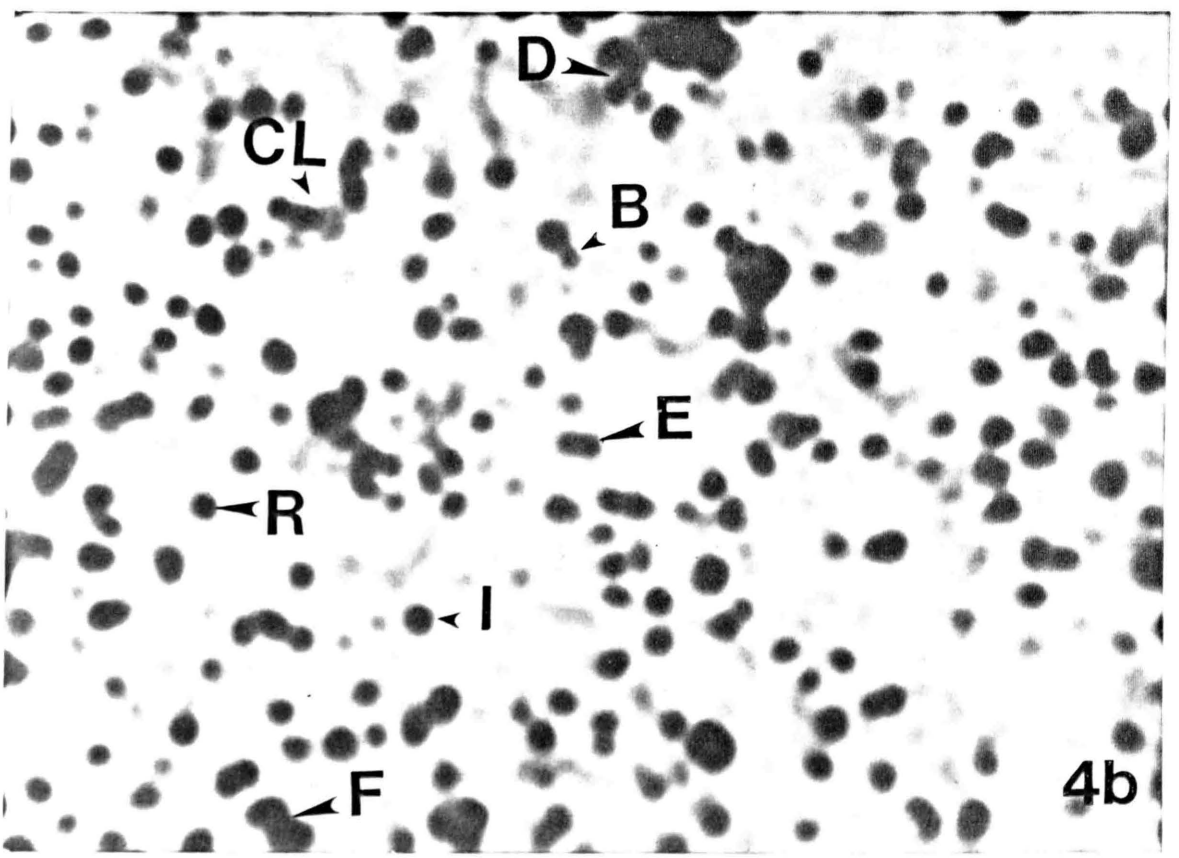
4a


D
CL
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R
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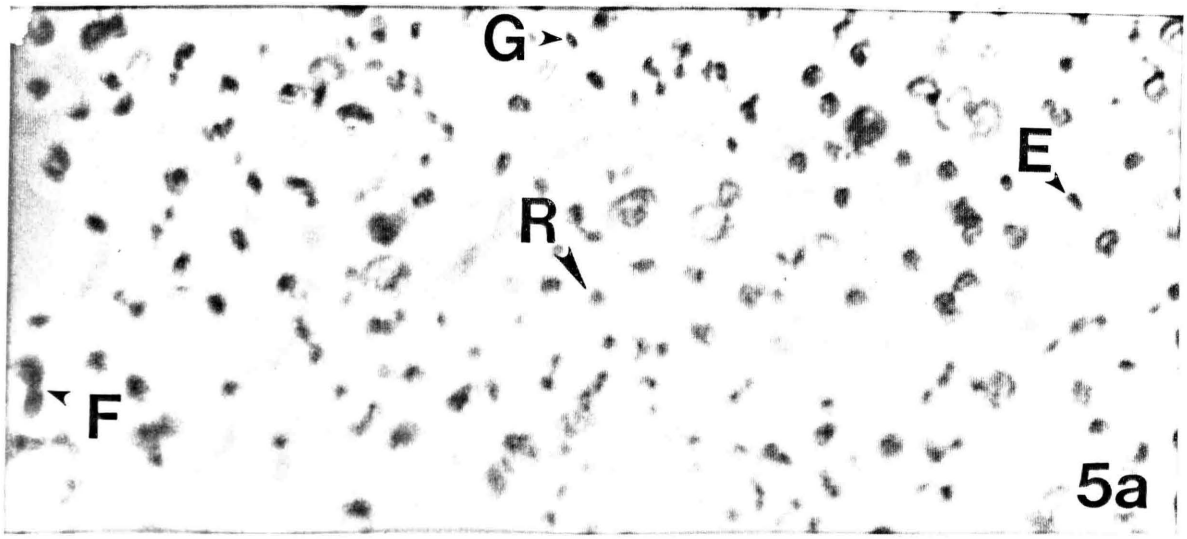
F
4b

Figure 5

Light micrographs of peroxisomes from serial sections of rat kidney incubated in DAB and stained with lead citrate. (5a) Control rats show numerous fused peroxisomes (F). G, Glycogen granules. X 1250. (5b) Rats treated with hydrogen peroxide reveal frequent fusions of peroxisomes (F). C, Cisterna of endoplasmic reticulum show intimate relationship with peroxisomes (P); G, Glycogen granules. X 1250. (5c) Peroxisomes from 800 R whole body gamma irradiated animals. P, Possible increase in size of some peroxisomes; G, Glycogen granules; F, Fusion of peroxisomes. X 1250. Peroxisomes in the above figures appeared round (R) and a few are ellipsoid (E) in shape.


G
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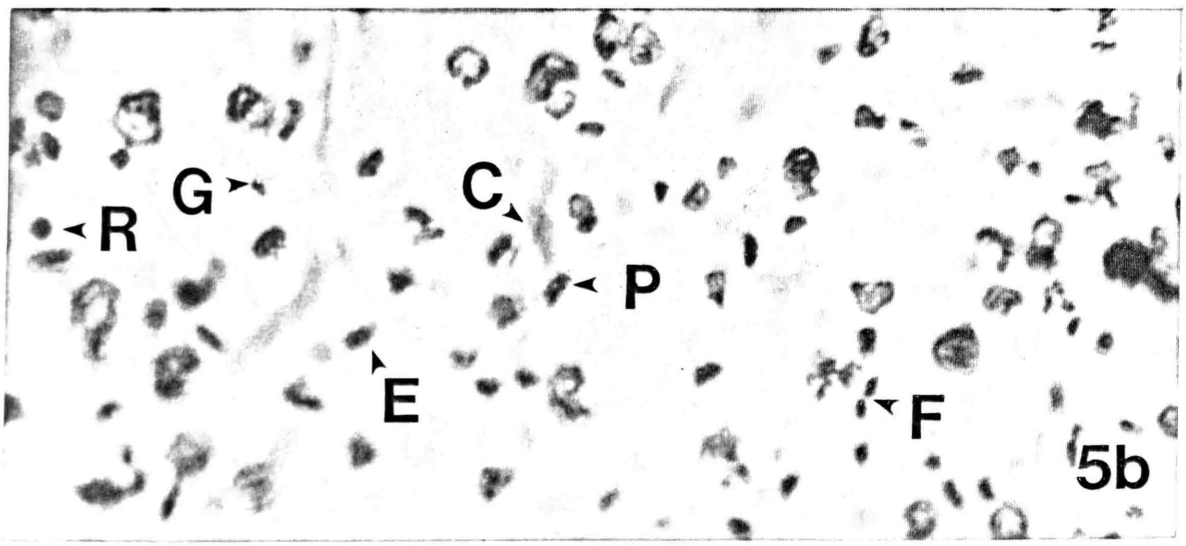
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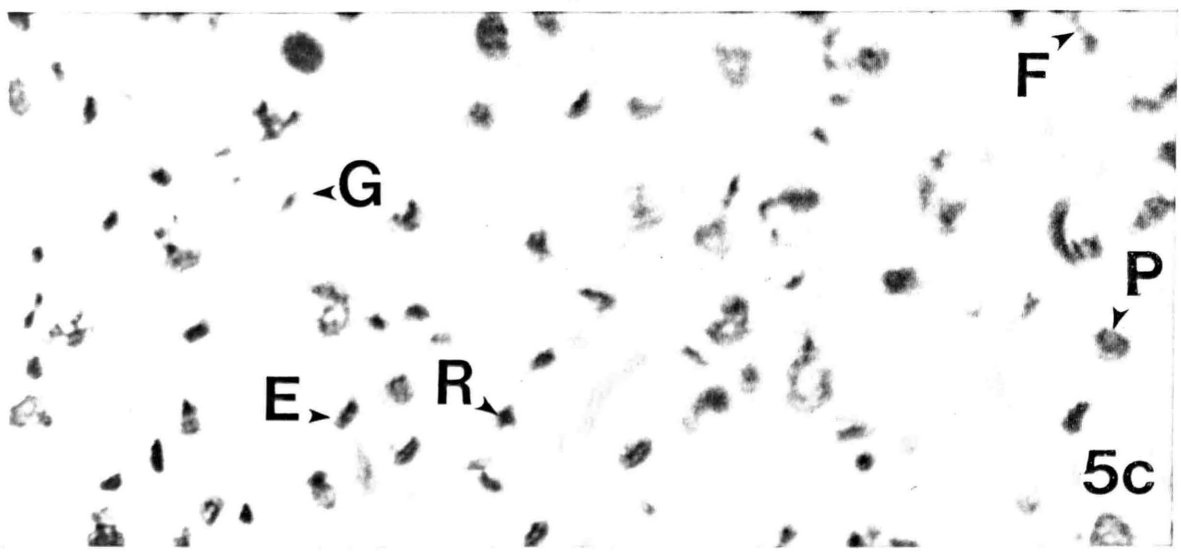
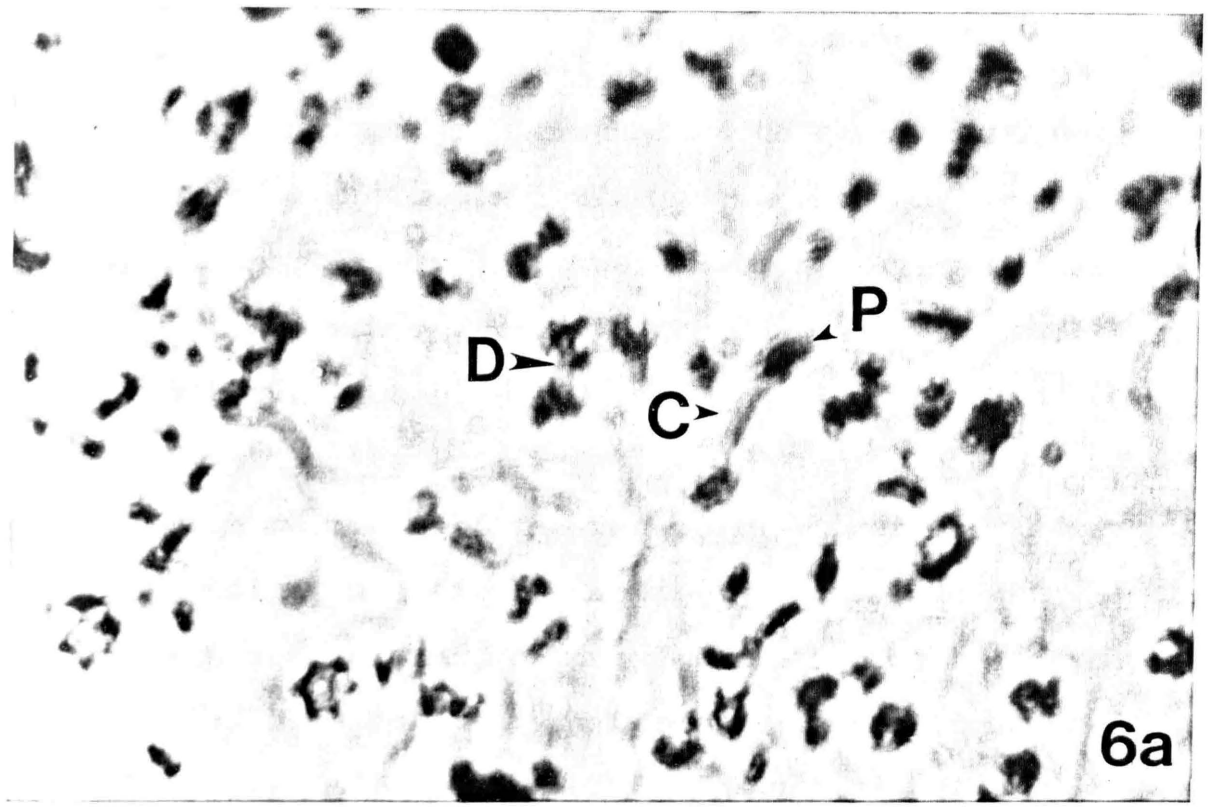

F
G
P
E
R
5c

Figure 6

Light micrographs of peroxisomes from tissues of rat kidney. (6a) Rats treated with MMC show membrane disruption of peroxisomes (D) throughout the examined areas. C, Cisterna of endoplasmic reticulum show intimate relationship with peroxisomes (P). X 2000. (6b) P, Peroxisomes of co-insult (MMC and 800 R gamma irradiation) treated animals. Micrographs show disruption of peroxisomal membrane (D). Many peroxisomes are left undisturbed or intact (I). Fusion of a few of these organelles (F) was observed. Many organelles are ellipsoid (E) in shape. G, Glycogen granules; C, Cisterna of endoplasmic reticulum. X 2000.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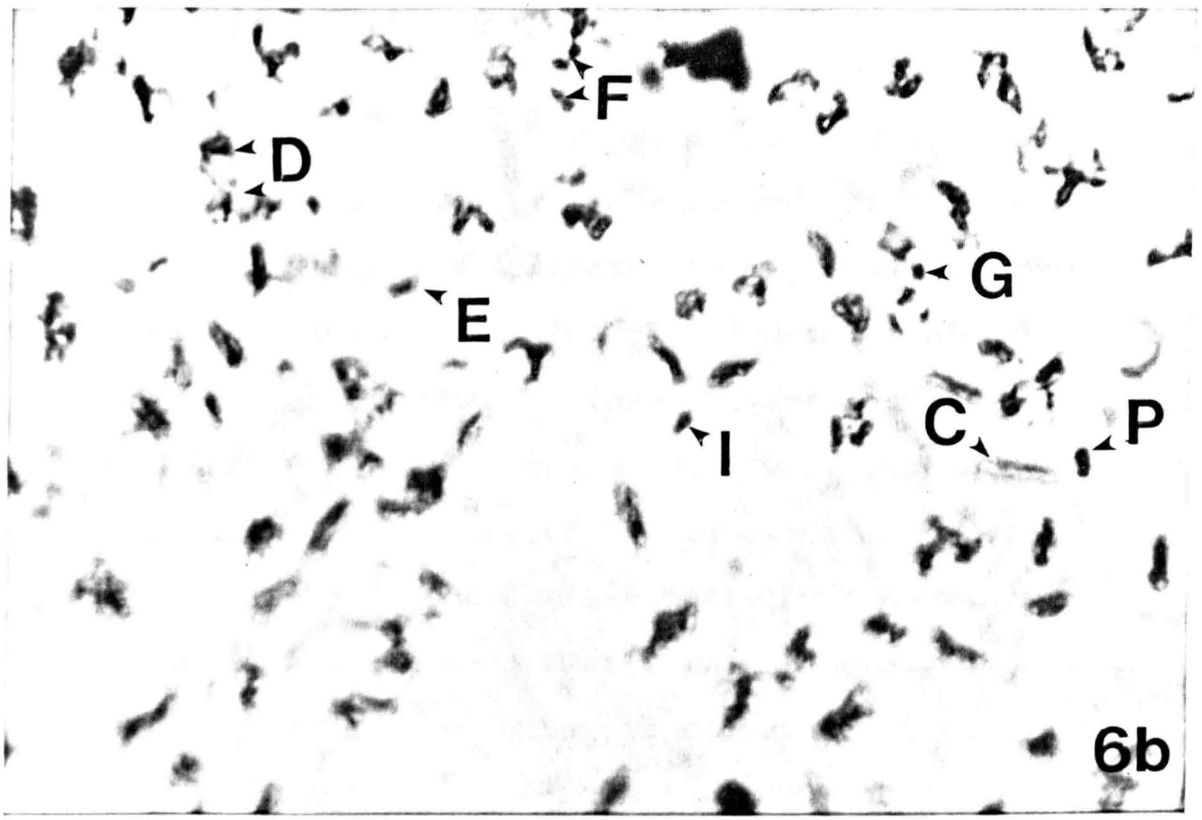
F
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6b

damaged peroxisomes with disruption of their membranes. Cisternae of endoplasmic reticulum were shown in an intimate relationship with some peroxisomes. Again a lesser degree of damage was observed in the co-insult treated rats in the MMC only group.

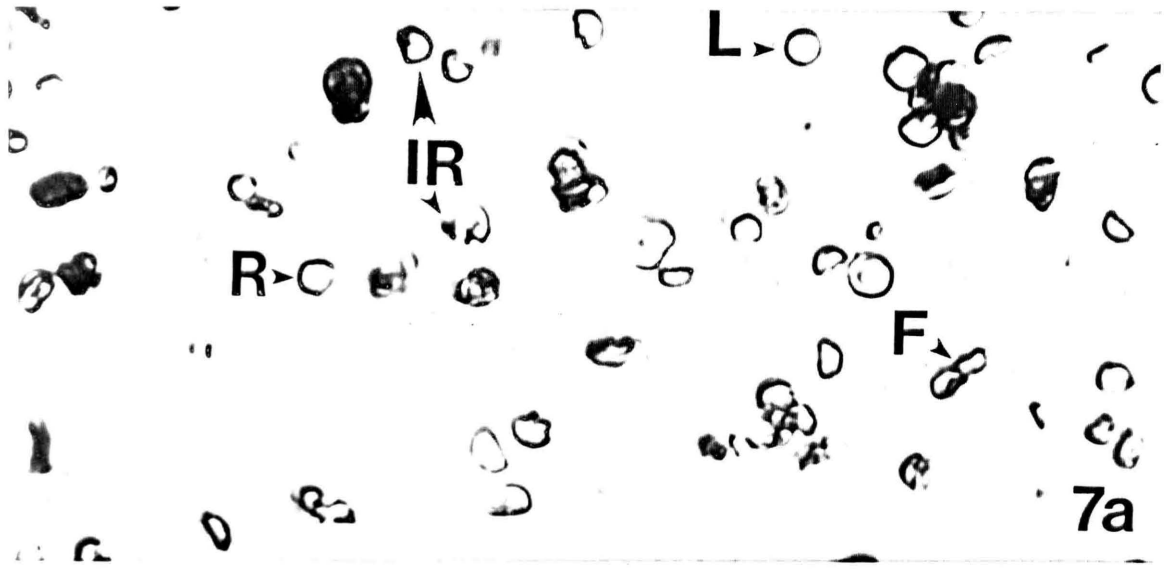
Light Microscopy of Lysosomes

Tissue sections of the rat brain, liver and kidney were incubated in cytidylic acid (CMP), stained with lead citrate thereafter, and examined morphologically for the presence of lysosomes and their characteristics by light microscopy.

Examinations of lysosomes in the nucleus arcuatus area of control rats (Fig. 7a) showed these organelles were uniform in size and round or irregular in shape. There was a precipitate of lead in the membrane and the matrix was homogenous and usually stained very lightly. Tissues from MMC treated animals (Fig. 7b) revealed membrane rupture in the majority of lysosomes. These organelles were darkly stained and irregular in shape. Few were observed to be in an intact form, with most of the lysosomal area darkly stained by the acid phosphatase activity. As was the case for peroxisomes, the lysosomes were frequently observed to be in close association with the cisternae of the endoplasmic reticulum. The co-insult of MMC and radiation

Figure 7

Light micrographs of lysosomes in the nucleus arcuatus of rat brain, incubated in CMP and stained with lead citrate. (7a) Control rats show many lysosomes (L) with lead precipitate in the lysosomal membrane. Lysosomes are homogenous in size and round (R) or irregular (IR) in shape. Note the uniform staining denisity of organelles. F, Fusion of lysosomes. X 2000. (7b) Lysosomes of MMC treated rats. Note damage to lysosomal membrane (D), illustrating possible action of MMC particles. C, Cisterna of endoplasmic reticulum in intimate relationship with lysosomes (L). X 2000. (7c) Co-insult animals, reveal damage of some lysosomal membrane (D). A, acid phosphatase activity in large area of the organelles; C, Cisternae of endoplasmic reticulum are clearly visible; I, Intact, undamaged lysosomes; F, Fusion of lysosomes; GP, Gap. X 2000.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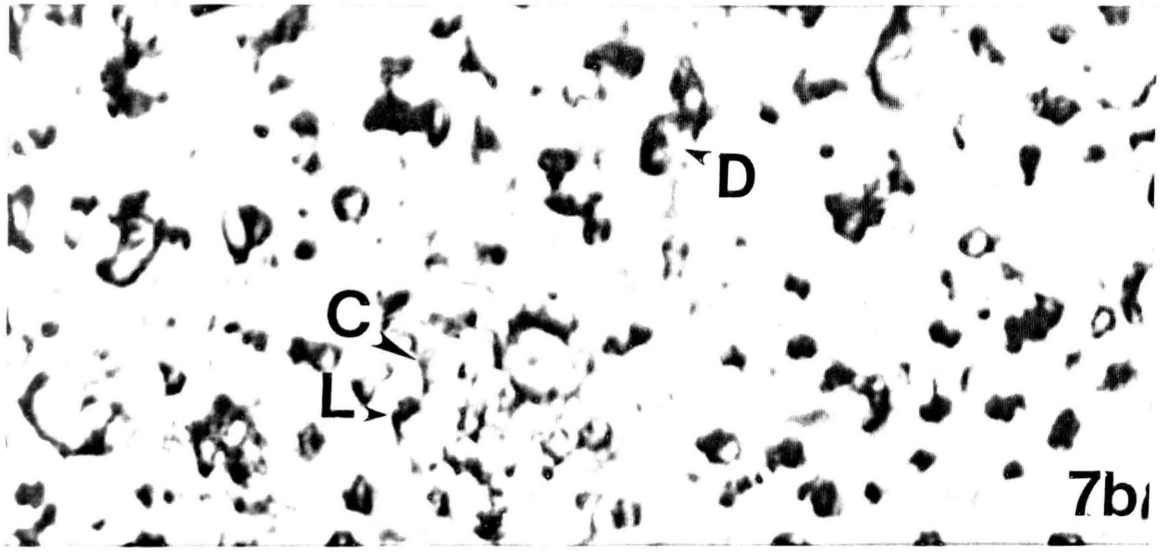
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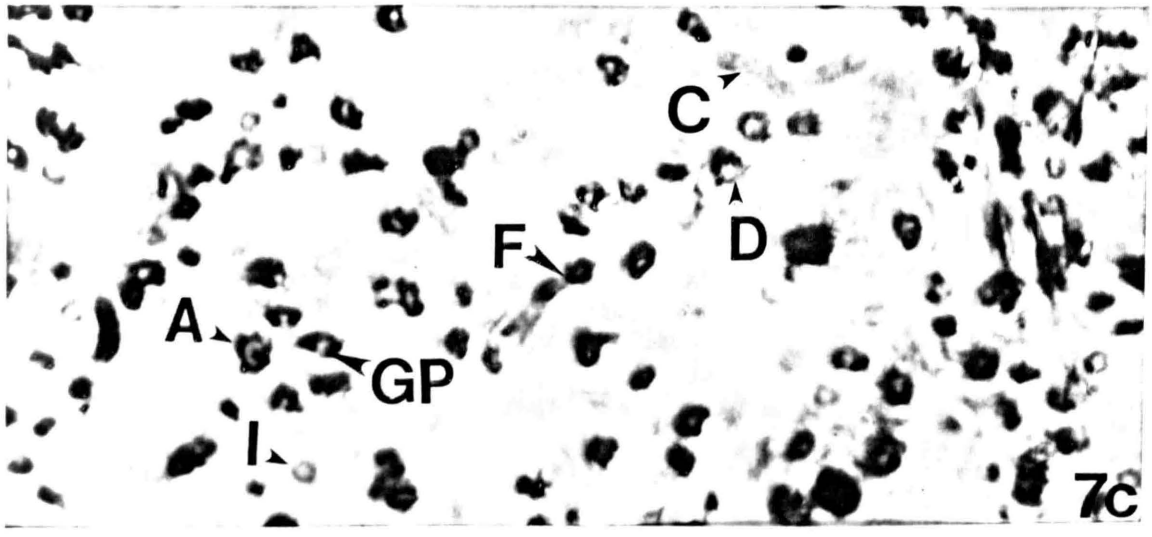
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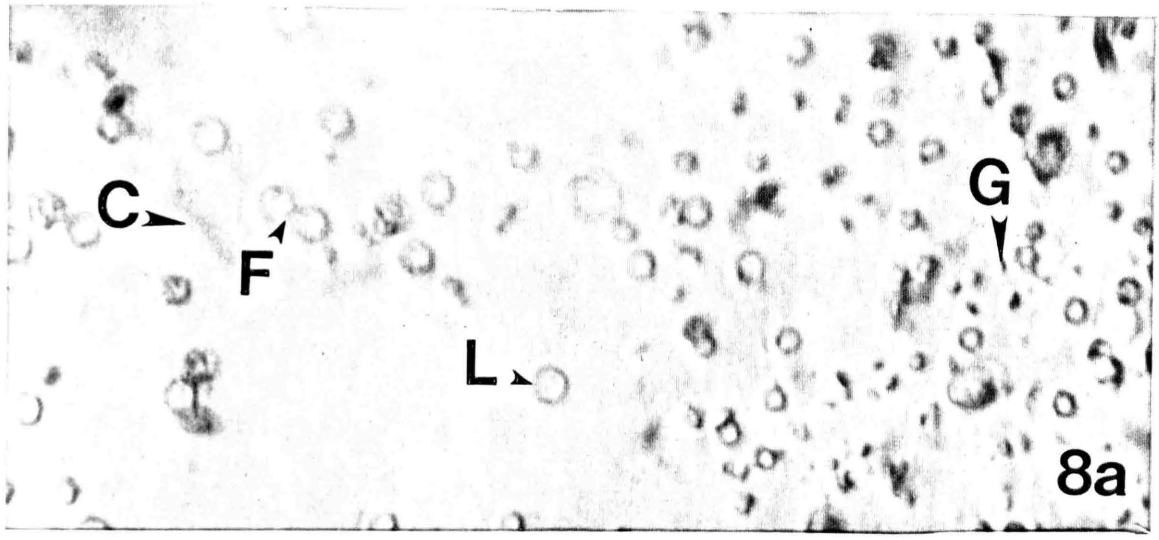
treated rats (Fig. 7c) showed a smaller number of lysosomes to be disrupted and their membrane ruptured. Again acid phosphatase activity was shown as dark stain, covering a wide area of the lysosomes. Invaginations in a few lysosomes were also observed, possibly indicating the location of action of MMC particles on the lysosomal membrane.

Serial sections of liver tissues from control animals (Fig. 8a) exhibited normal lysosomes. In contrast, hydrogen peroxide pretreated rats (Fig. 8b) revealed lysosomes of variable staining density, and irregular shape. Many of the lysosomes and those from 800 R whole body gamma irradiation treated rats (Fig. 8c) appeared slightly larger in size, and some were more darkly stained than the control, possibly due to a greater activity of the acid phosphatase enzyme within their compartments.

Liver tissue from methylmercuric chloride treated rats (Fig. 9a) showed, as previously observed in brain tissues, a similar pattern of disruption in the lysosomal membrane, revealing lysosomes as large and lumpy bodies. Some lysosomes were in an intimate relationship with the cisternae of endoplasmic reticulum. Glycogen granules were observed in the area of the damaged organelles. Co-insult treated animals (Fig. 9b) revealed rupture in the lysosomal membrane. A few large and lumpy bodies of lysosomes were observed. These organelles that were observed to be intact were

Figure 8

Light micrographs of lysosomes from rat liver, incubated in CMP and stained with lead citrate. (8a) Control rats reveal intact lysosomes (L). F, Fusion of two or more lysosomes; G, Numerous granules; C, Cisterna of endoplasmic reticulum. X 2000. (8b) Shows lysosomes (L) of hydrogen peroxide pretreated rats dense precipitate of lead citrate in the membrane. Note rupture of lysosomal membrane (D). F, Fusion of lysosomes; C, Cisterna of endoplasmic reticulum; G, Glycogen granules. X 2000. (8c) Shows lysosomes (L) from animals treated with 800 R whole body gamma irradiation. D, Possible damage of lysosomes; F, Fusion of two lysosomes; C, Cisternae of endoplasmic reticulum; A, Acid phosphatase activity in the lysosomes; G, Glycogen granules. X 2000.


C
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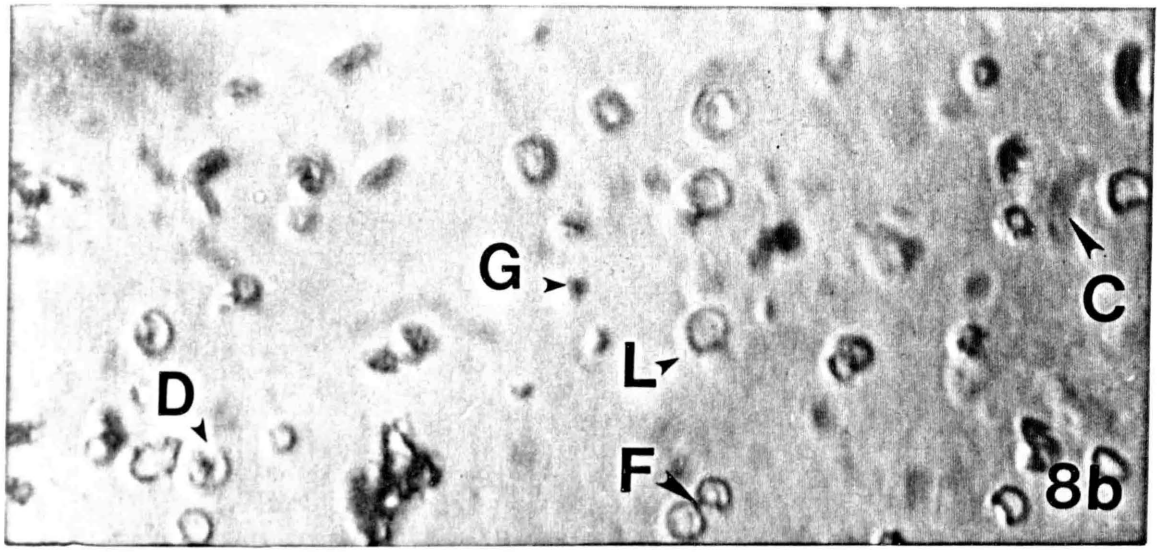
G
L
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C
8b

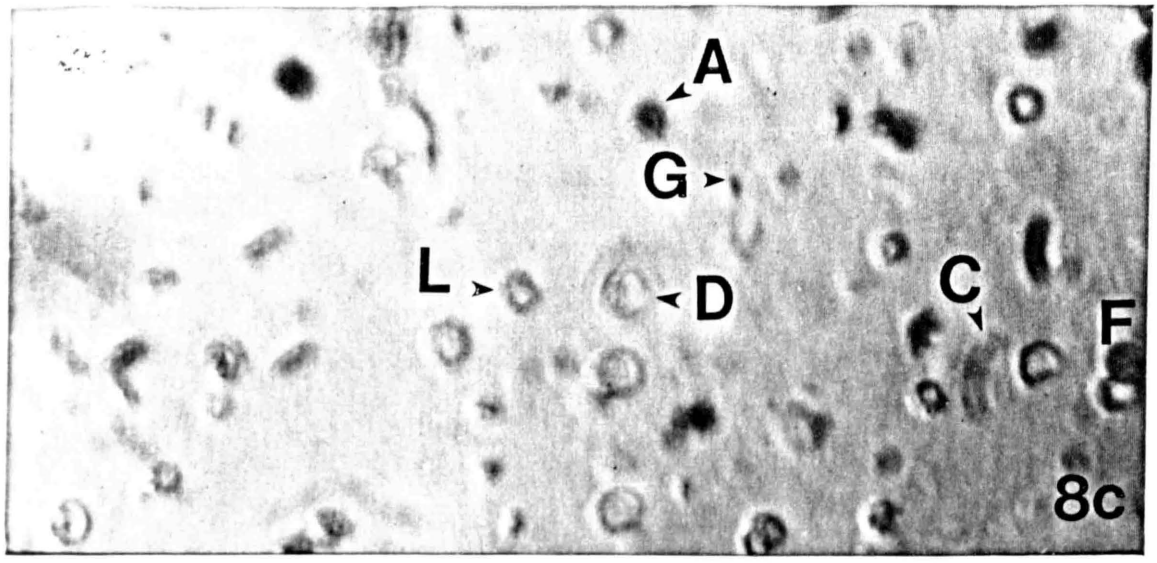
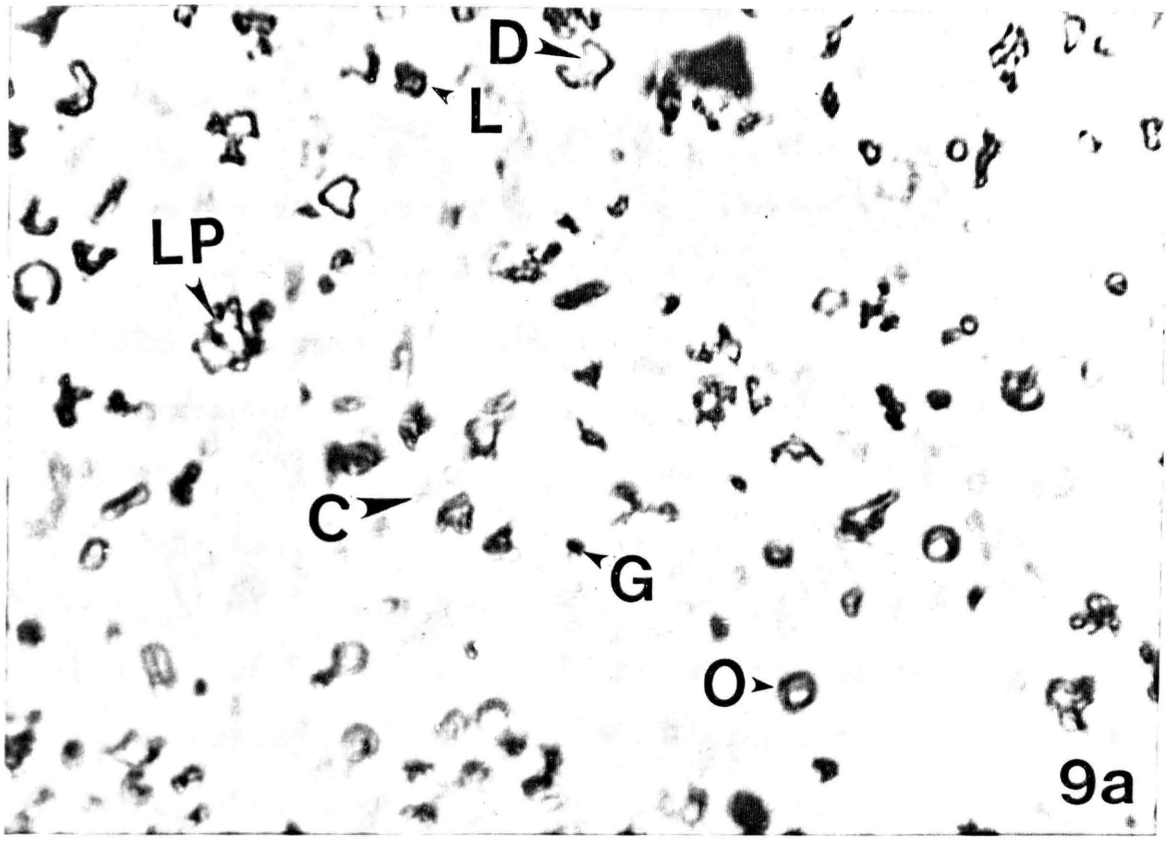
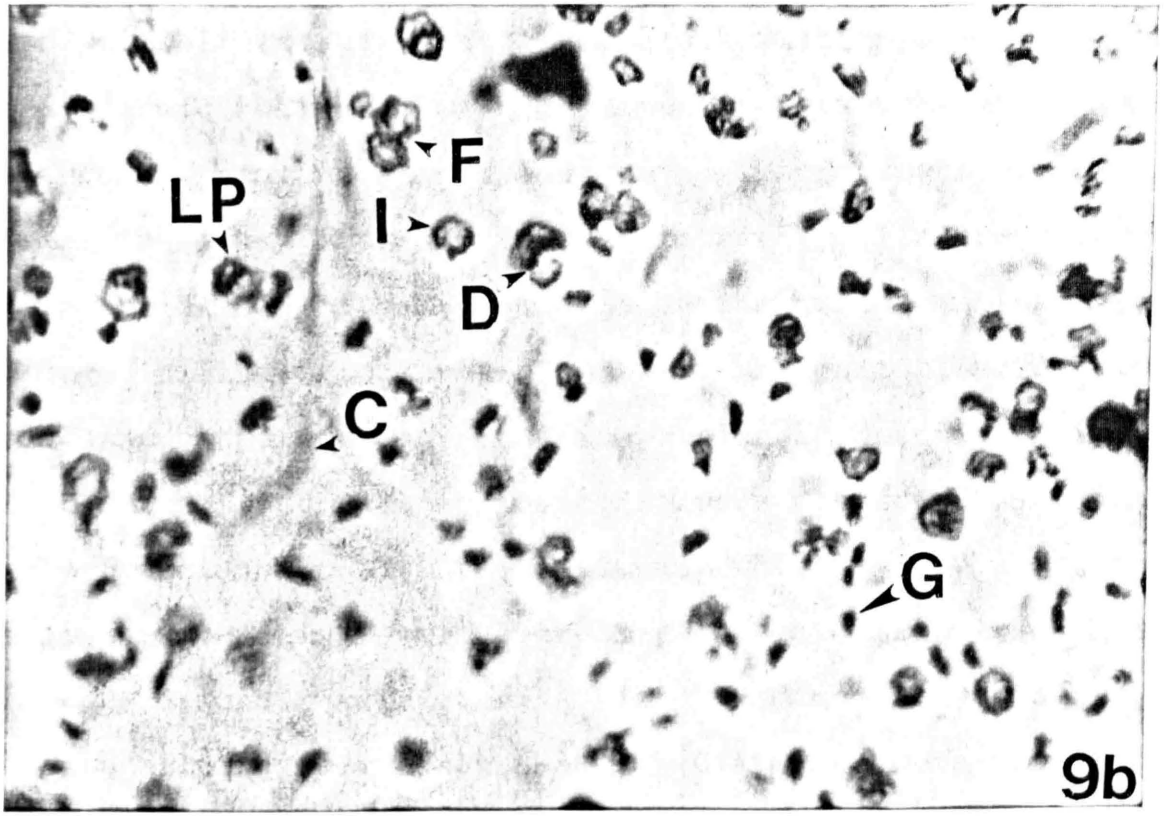

A
G
L
D
C
F
8c

Figure 9

Light micrographs of lysosomes from rat liver. (9a) Methylmercuric chloride treated rats show disruption of lysosomal membrane (D). Acid phosphatase activity covers large areas of the organelles (O) and in some includes the whole lysosome (L). In some cases several lysosomes appear fused and give a lumpy appearance (LP). C, Cisterna of endoplasmic reticulum; G, Glycogen granules. X 2000. (9b) Lysosomes from co-insult of MMC and gamma irradiated animals. Micrographs reveal membrane damage (D) of some organelles. Many lysosomes fused together appeared as lumpy body (LP). F, Fusion of two lysosomes; I, Intact lysosomes; G, Glycogen granules. X 2000.


D
L
LP
C
G
O
9a

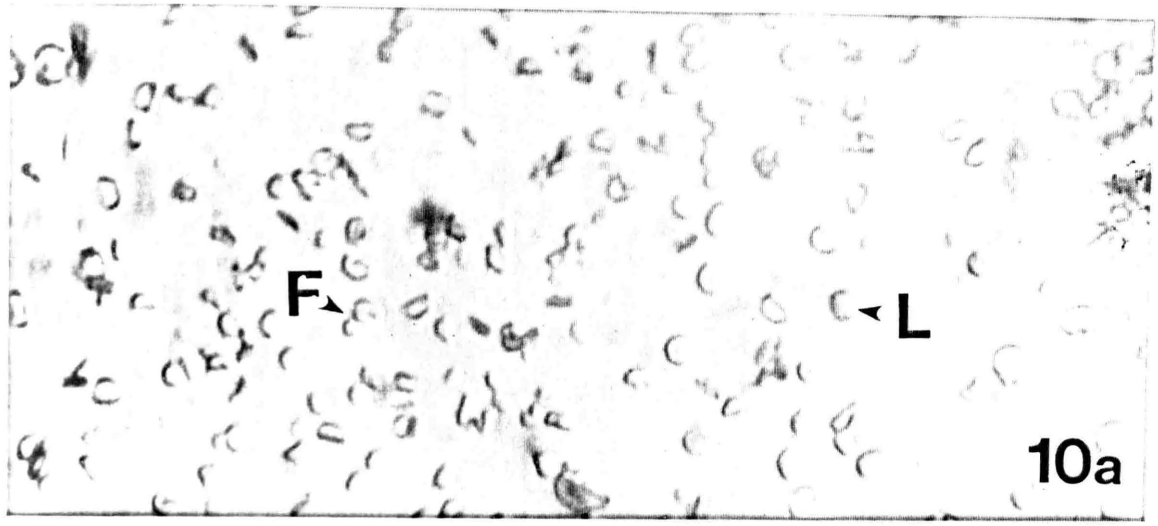

F
LP
I
D
C
G
9b

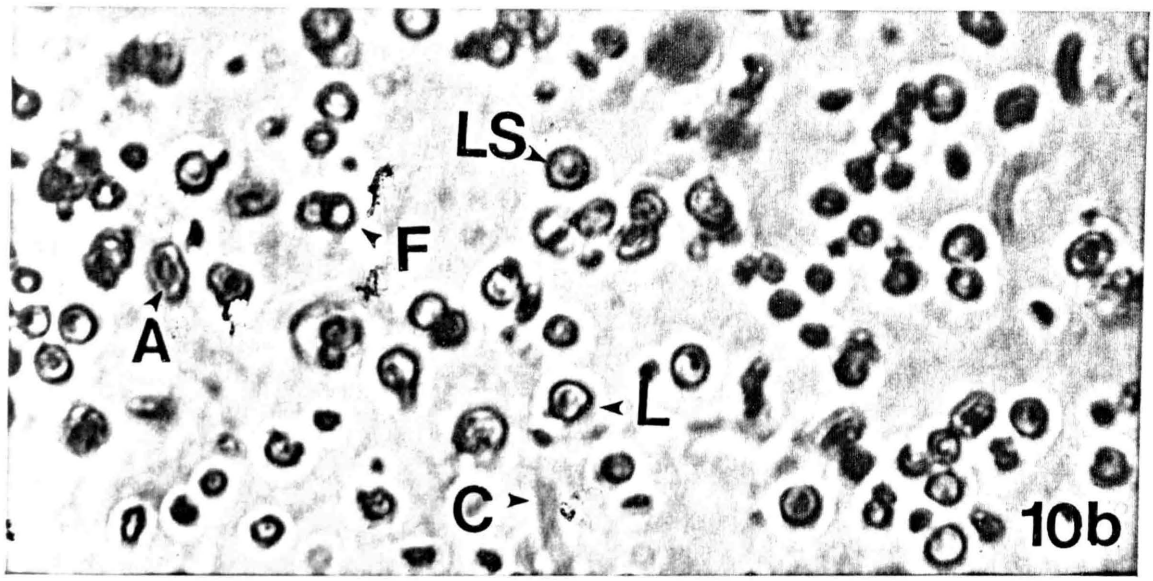
irregular in shape, and large areas of their compartment were shown as dark staining precipitate of lead citrate, indicating possible acid phosphatase activity.

Kidney tissues of control rats (Fig. 10a) exhibited normal lysosomes as round structures with lead citrate precipitate in the lysosome membrane. Kidney tissues of hydrogen peroxide pretreated rats (Fig. 10b) revealed lysosomes with acid phosphatase covering most of the lysosomal area; in some the reaction of the enzyme was centrally located, appearing as a dark spot. Gamma irradiated animals (Fig. 10c) showed the majority of the lysosomes as darkly stained organelles, with membrane damage to some of them and possible invagination in others. Cisternae of the endoplasmic reticulum were observed in larger areas in this treatment than in the previous ones. Micrographs of lysosomes in kidney from MMC treated rats (Fig. 11a) revealed membrane damage of all lysosomes in the areas examined. Damage inflicted to these tissues by MMC particles followed the same pattern as observed in previously described tissues. Cisternae of endoplasmic reticulum were observed in an intimate relationship with the lysosomes which showed acid phosphatase activity to cover large areas of the lysosomes. In the co-insult treated rats (11b), MMC action was observed in a smaller number of lysosomes. Cisternae were not as clearly observed as in the MMC treatment, and a greater

Figure 10

Light micrographs of lysosomes from kidney serial sections incubated in CMP and stained with lead citrate. (10a) Control rats show characteristics of normal, undisturbed lysosomes (L) as coarse opaque organelles, and the reaction of lead citrate with the lysosomal membrane. F, Fusion of lysosomes. X 2000. (10b) Hydrogen peroxide pretreated rats reveal some lysosomes larger in size (LS) than others. C, Cisterna of endoplasmic reticulum in intimate relationship to lysosomes (L). Numerous fusions of lysosomes (F). A, Acid phosphatase activity involves a wider area of organelles. X 2000. Animals treated with 800 R whole body gamma irradiation (10c). Micrographs shows C, Cisterna of endoplasmic reticulum in intimate relationship to lysosomes (L); B, Budding lysosomes; GP, Gap in lysosomal membrane; D, Damage to some lysosomal membrane. X 2000.


F
L
10a


LS
F
A
L
C
10b

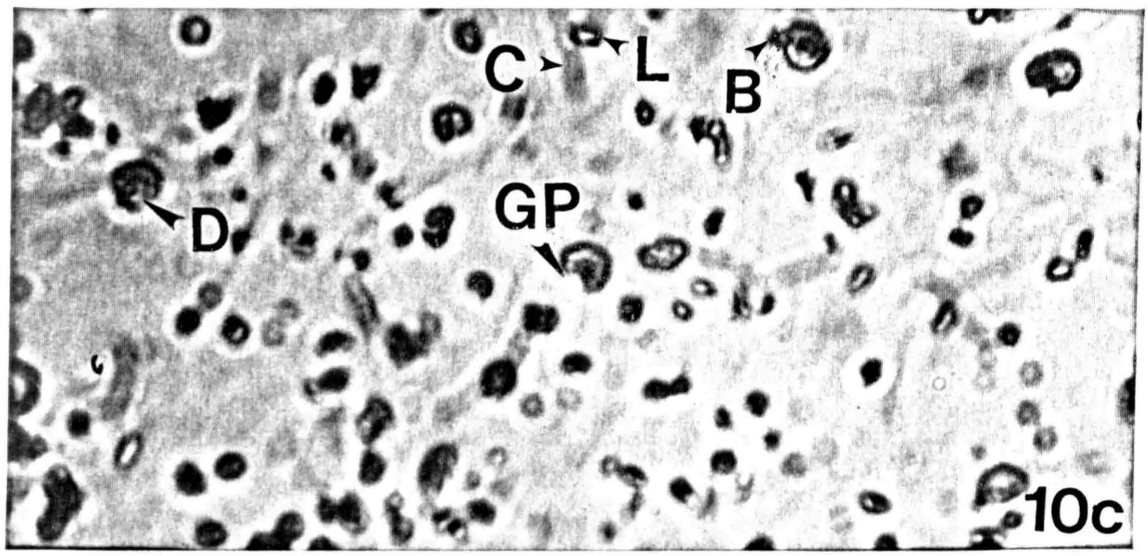
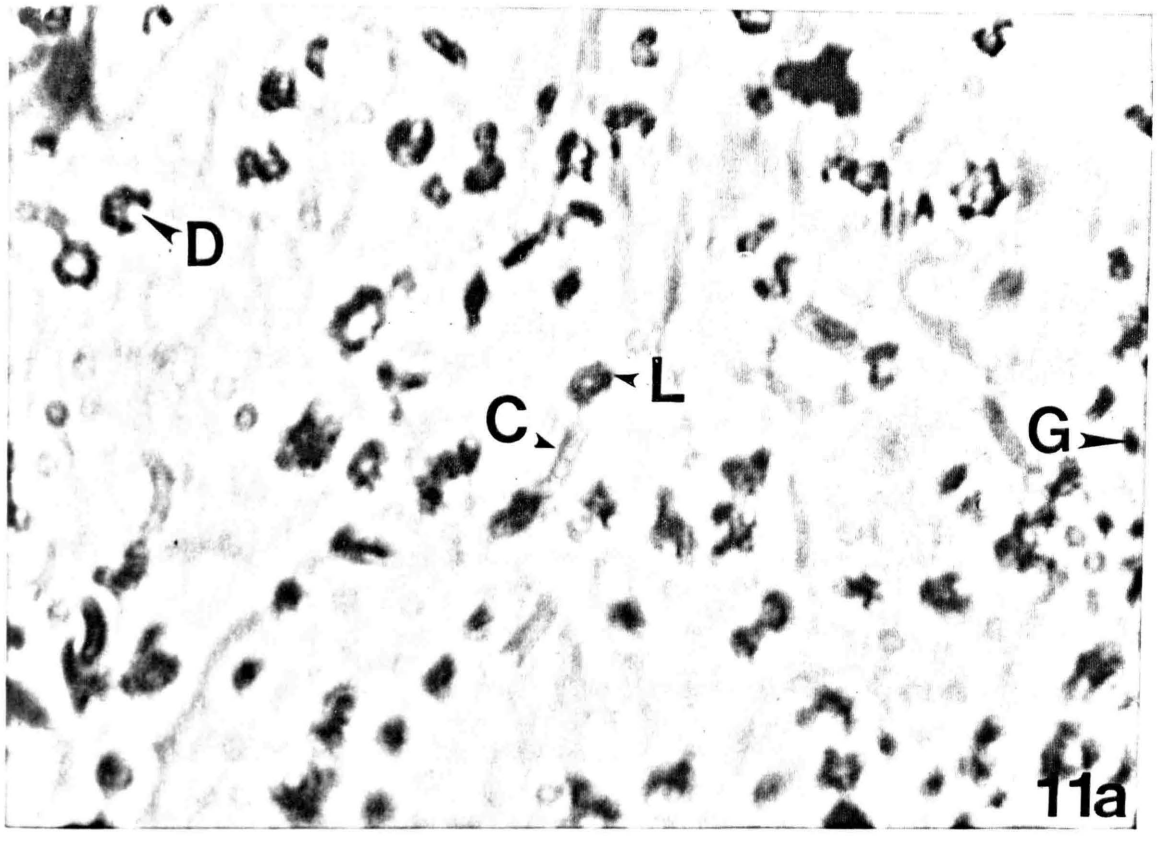
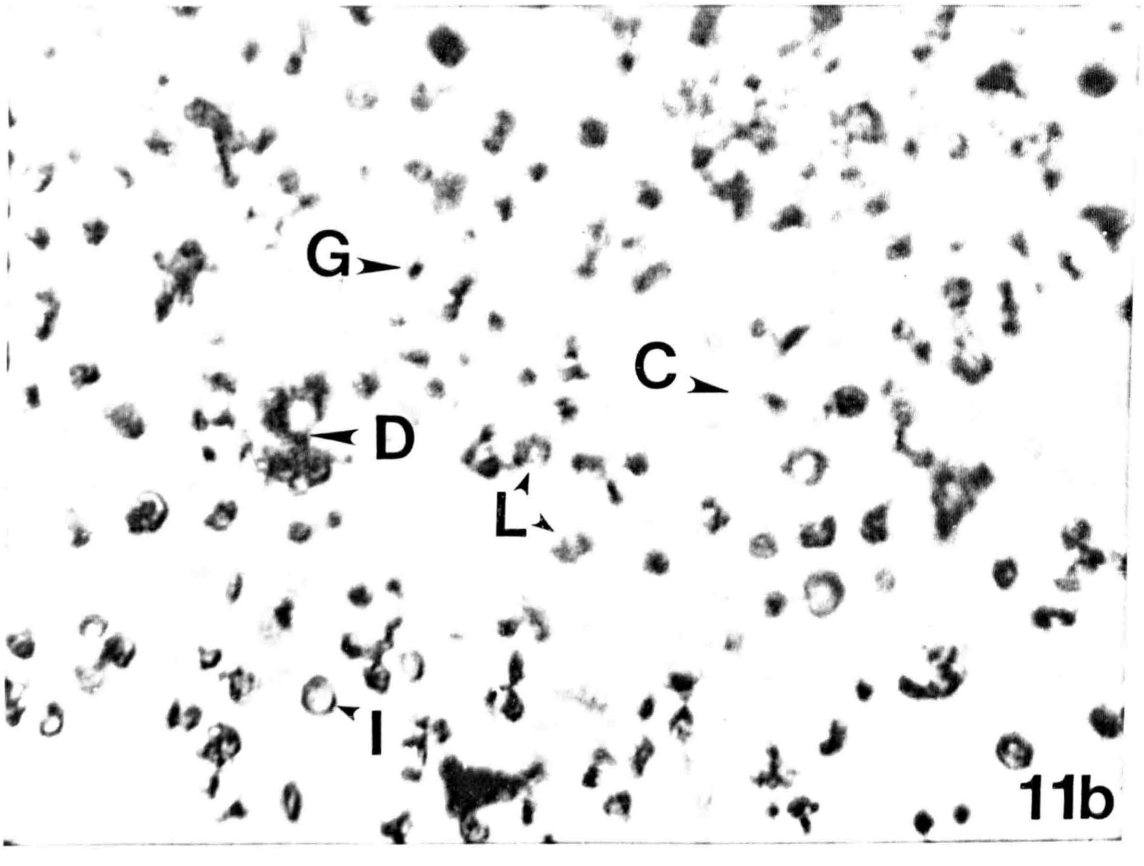

C
L
B
D
GP
10c

Figure 11

Light micrographs of lysosomes in serial sections of rat kidney. (11a) MMC treated rats show damage to lysosomal membrane (D), involving the entire observed section. C, Cisterna of endoplasmic reticulum in intimate relationship to lysosomes (L); Acid phosphatase activity covering large area of the lysosomes (L); G, Glycogen granules. X 2000. (11b) Co-insult of MMC and gamma irradiated animals, illustrating large number of damaged lysosomes (L), and rupture of their membrane (D). I, Intact lysosomes; C, Cisterna of endoplasmic reticulum; G, Glycogen granules. X 2000.



11a



11b

number of glycogen granules were observed in this group.

Serial sections of control rat brain, liver and kidney were incubated first in CMP to show lysosomes, and then in DAB medium to show peroxisomes in order to demonstrate the presence of both organelles in the same section. Figures 12a and 12b illustrate these organelles in two different fields of the nucleus arcuatus of the brain. Micrographs of rat brain (Fig. 13a), liver (Fig. 13b), and kidney (Fig.13c) sections of control groups, that were processed as above, then counterstained with haematoxylin also demonstrated peroxisomes and lysosomes in the same tissue section. The only noticeable difference between the two techniques was that more darkly stained organelles were evident after the latter treatment.

ANALYSIS OF THE NUMBER OF PEROXISOMES AND LYSOSOMES IN RAT BRAIN, LIVER AND KIDNEY

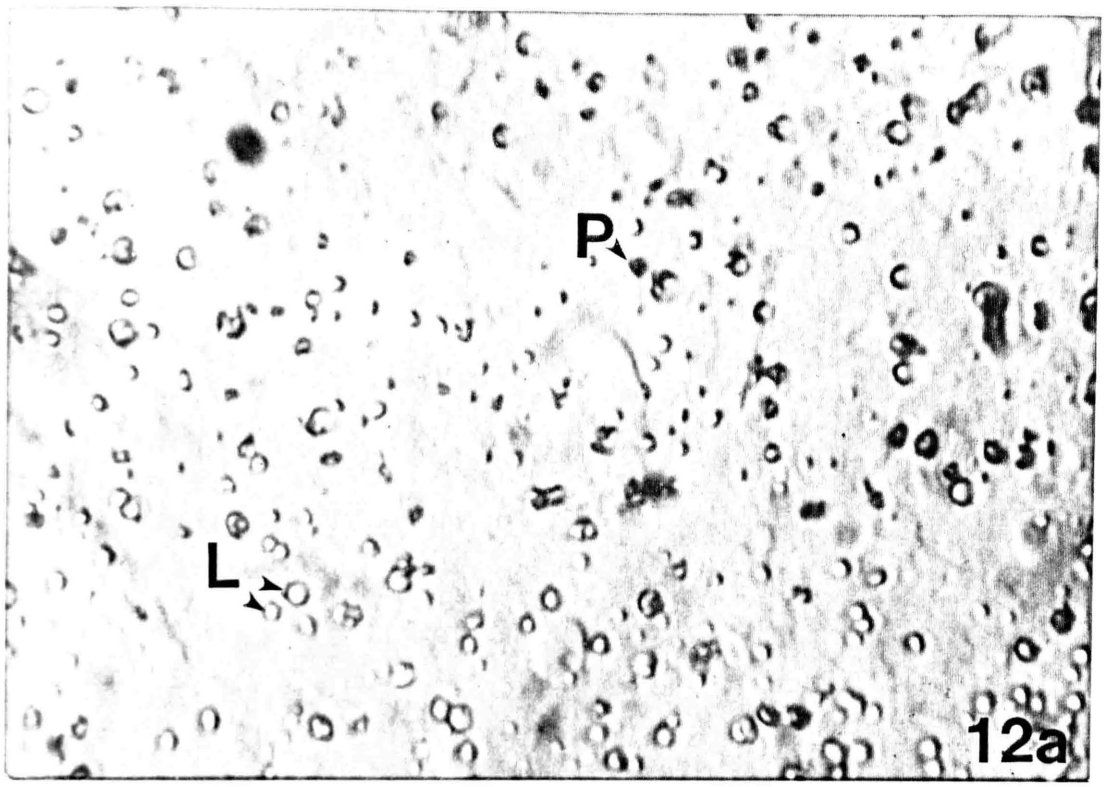
The total numbers of peroxisomes or lysosomes counted from the same tissue in the distilled water treated group of animals were more than those from the experimental groups; hydrogen peroxide, 800 R gamma irradiated, MMC or co-insult.

Peroxisome-like Organelles of the Nucleus Arcuatus

Counts of peroxisome-like (P-L) organelles in the nucleus arcuatus of the rat brain indicated a statistically significant decrease in the number of P-L organelles following all of the treatments, as compared to those of the distilled water treated animals (Table IV).

Figure 12

Light micrograph illustrating peroxisomes (P) and lysosomes (L) in the nucleus arcuatus of control rat brain. Figures 12a, 12b represent organelles from two different fields of the nucleus arcuatus. X 1260.


P
L
12a

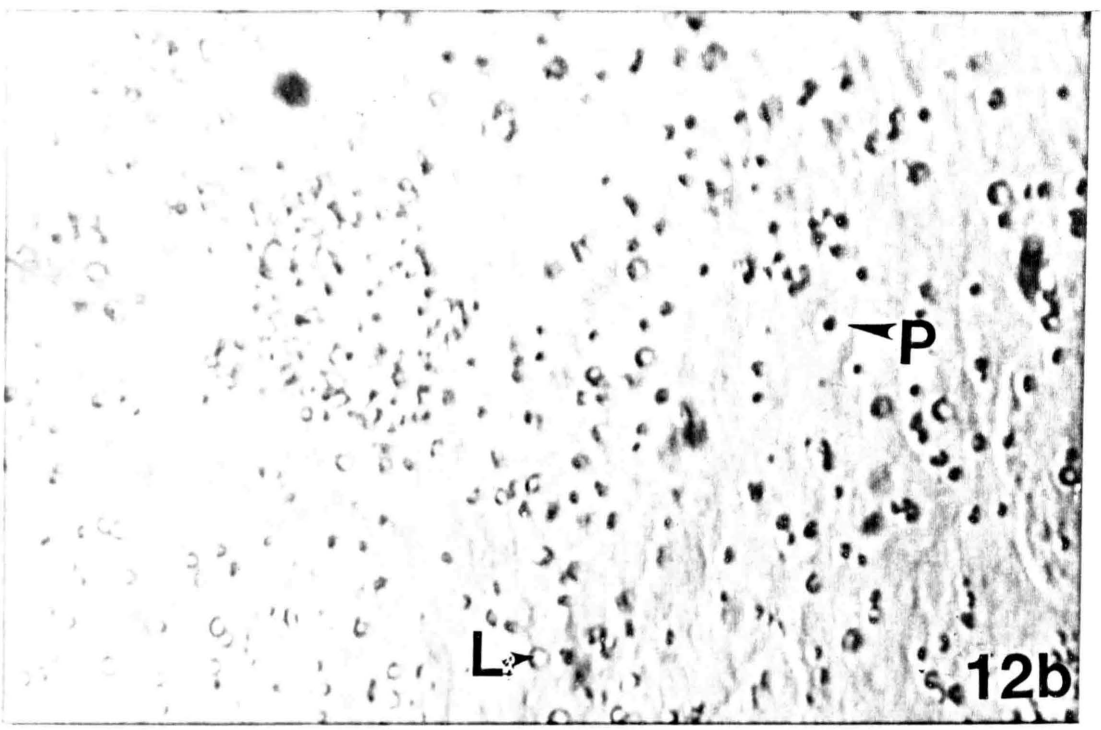
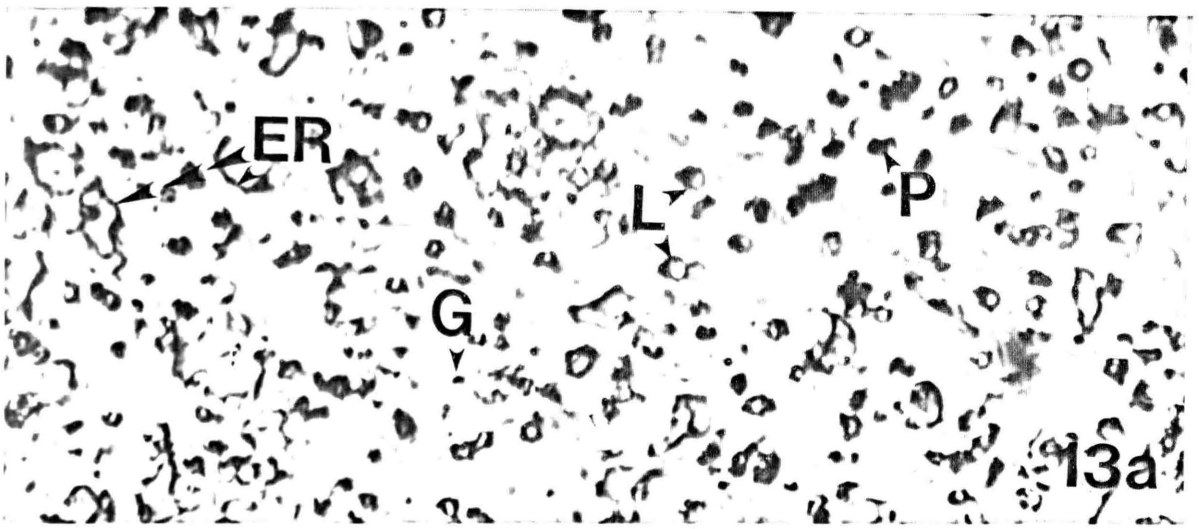
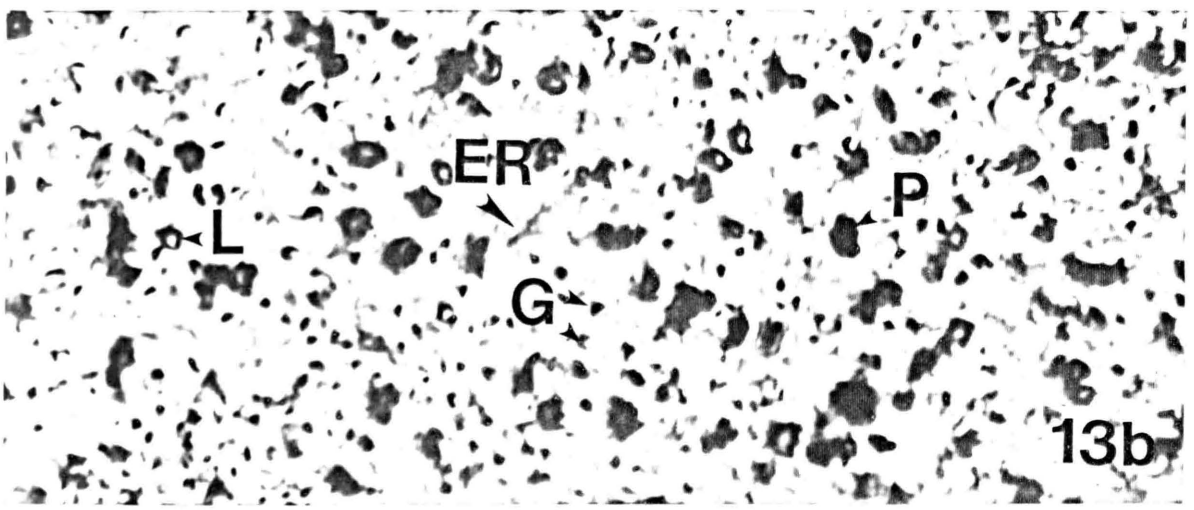

P
L
12b

Figure 13

Light micrograph of peroxisomes and lysosomes in serial sections of (13a) brain, (13b) liver, and (13c) kidney from control rats. All stained with lead citrate and counterstained with haematoxylin. Illustrating P, Peroxisomes; L, Lysosomes; G, Glycogen granules; ER, Rough endoplasmic reticulum. X 2000.


ER
L
P
G
13a


ER
P
L
G
13b

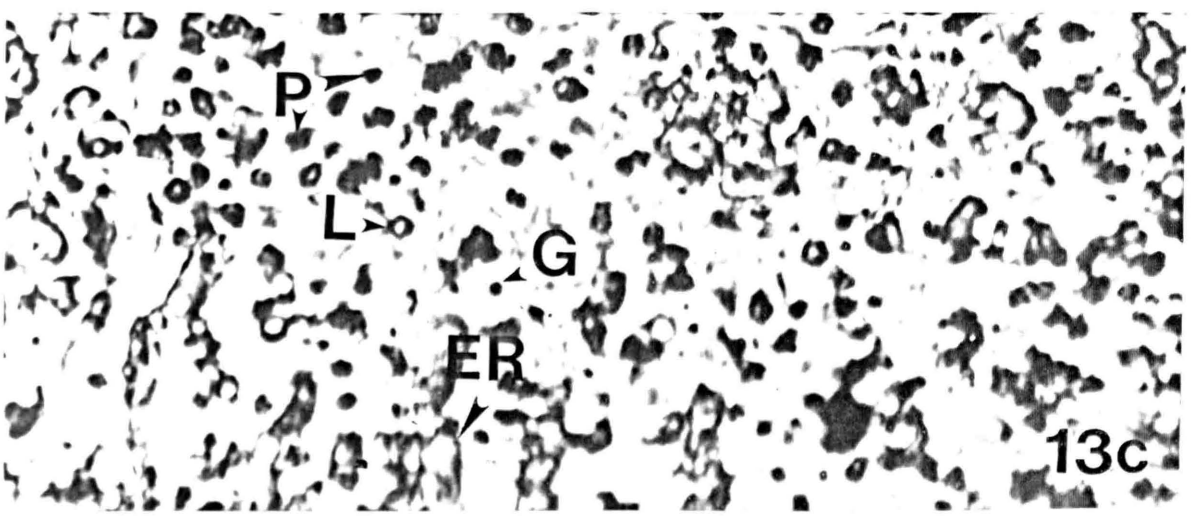

P
L
G
ER
13c

TABLE IV

Total numbers of peroxisome-like organelles, peroxisomes and lysosomes per area counted* in various tissues of male rats. Each value is a mean of 10 serial sections ± standard error.

Tissues	Organelles	Treatments				
		Hydrogen Peroxide	800 R Gamma-Irradiation	MMC	Co-Insult MMC Gamma-Irradiation	Distilled Water (Control)
Brain	Peroxisome-like	$136_{a} \pm 3$	$119_{a} \pm 4$	90 ± 3	$120_{a} \pm 4$	168 ± 7
	Peroxisomes	$123_{a,b} \pm 2$	$131_{b} \pm 3$	93 ± 2	$122_{a} \pm 2$	162 ± 2
	Lysosomes	$122_{a} \pm 2$	$116_{a} \pm 1$	72 ± 2	$119_{a} \pm 1$	153 ± 3
Liver	Peroxisomes	$135_{b} \pm 2$	$119_{a} \pm 4$	89 ± 5	$120_{a,b} \pm 4$	162 ± 3
	Lysosomes	$119_{a} \pm 2$	$112_{a} \pm 1$	89 ± 4	$117_{a} \pm 2$	145 ± 3
Kidney	Peroxisomes	$126_{a} \pm 2$	$123_{a} \pm 2$	83 ± 1	114 ± 1	146 ± 2
	Lysosomes	$100_{a} \pm 3$	$103_{a} \pm 2$	61 ± 2	$101_{a} \pm 2$	116 ± 1

* The area counted was 0.322 mm^2 for peroxisome-like organelles and 0.02 mm^2 for peroxisomes and lysosomes. a,b: Those values in the same row with the same subscript do not differ at the 5% level of significance as determined by least significant differences (Goldstein, 1965).

The greatest decrease was observed in the MMC treated animals which had only 54% as many P-L organelles as the control (Table V). The depression in numbers of P-L organelles in the hydrogen peroxide treated group was very similar to that observed in those treated with whole body gamma irradiation and the co-insult. The hydrogen peroxide treated group exhibited the least treatment effect, but was not significantly different from the two previously mentioned groups.

Peroxisomes of the Nucleus Arcuatus

Serial sections of rat brain tissues incubated in DAB medium and stained with lead citrate showed no significant difference at the 5% level of significance in the total numbers of peroxisomes from animals treated with whole body gamma irradiation (Table IV). The decrease in the total number of peroxisomes was greater in the co-insult treated animals than in the irradiated groups. The most obvious effect was a decrease in the total number of peroxisomes in rats treated with MMC. They had an average of 93 $\pm$ 10 peroxisomes per 0.02 mm^2.

Peroxisomes of Rat Liver

Total counts of peroxisome number in liver tissues of gamma irradiated animals and those treated with co-insults of MMC and gamma irradiation showed similar decreases (Table IV). The hydrogen peroxide treated rats revealed

TABLE V

Total number of peroxisomes and lysosomes of various tissue of male rats, expressed as percentage of control value.

Tissues	Organelles	Treatments			
		Hydrogen Peroxide	800 R Gamma-Irradiation	MMC	Co-insult MMC-Gamma-Irradiation
Brain	Peroxisome-like	81	71	54	71
	Peroxisomes	79	81	57	75
	Lysosomes	80	76	47	78
Liver	Peroxisomes	83	73	55	74
	Lysosomes	82	77	61	81
Kidney	Peroxisomes	86	84	57	78
	Lysosomes	86	89	53	87

the least treatment effects, these had 83% as many peroxisomes as the controls (Table V). Meanwhile the effect was clearly observed in the MMC treated group with 55% as many peroxisomes as the control.

Peroxisomes of the Rat Kidney

Counts of peroxisomes from 10 serial sections of rat kidney indicated a statistically significant decrease in the number of peroxisomes in all groups following treatments, as compared to the control animals. The greatest effect was shown in the MMC treated group, which had only 57% as many peroxisomes as the control (Table V). The hydrogen peroxide group and the gamma irradiated one showed approximately similar depressions, while the co-insult group had a smaller decrease, therefore was significantly different from the two previously mentioned groups.

Lysosomes of the Nucleus Arcuatus

Quantitative counts of lysosomes in the nucleus arcuatus of rat brain incubated in CMP medium and stained with lead citrate, indicated a statistically significant numerical decrease in the number of lysosomes following all of the treatments when compared to the control rats (Table IV). The greatest effect was observed in the MMC treated animals who had only 47% as many lysosomes as the control. The hydrogen peroxide treated, gamma irradiated, and co-insult groups exhibited the least treatment effect, with little

difference among groups.

Lysosomes of the Rat Liver

Serial sections of rat liver were examined for the presence of lysosomal organelles. The number of lysosomes in animals treated with hydrogen peroxide, gamma irradiation, and co-insult of MMC and radiation were not statistically significantly different (Table IV). Animals treated with MMC showed the greatest depression and had only 61% as many lysosomes as the control group (Table V).

Lysosomes of Rat Kidney

Total number of lysosomes counted from serial sections of kidney tissues indicated a very similar depression in animals treated with hydrogen peroxide, gamma irradiation, and co-insult of MMC and radiation. As was the case with the nucleus arcuatus and the liver, the major effect was more pronounced in the MMC treated animal, with only 53% as many kidney lysosomes as the control.

The Effect of Hydrogen Peroxide Pretreatment on Gamma Irradiation Induced Mortality

Lethality Experiments

None of the larger adult male rats treated in Experiment 1 died during the experimental 30 day period (Table VI).

The results obtained on the 90 day-old rats are presented in Table VII and Figure 14. A careful examination

TABLE VI

Percent survival of hydrogen peroxide or distilled water pretreated 170-day-old large mature rats 30 days after exposure to gamma irradiation.

Group No.	No. of Animals	Pretreatment*	Gamma-irradiation**	Percent Survival
I.	5	1.0 ml H_2O_2	775 R	100
II.	5	1.4 ml H_2O_2	775 R	100
III.	5	1.8 ml H_2O_2	775 R	100
IV.	5	1.8 ml H_2O	775 R	100
V.	5	1.8 ml H_2O_2	0	100
VI.	5	1.8 ml H_2O	0	100

* One intraperitoneal injection a day per animal for 5 days.

** Irradiation or sham irradiation administered 48 hours after the last injection.

TABLE VII

Percent survival of hydrogen peroxide or distilled water pretreated 90-day-old male rats 30 days after exposure to ^{60}Co gamma irradiation.

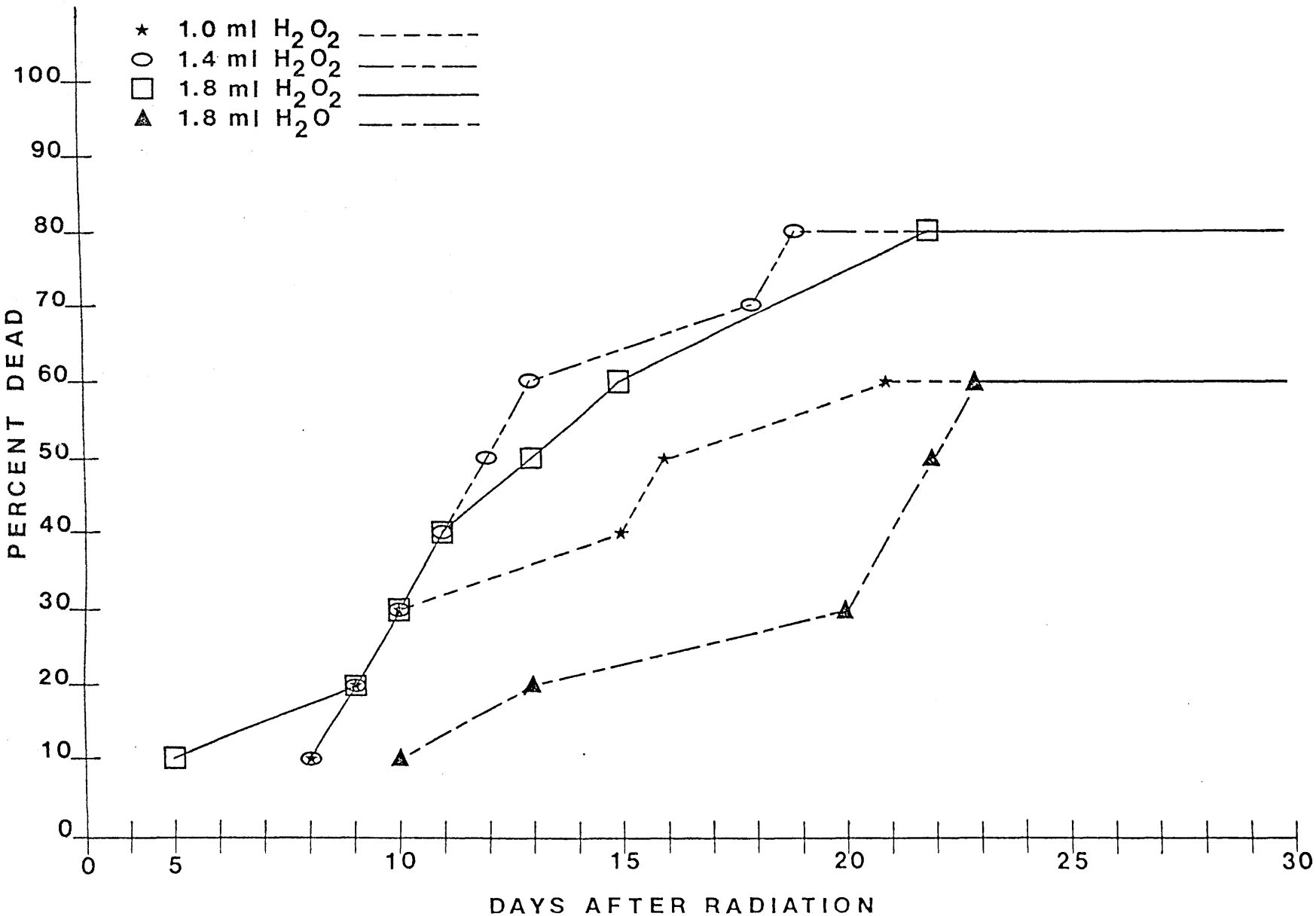
Group No.	No. of Animals	Pretreatment*	Gamma-irradiation Treatment**	Percent Survival
I.	10	1.0 ml H_2O_2	775 R	40
II.	10	1.4 ml H_2O_2	775 R	20
III.	10	1.8 ml H_2O_2	775 R	20
IV.	10	1.8 ml H_2O	775 R	40
V.	10	1.8 ml H_2O_2	0	100
VI.	10	1.8 ml H_2O	0	100

*One intraperitoneal injection a day per animal for 5 days.

**Irradiation or sham-irradiation administered 48 hours after the last injection.

Figure 14

Daily mortality presented as percent dead for 90-day-old adult rats during 30 days after hydrogen peroxide or distilled water pretreatments followed by 775 R of ^{60}Co gamma irradiation. Pretreatment consisted of one daily intraperitoneal injection for 5 days; irradiation was administered 48 hours after the last injection. Each group included 10 rats.


1.0 ml H_2O_2
1.4 ml H_2O_2
1.8 ml H_2O_2
1.8 ml H_2O
PERCENT DEAD
0
10
20
30
40
50
60
70
80
90
100
0
5
10
15
20
25
30
DAYS AFTER RADIATION

of the data revealed no significant differences between replicates within groups. In group I (1.0 ml of 1.5% hydrogen peroxide pretreated), 60% of the experimental animals died during the observation period; all deaths occurred by day 21 after exposure to gamma irradiation. In group II (1.4 ml of 1.5% hydrogen peroxide pretreated), 80% died during the first 19 days after exposure to gamma irradiation. In group III (1.8 ml of 1.5% hydrogen peroxide pretreated), 80% of the animals died within 22 days after they have been irradiated. In group IV (1.8 ml of distilled water pretreated), death occurred in 60% of the animals during 23 days after exposure to gamma irradiation.

The percentage survival in these groups was not significantly different (Table VII) and the pattern of death was quite similar (Fig. 14); although death occurred earlier in the peroxide treated groups. Therefore, the hydrogen peroxide pretreatment did not provide protection against the lethal effects of the acute gamma irradiation. In groups V and VI (pretreated with 1.8 ml of 1.5% hydrogen or 1.8 ml distilled water, respectively, and sham irradiated), none of the animals died during the observation period of 30 days.

DISCUSSION AND CONCLUSIONS

The current study is an extension of the work of Earhart (1975), who reported that hydrogen peroxide pretreatment protected against MMC toxicity. He also demonstrated an increase in the number of peroxisome-like (P-L) organelles in the nucleus arcuatus of rat brain following hydrogen peroxide pretreatment and postulated that the effect might be due to the proliferation of these organelles in the brain elicited by the effects of the radiation, which in turn protected against methylmercuric chloride toxicity. This research was conducted to provide evidence for Earhart's hypothesis. The results of this research will be discussed in relation to the hypothesized protective mechanism afforded by P-L organelles, and the possible involvement of peroxisomes and lysosomes in this mechanism.

P-L Organelles of the Nucleus Arcuatus

The greatest effect on numbers and morphology of P-L organelles was observed in the MMC treated group. Since this peroxisome system has been described to be rich in thiol and disulfide groups (Noda, 1959; Srebro, 1970; Srebro and Cichocki, 1971) MMC would be expected to have a greater affinity for the membranes of these organelles. The MMC was observed to cause the membranes to rupture (Fig. 1b, 1c) and possibly to release their contents of peroxidases, catalases, and/or their end-product such as hydrogen peroxide.

In the gamma radiation treated groups the important sulfhydryl bearing target molecules were damaged. No matter where these molecules were hit, the damage possibly migrated to the thiol groups, breaking their bonds and therefore causing some conformational changes in their structure, with an end-result of membrane damage (Srebro, 1970). Accordingly, if the molecules are attacked by radicals, it is primarily the sulfhydryl groups of the target molecules that are attacked.

In contrast with the increase in number of P-L organelles following hydrogen peroxide treatment observed by Earhart (1975) a reduction was observed in this study. Since the area measured in this study was greater than that measured by Earhart (1975), the counts obtained by Earhart were adjusted by multiplying by the ratio of the two areas in order to compare results. The total number of P-L organelles per unit area in control rats was less than that observed by Earhart. While Earhart observed a significant 32% increase in the number of P-L organelles due to hydrogen pretreatment and not a statistically significant 18% increase 7 days after 800 R whole body gamma radiation, a similar regime of hydrogen peroxide pretreatment or 800 R whole body gamma radiation 48 hours before sacrifice significantly decreased the number of organelles in the present study. These data tend to disagree with the work of Srebro (1969), who also observed a considerable increase in the number of chrome haematoxylin positive

periventricular glial cells on the 7th day post-irradiation per unit area (0.160 mm^2) of the nucleus arcuatus in 800 R whole body X-irradiated rats.

The reasons for the discrepancy between the results of Earhart (1975) and this study with regard to hydrogen peroxide pretreatment are not readily apparent, but may be due in part to the small sample size of 4 or 5 animals used by Earhart. Since the radiation only animals in this study were killed only 48 hours after treatment, to coincide with the co-insult group, damage that was apparent in this study may have been repaired and compensatory proliferation of the organelles may have occurred by 7 days, when the observations of Srebro (1969) and Earhart (1975) were made.

In this study, the co-insult of MMC and gamma radiation produced the same reduction in the number of organelles as radiation alone, which was less than the reduction caused by MMC alone (Table IV). In the co-insult group, the effects of radiation on the P-L organelles sensitized by the MMC treatment could have stimulated proliferation of the organelles, so that the damage seen 48 hours after radiation was not as great as that following MMC alone. However, sensitization by the MMC treatment was necessary since radiation alone reduced the number of organelles.

Peroxisomes and Lysosomes of the Brain, Liver and Kidney

These experiments to examine quantitatively the peroxisomes and lysosomes have revealed that all insults decreased

the number of these organelles. Histochemical analysis and morphological observations permitted further assessment of the pathologic spectrum and histochemical response of these organelles to be discussed in succeeding sections.

Peroxisomes of Various Tissues Examined

Peroxisomes induction expected by hydrogen peroxide pretreatment proposed by Earhart (1975), was not evident in the present study. Statistically significant reductions in the number of peroxisomes in the brain, liver and kidney were recorded from serial sections of hydrogen peroxide pretreated rats. This was possibly due to the poisonous nature of hydrogen peroxide in high concentrations. Peroxisomes have been shown to contain catalase and a variety of hydrogen peroxide-producing oxidase (De Duve and Baudhuin, 1966) capable of destruction of hydrogen peroxide. Hydrogen peroxide is decomposed by the enzyme catalase that is found in peroxisomes and in almost all living things. The following is a possible mechanism of action of hydrogen peroxide on peroxisomes:

H_2O_2	Catalase →	$HO_2 + H^{\cdot}$
$H^{\cdot} + H_2O_2$	→	$OH^{\cdot} + H_2O$
$OH^{\cdot}$ + Peroxisomal Organelle	→	Damage

The above mechanism indicates that a greater number of $OH^{\cdot}$ radicals are produced. As a result of the indirect action

of these radicals on the larger molecules in the cell, morphological damage to these organelles and resulting cellular damage was observed.

Animals treated with 800 R gamma irradiation also exhibited a significant reduction in the number of peroxisomes when compared to the control group. Light photomicrographs showed that some organelles were larger than others. Some were ellipsoid in shape, a phenomena not observed in control tissue; there was some variability in the matrix homogeneity. Since water constitutes approximately 65% of the body weight, it is feasible to propose the following mechanism of action of gamma irradiation;

$$H_2O \xrightarrow{\text{Gamma irradiation}} \bar{e}_{aq} \text{ (solvated electron in } H_2O), H\cdot, OH\cdot, H_2O_2, H_2, H_3O \text{ (Farahataziz, personal communication).}$$

$$\bar{e}_{aq} + H_2O_2 \longrightarrow OH\cdot + OH^-$$

$$OH\cdot + \text{Peroxisomal Organelle} \longrightarrow \text{Damage}$$

As in the previous proposed mechanism of hydrogen peroxide induced damage, OH· were produced in greater number, thus causing possibly similar damage to peroxisomes from this treatment regime. If, however, other molecules are present in the water which are capable of reacting with the free radicals H· and OH· , competition will exist for these radicals

between the reacting material and the molecular products. Accordingly, the H_2O_2 formed may not be destroyed, and thus undergoes further destructive reactions with the molecules of the cell.

Methylmercuric chloride action on peroxisomal organelles was clearly illustrated by a statistically significant decrease in the number of organelles and by histological examinations of organelles of the various tissues, which showed damage of the entire organelles, and, in some, rupture of their membrane. De Duve (1965), suggested that mercury particles interact with membrane thiol groups, and that a critical number of ligands cross-linked, thereby disrupting membrane continguity with resultant increased permeability. Moreover, difference in individual peroxisome responses can be attributed to morphological and biochemical parameters that may determine the relative availability of thiol groups.

Rats treated with the co-insult of MMC and 800 R gamma irradiation revealed the disruptive action of MMC in some of the peroxisomes, which possibly followed the same pattern of action as observed in the single insult treatment. The damage was indicated to the whole organelle or by rupture of their membrane, and in other instances invagination of some of these organelles was observed. The majority remained intact which was possibly due to the appartent induction of a protective mechanism against MMC destructive action. At some

point in this mechanism, binding of the mercurial particles to the thiol or sulfhydryl groups to the membranes of a majority of the organelles was prevented or reduced by direct and/or indirect effects of the ionizing radiation. The damage was also indicated by a decreased number of peroxisomes. The total numbers of these organelles counted were approximately similar to those for the hydrogen peroxide and gamma irradiated animals. These results support the argument that whole body gamma irradiation has afforded some protection against MMC toxicity, indicating a neutralization pattern in which the ionizing radiation effect appeared to override the toxic effect of MMC (Hupp et al., 1974, 1977). The mechanism is considered to be related to that of the single insult of gamma irradiation mentioned in earlier sections.

Disposal Mechanisms of Peroxisomes

Peroxisomal organelles damaged during treatment can be disposed of by at least one of the following ways. The first process is considered to be autophagic by nature; whereas, the second one is described as consisting of a "dissolution" or "atrophy" of individual peroxisomal organelles within the cytoplasmic matrix, without the involvement of sequestering membranes (Danpure and Taylor, 1974). A third process is described as proceeding by means of a retraction of peroxisomal organelles contents within the smooth endoplasmic reticulum (SER) compartment (Moody and Reddy, 1976); it is conceivable that the latter two processes are morphologically

interconnected. Histochemical procedures used in this study showed peroxisomal damage as a result of the MMC single insult and to a limited extent to the co-insult treatment.

Lysosomes of Various Tissues Examined

The total number of lysosomes in control rats was greater than that in any of the insult groups. Histological examination did not reveal any of the damage that was observed in the other groups.

Hydrogen peroxide pretreatment produced damaging effects on the lysosomes as indicated quantitatively by a decreased number of these organelles, and histologically by rupture of their membranes. It is possible that under other circumstances, a proliferative activity might result as a protective response mechanism to the initial damage caused by the peroxide. A similar decrease in the number of lysosomes was also observed in rats treated with whole body gamma irradiation. In the present study, the lysosomal changes found after this insult could lead to tissue damage, in possibly one or a combination of three ways: First, the radiation may directly damage or alter the lysosomes, which release acid hydrolases into the cytoplasm and possibly destroy parts of the cell; second, the ionizing radiation may damage both the lysosome and other parts of the cell simultaneously but independently; and third, the radiation may indirectly effect the lysosomes and directly damage other

parts of the cell, for example, a damaged nucleus may cause subsequent changes that are injurious to the lysosomes.

Ionizing radiation has been shown to initiate radical chains in preparations of polyunsaturated fatty acids (Mead, 1952). This type of initiation can take place in natural membranes and has been shown by Wills and Wilkinson (1966), who found that disruption of the lysosomal membrane with subsequent release of lysosomal enzymes could be brought about by radiation and other conditions leading to peroxide formation. In this study sublethal concentration and doses of the various agents were applied which could have initiated radical chain reactions in membrane lipids, and indirectly or directly resulted in damages to the lysosomes as cited earlier (Wills and Wilkinson, 1966). Meyer and Dannenberg (1970) have suggested that lysosomal enzyme changes after irradiation might be due to alteration in tissue composition in the tissue involved. In the liver this may be due to increased numbers of Kupffer cells, which may engulf and digest cells which have been damaged or killed by the radiation.

The effects of the deposition of MMC on lysosomes of the various tissues examined, in both the single insult and co-insult treatments, were detected from decreased numbers of organelles and from morphologically observable lysosomal damage in the form of membrane disruption or total

destruction. The damage was greater with the single insult, but MMC in both kinds of insults resulted in the leakage of acid hydrolases into the cytosol of the cell.

Mechanisms of Hydrolases Release from Lysosomes

Several mechanisms for the release of hydrolases from lysosomes have been proposed. Some workers suggested that labilization of the lysosomal membrane may result from direct attack of lipid peroxides on the membrane (Wills and Wilkinson, 1966). Others postulate that the permeability of lysosomal membranes following irradiation may be regulated by hormones (Rahman, 1963) or by mediators released from radio-sensitive lymphocytes (Aikman and Wills, 1974a). If lysosomal membrane permeability is controlled indirectly by the action of a particular hormone or other mediators following irradiation, it may be possible to correlate the changes in the lysosome number and/or morphology seen in this investigation with corresponding changes in tissues of a particular mediator post-irradiation. Such a conclusion would provide support for the concept of mediated control of lysosomal enzyme release following radiation exposure. Last, if the escape of hydrolases from radiation-damaged lysosomes is dose dependent, which may be the case since damage was indicated in a decreased number of peroxisomes and lysosomes in all treatments and morphologically observed very often in the single insult of MMC, it may be possible to use these

enzymes as biological indicators of radiation injury in an appropriate assay system.

Comparison of Peroxisomal and Lysosomal Counts in the Three Tissues Examined

Based on the percentage reduction from control values of the number of peroxisomes (Table V), it is concluded that a greater damage was produced in liver tissues in the 800 R whole body gamma irradiated rats than in brain and kidney tissues of the liver and brain was observed for the other treatments. Except for the MMC treatment, generally the damage to kidney tissue, as expressed by reduction in the number of peroxisomes, was less than in brain and liver tissue.

It appears logical to assume from the results (Table V). that there is a similar amount of damage caused to lysosomes in the brain by hydrogen peroxide, gamma irradiation, and co-insult treatments as far as the percentage reduction in number of these organelles are concerned. The same is true in kidney and liver tissues. The amount of damage to lysosomes caused by MMC was greater than for the other agents. The amount of MMC induced lysosomal damage in the brain and kidney was similar and was somewhat more than that observed in the liver (Table V).

The Effect of Hydrogen Peroxide Pretreatment on Gamma Irradiation-Induced Mortality

The hydrogen peroxide pretreatment used in this

mortality study was based upon evidence given in the literature that radiation-induced peroxides are responsible for the secondary effects of ionizing radiation (Srebro, 1969) and findings that protection was afforded by hydrogen peroxide pretreatment (Earhart, 1975).

The 100% survival response obtained in the preliminary study with large mature male rats (Table VI) was not expected. Since these rats were approximately 170 days old and weighed from 415 to 505 g, an age and/or size factor appeared to be involved in affording greater radioresistance to these large old rats, rather than the possible protection from hydrogen peroxide pretreatment since 100% survival occurred in rats not pretreated with hydrogen peroxide.

To investigate further the results obtained in the preliminary study another lethality experiment was conducted using two replicates of 30 mature 90 day old male rats, weighing from 250 to 300 g (Table VII). Results obtained from these experiments showed 60% to 80% mortality among hydrogen peroxide pretreated rats; while distilled water treated rats showed 60% mortality. Based upon these results the following conclusions were drawn.

1. Differences in the amount of hydrogen peroxide in the co-insult treatment (H_2O_2 and gamma irradiation) did not

produce any significant differences in survival.

2. Although survival of rats treated with distilled water (control) was 13% better than the mean of all hydrogen peroxide treated rats, the difference was not statistically significant ($0.1 < p < 0.05$).
3. Radiation induced death occurred earlier in the hydrogen peroxide treated rats, with a greater effect observed in the two groups receiving the higher doses of hydrogen peroxide pretreatment (Fig. 14).

Correlation of Histological and Mortality Studies

The attempt to correlate the histological study, which comprised recording the total number of peroxisomes and lysosomes, and by morphological observations of these organelles in the brain, liver and kidney of rats, to that of the radiation induced mortality rate of hydrogen peroxide treated animals yielded useful conclusions. The histological study provided conclusive evidence that hydrogen peroxide pretreatment did not cause a proliferative response or increase in the numbers of peroxisomes and/or lysosomes as previously was reported by Earhart (1975). Hydrogen peroxide did not protect against lethality in the whole body gamma irradiation study, a slightly greater death rate was observed in rats pretreated with hydrogen peroxide, than those with distilled water. The reduced number of peroxisomes and lysosomes might reduce the defense capability of

the cells, rather than provide protection. With a reduced number of these organelles, the cell would be more susceptible to damage caused by the peroxides produced as an indirect effect of radiation, as described earlier in this discussion. This is consistent with failure of hydrogen peroxide pretreatment to protect against radiation induced mortality. The earlier time of death and the slightly greater mortality of rats treated with peroxide and radiation indicated that the pretreatment reduced the radioresistance of the animals.

SUMMARY

The first part of the investigation reported in this dissertation was a histochemical study conducted to determine the effect of several agents on the number of peroxisome-like organelles in the nucleus arcuatus of the brain and the number of peroxisomes and lysosomes in the nucleus arcuatus of rat brain, liver and kidney. Serial sections, 7 micrometers thick, of the brain tissues were stained to show P-L organelles. Other sections of the brain, liver and kidney were incubated in diaminobenzidine medium and stained with lead citrate to show peroxisomes while other sections were incubated in cytidine monophosphoric acid and stained in lead citrate to show lysosomes. A counterstain of haematoxylin was also used to help differentiate peroxisomes from lysosomes in tissue sections treated to show both organelles.

Histochemical analyses and morphological observations revealed a significantly greater total number of the three organelles in all three tissues examined in animals treated with distilled water than in any of the other treatments. The largest decrease in the number of organelles was in animals treated with methylmercuric chloride. The possible proliferation of P-L organelles and the high number of these organelles in the nucleus arcuatus reported by Earhart (1975)

was not observed. A reduction in the toxic effects of MMC produced by whole body gamma radiation was demonstrated. In animals treated with MMC, histological examination of the tissues showed a similar pattern of effect in all three: rupture of organellar membrane and total destruction of some organelles. The same effect was observed, but to a lesser extent, in the MMC and gamma irradiation co-insult group.

The second part of the investigation was a lethality study designed to explore the hypothesis that hydrogen peroxide pretreatment provides protection against gamma irradiation.

A total of 60 mature male rats were used in the study. Animals were divided randomly into five groups. The experimental Groups I, II, and III, received five doses of 1.0, 1.4, or 1.8 ml of 1.5% hydrogen peroxide, respectively. Group IV received 5 doses of distilled water and all received 775 R gamma irradiation. Group V, and VI received five doses of 1.8 ml hydrogen peroxide and 1.8 ml distilled water, respectively and sham radiations. Deaths were recorded during a 30 day period. Results revealed 60% - 80% mortality among irradiated rats pretreated with hydrogen peroxide, while 60% mortality was obtained in irradiated animals pretreated with distilled water. Deaths occurred earlier in the hydrogen peroxide than in the distilled water treated animals. Therefore, it was concluded that

hydrogen peroxide pretreatment did not afford protection against 775 R whole body ^{60}Co gamma irradiation under the conditions of this study.

BIBLIOGRAPHY

Aikman, A.A., and Wills, E.D. 1974a. Studies on lysosomes after irradiation. Rad. Res. 57:416-430.

Aikman, A.A., and Wills, E.D. 1974b. A quantitative histochemical method for the study of lysosomal membrane permeability and acid phosphatase activity. Rad. Res. 57:403-415.

Altman, K.L., Gerber, G.B., and Okada, S. 1970. Radiation Biochemistry. Vol. II. Academic Press. N.Y. and London. pp. 68-71.

Barratt, G.M., and Wills, E.D. 1979. The effect of hypothermia and radiation on lysosomal enzyme activity of mouse mammary tumors. Europ. J. Cancer. 15:243-250.

Bitensky, L. 1962. The demonstration of lysosomes by the controlled temperature freezing-sectioning method. Quart. J. Micr. Sci. 103:205-209.

Brendeford, M. 1971. Intracellular distribution of inorganic and organic mercury in rat liver after exposure to methylmercury salts. Biochem. Pharm. 20:1101-1107.

Brun, A., and Brunk, U. 1970. Histochemical indications for lysosomal localization of heavy metals in normal rat brain and liver. J. Histochem. Cytochem. 18:820-827.

Brun, A., Abdulla, M., Ihse, I., and Samuelsson, B. 1976. Uptake and localization of mercury in the brain of rats after prolonged oral feeding with mercuric chloride. Histochemistry. 47:23-29.

Cafruny, E.J. 1968. The site and mechanism of action of mercurial diuretics. Pharmacol. Rev. 20:89-112.

Chang, L.W., and Hartmann, H.A. 1972. Ultrastructural studies of the nervous system after mercury intoxication. I. Pathological changes in the nerve cell bodies. Acta. Neuropathol. 20:122-138.

Chang, L.W., and Reuhl, K.R. 1977. Ultra-structural study of the latent effects of methylmercury on the nervous system after prenatal exposure. Environmental Res. 13:171-185.

Christensen, E.I. 1976. Rapid protein uptake and digestion in proximal tubule lysosomes. Kidney Int. 10:301-306.

Christensen, E.I., and Maunsbach, A.B. 1972. Intralysosomal digestion of lysozyme in renal proximal tubule cells. Kedney Int. 6:396-401.

Clarkson, T.W. 1972. The biological properties and distribution of mercury. Proc. Biochem. Soc. 130:61-63.

Cristofalo, V.J., and Kabakjian, J. 1975. Lysosomal enzymes and aging in vitro: Subcellular enzyme distribution and effect of hydrocortisone on cell life-span. Mech. Aging Dev. 4:19-28.

Danpure, C.J., and Taylor, D.M. 1974. The effect of the internally deposited plutonium-239 on the lysosomes of rat liver. Rad. Res. 59:679-692.

De Duve, C. 1963. General properties of lysosomes. The lysosome concept. In Ciba foundation symposium on lysosomes, edited by deReuck, A.U.S., and Cameron, M.P. Little, Brown and Co., Boston, Mass.

De Duve, C. 1965. The separation and characterization of subcellular particles. In: The Harvey Lectures, Series 59. Academic Press, New York, pp. 49-87.

De Duve, C., and Baudhuin, P. 1966. Peroxisomes (microbodies and related particles). Physiol. Rev. 46:323-357.

De Duve, C. 1973. Biochemical studies on the occurrence, biogenesis and life history of mammalian peroxisomes. J. Histochem. Cytochem. 21:941-948.

Dingle, J.T., and Barrett, A.J. 1968. The uptake of biologically active substance by lysosomes, abstracted. Biochem. J., 109:198-211.

D'Itri, F.M. 1972. The environmental mercury problem. CRC Press, The Chemical Rubber Co., Cleveland, Ohio.

Earhart, J.M. 1975. A study of the interaction of co-insult treatment with methylmercuric chloride and X-irradiation and demonstration of a peroxide induced protective mechanism. (Dissertation) Biology Department, North Texas State Univ.

Ericsson, J.L.E. 1964. Absorption and decomposition of homologous hemoglobin in renal proximal tubular cells. Acta Path. et microbiol. Scand., Suppl. 168.

Ericsson, J.L.E., and Trump, B.F. 1965. Electron microscopic studies of the epithelium of the proximal tubule of rat kidney. Lab. Invest. 14:1422-1456.

Ericsson, J.L.E., and Trump, B.F. 1966. Electron microscopic studies of the epithelim of the proximal tubule of the rat kidney. Lab. Invest. 15:1610-1633.

Farahataziz. 1980. Personal communications.

Finch, C.E. 1972. Enzyme activities, gene function and aging in mammals (review), Exp. Gerontol. 7:53-67.

Fowler, B.A., Brown, H.W., Lucier, G.W., and Beard, M.E. 1974. Mercury uptake by renal lysosomes of rats injesting methyl mercury hydroxide: Ultrastructural observations and energy dispersive x-ray analysis. Arch. Pathol. 98:297-308.

Friberg, L., and Vostal, J. 1972. Mercury in the environment. CRC Press, The Chemical Rubber Co., Cleveland, Ohio.

Gahan, R.B. 1965. Histochemistry of lysosomes. In: International review of cytology, edited by Bourne, G.H. and Danielli, E.W. Academic Press, New York, Vol. 21.

Gansler, H., Rouiller, C., and Schweiz, A. 1956. Modifications physiol. et path. du chondriome. Etude au micro electronique. Allgen. Pathol. Bakteriol. 19:217-221.

Goldstein, A. 1965. Biostatistics, an introductory text. Macmillan, New York.

Goldstone, A., Szabo, E., and Koenig, H. 1970. Isolations and characterizations of acidic lipoprotein in renal and hepatic lysosomes. Life Sciences, 9:607-616.

Goto, S., Takano, T., Mizuno, D., Makano, T., and Imaizumi, K. 1969. Again and location of acid ribonuclease in liver of various animals. J. Gerontol. 24:305-308.

Gouiter, M., and Gouiter, R. 1962. Acid deoxyribonuclease and acid phosphatase activities in regenerating rat liver after whole-body X irradiation. Rad. Res., 16:728-734.

Goyer, R.A. 1968. The renal tubule in lead poisoning: I. Mitochondrial swelling and aminoaciduria. Lab. Invest. 19:71-77.

Haley, T.J., and Snider, R.S. 1962. Response of the nervous system to ionizing radiation: Proceeding of an international symposium held at Northwestern University Medical School, Chicago, Illinois, Sept. 7-9, 1960. Academic Press, New York.

Hand, A.R. 1973. Morphologic and cytochemical identification of peroxisomes in the rat parotid and other exocrine glands. J. Histochem. Cytochem. 21:131-141.

Hand, A.R. 1974. Peroxisomes (microbodies) in striated muscle cells. J. Histochem. Cytochem. 22:207-209.

Herzog, V.K., and Fahimi, H.D. 1974. Peroxisomes in mouse myocardium, biochemical identification. J. Cell Biol. 63:136A.

Herzog, V.K., and Fahimi, H.D. 1976. Identification of peroxisomes (microbodies) in mouse myocardium. J. Molec. Cell Cardiol. 8:271-281.

Hicks, L., and Fahimi, H.D. 1977. Peroxisomes (microbodies) in the myocardium of rodents and primates. Cell Tiss. Res. 175:467-481.

Hruban, Z., and Rechicigl, M. 1969. Microbodies and related particles: morphology, biochemistry, and physiology. Int. Rev. Cytol., Suppl. I. Academic Press, New York and London.

Hruban, Z., Vigil, E.L., Slesers, A., and Hopkins, E. 1972. Microbodies: Constituent organelles of animal cells. Lab. Invest. 27:184-191.

Humason, G.L. 1972. Animal tissue techniques. W.H. Freeman, San Francisco.

Hunter, D. 1943. Industrial toxicology. Quarterly of Medicine. 12:185-258.

Hupp, E.W., Day, H.D., Hardcastle, J., and Hines, J. 1974. Interaction between methylmercury and radiation effects on nervous systems. Interim progress report to environmental protection agency. TWU, Denton, Texas.

Hupp, E.W., Hardcastle, J., Hines, J., and Minnich, J. 1977. Interaction between methylmercury and radiation effects on nervous system. National Technical Information Service. Springfield, Va.

Itabashi, M., Mochizuki, Y., and Tsukada, H. 1977. Changes in peroxisomes in pre-neoplastic liver of rats induced by 3-methyl-4-dimethylamino azobenzene. Cancer Res. 37:1035-1043.

Katsuki, S., Hirai, S., and Terao, T. 1957. On the disease of central nervous system in Minamata District with unknown etiology, with special references to the clinical observation. Kumamoto Igakkai Zasshi. 31, Suppl. 23:110-121.

Klein, R., Herman, S.P., Bullock, B.C., and Talley, F. A. 1973. Early functional and pathological changes in rat kidney during methyl mercury intoxication. Arch. Pathol. 96:83-90.

Kocmiersha, D., and Rodzka, P. 1972. Investigations on post-radiation hydrolytic activity of isolated perfused liver. Strahlentherapie. 143:705-710.

Konig, J.F., and Klippel, R.A. 1970. The rat brain, a stereotaxic atlas of the forebrain and lower parts of the brain stem. R.E. Kreiger, Huntington, New York.

Madsen, K., Hansen, J.C., and Maunsbach, A.B. 1976. Demonstration of mercury and iron in kidney lysosomes. In Proceedings of the ninth congress of the Nordic society of cell biology, ed. by Bierring F., Odense University Press, Odense, Denmark. pp. 129-138.

Madsen, M.K., and Christensen, E.I. 1978. Effect of mercury on lysosomal protein digestion in the kidney proximal tubule. Lab. Invest. 38:165-174.

Maunsbach, A.B. 1973. Ultrastructure of the proximal tubule. In Handbook of Physiology, edited by Orloff, J., and Berliner, R.W. Sect. 8: Renal Physiology. Washington, D.C. American Physiological Society. pp. 31-46.

Mead, J.F. 1952. In "Autoxidation and Antioxidants" W.O. Lundberg, ed., Vol. I. Wiley (Interscience), New York. p. 299.

Mego, J.L., and Barnes, J. 1973. Inhibition of heterolysosome formation and function in mouse kidneys by injection of mercuric chloride. Biochem. Pharmacol. 22:373-379.

Meyer, O.T., and Dannenberg, A.M. 1970. Radiation injection and macrophage function. II. Effect of whole body radiation on the number of pulmonary aveolar macrophages and their levels of hydrolytic enzymes. J. Teticuloendothel. Soc. 7:79-90.

Miyakawa, T., and Deshimaru, M. 1969. Electron microscopical study of experimentally induced poisoning due to organic mercury compound. Mechanism of development of a morbid change. Acta Neuropath. 14:126-137.

Miyakawa, T., Deshimaru, M., Sumiyoshi, S., Teraoka, A., Udo, N., Mattori, E., and Tatetsu, S. 1970. Experimental organic mercury poisoning--pathological changes in peripheral nerves. Acta. Neuropath. 15:45-51.

Moody, D.E., and Reddy, J.K. 1976. Morphometric analysis of the ultra-structural changes in rat liver induced by the peroxisome proliferator SaH 42-347. J. Cell Biol. 71:768-780.

Noda, H. 1959. On the gomoriphil findings other than the neurosecretory system in the observation of the hypothalamo-hypophyseal system. Ganma. J. Med. Sci. 8:223-232.

Novikoff, A., and Shin, W. 1964. The endoplasmic reticulum in the Golgi zone and its relations to microbodies, Golgi apparatus, and autophagic vacuoles in rat liver cells. J. Microsc. (Paris). 3:198-209.

Novikoff, A.B., and Goldfischer, S. 1969. Visualization of peroxisomes and mitochondra with diaminobenzidine. J. Histochem. and Cytochem. 17:675-680.

Novikoff, A.B., and Novikoff, P.M. 1973. Microperoxisomes. J. Histochem. Cytochem. 21:963-966.

Overgaard, K., and Overgaard, J. 1972. Investigations on the possibility of a thermic tumor therapy. I. Short wave treatment of a transplanted isologous mouse mammary Carcinoma. Europ. J. Cancer. 8:65-69.

Pearse, A., and Everson, G. 1960. Histochemistry, theoretical and applied. Little, Brown and Co., Boston, Mass.

Rahman, Y.E. 1963. The effect of X-irradiation on the fragility of rat spleen lysosomes. Rad. Res. 20:741-750.

Reddy, J., and Krishnakantha, T. 1975. Hepatic peroxisome proliferation: Induction by two novel compounds structurally unrelated to clofibrate. Sci. 190:787-789.

Rene, A.A., Darden, J.H., and Parker, J.L. 1971. Radiation-induced ultra-structural and cytochemical changes in lysosomes. Lab. Invest. 25:230-239.

Reynold, E.S. 1962. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17:202-212.

Roth, J.S., and Hilton, S. 1968. The effect of whole-body X-irradiation on the distribution of acid deoxyribonuclease and B-galactosidase in subcellular fractions of rat spleen. Rad. Res. 19:42-49.

Rouiller, C., and Bernard, W. 1956. Microbodies and the problem of mitochondrial regeneration in liver cells. J. Biophys. Biochem. Cytol. 2, (Suppl. 25), 355.

Sahaphong, S., and Trump, B.F. 1971. Studies of subcellular injuries in isolate kidney tubule of the flounder. V. Effect of inhibiting sulfhydryl groups of plasma membrane with the organic mercurials PCMB (Parachloromercuribenzoate). Am. J. Path. 63:277-285.

Shibko, S., and Tappel, A.L. 1965. Rat-kidney lysosomes: Isolation and properties. Biochem. J. 95:731-737.

Shingo, A., Hideoh, K., Etsuko, H., and Hideo, S. 1979. Changes intracellular activities of lysosomal enzymes in tissues of rats during aging. Mechanisms of Aging and Development. 10:81-92.

Srebro, Z. 1969. A comparative and experimental study of the gomori positive glia. Folia Biologica. 17:177-192.

Srebro, Z. 1970. The ependyma, the cysteine-rich complex containing periventricular glia, and the subfornical organ in normal and X-irradiated rats and mice. Folia Biologica. 18(4):327-342.

Srebro, A.R., and Cichocki, T. 1971. A system of periventricular glia in brain characterized by large peroxisome-like organelles. Acta. Histochem. 41:108-114.

Srebro, A.R., Slebodzinski, A., and Szirmai, E. 1970. Disease in hypo, and hyperthyreotic rats. A gressologie II. Rad. Res. 56:343-356.

Staubli, W., Schweizer, J.S., and Weibel, E.R., 1977. The proliferative response of hepatic peroxisomes of neonatal rats to treatment with Su-13-437 (Nafenopin). J. Cell Biol. 74:665-689.

Sternlieb, I., and Goldfischer, S. 1976. Heavy metals and lysosomes. In lysosomes in biology and pathology, edited by Dingle, J.T., and Dean, R.T., Vol. 5, North-Holland Publishing Co., Amersterdam, p. 185.

Sternlieb, I., and Quintana, N. 1977. The peroxisomes of human hepatocytes. Laboratory Invest. 36:140.149.

Straus, W. 1964. Cytochemical observations on the relationship between lysosomes and phagosomes in kidney and liver by combined staining for acid phosphatase and intravenously injected horse-radish peroxidase. J. Cell Biol. 20:497-508.

Stuve, J., and Galle, P. 1970. Role of mitochondria in the handling of gold by the kidney: A study by electron microscopy and electron-probe micronalysis. J. Cell Biol. 44:667-676.

Webb, J.L. 1966. In Enzyme and metabolic inhibitors. Edited by Webb, J.L. Vol 2. Academic Press, New York. p. 729.

Wilson, P. 1973. Enzyme changes in aging mammals (review). Gerontologia, 19:79-125.

Wills, E.D. 1966. Renal tubular lesions caused by mercuric chloride: Electron microscopic observations. Am. J. Pathol. 52:1225.

Wills, E.D., and Wilkinson, A.E. 1966. Release of enzymes from lysosomes by irradiation and the relations of lipid peroxide formation to enzyme release. Biochem. J. 99:657-669.