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# Mechanism of *p*-Hydroxybenzoate Ester-induced Mitochondrial Dysfunction and Cytotoxicity in Isolated Rat Hepatocytes

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**ABSTRACT.** The relationship between the metabolism and the cytotoxic effects of the alkyl esters of *p*-hydroxybenzoic acid (parabens) has been studied in freshly isolated rat hepatocytes. Incubation of hepatocytes with propyl-paraben (0.5 to 2.0 mM) elicited a concentration- and time-dependent cell death that was enhanced when enzymatic hydrolysis of propyl-paraben to *p*-hydroxybenzoic acid was inhibited by a carboxylesterase inhibitor, diazinon. The cytotoxicity was accompanied by losses of cellular ATP, total adenine nucleotide pools, and reduced glutathione, independently of lipid peroxidation and protein thiol oxidation. In the comparative toxic effects based on cell viability, ATP level, and rhodamine 123 retention, butyl- and isobutyl-parabens were more toxic than propyl- and isopropyl-parabens, and ethyl- and methyl-parabens and *p*-hydroxybenzoic acid were less toxic than propyl-paraben. The addition of propyl-paraben to isolated hepatic mitochondria reduced state 3 respiration with $NAD^+$-linked substrates (pyruvate plus malate) and/or with an FAD-linked substrate (succinate plus rotenone), whereas state 3 respiration with ascorbate plus tetramethyl-*p*-phenylenediamine (cytochrome oxidase-linked respiration) was not affected significantly by propyl-paraben. Further, the addition of these parabens caused a concentration-dependent increase in the rate of state 4 oxygen consumption, indicating an uncoupling effect. The rate of state 3 oxygen consumption was inhibited by propyl-paraben, butyl-paraben, and their chain isomers. These results indicate that a) propyl-paraben-induced cytotoxicity is mediated by the parent compound rather than by its metabolite *p*-hydroxybenzoic acid; b) the toxicity is associated with ATP depletion via impairment of mitochondrial function related to membrane potential and/or oxidative phosphorylation; and c) the toxic potency of parabens to hepatocytes or mitochondria depends on the relative elongation of alkyl side-chains esterified to the carboxyl group of *p*-hydroxybenzoic acid. BIOCHEM PHARMACOL **55**;11:1907–1914, 1998. © 1998 Elsevier Science Inc.

**KEY WORDS.** *p*-hydroxybenzoate esters; parabens; mitochondrial dysfunction; cytotoxicity; rat hepatocytes; antimicrobial preservative

Alkyl esters of *p*-hydroxybenzoic acid, which are commonly called parabens§, are used widely as antimicrobial preservatives in pharmaceuticals, cosmetic products, processed foods, and beverages. Because of their widespread use, the potential toxicity of parabens has been investigated *in vivo* and *in vitro* to assess various toxicological properties, i.e. acute and chronic toxicity [1–3], carcinogenicity [4, 5], mutagenicity [6], teratogenicity [7], cytogenetic effects [8], and irritation and sensitization [3, 9]. Despite their presumed low toxicity, parabens exert a variety of effects on tissues and cell functions. It has been reported that parabens (a) inhibit nucleic acid synthesis via inhibition of many enzyme systems in microorganisms and embryonic mouse fibroblasts [10, 11], (b) cause an acute myocardial depression accompanied by hypotension in dogs [1], (c) are potent inhibitors of ciliary activity in cultures of ferret tracheal rings [12], (d) induce hemolysis in rabbit and human erythrocytes [13], and (e) act as modulators for both voltage- and ligand-gated channels in peripheral neuronal cells [14].

The metabolism and disposition of parabens have been studied extensively in a wide variety of animal species including humans [3]. In general, parabens after oral administration in rats are quickly absorbed from the gastrointestinal tract and are easily hydrolyzed to *p*-hydroxybenzoic acid by esterases in organs [15]. It is known that *p*-hydroxybenzoic acid itself is an intermediate in the metabolic sequences of ubiquinones, tyrosine, and other cellular components *in vivo*. Using *in vitro* assays, it has been

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§ *Abbreviations:* diazinon, phosphorothioic acid *O*,*O*-diethyl-*O*-[6-methyl-2-(1-methylethyl)-4-pyrimidinyl]ester; Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid); MDA, malondialdehyde; parabens, alkyl esters of *p*-hydroxybenzoic acid; and TMPD, tetramethyl-*p*-phenylenediamine.

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found that esterases in the liver and kidneys are extremely efficient in hydrolyzing parabens [16]. Consequently, *p*-hydroxybenzoic acid derived from parabens is mainly conjugated as glucuronides, sulfate, and hippuric acid, which are excreted rapidly in the urine [15, 17]. Despite the known information about the metabolic pathway of parabens, extensive studies have not investigated the relationship between the metabolism and the cytotoxic effects of parabens. The isolated cell system serves as a very useful system to address the issues of intracellular target sites and temporal sequences leading to cell damage caused by chemicals and drugs. In the present study, we investigated the action of parabens on freshly isolated rat hepatocytes and isolated rat mitochondria, and we will discuss the mechanisms of the toxic effects of parabens.

## MATERIALS AND METHODS

### *Materials*

The chemical compounds used were obtained from the following companies: *p*-hydroxybenzoic acid and its esters (parabens): methyl-, ethyl, propyl-, isopropyl-, butyl-, and isobutyl-*p*-hydroxybenzoates (purities of >99%) from the Tokyo Kasei Co.; GSH, adenine nucleotides, rhodamine 123, and bovine serum albumin from the Sigma Chemical Co.; and diazinon (purity of >99%) and collagenase from Wako Pure Chemicals. All other chemicals were of the highest purity commercially available.

### *Isolation and Incubation of Hepatocytes*

Male F344/DuCrj (240–260 g) rats were obtained from Charles River Japan Inc. and were housed in wire-bottom cages. The rats were allowed food (CE-2, Clea Japan Inc.) and water *ad lib.* before hepatocytes were prepared. The hepatocytes were isolated by collagenase perfusion of the liver, as described previously [18]. Hepatocyte viability was assessed by Trypan Blue exclusion, and initial cell viability in each experiment was more than 85%.

Hepatocytes ($10^6$ cells, approximately 2.2 mg protein/mL) were suspended in Krebs–Henseleit buffer, pH 7.4, containing 12.5 mM of HEPES and 0.1% albumin. All incubations were performed in rotating, round-bottomed flasks at 37°, under a constant flow of humidified carbogen (95% $O_2$/5% $CO_2$). Reactions were started by the addition of parabens dissolved in DMSO (final concentration, <1%). Corresponding control groups received an equivalent volume of DMSO. In some experiments using diazinon, the compound (100 μM) dissolved in DMSO was added to the hepatocyte suspension 15 min before the addition of propyl-*p*-hydroxybenzoate (propyl-paraben). Aliquots of incubation mixture were taken at intervals for the determination of cell death and the concentrations of intracellular adenine nucleotides, GSH, protein thiols, protein, MDA, parabens and *p*-hydroxybenzoic acid.

### *Preparation of Liver Mitochondria and Measurement of Respiration Rates of Mitochondria*

Liver mitochondria were isolated from male F344/DuCrj rats by differential centrifugation in medium containing 0.25 M of sucrose, 5 mM of Tris–HCl, pH 7.4, and 1 mM of EDTA [19]. EDTA was omitted for the final wash and resuspension. The rate of oxygen consumption was measured polarographically with a Clark-type oxygen electrode (model 5300; Yellow Springs Instrument) at 25° in the presence (state 3) and after exhaustion (state 4) of 50 μM of ADP [19]. Respiration buffer (3 mL, pH 7.4) contained 0.2 M of sucrose, 20 mM of KCl, 3 mM of $MgCl_2$, and 5 mM of potassium phosphate. The respiration substrates were 5 mM of pyruvate plus 0.5 mM of malate, 5 mM of succinate containing 1 μM of rotenone for inhibition of $NAD^+$-linked oxidation, or 1 mM of ascorbate plus 50 μM of TMPD containing 1 μM of rotenone/50 nM of antimycin A for inhibition of electron transport at complex III (cytochrome *c* reductase), and the amount of mitochondria was 1 mg of protein/mL. The respiratory control index was calculated as the ratio of state 3/state 4 respiration.

### *Determination of Mitochondrial Membrane Potential*

Mitochondrial membrane potential ($\Delta\Psi$) in hepatocytes was determined with rhodamine 123, a fluorescence probe, which selectively distributes into mitochondria with an intact membrane potential and is retained in the mitochondria [20]. Hepatocytes were incubated with 1 μM of rhodamine 123 at 37° for 10 min before the addition of propyl-paraben or other parabens at a concentration of 2 mM. After 60 min, aliquots of incubation mixture were centrifuged at 50 *g* for 3 min to remove the supernatant. The hepatocyte pellets were washed with Krebs–Henseleit buffer and then centrifuged again. The pellets obtained were resuspended in Krebs–Henseleit buffer containing 0.1% Triton X-100. After 10 min, the samples were centrifuged at 1600 *g* for 5 min to sediment any cellular debris. The rhodamine 123 concentration in the supernatant was measured in a CytoFluor 4000 fluorescence plate reader (PerSeptive Biosystems) with filters set for 485-nm excitation and 530-nm emission. The results are expressed as percentages of the fluorescence values for the control (untreated) hepatocytes.

### *Biochemical Assays*

Adenine nucleotides (ATP, ADP, and AMP) in hepatocytes were measured using HPLC, according to the procedure of Jones [21]. Cellular GSH levels were determined by HPLC essentially as described by Reed *et al.* [22]. Reduced protein thiol concentrations were determined using Ellman's reagent, as described previously [23]. Protein was determined by the method of Lowry *et al.* [24], using bovine serum albumin as the standard. MDA was measured as thiobarbituric acid-reactive products, as described previ-

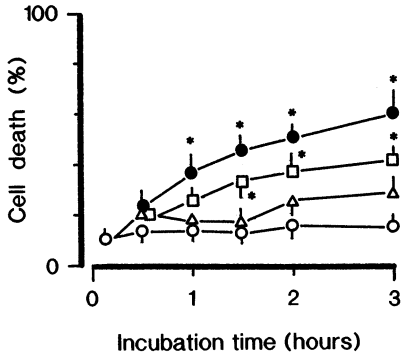


**FIG. 1. Effects of propyl-paraben on cell death in isolated rat hepatocytes. Hepatocytes were incubated at $10^6$ cells/mL in Krebs–Henseleit buffer, pH 7.4, with no addition (○), or with 0.5 mM (△), 1.0 mM (□), and 2 mM (●) propyl-paraben, as described in Materials and Methods. Results are expressed as the means ± SEM of three separate experiments. An asterisk (*) indicates a significant difference from values for untreated hepatocytes ($P < 0.05$).**

ously [25]. Cell death of hepatocytes was assessed by Trypan Blue (0.16%, w/v) uptake under a light microscope. Blebbing of hepatocytes was expressed as the percentage of Trypan Blue-excluding cells that exhibited multiple surface protrusions.

### *Determination of Parabens and p-Hydroxybenzoic Acid by HPLC*

An equal volume of chilled methanol was added to the cell suspension, and then the mixture was filtered through a membrane cartridge (pore size, 0.45 μM). The eluent was injected onto an analytical TSKgel ODS-120T column (4.6-mm i.d. × 250 mm, 5-μm particle size; Tosoh Co.) equipped with a UV absorbance detector (260 nm). The mobile phase was methanol/0.1 M of ammonium dihydrogen phosphate (50:50, by volume, pH 5.3), and the flow rate was 1.0 mL/min. *p*-Hydroxybenzoic acid was identified by co-chromatography or by comparison of its HPLC retention time with the authentic compound. The recoveries for parabens and *p*-hydroxybenzoic acid extracted from hepatocyte suspensions were more than 85%.

### *Statistical Analysis*

Statistically significant differences among several treatment groups were observed using ANOVA followed by Dunnett's *t*-test.

## RESULTS

### *Toxic Effects of Parabens on Rat Hepatocytes*

Incubation of rat hepatocytes with propyl-paraben (0.5 to 2.0 mM) caused a concentration- and time-dependent cell killing (Fig. 1). To elucidate the mechanism of this toxicity, effects on several parameters related to hepatocyte viability were investigated during a 3-hr exposure to the compound.

Moreover, to determine whether the paraben itself or a product of its hydrolytic cleavage was the active principle, experiments were carried out in the presence and absence of diazinon, an inhibitor of esterases. Treatment with 1 mM of propyl-paraben alone caused a decrease in the level of ATP (Fig. 2C) and the total content of adenine nucleotides (Fig. 2D) after 1 to 1.5 hr; significant cell death was seen after 1.5 hr (Fig. 2A). In a preliminary experiment, propyl-paraben did not react with ATP in the absence of hepatocytes. However, there was no surface blebbing (Fig. 2B) and no change in either the content of GSH (Fig. 2E), protein thiols (Fig. 2F), or the mitochondrial membrane potential (data not shown) even after 3 hr of incubation.

Diazinon (100 μM) on its own had no effect on any of the parameters measured (Fig. 2A–F). Exposure of hepatocytes to both propyl-paraben (1 mM) and diazinon (100 μM) enhanced and accelerated cell killing as well as the loss of cellular ATP and total adenine nucleotides. The abrupt depletion of ATP levels resulted in transient concomitant increases in levels of ADP and AMP. At 60 min, the levels of the three nucleotides were, respectively, 1.0, 7.0, and 13 nmol/$10^6$ cells; these values declined steadily thereafter. There was detectable surface membrane blebbing at the early time points (0.5 and 1 hr; Fig. 2B) and a gradual loss of GSH (Fig. 2E) beginning at 0.5 hr. The mitochondrial membrane potential was reduced to approximately 65% of control after 1 hr. However, there was no change in the protein thiol content of cells even at the end of the incubation. MDA levels were not affected by any of the treatments. After 3 hr the values obtained were (in nmol/$10^6$ cells): control, 0.42 ± 0.07; diazinon, 0.44 ± 0.07; propyl-paraben, 0.43 ± 0.06; and diazinon plus propyl-paraben, 0.41 ± 0.06.

The increased effectiveness of propyl-paraben in the presence of esterase inhibitors has suggested that the compound itself rather than products of its hydrolysis was responsible for the observed toxicity. To confirm this suggestion the concentration of *p*-hydroxybenzoic acid was measured simultaneously with that of the parent molecule in hepatocyte suspensions. It can be seen (Fig. 3) that in the absence of diazinon almost all of the paraben added to the incubation was hydrolyzed within 0.5 hr, and this was accompanied by the appearance of a corresponding amount of *p*-hydroxybenzoic acid. The concentration of the latter then gradually declined with time. In hepatocytes treated with the inhibitor, the loss of propyl-paraben was small and slow and so was the formation of *p*-hydroxybenzoic acid; at 3 hr 80% of the parent compound was still present in the suspension. Propyl-paraben itself was stable when incubated under carbogen gas for 3 hr at 37° in Krebs–Henseleit buffer without hepatocytes. Furthermore, HPLC analysis has shown that diazinon neither reacted directly with propyl-paraben nor affected the loss of the ester when added to the buffer.

The relative toxicities of various parabens (2 mM) were compared by measuring their effects on cell killing, the levels of ATP and total adenine nucleotides, and the

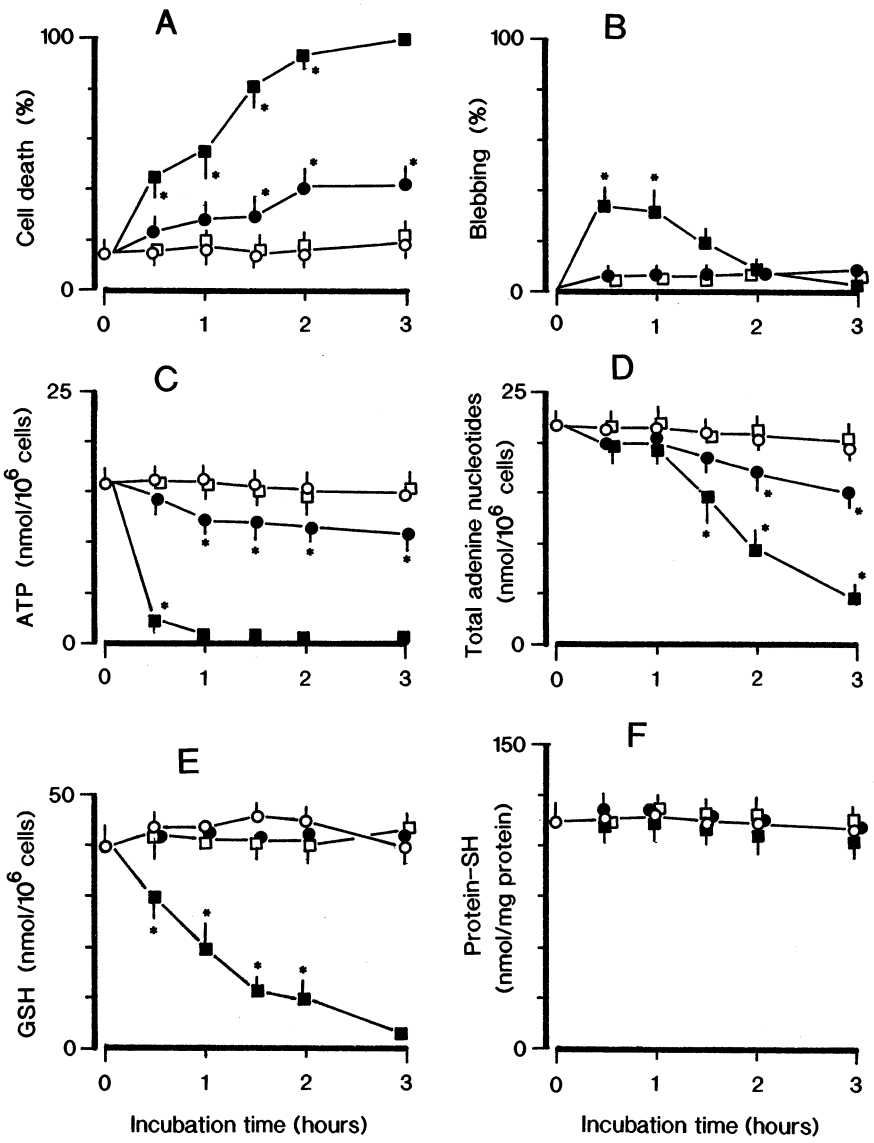


FIG. 2. Effects of the esterase inhibitor diazinon on cell death (A), cell blebbing (B), levels of intracellular ATP (C), total adenine nucleotides (D), GSH (E), and protein thiols (F) in isolated rat hepatocytes treated with propyl-paraben (1.0 mM). Hepatocytes pretreated with diazinon (100 μM) were incubated with 1.0 mM of propyl-paraben. Key: (○) no addition; (□) 100 μM diazinon; (●) 1.0 mM of propyl-paraben; and (■) 100 μM of diazinon plus 1.0 mM of propyl-paraben. Results are expressed as the means ± SEM of three separate experiments. An asterisk (*) indicates a significant difference from values for untreated hepatocytes ($P < 0.05$).

mitochondrial membrane potential ($\Delta\Psi$) during a 1-hr incubation with hepatocyte suspensions (Table 1). Methyl- and ethyl-parabens exhibited very low toxicities: the only significant changes were decreases in the levels of ATP and total adenine nucleotides. Much larger alterations were seen with propyl- and isopropyl-parabens, which induced a 50% decrease in cell viability and mitochondrial membrane potential, and a drastic fall in the levels of ATP to 2–3 nmole/$10^6$ cells; total adenine nucleotides were reduced by about 25%. The most toxic were butyl- and isobutyl-

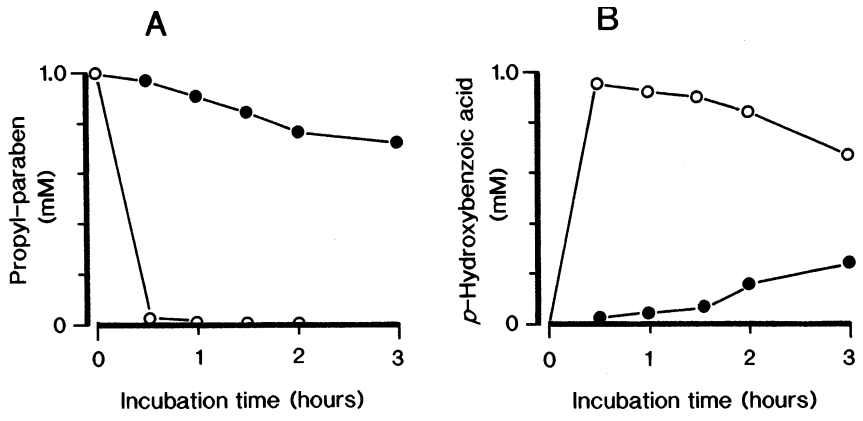


FIG. 3. Changes in the levels of propyl-paraben (A) and its metabolite *p*-hydroxybenzoic acid (B) in hepatocyte suspensions. Hepatocytes pretreated with diazinon (100 μM) were incubated with 1.0 mM of propyl-paraben. Key: (○) propyl-paraben alone; and (●) diazinon plus propyl-paraben. Values are means of duplicate experiments differing by less than 10% from each other.

**TABLE 1. Comparative cytotoxicity of parabens and *p*-hydroxybenzoic acid on isolated rat hepatocytes**

| Treatments (2 mM) | Cell death (%) | ATP (nmol/$10^6$ cells) | Adenine nucleotide pools (nmol/$10^6$ cells) | Mitochondrial $\Delta\Psi$ (% of control) |
|---|---|---|---|---|
| None (control) | 21 ± 4 | 15.1 ± 0.9 | 20.7 ± 3.5 | 100 |
| *p*-Hydroxybenzoic acid | 23 ± 8 | 14.1 ± 1.5 | 19.9 ± 3.1 | 96.3 |
| Methyl-paraben | 29 ± 5 | 11.0 ± 2.6* | 19.3 ± 1.9 | 93.3 |
| Ethyl-paraben | 32 ± 6 | 9.7 ± 2.0* | 15.7 ± 2.8* | 91.5 |
| Propyl-paraben | 50 ± 4* | 2.1 ± 0.3* | 15.7 ± 3.0* | 48.4 |
| Isopropyl-paraben | 47 ± 7* | 3.3 ± 0.6* | 16.6 ± 2.4* | 55.1 |
| Butyl-paraben | 88 ± 4* | 0.3 ± 0.2* | 7.1 ± 1.8* | 39.3 |
| Isobutyl-paraben | 98 ± 2* | 0.2 ± 0.1* | 7.1 ± 0.7* | 37.1 |

Hepatocytes ($10^6$ cells/mL) without diazinon were incubated with parabens and *p*-hydroxybenzoic acid (2 mM) in Krebs–Henseleit buffer at 37°, as described in Materials and Methods. Cytotoxicity was determined at 60 min. The values of cell death, ATP, and adenine nucleotides are expressed as the means ± SEM of three experiments, and the values of mitochondrial $\Delta\Psi$ are the means of two experiments.

*Significantly different from values for untreated hepatocytes ($P < 0.05$).

parabens, which caused 88–98% cell death, almost total loss of ATP, and reductions in the total adenine nucleotide pool and mitochondrial membrane potential of approximately 60%. There were no significant differences in toxicity between chain isomers, i.e. iso-alkyl and normal-alkyl parabens. Control experiments have shown that propyl-, butyl-, and isobutyl-parabens added to hepatocyte suspensions were converted rapidly to *p*-hydroxybenzoic acid and were undetectable after 1-hr incubations. Moreover, propyl-, isopropyl-, butyl-, and isobutyl-alcohols, at 2 mM, had no effect on either hepatocyte viability or ATP levels (data not shown).

### *Toxic Effects of Parabens in Isolated Hepatic Mitochondria*

Table 2 shows the effect of propyl-paraben (0.5 mM) on mitochondria respiring in state 3 (i.e. in the presence of ADP) with substrates specific for site I (NAD-linked, pyruvate plus malate), site II (FAD-linked, succinate), and site IV (ascorbate plus TMPD). It can be seen that oxygen uptake was reduced by 36% with an NAD-linked substrate and by 29% with succinate, while that with ascorbate plus TMPD was not affected.

Comparative toxicities of various parabens were evaluated by measuring state 3 and 4 respiration with succinate (+ rotenone) as substrate at 2–3 different concentrations of the compounds. The results displayed in Table 3 can be briefly summarized as follows: (i) oxygen uptake in state 4 was increased by parabens, whereas that in state 3 was decreased; consequently, the respiratory control index was reduced; (ii) effectiveness of longer-chain alkyl-parabens was greater than that of the short-chain molecules; and (iii) there was no significant difference in toxicity between chain isomers.

## DISCUSSION

The results obtained in the present study show that in hepatocyte suspensions propyl-paraben caused a concentration- and time-dependent decrease in the levels of ATP and GSH, which was accompanied by a loss of cell viability. In isolated hepatic mitochondria, the compound inhibited both $NAD^+$- and FAD-linked respiration and, with succinate as substrate, acted as an uncoupler of oxidative phosphorylation. The mitochondrial membrane potential was reduced in both the isolated organelles and in hepatocytes *in situ*. Of the other parabens tested, methyl- and ethyl-derivatives were almost non-toxic, while butyl- and isobutyl-parabens elicited stronger effects than the propyl- and isopropyl-derivatives.

We have found that propyl-paraben was essentially stable in Krebs–Henseleit buffer, whereas in the presence of hepatocytes it was broken down rapidly to *p*-hydroxybenzoic acid and propyl alcohol in a reaction sensitive to an

**TABLE 2. Effect of propyl-paraben on respiratory activity of mitochondria supplied with specific substrates of the different oxidation complexes of the respiratory chain**

| Substrates | Respiratory activity of state 3 (ng of oxygen/mg protein/min) | | |
|---|---|---|---|
| | None* | Propyl-paraben* | % Inhibition |
| Pyruvate (5 mM) + malate (0.5 mM) | 53.0 ± 2.9 | 33.9 ± 2.1† | 36.1 |
| Succinate (5 mM) | 76.3 ± 4.1 | 54.4 ± 2.5† | 28.7 |
| Ascorbic acid (1 mM) + TMPD (50 μM) | 41.7 ± 1.8 | 39.2 ± 1.5 | 6.0 |

Mitochondria (1 mg of protein/mL) were preincubated in 3 mL of respiration buffer, containing either 5 mM of pyruvate plus 0.5 mM of malate, 5 mM of succinate containing 1 μM of rotenone, or 1 mM of ascorbic acid plus 50 μM of TMPD containing 1 μM of rotenone/50 nM of antimycin A, for 1.5 min at 25°. For the measurement of state 3 respiration, propyl-paraben (0.5 mM) was incubated with mitochondria for 1.5 min before the addition of ADP (50 μM).

*Values are the means ± SEM of three experiments.

†Significantly different from values for untreated mitochondria ($P < 0.05$).

**TABLE 3. Effects of parabens on mitochondrial respiration**

| Treatments | Concentration (mM) | Mitochondrial respiration (ng oxygen/mg protein/min) State 4 | State 3 | RCI* |
|---|---|---|---|---|
| None | 0 | 23.8 ± 0.9 | 77.3 ± 4.0 | 3.24 |
| *p*-Hydroxybenzoic acid | 0.50 | 22.5 ± 1.6 | 63.1 ± 1.9 | 2.80 |
| | 1.00 | 28.7 ± 1.4† | 64.8 ± 2.3† | 2.26 |
| Methyl-paraben | 0.50 | 31.8 ± 3.0 | 69.0 ± 3.0 | 2.17 |
| | 0.75 | 31.5 ± 1.6 | 66.9 ± 2.4 | 2.12 |
| | 1.00 | 35.2 ± 1.3† | 63.7 ± 2.3† | 1.81 |
| Ethyl-paraben | 0.50 | 34.8 ± 1.1 | 62.7 ± 2.5 | 1.80 |
| | 0.75 | 34.6 ± 2.5 | 60.3 ± 2.8 | 1.74 |
| | 1.00 | 38.6 ± 2.0† | 58.7 ± 1.6† | 1.52 |
| Propyl-paraben | 0.10 | 28.5 ± 1.7 | 67.8 ± 3.3 | 2.38 |
| | 0.25 | 28.9 ± 1.9 | 64.6 ± 1.7 | 2.24 |
| | 0.50 | 40.3 ± 2.6 | 55.4 ± 2.0 | 1.37 |
| Isopropyl-paraben | 0.10 | 29.9 ± 0.8 | 66.3 ± 3.1 | 2.21 |
| | 0.25 | 31.4 ± 1.2 | 58.0 ± 2.4 | 1.85 |
| | 0.50 | 37.3 ± 1.5 | 48.8 ± 0.9 | 1.31 |
| Butyl-paraben | 0.05 | 30.3 ± 1.7 | 57.9 ± 1.7 | 1.91 |
| | 0.10 | 29.8 ± 0.6 | 63.5 ± 1.4 | 2.13 |
| | 0.25 | 50.4 ± 2.2 | 50.4 ± 1.8 | 1.00 |
| Isobutyl-paraben | 0.05 | 32.4 ± 1.8 | 69.4 ± 3.1 | 2.14 |
| | 0.10 | 36.4 ± 0.6 | 63.8 ± 1.5 | 1.75 |
| | 0.25 | 58.9 ± 2.0 | 58.7 ± 2.0 | 1.00 |

Mitochondria (1 mg of protein/mL) were preincubated in 3 mL of respiration buffer, containing succinate (5 mM) and rotenone (1 μM), for 1.5 min at 25° (see Materials and Methods). For the measurement of state 3 respiration, parabens and *p*-hydroxybenzoic acid were incubated with mitochondria for 1.5 min before the addition of ADP (50 μM). Values are the means ± SEM of three determinations, except where noted otherwise.

*The respiratory control index (RCI) was calculated as the ratio of state 3/state 4 respiration.

†Means ± range of two determinations.

inhibitor of esterases, diazinon (Fig. 3). Such a behavior indicates that the compound is hydrolyzed enzymatically by hepatic carboxylesterases (EC 3.1.1.1), a group of β-esterases that act upon a wide range of xenobiotic ester substrates. Because the effectiveness of propyl-paraben was enhanced markedly by the presence of diazinon while neither *p*-hydroxybenzoic acid nor the alcohol derived from the ester exhibited any significant toxicity, one can conclude that the paraben itself and not products of its hydrolysis is the toxic compound. The same holds true for the longer side-chain paraben esters, which were also converted efficiently to nontoxic molecules in the presence of hepatocytes. It is interesting that in spite of the rapid detoxification of the parent compounds the propyl- and butyl-derivatives were highly cytotoxic at early time points, which suggests that some initial cellular interactions may play a critical role in the onset of irreversible lethal alteration of cell function.

An analysis of comparative toxicities of various paraben esters shows that their cytotoxicity depends on the relative length, or molecular weight, of the alkyl side chains esterified to the carboxyl group of *p*-hydroxybenzoic acid (Table 1). The introduction and elongation of these side-chains led to an increase in the hydrophobicity of the parent molecules. The solubilities of methyl-, ethyl-, propyl-, isopropyl-, butyl-, and isobutyl-parabens in water at 25° are 16.4, 10.2, 2.70, 4.88, 1.03, and 1.80 mM, respectively, while that of *p*-hydroxybenzoic acid is 58 mM [26]. This means that the longer chain esters would partition preferentially into the interior hydrophobic portion of biological membranes rather than into their polar hydrophilic phase. It has been reported that such behavior is characteristic of phenolic antioxidants, butylated hydroxytoluene, and butylated hydroxyanisole, which exert cytotoxic effects via inhibition of mitochondrial energy production in a process involving the hydroxy group [27]. A study of the relationship between the structure of parabens and their antimicrobial potency has shown that the alkyl side-chain and the *p*-hydroxy group are necessary for cytotoxicity [28]. In agreement with the latter work, Shiralkar *et al.* [10] found that the presence of an esterase, which was able to hydrolyze the ester linkage of the parabens, could ensure microorganism survival, even in the presence of these toxins. Taken together, these results indicate that the phenolic hydroxy group is essential for the inhibition of mitochondrial respiration, while the alkyl group esterified to hydroxybenzoic acid enhances mitochondrial dysfunction by increasing the hydrophobicity of the molecule.

The mechanism of the toxicity of the parabens' is not clear. The addition of propyl-paraben to Krebs–Henseleit buffer did not elicit an increase in oxygen consumption, which indicates that autoxidation via formation of superoxide anion radicals does not occur readily [29]. The latter

observation and our finding that the ester did not cause accumulation of MDA, an index of lipid peroxidation, in hepatocyte suspensions suggest that formation of free radicals, a major mechanism in the onset of tissue damage induced by some chemicals [30], is unlikely to be involved in the cytotoxicity of the parabens explored in this work.

The data of the present study show that mitochondria are an important target for the action of parabens: these compounds cause uncoupling of oxidative phosphorylation, inhibit $NAD^+$- and FAD-linked mitochondrial respiration, and reduce mitochondrial membrane potential (Tables 1–3). The effectiveness of various derivatives is directly proportional to their hydrophobicity. Because mitochondria in hepatocytes, as in other cells, are the main site of energy production, impairment of their function results in a decrease in the rate of cellular ATP synthesis and, thus, the nucleotide level. Mitochondrial dysfunction and its consequences are common mechanisms of cytotoxicity caused by a wide range of chemicals and a number of pathological conditions [31–33].

Propyl-paraben produced blebbing of the plasma membrane, which has been recognized by previous investigators as an early event in chemically induced toxicity and hypoxic damage in hepatocytes [32]. The formation of "blebs" may be causally related to depletion of ATP because the latter is necessary for polymerization of microtubules and microfilaments, which are involved in the interactions between the cytoskeleton and plasma membrane [34]. It has also been suggested that the phenomenon can be caused by either a collapse of the mitochondrial membrane potential or an elevation of cytosolic free $Ca^{2+}$ [34]. The former can result from an impairment of electron transport and uncoupling, both of which were observed in the present work. Collapse of the mitochondrial membrane potential can lead, in turn, to the inability of mitochondria to take up calcium from the cytosol with a consequent rise in its level. A decrease in cellular ATP levels would also adversely affect transport of calcium into the endoplasmic reticulum, which is an energy-requiring process. A rise in cytosolic free $Ca^{2+}$ induced by parabens has been found by Sone *et al.* [35] in smooth muscle from guinea pig ileum, although in this case it was reported to be the result of activation of a calcium channel.

If parabens do indeed open plasma membrane calcium channels and increase intracellular $Ca^{2+}$, one may offer an alternative hypothesis that could explain, in part, mitochondrial dysfunction. It is possible that these compounds, like some other chemicals [36], induce a calcium-dependent increase in permeability of the mitochondria inner membrane to small ions and molecules (a so-called "permeability-transition") which leads to the collapse of mitochondrial membrane potential and swelling of the organelles [37–40]. If this occurs, it could aggravate the damage caused by direct inhibition of electron transport. However, more work is needed to prove or disprove this suggestion.

In conclusion, the present study shows that hydrophobic alkyl esters of *p*-hydroxybenzoic acid are cytotoxic, most likely through impairment of mitochondrial function and a consequent decrease in the cellular level of ATP. The insufficient supply of ATP, in turn, limits activities of all energy-requiring reactions and eventually leads to cell death.

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