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Hydroxylation of salicylate by microsomal fractions and cytochrome *P*-450

Lack of production of 2,3-dihydroxybenzoate unless hydroxyl radical formation is permitted

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Attack by hydroxyl radicals (·OH) upon salicylate (2-hydroxybenzoate) leads to formation of both 2,3-dihydroxybenzoate (2,3-DHB) and 2,5-dihydroxybenzoate (gentisate, 2,5-DHB). It has been suggested that formation of 2,3-DHB from salicylate is a means of monitoring ·OH formation. Production of 2,3-DHB and 2,5-DHB by liver microsomal fractions and isoforms of cytochrome *P*-450 was investigated. Liver microsomes prepared from variously treated rats and rabbits catalysed the formation of 2,5-DHB but not 2,3-DHB. Formation of 2,5-DHB was inhibited by CO, metyrapone and SKF-525A, but not by the ·OH scavengers mannitol and formate or by the iron chelator desferrioxamine. Purified *P*-450s IIE1, IIB4 or IA2 from rabbit liver microsomes, reconstituted together with NADPH–cytochrome *P*-450 reductase, led to formation of equal amounts of 2,3-DHB and 2,5-DHB in reactions that were almost completely inhibited by mannitol or formate. Addition of Fe^{3+}/EDTA either to microsomes or to membranes containing reconstituted *P*-450 caused formation of approximately equal amounts of 2,3-DHB and 2,5-DHB, consistent with an ·OH-dependent attack on salicylate. The data indicate that the microsomal *P*-450 system catalyses hydroxylation of salicylate to 2,5-DHB, but not formation of 2,3-DHB. Hence measurement of 2,3-DHB might provide a means of monitoring ·OH formation. Care must be taken in studies of substrate hydroxylation by microsomes or reconstituted *P*-450 systems to avoid artefacts resulting from ·OH generation.

INTRODUCTION

Aspirin (*O*-acetylsalicylic acid) is a widely used analgesic for self-medication, and larger doses are sometimes used in the treatment of rheumatoid arthritis. There is also considerable interest in the use of aspirin as a prophylactic agent against thrombotic vascular disease. Despite its widespread use, our knowledge of enzymes involved in aspirin metabolism is limited [1–3].

Ingested aspirin is quickly hydrolysed to salicylate in the human body. Salicylate is excreted from the body in one of the following ways: (i) conjugated with glycine, in the form of salicyluric acid, or with glucuronic acid, (ii) in the hydroxylated form as gentisic acid (2,5-dihydroxybenzoic acid), or (iii) as unchanged salicylate [1–3]. The therapeutic actions of aspirin may involve not only its well-established ability to inhibit cyclo-oxygenase [4], but also its effects on neutrophil activation [5] and leukotriene production [6]. Aspirin and salicylate are also powerful scavengers [7,8] of highly reactive hyroxyl radicals (·OH), which are thought to contribute to tissue damage at sites of inflammation (reviewed in [9]). The concentrations of salicylate in the body fluids of patients with rheumatoid arthritis taking therapeutic doses of aspirin can be sufficiently high for scavenging of ·OH by salicylate *in vivo* to be feasible [10].

Attack by ·OH upon salicylate under physiological conditions produces, as major products, approximately equimolar amounts of 2,3- and 2,5-dihydroxybenzoic acids (2,3-DHB and 2,5-DHB) [10,11]. It has, therefore, been suggested [10–12] that measurement of these hydroxylated products, especially 2,3-DHB [13], might be used as a method to measure ·OH in organelles, cells and organs, and even *in vivo*. Thus elevated concentrations of 2,3-DHB have been observed in the body fluids of rheumatoid patients taking aspirin [10] and also in subjects consuming excess alcohol (R. A. Ward & T. J. Peters, personal communication), consistent with the increased oxidative stress known to occur in rheumatoid disease [9] and as a consequence of excess ethanol consumption [9,14].

Although 2,5-DHB is thought to be an enzyme-produced metabolite of salicylate [1–3], its precise metabolic origin is unclear. The cytochrome *P*-450 system is a possibility, since salicylate has been reported to induce cytochrome *P*-450 [15]. By contrast, 2,3-DHB has not been reported to be an enzyme-produced metabolite of salicylate [1–3], although the ability of cytochromes *P*-450 to produce either 2,3- or 2,5-DHB has not been studied.

In the present paper, we have investigated whether the liver microsomal mixed-function oxidase systems might be able to convert salicylate into 2,3- or 2,5-DHB. Various forms of cytochrome *P*-450 have separate, but overlapping, substrate specificities, and can be induced by ligands to the enzymes and by other endogenous and exogenous factors. We have used both liver microsomal fractions from control and treated rats and several different purified forms of cytochrome *P*-450 (incorporated into reconstituted vesicles) as enzyme systems.

MATERIALS AND METHODS

Reagents

H.p.l.c.-grade solvents were obtained from BDH Chemicals, and aromatic compounds were from Aldrich. Standard solutions

Abbreviation used: DHB, dihydroxybenzoate.

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of 2,3-DHB and 2,5-DHB were made up in h.p.l.c.-grade water containing 50 mM-HCl, to a maximum concentration of 10 mM. Desferrioxamine (Desferal) was from CIBA–Geigy. Metyrapone was kindly donated by Professor Sten Orrenius (Karolinska Institute, Stockholm, Sweden) and SKF-525A was kindly donated by Smith, Kline and French.

Methods

Microsomal fractions were isolated from the livers of untreated rabbits or rabbits that had been pre-treated with imidazole (200 mg/kg for 3 days) or phenobarbital (70 mg/kg for 3 days) as described in [16]. Rat liver microsomes were prepared according to Eliasson *et al.* [17] from untreated animals or from animals pre-treated with phenobarbital (80 mg/kg for 3 days), dimethyl sulphoxide (200 mg/kg, twice daily for 3 days), isoniazid (150 mg/kg for 3 days) or acetone (5 ml/kg, intragastric, for 2 days), as described in [18]. The cytochrome *P*-450 content of microsomes was measured as described in [16,17].

Phenobarbital-inducible cytochrome *P*450IIB4, ethanol-inducible cytochrome *P*450IIE1 and β-naphthoflavone-inducible cytochrome *P*450IA2 were isolated from liver microsomes of rabbits that had been treated with phenobarbital, acetone or 3-methylcholanthrene as previously described [6]. NADPH:cytochrome *P*-450 reductase was purified from rabbit or rat liver microsomes as described in [6]. Monolamellar membrane vesicles containing phospholipids, NADPH:cytochrome *P*-450 reductase and cytochrome *P*-450 in a molar ratio of 1200:1:4 or 1200:1:1 were prepared according to Ingelman-Sundberg & Glaumann [19]. Incubations were carried out in a final volume of 1 ml and contained 50 mM-KH_2PO_4/KOH buffer, pH 7.4, microsomes (1 mg of protein), salicylic acid adjusted to pH 7.4 (1 mM) and (unless otherwise stated) desferrioxamine (100 μM). Concentrations given are final concentrations in the reaction mixtures.

In some experiments, microsomes were replaced by membrane vesicles containing 0.15 nmol of cytochrome *P*-450 and NADPH:cytochrome *P*-450 reductase. Buffers were freed of contaminating metal ions as far as possible by passing them through a column of Chelex 100. Reactions were started by adding 0.25 mg of NADPH, reaction mixtures were incubated at 37 °C for the times stated and the reaction was stopped by adding 100 μl of 1 M-HCl. Controls were performed in which NADPH was added after the HCl. The amounts of product formed increased proportionally to the time of incubation for times up to 15 min (results not shown). 2,3-DHB and 2,5-DHB were determined by h.p.l.c., using slight modifications of the method of Grootveld & Halliwell [10], as described in full in the legend to Fig. 1.

RESULTS

Metabolism of salicylate by microsomal fractions

In order to evaluate the participation of different forms of cytochrome *P*-450 in the metabolism of salicylate, liver microsomes were isolated from rabbits and rats that had been pre-treated with various compounds to cause induction of different forms of *P*-450 (reviewed in [20]). Measurement of the *P*-450 content of the microsomes confirmed that induction was achieved (Table 1). Isolated liver microsomal fractions are known to produce $O_2^{\cdot-}$ and H_2O_2 which can, in the presence of contaminating iron ions, form ·OH (reviewed in [21]). In order to inhibit this, buffers were pre-treated on a Chelex column and reaction mixtures also contained desferrioxamine, which usually inhibits iron-ion-dependent ·OH generation (reviewed in [22]). Control experiments showed that desferrioxamine did not inhibit *P*-450-dependent reactions.

Table 1 shows that microsomes in the presence of NADPH hydroxylated salicylate into 2,5-DHB. No hydroxylation occurred in the absence of NADPH or when NADPH was added after the acid used to terminate the reaction. Despite the formation of 2,5-DHB, no significant amounts of 2,3-DHB could be detected in the reaction mixtures and there were no 'extra' peaks on the h.p.l.c. chromatograms (e.g. see Fig. 1), suggesting that other isomeric dihydroxybenzoates (e.g. 2,4-DHB) had not been formed either. Fig. 1 shows that good resolution of the isomers was achieved with the h.p.l.c. conditions used.

Microsomes from phenobarbital-, imidazole- or acetone-treated animals formed the most 2,5-DHB (when expressed per unit protein), whereas microsomes from untreated rats or from rats treated with isoniazid were less active in generating 2,5-DHB. However, calculation of the results on a unit cytochrome *P*-450 basis showed that only small differences were evident between the different types of microsomes (Table 1, last two columns), suggesting a lack of specificity of the different types of *P*-450 for salicylate. In order to confirm that *P*-450 was indeed involved in the 2,5-DHB generation, the effects of inhibitors were studied. Table 2 shows that SKF-525A and metyrapone were powerful inhibitors of hydroxylation. CO produced a weaker but still significant inhibitory effect.

In separate experiments, the K_m of microsomal cytochrome *P*-450 for salicylate was evaluated; microsomes from untreated rats were incubated with 20, 50, 100, 200, 500 or 1000 μM-salicylate. The results gave linear Lineweaver–Burk plots with an apparent K_m value of 400 μM (± 10%). A similar value was obtained using microsomes from rats pre-treated with phenobarbital.

Contribution of ·OH to salicylate metabolism

The ·OH scavengers mannitol (tested up to 500 mM) and sodium formate (tested up to 100 mM) had no effect on the generation of 2,5-DHB by microsomal fractions. However, if desferrioxamine was omitted from the incubation mixtures, the amount of 2,5-DHB formed was not significantly altered, but

Table 1. NADPH-dependent metabolism of salicylate in liver microsomes from variously treated rats and rabbits

Incubations were performed for 15 min at 37 °C with microsomes corresponding to 1 mg of protein as described in the Materials and methods section. 2,3- and 2,5-DHB were measured as described in the legend to Fig. 1. The results shown are mean values from duplicate incubations performed with liver microsomes from two (rabbit) or four (rat) different animals. The results from separate incubations with the same microsomes varied by ⩽ 10%.

Microsomes (nmol of *P*-450 per mg of protein)	Amount of product formed (nmol/min per mg of protein)		(nmol/min per mg of cytochrome *P*-450)	
	2,3-DHB	2,5-DHB	2,3-DHB	2,5-DHB
Rabbit				
Untreated (0.64)	< 0.01	0.09	< 0.01	0.14
Phenobarbital (2.08)	< 0.01	0.25	< 0.01	0.12
Imidazole (1.60)	< 0.01	0.16	< 0.01	0.10
Rat				
Untreated (0.51)	< 0.01	0.056	< 0.01	0.11
Acetone (1.71)	0.01	0.12	< 0.01	0.07
Isoniazid (0.54)	< 0.01	0.043	< 0.01	0.08
Dimethyl sulphoxide (0.72)	< 0.01	0.065	< 0.01	0.09
Phenobarbital (1.86)	0.015	0.13	< 0.01	0.07

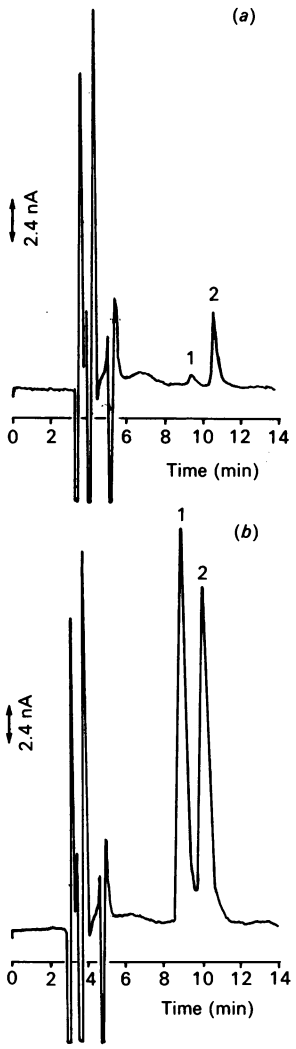

Fig. 1 H.p.l.c. analysis of product formation after incubation of salicylate (1 mM) with microsomes from control rats (*a*) in the presence of desferrioxamine, and of microsomes in the presence of $FeCl_3$/EDTA with no desferrioxamine added (*b*)

A Spherisorb 5 μ ODS column was eluted with [30 mM-sodium citrate/27.7 mM-acetate buffer (pH 4.75)]/methanol (97:3, v/v) at a flow rate of 0.9 ml/min. An electrochemical detector (EDT LCA 15) equipped with a glassy carbon electrode and an Ag/AgCl reference electrode was used. The detector potential was set at 0.65 V and maximum sensitivity was 30 nA. Peak 1, 2,3-DHB; peak 2, 2,5-DHB.

small and variable amounts of 2,3-DHB could be detected. Tables 3 and 4 show some representative data. Addition of the ·OH scavengers mannitol (final concentrations 200 mM or 500 mM) or sodium formate (20 mM or 100 mM) markedly inhibited the generation of 2,3-DHB, but had much smaller effects on the formation of 2,5-DHB (Table 4). Addition of a redox-active source of ion ions ($FeCl_3$/EDTA) to the reaction mixtures to allow more ·OH generation [21] markedly increased the amount of 2,3-DHB detected. Fig. 1 shows a representative h.p.l.c. chromatogram. In the presence of only 1 μM-Fe^{3+}/EDTA, comparable amounts of 2,3- and 2,5-DHB were formed. At 10 μM-Fe^{3+}/EDTA, the rates of formation of 2,3- and 2,5-DHB had increased 5–10-fold. Formation of these increased amounts of 2,3- and 2,5-DHB was inhibited by addition of 500 mM-mannitol or 100 mM-sodium formate (results not shown).

Table 2. Effect of cytochrome *P*-450 inhibitors on the NADPH-dependent formation of 2,5-DHB from salicylate by liver microsomal fractions

Microsomes from untreated rats were used in these studies and the data represent means ± S.D. from three different experiments, each performed in triplicate (i.e. $n = 9$). CO was bubbled through the microsomal suspension for 10 min before incubation. SKF-525A and metyrapone were added to give a final concentration of 1 mM in the reaction mixtures. Incubations were performed as described in the legend to Table 1.

Conditions	Rate of formation (nmol/min per mg of protein) 2,3-DHB	2,5-DHB
Control	< 0.01	0.036 ± 0.004
+CO	< 0.01	0.027 ± 0.000
+SKF-525A	< 0.01	0.010 ± 0.006
+Metyrapone	< 0.01	0.015 ± 0.001

Table 3. Effect of Fe^{3+}/EDTA (1:2) on the rate of formation of 2,3-DHB and 2,5-DHB from salicylate by microsomes isolated from phenobarbital-treated rats

The values represents means ± S.D. of three different experiments performed in duplicate ($n = 6$). Reaction mixtures are as described in the Materials and methods section, except that desferrioxamine was omitted from the reaction mixture. Fe^{3+} and EDTA were pre-mixed before addition to the reaction mixtures.

Fe/EDTA (μM)	Amount of product formed (nmol/min per mg of protein) 2,3-DHB	2,5-DHB
0	0.009 ± 0.003	0.038 ± 0.007
1	0.057 ± 0.006	0.084 ± 0.011
5	0.226 ± 0.040	0.210 ± 0.040
10	0.391 ± 0.092	0.311 ± 0.030

Table 4. Effect of ·OH scavengers on the rate of formation of 2,3-DHB and 2,5-DHB by rat liver microsomal fractions incubated in the absence of desferrioxamine

Results are means of two experiments performed in duplicate, and assays agreed to within 10%. Reaction mixtures were as described in the legend to Table 3, except that Fe^{3+}/EDTA was not added. Scavengers were added to give the final concentrations stated. Percentage inhibitions of product formation are given in parentheses.

Scavenger added	Amount of product formed (nmol/min per mg of protein) 2,3-DHB	2,5-DHB
None	0.012	0.061
Mannitol (200 mM)	0.006 (50%)	0.050 (18%)
Mannitol (500 mM)	0.007 (42%)	0.044 (28%)
Sodium formate (20 mM)	0.000 (100%)	0.053 (13%)
Sodium formate (100 mM)	0.003 (75%)	0.072 (0%)

Table 5. Salicylate metabolism by reconstituted membrane vesicles containing NADPH:cytochrome *P*-450 reductase and various forms of cytochrome *P*-450

Salicylate (1 mM) was incubated at 37 °C with vesicles containing 0.15 nmol of *P*-450 for 17 min. The vesicles contained phospholipids, NADPH:cytochrome *P*-450 reductase and cytochrome *P*-450 in a molar ratio of 1200:1:4. Other conditions were as described in the Materials and methods section. Results are the means of two incubations, performed in duplicate, and analyses differed by $\leqslant 10\%$.

Type of vesicles	Amount of product formed (nmol/min per nmol of cytochrome *P*-450)	
	2,3-DHB	2,5-DHB
No *P*-450 (reductase only)*	0.006	0.006
*P*450IIB4	0.062	0.065
*P*450IIE1	0.10	0.091
*P*450IA2	0.13	0.098

* Results are expressed as nmol of product formed/0.25 nmol of NADPH:cytochrome *P*-450 reductase.

Studies on reconstituted systems

Purified forms of cytochrome *P*-450 were incorporated into phospholipid membrane vesicles together with NADPH:cytochrome *P*-450 reductase. When both components were present, NADPH-dependent formation of hydroxylated products from salicylate was observed, but omission of NADPH, *P*-450 or its reductase inhibited salicylate hydroxylation. Approximately equal amounts of 2,3-DHB and 2,5-DHB were formed and no marked difference in the rate of DHB formation was observed between vesicles containing phenobarbital-inducible *P*450IIB4, ethanol-inducible *P*450IIE1 or 3-methylcholanthrene-inducible *P*450IA2 (Table 5). Formation of both dihydroxybenzoates was largely inhibited by the ·OH scavengers mannitol and formate. Addition of Fe^{3+}/EDTA increased the amounts of both dihydroxybenzoates detected, and the increased formation was again almost completely inhibited by formate and mannitol (Table 6). The hydroxylation properties of these reconstituted systems towards other *P*-450 substrates have already been described in detail in the literature [16,19].

DISCUSSION

Our results indicate that salicylate is metabolized by liver microsomal fractions to give 2,5-DHB. The *P*-450 system is probably responsible, given the inhibitory effects of SKF-525A and metyrapone. Hence, *P*-450 may generate the 2,5-DHB formed when salicylate is administered to humans [1–3,23]. Formation of 2,5-DHB was not inhibited by desferrioxamine or by ·OH scavengers (if desferrioxamine was present). Although the rate of 2,5-DHB production per unit protein differed between microsomes isolated from animals pre-treated with different compounds, there was apparently no great specificity in 2,5-DHB production, expressed per unit *P*-450, in microsomes from eight different liver sources representing most of the known types of *P*-450. The affinity of microsomes for salicylate as a substrate was fairly low (K_m about 0.4 mM in microsomes from untreated or phenobarbital-pretreated rats).

None of the microsomal systems tested produced any significant amount of 2,3-DHB when precautions were taken to avoid generation of ·OH in the reaction mixtures (Chelex treatment of buffers, addition of desferrioxamine). It is noteworthy that ethanol-inducible *P*-450 failed to generate 2,3-DHB, even though ethanol administration has been shown to increase concentrations of 2,3-DHB in humans taking aspirin (R. A. Ward & T. J. Peters, personal communication). However, omission of desferrioxamine, and especially addition of $FeCl_3$/EDTA (an excellent promoter of ·OH generation [21,24]), caused formation of 2,3-DHB which was largely inhibited by ·OH scavengers. Under these reaction conditions, some of the 2,5-DHB formation measured was also inhibited by ·OH scavengers, presumably because attack of ·OH upon salicylate generates both isomers [10–13].

The reconstituted *P*-450 systems studied formed 2,3-DHB and 2,5-DHB in approximately equal amounts. Formation of both products was almost completely inhibited by ·OH scavengers and much increased by addition of low concentrations of Fe^{3+}/EDTA. It seems that *P*450IIE1- and IIB4-dependent hydroxylation of salicylate in reconstituted systems is largely or entirely due to generation of ·OH, and is not the result of specific *P*-450-dependent catalysis. Indeed, reconstituted systems have

Table 6. Effect of ·OH scavengers on the rate of *P*-450-dependent hydroxylation of salicylate in reconstituted membrane vesicles containing either *P*450IIE1 or *P*450IIB4

The vesicles contained *P*-450/reductase/phospholipids at a molar ratio of 1:1:1200. Final concentrations of the various additions are given. Values in parentheses indicate percentage inhibition of product formation.

Addition to reaction mixture	Amount of product formed (nmol/min per nmol of *P*-450)			
	2,3-DHB		2,5-DHB	
	IIE1	IIB4	IIE1	IIB4
None	0.41	0.17	0.37	0.18
Mannitol (50 mM)	0.20 (52)	0.044 (74)	0.11 (70)	0.075 (58)
Mannitol (200 mM)	0.09 (78)	0.022 (87)	0.16 (57)	0.049 (73)
Formate (5 mM)	0.22 (47)	0.089 (48)	0.18 (51)	0.12 (34)
Formate (20 mM)	0.09 (78)	–	0.13 (65)	–
Fe^{3+}/EDTA (5 μM)	3.93	2.57	4.95	2.42
+Mannitol (50 mM)	0.53 (87)	0.33 (87)	0.49 (90)	0.35 (86)
+Mannitol (200 mM)	0.18 (95)	0.11 (96)	0.18 (96)	0.09 (96)
+Formate (5 mM)	1.82 (54)	1.42 (45)	1.40 (72)	1.53 (37)
+Formate (20 mM)	0.82 (79)	0.50 (81)	0.67 (86)	0.51 (79)

previously been shown [16] to generate oxygen radicals at rates consistent with the rates of salicylate hydroxylation observed in the present investigation. A surprising observation is that this ·OH generation occurred even though desferrioxamine was present in the reaction mixture at concentrations sufficient to suppress the reaction of ·OH with salicylate in intact microsomes. Perhaps the metal ions needed for ·OH formation in the reconstituted systems are attached to the proteins and/or liposomal lipids and are not accessible to chelation by desferrioxamine, or the rate of transfer of these metal ions to desferrioxamine is slow, as has been demonstrated with certain physiological iron chelates [25,26].

Our results show that 2,5-DHB may arise by metabolism of salicylate by the endoplasmic reticulum *in vivo*, whereas 2,3-DHB probably does not. This is consistent with the proposal that formation of 2,3-DHB from salicylate *in vivo* may represent a product of free radical attack [10]. Previous reports that formation of 2,3-DHB is increased in humans known to be under oxidative stress [10,13] and in animals exposed to ionizing radiation or to elevated oxygen concentrations [27] are also consistent with this view. Our results additionally demonstrate the ease with which artefacts can arise when microsomes or reconstituted microsomal systems are used to study hydroxylation of substrates, unless careful precautions are taken to inhibit the generation of ·OH or to scavenge it in the reaction mixtures.

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