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Oxidative Decarboxylation of Benzoate to Carbon Dioxide by Rat Liver Microsomes: A Probe for Oxygen Radical Production during Microsomal Electron Transfer†

Gary W. Winston and Arthur I. Cederbaum*

ABSTRACT: The oxidative decarboxylation of [7-^{14}C]benzoate has been used by others to evaluate ·OH production by phagocytes and during the xanthine–xanthine oxidase reaction. The current report concerns the use of benzoate as a probe for the detection of oxy radicals generated by another biological system, NADPH-dependent, microsomal electron transfer. $^{14}CO_2$ was produced from [7-^{14}C]benzoate by rat liver microsomes in a Chelex-treated incubation medium. The reaction was dependent upon an NADPH-generating system and intact microsomes. $^{14}CO_2$ was augmented nearly 10-fold in the presence of azide, an inhibitor of catalase, suggesting that H_2O_2 may serve as a precursor of ·OH. External addition of H_2O_2 also increased benzoate decarboxylation. The ·OH scavengers mannitol, dimethyl sulfoxide, 2-oxo-4-(methylthio)butyric acid, and ethanol inhibited $^{14}CO_2$ production. Kinetic studies suggested that the scavengers compete with benzoate for a common intermediate or site. Iron–ethylenediaminetetraacetic acid (EDTA), which catalyzes the decomposition of H_2O_2 resulting in the generation of ·OH (Fenton-type reaction), stimulated $^{14}CO_2$ production in a dose-dependent manner. Metyrapone and SKF-525A, typical inhibitors of mixed-function oxidase activity, did not significantly affect the decarboxylation of benzoate. Organic hydroperoxides such as cumene or *tert*-butyl hydroperoxide, which are capable of catalyzing the metabolism of certain drugs in the absence of NADPH, did not catalyze benzoate decarboxylation. These results disassociate the overall metabolism of benzoate from typical substrates of the mixed-function oxidase system and suggest that discrete pathways for microsomal metabolism of drugs and ·OH scavengers exist. The autoxidation of ascorbate by Fe^{3+} readily promoted benzoate decarboxylation in a manner consistent with a role for ·OH. These data suggest that $^{14}CO_2$ production from [7-^{14}C]-benzoate serves as a sensitive, simple, and efficacious probe for the production of an oxidative radical or radicals generated during microsomal electron transport. This oxidant appears to resemble ·OH in its oxidizing properties.

Earlier investigations have established that benzoic acid was decarboxylated upon interaction with hydroxyl radicals (·OH)[1] generated by radiolysis of aqueous solutions (Matthews & Sangster, 1965; Hoigne & Bader, 1975). More recently, Sagone et al. (1980) extended this concept by demonstrating that benzoate also was decarboxylated by ·OH that was generated during the coupled oxidation of xanthine by xanthine oxidase and by ·OH or a similar oxidant generated by granulocytes during the phagocytosis of zymosan particles. The production of $^{14}CO_2$ from [7-^{14}C]benzoate was used to investigate the mechanism of ·OH generation by phagocytic cells (Sagone et al., 1980).

The current report concerns the use of benzoate as a probe for the detection of oxy radicals generated by another biological system, NADPH-dependent, microsomal electron transfer. Microsomes produce $O_2^{-}\cdot$ and H_2O_2 during electron transport (Hildebrandt & Roots, 1975; Thurman et al., 1972; Aust et al., 1972; Prough & Masters, 1973; Nordblom & Coon, 1977; Dybing et al., 1976; Fong et al., 1973). Two possible loci of $O_2^{-}\cdot$ production are the autoxidation of oxycytochrome P-450 and the autoxidation of reduced NADPH–cytochrome P-450 reductase. H_2O_2 is produced from the spontaneous dismutation of $O_2^{-}\cdot$. H_2O_2 can serve as a precursor of ·OH via either a Fenton reaction (Walling, 1975) or an iron-catalyzed Haber–Weiss reaction (McCord & Day, 1978, Halliwell, 1978). During microsomal electron transport, ethylene was produced from methional or from KTBA, whereas methane gas was generated from Me_2SO (Cohen & Cederbaum, 1979, 1980). The oxidation of these ·OH scavengers to products known to arise from their interaction with ·OH suggested that microsomes generate an oxy radical with oxidizing properties similar to those of ·OH. However, ethylene production from methional or KTBA is not specific for the detection of ·OH (Pryor & Tang, 1978), and recent results indicate that formaldehyde, not methane, represents a major product of the interaction of Me_2SO with ·OH (Klein et al., 1981). In view of the fact that benzoate was shown to inhibit the oxidation of alcohols by the microsomes, a system dependent on the interaction of the alcohols with metabolically generated ·OH (Cederbaum et al., 1978, 1979, 1981), it appeared that microsomes might be capable of catalyzing the decarboxylation of benzoate. The data reported herein demonstrated that $^{14}CO_2$ production from [7-^{14}C]benzoic acid

† From the Department of Biochemistry, Mount Sinai School of Medicine of the City University of New York, New York, New York 10029. *Received December 22, 1981.* Supported by U.S. Public Health Service Grant AA-04413 and Research Career Development Award 2K02-AA00003 (A.I.C.) from the National Institute on Alcohol Abuse and Alcoholism.

[1] Abbreviations: ·OH, hydroxyl radical or a species with the oxidizing power of the hydroxyl radical; Me_2SO, dimethyl sulfoxide; KTBA, 2-oxo-4-(methylthio)butyric acid; $O_2^{-}\cdot$, superoxide anion radical; EDTA, ethylenediaminetetraacetic acid.

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Table I: $^{14}CO_2$ Production from [7-^{14}C]Benzoate during Iron-Catalyzed Oxidation of Ascorbate[a]

additions	concn (mM)	production of $^{14}CO_2$ (nmol) 2.5 min	5.0 min	10 min	20 min
none		57.9 ± 4.8	125.1 ± 7.2	232.5 ± 18.6	362.0 ± 24.3
Me_2SO	10	41.7 ± 6.3	81.3 ± 6.3	160.2 ± 17.1	336.0 ± 25.5
	30	20.1 ± 5.4	24.3 ± 3.9	51.6 ± 6.6	108.6 ± 6.0
	100	1.2 ± 0.9	6.6 ± 5.4	15.6 ± 3.3	48.3 ± 11.4
KTBA	10	37.5 ± 4.2	85.5 ± 9.9	151.2 ± 11.1	240.0 ± 12.6
	30	7.8 ± 4.2	16.5 ± 3.0	44.1 ± 6.0	72.3 ± 6.9
	100	0.6 ± 0.3	3.3 ± 1.2	6.0 ± 1.8	17.4 ± 2.7

[a] The reaction was carried out in 25-mL Erlenmeyer flasks containing 100 mM KP_i buffer, pH 7.4, 0.1 mM EDTA, 167 μM iron-EDTA (bis complex), 2.0 mM ascorbic acid, and 10 mM benzoate containing 0.15 μCi of [7-^{14}C]benzoate. The reaction was started by the addition of ascorbate and was terminated by the addition of 100 units of catalase. Values represent the mean ± SEM for three experiments, carried out in duplicate.

serves as a sensitive, simple, and efficacious means of monitoring oxygen radicals that are generated during microsomal electron transport.

Experimental Procedures

Liver microsomes were prepared from male Sprague-Dawley rats according to previously described techniques (Cederbaum et al., 1978). The standard reaction mixture consisted of 100 mM potassium phosphate buffer, pH 7.4, 10 mM $MgCl_2$, 0.1 mM EDTA, 10 mM potassium pyrophosphate, 0.3 mM $NADP^+$, 10 mM glucose 6-phosphate, 7 units of glucose-6-phosphate dehydrogenase, 5–7 mg of microsomal protein, and 10 mM sodium benzoate containing trace amounts of [7-^{14}C]benzoate (New England Nuclear, final specific activity 5.5 μCi/mmol) in a final volume of 3.0 mL. When present, sodium azide was at 1.0 mM. The reactions were carried out in duplicate or triplicate 25-mL Erlenmeyer flasks fitted with gas-sealing, rubber, center-well caps in a shaking water bath at 37 °C. The reaction was initiated by the addition of the NADPH-generating system (glucose 6-phosphate plus glucose-6-phosphate dehydrogenase) and terminated by injecting 0.3 mL of 70% perchloric acid through the rubber cap with a syringe. All buffers and water used in these experiments were passed through a Chelex 100 (Bio-Rad) column to remove contaminating iron. Following cessation of the reaction, 0.3 mL of hyamine hydroxide was added to the center well with a syringe directly through the rubber caps. The flasks were allowed to incubate for 1 h to enable absorption of the CO_2 into the center well. The center wells were then removed and placed directly into scintillation vials containing 10 mL of Econofluor (New England Nuclear) and shaken vigorously to disperse evenly the hyamine hydroxide. The radioactivity was counted on a Beckman LS 9000 liquid scintillation counter by using automatic quench control and random coincidence monitoring. All values were corrected for "zero-time" controls which contained the acid added before the NADPH-generating system.

Aminopyrine demethylase activity was assayed by replacing the benzoate with 10 mM aminopyrine and assaying for the production of formaldehyde by the method of Nash (1953). In some experiments, the NADPH-generating system was replaced by organic hydroperoxides. Conditions were the same except that the reactions were initiated by the addition of either 0.5 mM cumene hydroperoxide or 2.5 mM *tert*-butyl hydroperoxide. In some experiments, hydroxyl radicals were generated at 37 °C by a chemical system, namely, the autoxidation of ascorbate by iron (Cohen, 1977). Flasks contained 100 mM potassium phosphate buffer, pH 7.4, 0.1 mM EDTA, 167 μM Fe–EDTA (bis complex), 2.0 mM ascorbic acid, and 10.0 mM benzoate containing 0.15 μCi of [7-^{14}C]benzoate in a final volume of 3.0 mL. The reaction was started by the addition of ascorbate and was terminated at time intervals by the addition of 100 units of catalase. $^{14}CO_2$ production was determined as described above. All reagents were of analytical grade from regular commercial sources. SKF-525A was a generous gift from Smith Kline & French (Philadelphia, PA). Statistical analyses were performed by using the Student's *t* test.

Results

Oxidative Decarboxylation of Benzoate by Ascorbate–Iron–EDTA. Initial experiments to study the decarboxylation of [7-^{14}C]benzoate upon interaction of benzoate with the hydroxyl radical or an oxygen radical with similar oxidizing power were carried out by using a chemical model ·OH-generating system, namely, the autoxidation of ascorbate by iron. This system catalyzed production of $^{14}CO_2$ from [7-^{14}C]-benzoate (Table I). $^{14}CO_2$ production increased over the 20-min time course and remained essentially linear for 10 min. The precursor of ·OH during the iron–ascorbate reaction appears to be H_2O_2, which is decomposed to ·OH in the presence of ferrous iron (reduced by ascorbate) according to the Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow \cdot OH + OH^- + Fe^{3+} \quad (1)$$

Therefore, the addition of catalase, which catalyzes the decomposition of H_2O_2

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2 \quad (2)$$

should prevent the decarboxylation of benzoate. $^{14}CO_2$ production from benzoate was completely prevented by the addition of 100 units of catalase. Further, when catalase was added at zero time or when the experiment was carried out under anaerobic conditions, benzoate was not decarboxylated (data not shown).

For implication of a role for ·OH in the decarboxylation of benzoate by the iron–ascorbate system, the effect of competing ·OH scavengers was evaluated. Me_2SO and KTBA inhibited $^{14}CO_2$ production in a dose-dependent manner over the entire time course studied (Table I). Thus, benzoate decarboxylation appeared to be a veracious probe for detecting the oxidizing species produced by a chemical model ·OH-generating system.

Oxidative Decarboxylation of Benzoate by Rat Liver Microsomes. Similar experiments were carried out with rat liver microsomes. In the presence of azide, which was added to inhibit the activity of catalase (present as a contaminant in isolated microsomes), microsomal decarboxylation of benzoate was linear over a 40-min time period (Figure 1). H_2O_2 is generated upon oxidation of NADPH and most likely represents the precursor of ·OH in this system during microsomal electron transport (Cederbaum et al., 1978, 1979; Cohen & Cederbaum, 1979, 1980). In the presence of 1.0 mM sodium

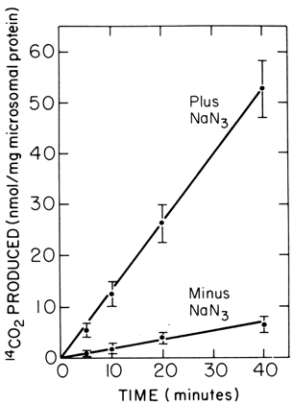

FIGURE 1: Time course for production of $^{14}CO_2$ from [7-^{14}C]benzoate during NADPH-dependent electron transport by rat liver microsomes. Experiments were carried out as described under Experimental Procedures either in the absence or in the presence of 1.0 mM azide. Results are the mean ± SEM from three experiments.

Table II: Effect of Exogenous H_2O_2 on $^{14}CO_2$ Production from [7-^{14}C]Benzoate during Microsomal Electron Transport[a]

concn of exogenous H_2O_2 (mM)	$^{14}CO_2$ generation [nmol min^{-1} (mg of microsomal protein)$^{-1}$]	effect of H_2O_2 (%)
0	1.13 ± 0.07	
0.1	1.55 ± 0.18	+37[b]
0.3	1.76 ± 0.14	+63[c]
1.0	2.23 ± 0.24	+97[c]
3.0	1.63 ± 0.32	+44[b]

[a] Experiments were carried out as described under Experimental Procedures in the presence of the indicated concentration of H_2O_2. Azide was present at a final concentration of 1.0 mM. Data represent the mean ± SEM for 9–12 determinations on three to four microsomal preparations. [b] $P < 0.05$. [c] $P < 0.01$.

azide (a dose sufficient to inhibit contaminating catalase >95%) H_2O_2 should accumulate and thereby serve to generate ·OH. Indeed, azide strikingly stimulated the production of $^{14}CO_2$ (Figure 1), an observation consistent with the hypothesis that H_2O_2 served as a precursor of ·OH production by microsomes. Most other microsomal experiments were routinely carried out in the presence of 1.0 mM azide.

Further support for the precursor nature of H_2O_2 is presented in Table II. External addition of H_2O_2 in the range of 0.1–1.0 mM produced a concentration-dependent increase in the rate of CO_2 production from benzoate. It should be pointed out that H_2O_2 alone, in the absence of NADPH, did not support benzoate decarboxylation. Therefore simultaneous electron transfer, in addition to H_2O_2, is required to promote the decarboxylation of benzoate. The extent of stimulation by H_2O_2 was decreased at 3 mM H_2O_2, probably due to oxidative damage to the microsomes at elevated levels of H_2O_2.

Control experiments indicate that benzoate was not decarboxylated in the absence of microsomes or when microsomes were denatured either by acid treatment (zero-time controls) or by boiling (Table III). NADPH-dependent microsomal electron transport also was required as CO_2 was not produced in the absence of the NADPH-generating system (Table III).

Effect of Hydroxyl Radical Scavengers on Decarboxylation of Benzoate. For further implication of a role for ·OH in the microsomal oxidation of benzoic acid, the effect of competing ·OH scavengers on the production of $^{14}CO_2$ was studied. The scavengers utilized were KTBA, Me_2SO, mannitol, and ethanol. The results of these experiments are presented in Figure 2. $^{14}CO_2$ production from [7-^{14}C]benzoate was inhibited by

Table III: Control Experiments for Oxidative Decarboxylation of Benzoate by Rat Liver Microsomes[a]

experimental conditions	$^{14}CO_2$ production from [7-^{14}C]benzoate (dpm/mL)[b]
zero time	40.7 ± 13.2
boiled microsomes	36.9 ± 19.8
no microsomes	37.0 ± 10.0
no NADPH-generating system	71.0 ± 8.1
complete microsomal system	1382.0 ± 159.0

[a] Experiments were carried out as described under Experimental Procedures and represent the mean ± SD for three experiments. Zero time refers to flasks containing perchloric acid added before the NADPH-generating system. Results are for a 20-min reaction period. Microsomal protein was 5–7 mg/flask. [b] 12.9 dpm was equivalent to 1 nmol of $^{14}CO_2$.

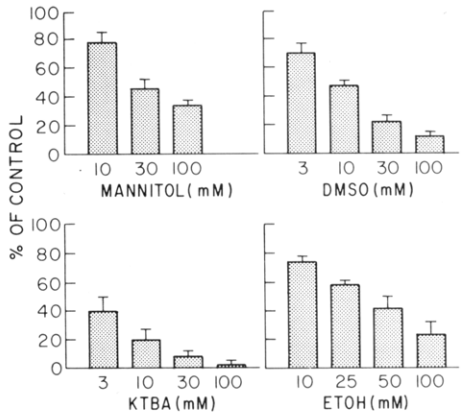

FIGURE 2: Effects of hydroxyl radical scavenging agents on production of $^{14}CO_2$ from [7-^{14}C]benzoate by rat liver microsomes. $^{14}CO_2$ production from 10 mM benzoate was assayed in the presence of the indicated concentrations of competitive hydroxyl radical scavenging agent: mannitol, Me_2SO (DMSO), KTBA, or ethanol (EtOH). Results are from three experiments and refer to the effect of the competitive scavenger on control rates of $^{14}CO_2$ production. Control values were 1.7 ± 0.2 nmol min^{-1} (mg of microsomal protein)$^{-1}$.

each of the competing scavengers in a dose-dependent manner. The patterns of inhibition were noted to reflect the relative potency of the respective scavengers in interacting with ·OH. For example, Me_2SO, which has a rate constant of 7×10^9 M^{-1} s^{-1} (Anbar & Neta, 1967), was considerably more inhibitory than ethanol, which has a rate constant of about 1.1×10^9 M^{-1} s^{-1} (Anbar & Neta, 1967) (Figure 2).

For determination of the kinetics of inhibition, the concentration of benzoate was varied from 2 to 20 mM in the absence and presence of either 10 mM Me_2SO or 6 mM KTBA. The apparent K_m for benzoate was about 5.5 mM, while V_{max} was about 2.2 nmol of CO_2 produced min^{-1} (mg of microsomal protein)$^{-1}$ (Figure 3). KTBA and Me_2SO had no effect on the V_{max} but increased the K_m values for benzoate to 35 and 13 mM, respectively (Figure 3). These results indicate that Me_2SO and KTBA were competitive inhibitors; i.e., the scavengers were competing with benzoate for binding or for a common intermediate, e.g., ·OH.

Effect of Iron–EDTA on Decarboxylation of Benzoate. Iron is believed to stimulate the production of ·OH in biological systems through two probable mechanisms, either the ferrous-catalyzed decomposition of H_2O_2 (Fenton reaction, eq 1) or an iron-catalyzed Haber–Weiss reaction (McCord & Day, 1978; Halliwell, 1978):

$$Fe^{3+}\text{–chelate} + O_2^{-}\cdot \rightarrow O_2 + Fe^{2+}\text{–chelate} \quad (3)$$

$$Fe^{2+}\text{–chelate} + H_2O_2 \rightarrow \cdot OH + OH^- + Fe^{3+}\text{–chelate} \quad (4)$$

Table IV: Comparison of Effect of SKF-525A or Metyrapone on Oxidation of Benzoate and Aminopyrine by Rat Liver Microsomes[a]

addition	concn (mM)	benzoate decarboxylation [nmol of $^{14}CO_2$ min^{-1} (mg of microsomal protein)$^{-1}$]	effect of addition (%)	aminopyrine demethylation [nmol of formaldehyde min^{-1} (mg of microsomal protein)$^{-1}$]	effect of addition (%)
control		2.1		9.5	
SKF-525A	0.1	2.0	−5	5.8	−39
	0.3	2.0	−5	4.4	−54
	1.0	1.8	−14	2.9	−68
metyrapone	0.3	2.2	+5	5.0	−47
	1.0	2.0	−5	3.4	−64
	3.0	1.8	−14	2.3	−76

[a] $^{14}CO_2$ production from [7-^{14}C]benzoate or formaldehyde production from aminopyrine was assayed as described under Experimental Procedures in the absence or presence of the indicated concentrations of either SKF-525A or metyrapone. Results are the mean from two experiments, carried out in duplicate.

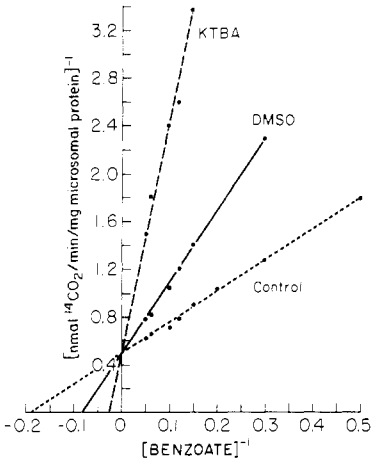

FIGURE 3: A representative Lineweaver–Burk plot to determine kinetics of inhibition of $^{14}CO_2$ production from [7-^{14}C]benzoate by 6 mM KTBA or by 10 mM Me_2SO (DMSO). The concentration of benzoate was varied from 2 to 20 mM in the absence or presence of KTBA or Me_2SO, and $^{14}CO_2$ production was assayed. Three Lineweaver–Burk plots were performed, giving an average K_m of 5.2 ± 0.4 mM and an average V_{max} of 2.4 ± 0.2 nmol min^{-1} (mg of protein)$^{-1}$.

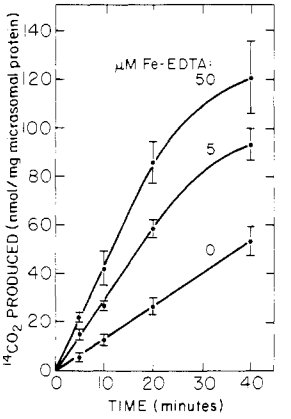

FIGURE 4: Effect of iron–EDTA on production of $^{14}CO_2$ from [7-^{14}C]benzoate by rat liver microsomes. The oxidation of benzoate was assayed in the absence or presence of either 5 or 50 μM iron–EDTA. The iron–EDTA was added as a 1:2 mixture of ferrous ammonium sulfate in Na_4EDTA. Results are from three experiments carried out in duplicate from three microsomal preparations.

The sum of eq 3 and 4 is eq 5, the Haber–Weiss reaction (Haber & Weiss, 1934):

$$O_2^{-}\cdot + H_2O_2 \rightarrow \cdot OH + OH^- + O_2 \qquad (5)$$

If production of CO_2 from benzoate reflects an interaction with ·OH generated by the microsomes, it can be anticipated that external iron should increase the production of ·OH and, subsequently, increase the decarboxylation of benzoate. As can be seen from Figure 4, iron–EDTA stimulated this production of CO_2 from benzoate during microsomal electron transport over the time course studied.

Effect of Mixed-Function Oxidase Inhibitors and Organic Hydroperoxides on Decarboxylation of Benzoate. Small aromatic compounds are known to be suitable substrates for the cytochrome P-450 mediated mixed-function oxidase system. Therefore, it was possible that benzoate decarboxylation might reflect metabolism of the benzoate by the classical cytochrome P-450 dependent, mixed-function oxidase pathway and not by ·OH; i.e., benzoate may serve as a typical substrate for cytochrome P-450. So that this possibility could be tested, the effects of two mixed-function oxidase inhibitors, metyrapone and SKF-525A, on benzoate decarboxylation were compared to their effects on the demethylation of aminopyrine, a typical substrate of the mixed-function oxidase system. Table IV shows that aminopyrine demethylase activity was inhibitied by metyrapone and SKF-525A in a concentration-dependent manner. However, no significant inhibition of CO_2 production from benzoate was observed in the presence of concentrations of SKF-525A or by metyrapone, which strikingly inhibited the metabolism of aminopyrine (Table IV). These results disassociate the overall metabolism of benzoate from typical substrates of the mixed-function oxidase system.

Organic hydroperoxides are capable of catalyzing the metabolism of certain drugs by cytochrome P-450 (Rahimtula & O'Brien, 1974, 1977). There is no requirement for NADPH, NADPH–cytochrome P-450 reductase, or molecular oxygen in the organic hydroperoxide catalyzed system, as the organic hydroperoxide appears to donate an atom of oxygen directly to cytochrome P-450 to form an activated oxygenated complex of cytochrome P-450. Cumene or *tert*-butyl hydroperoxide catalyzed the production of formaldehyde from aminopyrine (Figure 5). However, levels of *tert*-butyl hydroperoxide and cumene hydroperoxide capable of supporting aminopyrine demethylase activity were not capable of supporting benzoate decarboxylation (Figure 5). The inability of the hydroperoxides to support benzoate decarboxylation is in contrast to the effectiveness of the NADPH in catalyzing CO_2 production from benzoate (Figure 5). These results, plus those described in Table IV, suggest that the overall mechanisms for the NADPH-dependent oxidation of benzoate (and, hence, production of ·OH) could be differentiated from the oxidation of typical substrates of the microsomal mixed-function oxidase system.

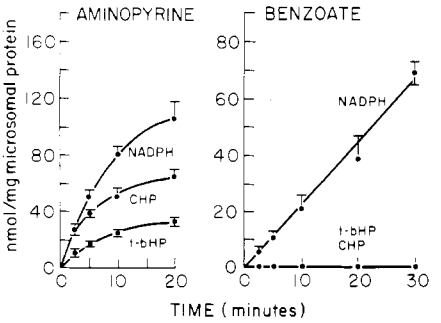

FIGURE 5: Comparison of abilities of organic hydroperoxides [cumeme hydroperoxide (CHP) or *tert*-butyl hydroperoxide (t-bHP)] to catalyze oxidative decarboxylation of benzoate or oxidative demethylation of aminopyrine. Oxidation of benzoate or of aminopyrine was assayed in the presence of either (a) an NADPH-generating system (10 mM glucose 6-phosphate plus 0.3 mM $NADP^+$ plus 7 units of glucose-6-phosphate dehydrogenase), (b) 0.5 mM cumene hydroperoxide, or (c) 2.5 mM *tert*-butyl hydroperoxide. Results refer to either nanomoles of formaldehyde (aminopyrine) or nanomoles of $^{14}CO_2$ (benzoate) produced per milligram of microsomal protein and represent the mean ± SEM from three duplicate experiments with three microsomal preparations.

Discussion

The present report characterizes the ability of rat liver microsomes to decarboxylate benzoic acid during NADPH-dependent electron transport. The decarboxylation reaction was found to be competitively inhibited by ·OH scavengers and stimulated by azide, H_2O_2, and iron–EDTA. Rat liver microsomes also catalyze the metabolism of Me_2SO, KTBA, and *tert*-butyl alcohol, all known to be ·OH scavengers (Cohen & Cederbaum, 1979, 1980; Cederbaum & Cohen, 1980). Oxidative decarboxylation of benzoate occurs in model ·OH-generating systems such as the iron-catalyzed oxidation of ascorbic acid (Table I) and the coupled oxidation of xanthine by xanthine oxidase (Sagone et al., 1980). The production of $^{14}CO_2$ from [7-^{14}C]benzoic acid appears to be a result of an interaction of the benzoate with ·OH or a species with the oxidizing power of ·OH that is generated during microsomal electron transport.

The experiments with benzoate provide an insight into the mechanism of ·OH production by the microsomes. H_2O_2 appears to be the precursor of ·OH in biological systems. A possible mechanism for the production of ·OH during microsomal electron transport may be via H_2O_2 production when NADPH is oxidized by NADPH–cytochrome P-450 reductase and cytochrome P-450. The results of Figure 1 show that in the presence of the catalase inhibitor azide, CO_2 production from benzoate is augmented nearly 10-fold. Results of Table II show that externally added H_2O_2 also stimulates the decarboxylation of benzoate. These results are consistent with the idea that H_2O_2 serves as the precursor for the oxygen radical species involved in the decarboxylation reaction. External addition of H_2O_2 was previously shown to stimulate the oxidation of several alcohols by the microsomes (Cederbaum et al., 1978, 1981).

Careful titration with H_2O_2 is required because added H_2O_2 may damage the microsomal membrane. Indeed, a waning stimulation at 3.0 mM added H_2O_2 is observed. In view of this damage, a theoretically greater stimulation of benzoate decarboxylation by H_2O_2 then 2-fold could have ensued. In experiments in which H_2O_2 is added without the NADPH-generating system, no decarboxylation is observed (data not shown). Thus, the reacting oxygen radical species appears to depend on the presence of both H_2O_2 and some reduced component(s) of the microsomal electron transport system.

It is possible that H_2O_2 may serve as a precursor of ·OH via a Fenton reaction or a modified, i.e., iron catalyzed, Haber–Weiss reaction with endogenous iron–chelates. One function of microsomal electron transport may be to maintain iron in the reduced state. Externally added iron–EDTA stimulates the decarboxylation of benzoate (Figure 4). Iron–EDTA previously was shown to stimulate the oxidation of alcohols by rat liver microsomes in a reaction that was sensitive to inhibition by competing ·OH scavengers (Cederbaum et al., 1980, 1981). The conditions described herein may be appropriate for a Fenton or modified Haber–Weiss reaction to occur with microsomal iron. One possible endogenous iron–chelate in microsomes may be cytochrome P-450. Recently, it was demonstrated that the rate at which Me_2SO and KTBA were oxidized by microsomal preparations from various tissues correlated with both the relative content of cytochrome P-450 and the relative specific activities of the NADPH–cytochrome P-450 reductase from these tissues (Winston & Cederbaum, 1982). Other sources of microsomal iron (heme or nonheme) may participate in catalyzing the production of ·OH from H_2O_2. Adventitious iron in the microsomes, if present, could catalyze the generation of ·OH. In view of the fact that the water and buffers are routinely treated with Chelex 100, it is unlikely that contaminating iron in the solutions represent a significant source of iron available to the microsomes.

Experiments using benzoate as a probe for the detection of the production of ·OH or other oxy radicals by the microsomes indicate that two discrete pathways for drug metabolism and the metabolism of ·OH scavengers exist. This conclusion is based upon the observation that inhibitors of drug metabolism do not inhibit the oxidation of benzoate (Table IV) and the fact that organic hydroperoxides, which are capable of supporting the metabolism of aminopyrine, do not support CO_2 production from benzoate (Figure 5). In previous experiments, it was noted that typical ·OH scavengers such as benzoate, mannitol, Me_2SO, or KTBA did not block the metabolism of aminopyrine or aniline by the microsomes (Cederbaum et al., 1978; Cohen & Cederbaum, 1980). Taken as a whole, these experiments disassociate the overall metabolism of ·OH scavengers by microsomes from the metabolism of typical substrates of the mixed-function oxidase pathway. It is possible that the H_2O_2 that serves as the precursor of ·OH is generated from NADPH–cytochrome P-450 reductase and not cytochrome P-450. Reduction of microsomal iron via the reductase directly or via $O_2^{-}\cdot$ produced from the autoxidation of reduced reductase would then set up conditions appropriate for a Fenton or an iron-catalyzed Haber–Weiss reaction to generate ·OH. Experiments are currently under way to study the oxidation of benzoate and other ·OH scavengers by purified preparations of cytochrome P-450 and the reductase in order to determine the source of H_2O_2 and iron and the actual role of cytochrome P-450 in the production of ·OH.

Is the decarboxylation of benzoate specific for the detection of ·OH or do other oxy radicals or oxidants promote the production of CO_2 from benzoate? Among the latter to be considered are $O_2^{-}\cdot$, singlet oxygen, H_2O_2, hydroperoxides, or alkoxy radicals (·OR). Evidence has been presented by others to show that a charged species such as $O_2^{-}\cdot$ was not a decarboxylating species (Matthews & Sangster, 1965). Azide is a potent singlet oxygen quencher, which was maintained at 1.0 mM in all of the assays using microsomes described herein. Azide stimulated the production of CO_2 (Figure 1). Therefore, singlet oxygen is unlikely to be causing the observed decarboxylation. H_2O_2 alone, in the absence of NADPH, did not

promote the decarboxylation of benzoate; neither did organic hydroperoxides (Figure 5). In preliminary experiments, we attempted to generate an alkoxy radical from cumene or *tert*-butyl hydroperoxide in the presence of a ferrous–chelate

$$Fe^{2+}\text{–chelate} + ROOH \rightarrow RO\cdot + OH^- + Fe^{3+}\text{–chelate} \quad (6)$$

in analogy to the Fenton reaction (eq 1). This system actively promoted the production of ethylene from KTBA and of acetaldehyde from ethanol. However, CO_2 was not produced when benzoate was the substrate. If H_2O_2 replaced the ROOH (thereby generating ·OH), CO_2 was produced in good yield. These experiments are currently being extended; however, these initial observations suggest that benzoate may not be decarboxylated by RO·. Consequently, CO_2 production from benzoate may serve as a relatively more specific probe for the detection of ·OH than methional, KTBA, or alcohols.

In summary, the present report characterized the ability of rat liver microsomes to decarboxylate benzoate during NADPH-dependent microsomal electron transport. It would appear that the production of CO_2 during the metabolism of benzoic acid by rat liver microsomes is due to the interaction of benzoate with ·OH or a species with the oxidizing power of ·OH that is generated during microsomal electron transport. Thus, benzoate decarboxylation may be a useful technique to detect the production of ·OH during microsomal electron transport and by other biological systems and to evaluate the role of ·OH in these systems.

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