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THE EFFECT OF PHENYL *TERT*-BUTYL NITRONE (PBN) ON CCl_4-INDUCED RAT LIVER INJURY DETECTED BY PROTON MAGNETIC RESONANCE IMAGING (MRI) *IN VIVO* AND ELECTRON MICROSCOPY (EM)

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Acute intoxication by CCl_4 induces morphological changes in rat liver which are readily detectable by ^{1}H-NMR imaging techniques *in situ*. Two to four hours after the administration of CCl_4, regions of high proton signal intensity are observed in the centrilobular region of the liver. The regions of high signal intensity are attributed to the formation of local edema as a result of CCl_4-induced damage. Electron microscopy of the high intensity regions of CCl_4 treated liver sections revealed characteristic subcellular changes which include the disappearance of ribosomes from the rough endoplasmic reticulum (RER), the fragmented appearance of the smooth endoplasmic reticulum (SER), formation of vacuoles in the cisternae and swelling of the mitochondria. Pretreatment of rats with α-phenyl-*tert*-butyl nitrone (PBN), a free radical spin trap, prior to halocarbon exposure, was found to reduce the halocarbon-induced edema in the liver. Electron microscopy of the PBN pretreated CCl_4 exposed rat liver sections revealed no observable changes in subcellular organization when compared to controls.

KEY WORDS: CCl_4, free radicals, rat liver, spin trapping, PBN, MRI, EM.

ABBREVIATIONS: PBN = α-phenyl *tert*-butyl nitrone; MRI = magnetic resonance imaging; EM = electron microscopy.

INTRODUCTION

The free radical metabolism of carbon tetrachloride (CCl_4) in rat liver or in rat liver microsomal preparations has become one of the most intensely studied xenobiotic systems from the point of developing the *in vitro* and *in vivo* spin trapping technique. The first radical detected[1] and positively identified[2] was the trichloromethyl radical. Later it was clearly established that oxy- and carbon-centered radicals[3,4] were also formed and a partial structure for the latter has been published.[5] Depending on the conditions chosen for the detection of the spin adducts[6] the carbon dioxide radical ion is also formed in substantial quantities.[7] For most of these studies α-phenyl-*tert*-butyl nitrone (PBN) has been used but 5,5-dimethylpyrroline-N-oxide (DMPO) can also be effective.[8] Since mass spectrometric methods have been shown to be suitable for determining the structure of certain PBN spin adducts,[9] experiments are presently underway to assign the structure of oxy- and carbon-centered radical adducts found in CCl_4 metabolism in the presence of PBN.[10] It is anticipated that as better spin traps are developed a more detailed picture of this xenobiotic system will evolve.

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It has been our objective to investigate other methods for the study of *in vivo* free radical events. Since ESR spectroscopy of whole animals is still beyond the reach of the experimentalist we have turned to NMR imaging and *in vivo* NMR spectroscopy. The postulate is that if an effect can be found as a result of CCl_4(or any xenobiotic) on rat liver (or any part of any animal) which is altered by spin traps or other free radical "inhibitors" an indirect method could be developed to study such systems *in vivo* non-invasively.

Recently, we have used ^1H magnetic resonance imaging (MRI) in conjunction with ^1H localized NMR spectroscopy to observe the formation of local edema in the centrilobular region of rat liver following acute exposure to CCl_4.[11] This effect was also detected with the use of ^{23}Na MRI.[12] In addition, we have used *in vivo* ^{31}P NMR spectroscopy to determine that acute intoxication by CCl_4 causes an intracellular acidosis from pH 7.29 to 7.05 (20 hr. after exposure), as well as, increases the amounts of phosphomonoesters (PME), particularly phosphocholine.[13]

MATERIALS AND METHODS

(1) Magnetic resonance imaging

Wistar rats (200–250 g) were administered two doses of CCl_4 one hour apart (160 μl/kg body weight, with 5% Emulphor in 0.85% saline) via i.p. injection and subsequently anaesthetized with sodium pentobarbital (Somnotol; 1 ml/2.27 kg body weight; i.p. injection). PBN (25 mg/200 g rat in a 0.07 M pH 7.4 phosphate buffer and corn oil) was administered i.p. 30 min prior to the administration of CCl_4 as above.

NMR measurements were made with the use of a SIS 2.0 T/31 cm bore imaging spectrometer (Spectroscopy Imaging Systems Corp., Fremont, Calif., USA).

The anaesthetized rats were placed on a respiratory gating device. A balloon stretched over a piece of curved plastic moulding was placed under the rat. The inspiration of the rat caused an increase in pressure within the balloon which was transferred via an increase in air pressure within a length of tubing to a pressure transducer. This increase in pressure during inspiration was used to trigger acquisition of phase-encoding steps in the imaging sequence. Multiple slices were taken in the transverse plane using a T_E of 20 ms. The image slices were 4.7 mm thick, field of view was 8 × 8 cm with 256 phase-encoding steps, 2 acquisitions per step, and 512 frequency encoding points.

Electron microscopy

Rats were euthanized with an overdose of sodium pentobarbital (i.p.). The livers were perfused via the hepatic protal vein with a universal electron microscopy fixative solution consisting of 2.5% glutaraldehyde and 2.5% paraformaldehyde in an isotonic phosphate buffer (pH 7.2). Appropriate small sections were removed and immersed in the above fixative. The tissue fragments were subsequently post-fixed with 2.0% OsO_4 in phosphate buffer and embedded in Epon. Sections (1 μm thick) were cut with a Reichert ultramicrotome OMU3 followed by Toluidine blue staining. Ultrathin sections were cut from the areas selected by light microscopic examination and mounted on copper grids. These sections were contrasted with alcoholic uranyl acetate and lead citrate and subsequently examined on a JEOL 100-S transmission electron microscope.

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RESULTS AND DISCUSSION

Transverse ^1H images, through the liver, taken 3–4 hours after CCl_4 administration, indicated localized regions of the liver with dramatically increased proton signal intensities (Figure 1b) compared to a control rat (Figure 1a). This region of the liver corresponds to the centrilobular location where the hepatic portal vein initally enters the liver. $BrCCl_3$ (bromotrichloromethane), a more potent hepatotoxin, was found to produce a more dramatic localized high proton intensity region 1–2 hours after administration (Figure 1c). The increased localized proton signal intensities for both CCl_4 and $BrCCl_3$ treated rat livers was observed on numerous trials (n = 5 for the CCl_4 studies, and n = 3 for the $BrCCl_3$ studies). It was determined from previous experiments[11] with the use of localized proton spectroscopy in the regions of apparent tissue damage that the increase in observed proton signal intensity in the images after halocarbon administration is due to a change in the water proton resonance and not a change in the fatty acid methylene proton resonance. The increased water proton signal intensity was found to depend on both a larger water T_2 (transverse relaxation time constant) as well as an increase in the concentration of water in comparison to controls. The values of T_2 for water in a given tissue are strongly dependent on the amount of 'bound' water present.[14] Therefore the increase in water T_2 and concentration in the high signal intensity regions of the image, are likely due to local edema as a result of the halocarbon-induced tissue damage. Areas of the liver with no apparent edema indicated no change in water T_2 when compared to a control liver.

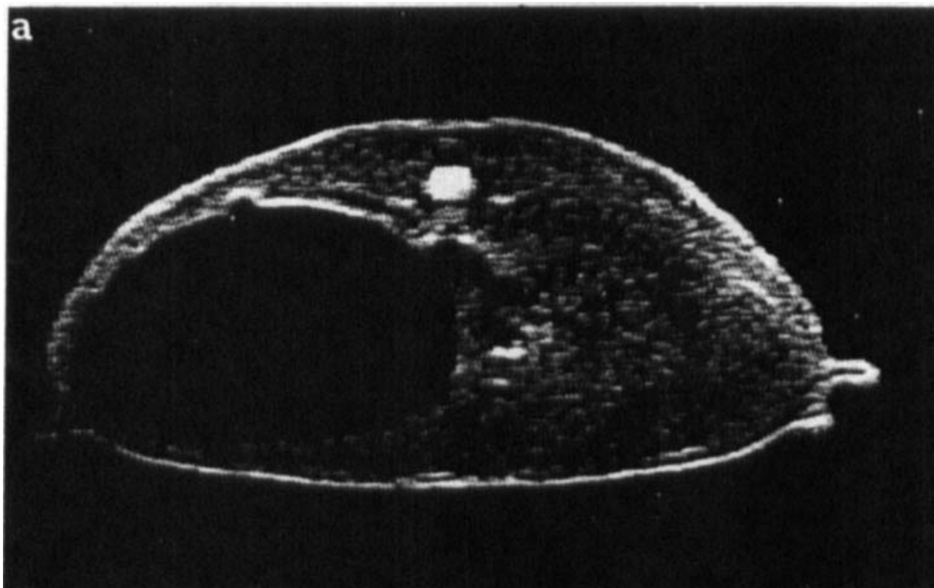

FIGURE 1 (a) Transverse proton magnetic resonance image slice through the liver of a normal rat *in vivo* using respiratory gating. The stomach is clearly visible as a region of lower intensity in the upper left part of the image and the spinal cord is a bright region near the top of the image. The liver is the relatively homogenous region below the spinal cord and to the right of the stomach. At the extreme left and right positions of the image are folds of skin as a result of taping down to immobilize the animal.

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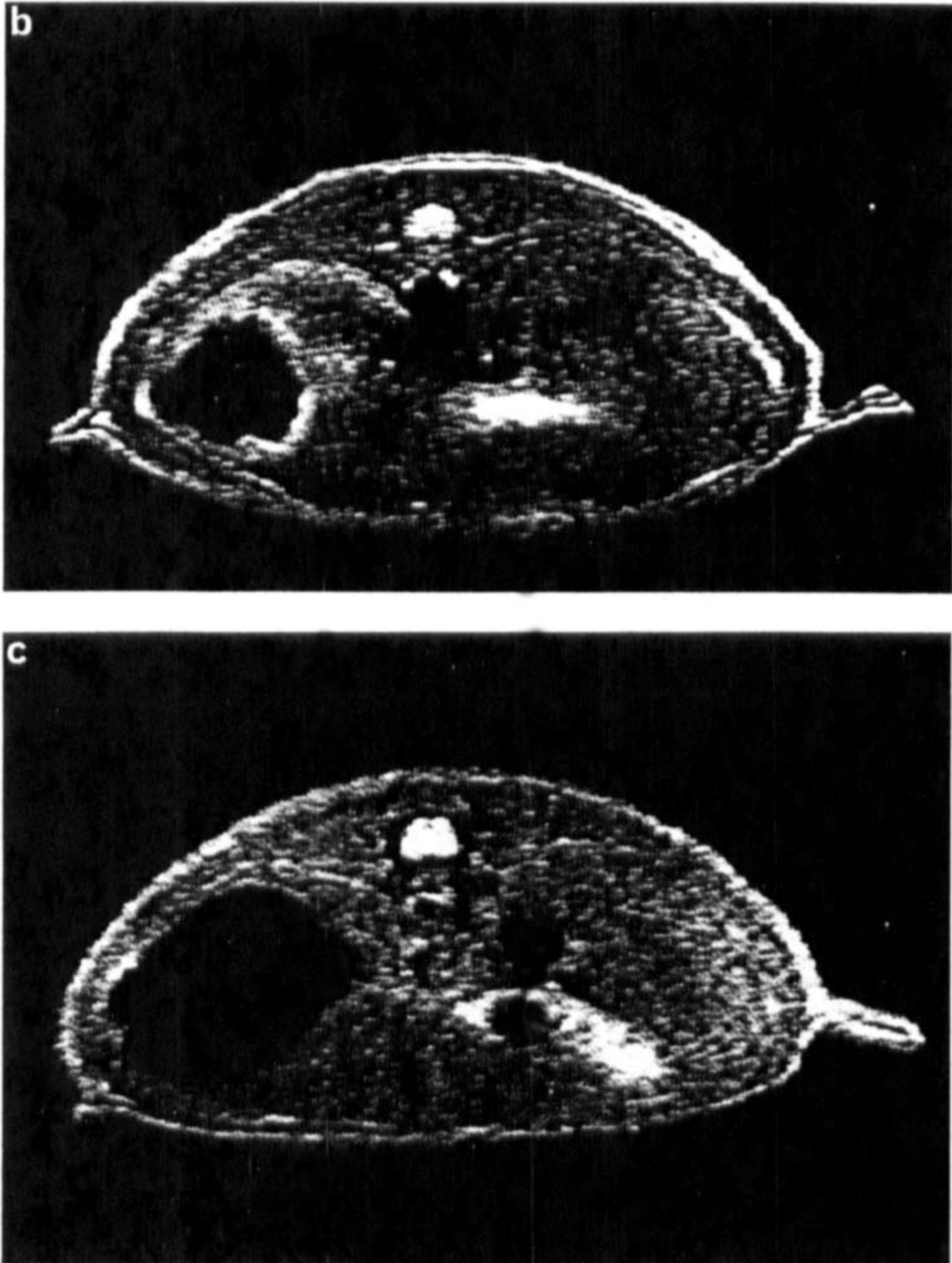

FIGURE 1 (b) An analogous image to that in Figure 1a was obtained 3–4 hours after CCl_4 treatment. Note the area of high signal intensity in the liver to the right of the stomach. (c) Transverse slice through the liver 2 hours after $BrCCl_3$ treatment. Note the area of high signal intensity to the right of the stomach surrounding the hepatic portal vein.

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Electron microscopy of liver sections of halocarbon treated rats taken from the regions of high proton signal intensities in the images indicated numerous characteristic subcellular changes which have been reported previously by various investigators.[15,16,17] These changes include the formation of vacuoles in the cisternae, the disappearance of ribosomes from the RER, the fragmented appearance of the SER (normally in parallel arrays closely oriented to the mitochondria), swelling and degranulation of the mitochondria, and a loss of the normally present dense particles in the Golgi apparatus (as shown in Figure 2). Figure 2c shows a sectioned portion of the apparently damaged edematous area of the liver from a rat exposed to $BrCCl_3$ a substantially more potent hepatotoxin than CCl_4 indicating the most extreme case. A substantial increase in vacuole formation is observed which may explain the increase in 'free' water or edema that is detected in the MRI images of halocarbon treated animals. The subcellular damage observed from CCl_4 exposure (Figure 2b) is not as dramatic as that seen for the $BrCCl_3$ treated rat liver section, but does demonstrate quite significant ultrastructural disorganization in comparison to the control (Figure 2a). EM of a non-edematous region of a halocarbon-treated rat liver showed no significant changes in comparison to those of control rat liver sections (results not shown).

Pre-administration of PBN 30 min prior to the administration of CCl_4 resulted in a substantial decrease in signal intensity of the halocarbon induced region of the liver

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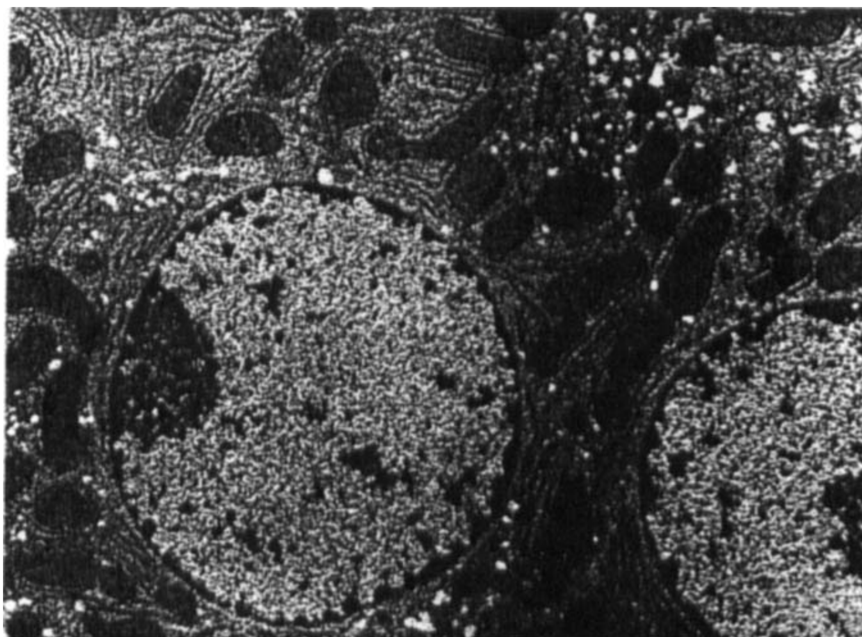

FIGURE 2 (a) Electron micrograph (× 5,000 magnification) depicting hepatocytes from a control rat. Cytoplasm of the hepatocytes includes slender mitochondria, stacks of granular endoplasmic reticulum, some smooth endoplasmic reticulum and peribiliary distribution of lysosomes.

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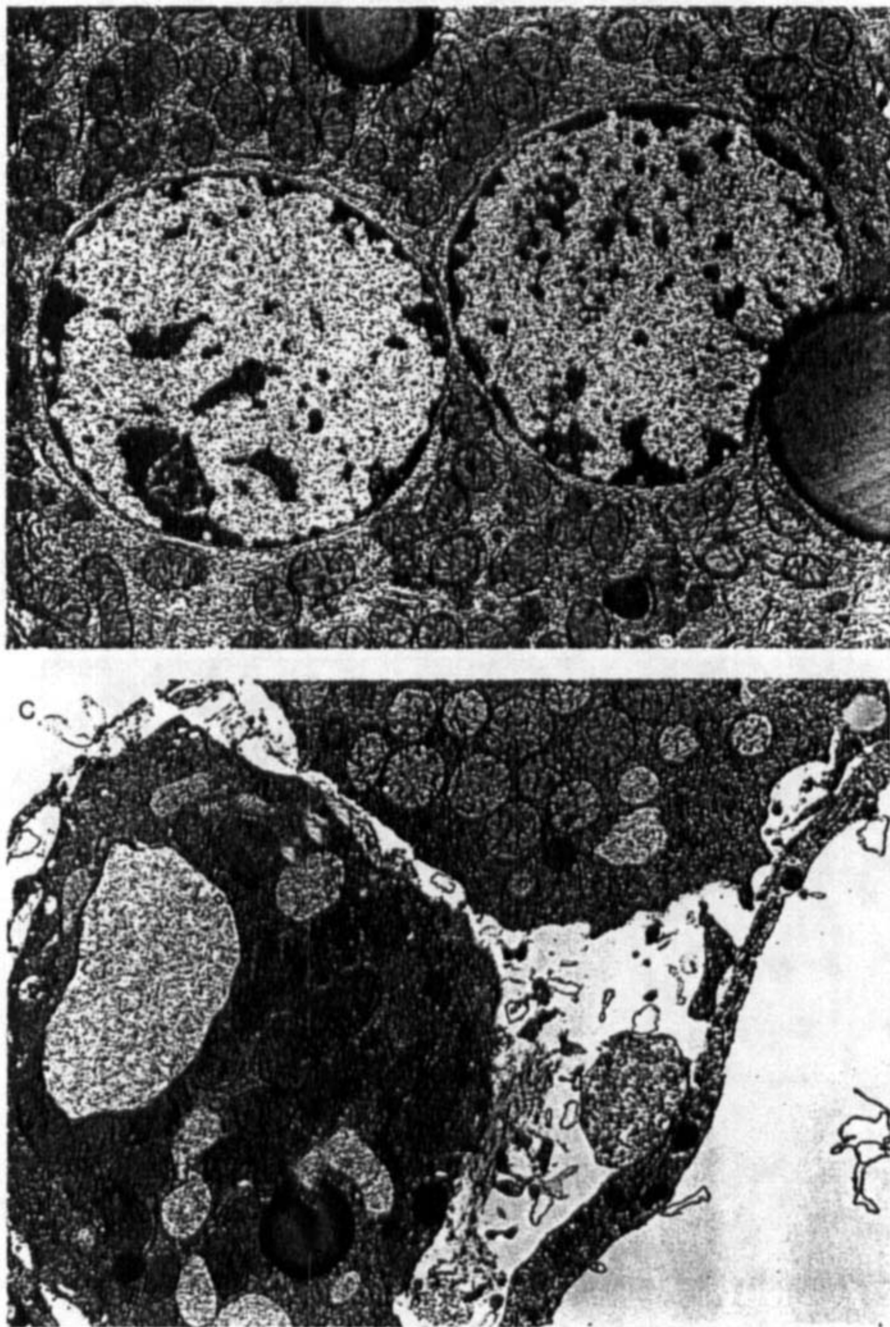

FIGURE 2 (b) Electron micrograph (× 5,000) of hepatocytes from CCl_4 treated rats indicating apparent swollen mitochondria and the appearance of some lipid droplets in the cytoplasm. (c) An electron micrograph (× 5,000) of a section of a hepatic plate from a $BrCCl_3$ treated rat showing an accentuated perisinusoidal space, portions of hepatocytes containing vesicles of various sizes and swollen mitochondria.

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as observed in Figure 1 (see Figure 3b). In both the ^{1}H-NMR transverse image and the electron micrograph of a liver section from a PBN pretreated CCl_4 exposed rat (a similar region to that of the damaged area of a halocarbon treated rat), there are no discernable morphological changes as compared to those observed for the control rat (see Figure 3a and b for results from ^{1}H-NMR imaging and Figure 4a and b for EM studies). Since PBN is a trap for free radical species, this result demonstrates that the high intensity or edematous regions of the livers of rats treated with halocarbons (as detected by ^{1}H-NMR imaging) are possibly related to the damage initiated as a result of the formation of free radical intermediates from the metabolism of these compounds in the liver. The prevention of halocarbon-induced edema formation (as observed by MRI) by pretreatment with PBN 30 min prior to halocarbon exposure has been observed on numerous occurences ($n = 4$).

It should be noted that PBN when administered alone in high concentrations (> 28 mM) also produces a region of high proton signal intensity in the liver (Figure 3c). Electron microscopy of this high intensity region indicates some ultrastructural changes, particularly an alteration in the cytoplasmic matrix depicted as a decrease in density (rarefaction). These results are in accordance with a study by Slater *et al.* (1986) on the adverse effects of spin traps on isolated rat hepatocytes, which indicated that concentrations of PBN greater than 25 mM moderately affected liver cell integrity resulting in a slightly reduced cell viability.[18] More studies are required to further understand the effect of high concentrations of PBN in the liver.

In summary, these studies demonstrate the sensitivity and advantage of using NMR imaging *in vivo* noninvasively in studying the response of biological systems to a

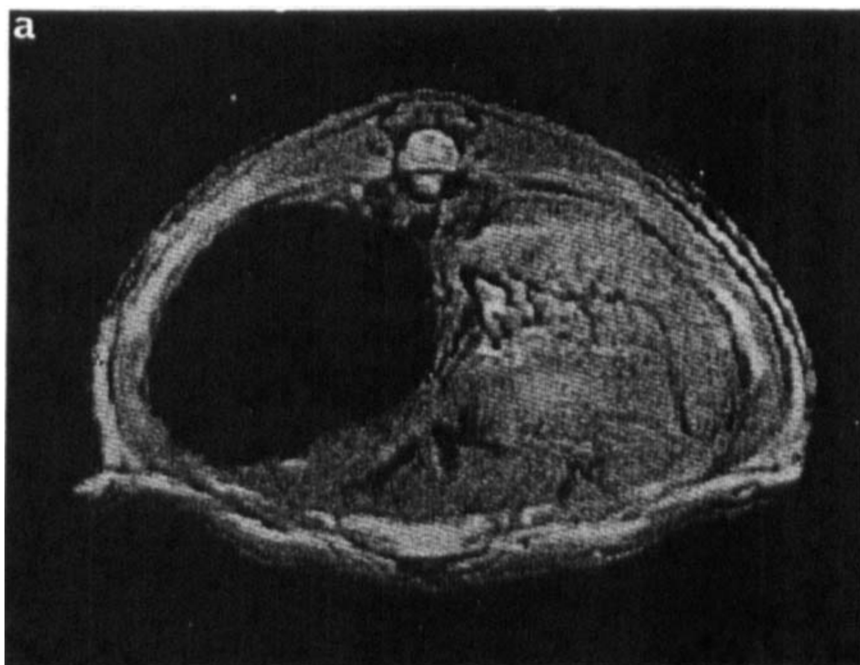

FIGURE 3 (a) Normal rat liver transverse proton magnetic resonance image as described in Figure 1a.

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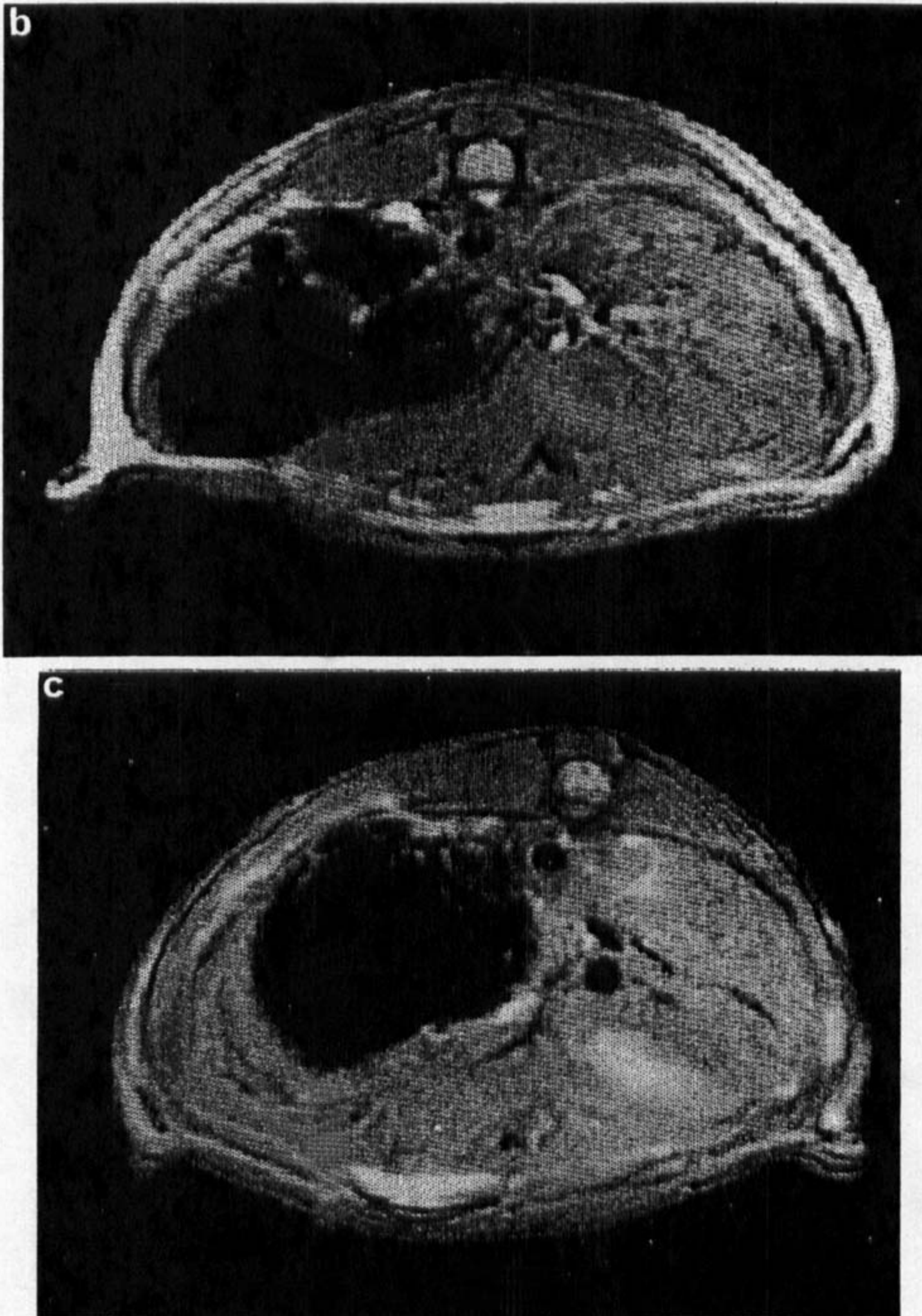

FIGURE 3 (b) Transverse image slice through a rat liver 2 hours after PBN (25 mg/200 g rat) and CCl_4 treatment. Note that after PBN treatment there is no region of high intensity as observed in Figure 1b. (c) Transverse slice through the liver 2 hours after with PBN (50 mg/200 g rat) alone. Note that if PBN is administered alone there is a slight region of high intensity present.

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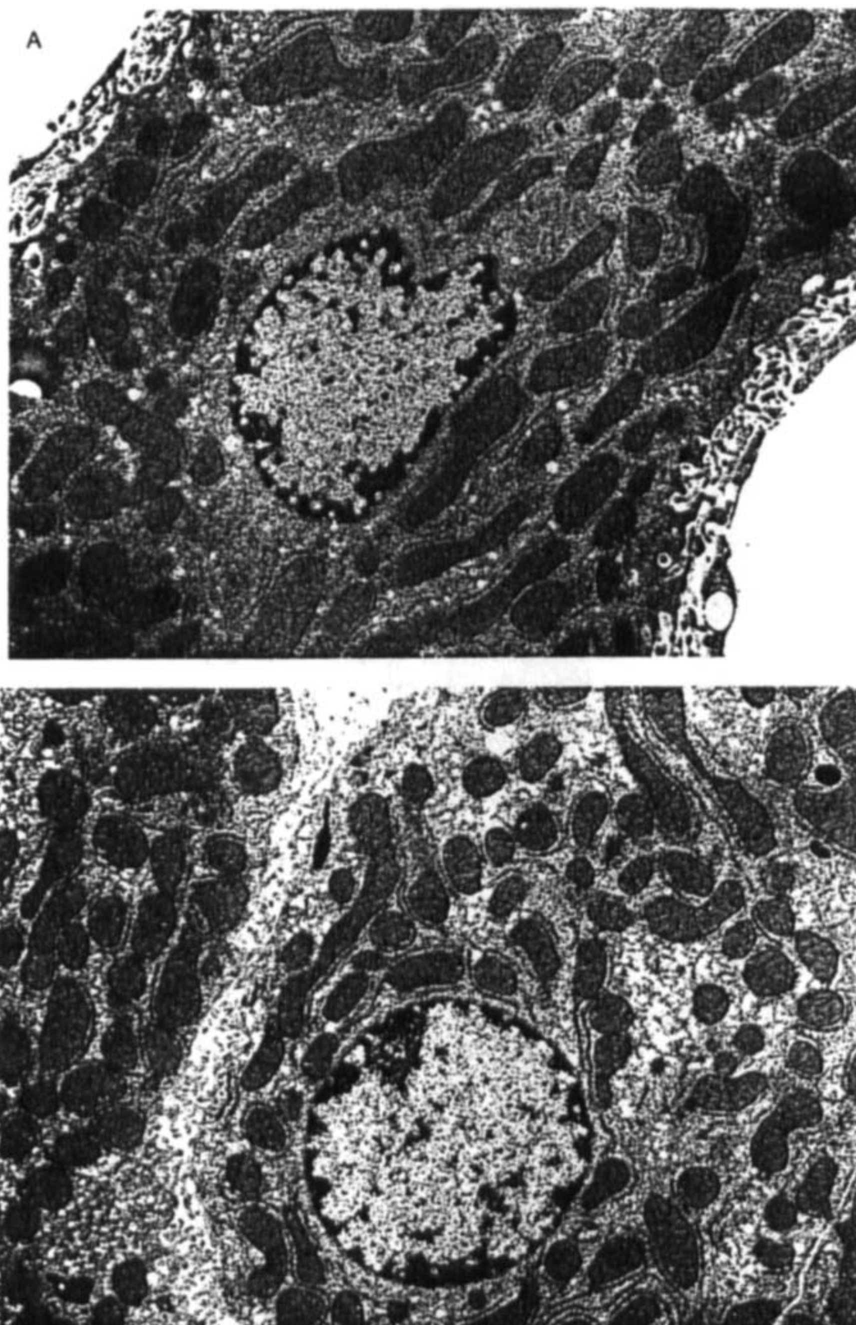

FIGURE 4 (a) Electron micrograph (× 5,000) depicting sections of hepatocytes from a control rat as described in Figure 2a. (b) Electron micrograph (× 5,000) of hepatocytes from CCl_4 treated rats that have been pretreated with PBN. Normal appearance of the hepatocytes is observed. Mitochondria are slender and their matrix shows medium electron density.

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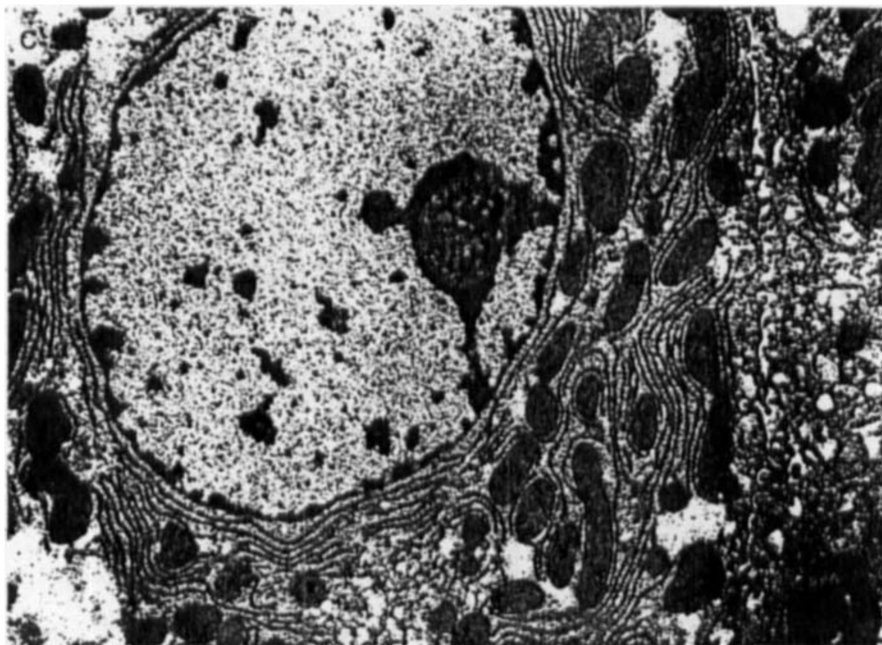

FIGURE 4 (c) Electron micrograph (× 5,000) of hepatocytes from PBN (50 ng) treated rats depicting normal appearance of the mitochondria and stacks of granular endoplasmic reticulum. Note that there is a mild alteration of the cytoplasmic matrix (rarefaction) observed.

toxicological challenge while at the same time monitoring an alteration of the response with appropriate free radical inhibitors.

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