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The Formation of Catechols by Consecutive Hydroxylations: A Study of the Microsomal Hydroxylation of Butamoxane

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SUMMARY

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The hydroxylation of butamoxane [2-(butylaminomethyl)-1,4-benzodioxane] both *in vivo* and *in vitro* by an enzyme derived from rat liver yields a mixture of 6-hydroxybutamoxane and 7-hydroxybutamoxane in a ratio of approximately 2:1. In addition, the microsomal oxidation of butamoxane *in vitro* yields the catechol 6,7-dihydroxybutamoxane. This same catechol is produced by the hydroxylation of either 6-hydroxybutamoxane or 7-hydroxybutamoxane. A series of ^{18}O studies are described which support the concept that 6,7-dihydroxybutamoxane is formed via two consecutive hydroxylations rather than via a dihydrodiol or an endoperoxide.

INTRODUCTION

The major route of metabolism of butamoxane [2-(butylaminomethyl)-1,4-benzodioxane] in dogs and rats is through aromatic hydroxylation followed by conjugation and elimination (1). The site of hydroxylation was shown to be position 6 and/or 7 of the benzodioxane nucleus. Recently Jack *et al.* (2) reported that the major route of metabolism in man of a chemically related compound, guanoxan, is via hydroxylation at position 6 and/or 7 of the benzodioxane moiety. A similar route of metabolism was reported for ethoxybutamoxane (3) in man.

Our studies on the fate of butamoxane in the rat were undertaken to define more clearly the nature of the oxidation reaction leading to the hydroxylated benzodioxane derivatives. During these studies it was observed that a minor product of microsomal oxidation was a catechol derivative of butamoxane. The mechanism of the formation of this metabolite has been examined using $^{18}O_2$ tracer studies. The results indicate that the catechol is formed by two consecutive hydroxylation reactions, each of which utilizes molecular oxygen and has the properties of a monooxygenase reaction.

METHODS

All cofactors were purchased from Boehringer/Mannheim. $^{18}O_2$ (93 atom %) was obtained from Miles Laboratories. The 6- and 7-hydroxybutamoxanes were generous gifts of Professor A. Funke, Institut Pasteur, France (4). The 5- and 8-hydroxy derivatives of butamoxane were prepared as described previously (1). Heptafluorobutyric anhydride was obtained from Pierce Chemicals.

Incubation in vitro. Livers were obtained from 24-hr-fasted rats killed by decapitation. Liver microsomes were prepared by differential centrifugation of 20% liver homoge-

nates prepared in 0.25 M sucrose (5). The 100,000 × *g* pellet was resuspended in the original volume of 0.05 M phosphate buffer, pH 7.4.

NADPH was prepared *in situ* by the addition of 0.5 mM NADP, 5 mM DL-isocitrate, 0.01 mM $MnCl_2$, and 0.1 mg of isocitrate dehydrogenase (pig heart, 2 units/mg, Boehringer/Mannheim). Incubations were performed at 37° for 30 min, during which the hydroxylation reactions were linear.

Incubations were terminated by the addition of 0.5 volume of acetone. The precipitated protein was removed by centrifugation, and then 1 ml of 1 N Na_2CO_3, which had been adjusted to pH 9 with HCl, was added. The solution was extracted with three 1-ml portions of ethyl acetate. The combined ethyl acetate extracts were then extracted with two 1-ml portions of 0.1 N HCl. The HCl extracts were combined, brought to pH 9 with 1 N Na_2CO_3, and then re-extracted with three 1-ml portions of ethyl acetate. The ethyl acetate extract was then reduced to dryness.

Analysis. The dried extracts were dissolved in 0.2 ml of a 1:1 mixture of heptafluorobutyric anhydride and acetonitrile. After standing at room temperature for 1 hr, the solutions were evaporated under nitrogen, and 0.2 ml of acetonitrile was added. The solutions were then analyzed by gas chromatography.

Gas chromatographic analyses were performed on a Hewlett-Packard 5750 B gas chromatograph, using a flame ionization detector. A 4-foot column was packed with 3% UC W-98 on Gas-Chrom Q (Applied Science Laboratories). The flame detector and flash heater were maintained at 230°. The helium flow rate was 60 ml/min. The oven temperature was raised from 180° to 220° at a rate of 10°/min, starting at the time of injection.

Mass spectra were obtained using an LKB 9000 combined gas chromatograph–mass spectrometer equipped with a similar gas chromatography column.

RESULTS AND DISCUSSION

Gas chromatographic analysis of the heptafluorobutyrate derivatives of butamoxane and its four isomeric hydroxyl derivatives is shown in Fig. 1. Samples of authentic 6- and 7-hydroxybutamoxane became available through the work of Funke *et al.* (4). Each of the isomers is clearly distinguished by its retention time. Using this method of analysis, we determined the position of hydroxylation of butamoxane after metabolism *in vitro* and *in vivo*. Analysis of the urine of rats dosed with butamoxane (10 mg/kg intraperitoneally) is shown in Table 1. An average of 78% of the drug was excreted as a mixture of 6- and 7-hydroxybutamoxane. No 5- or 8-hydroxybutamoxane was detected. Over 80% of the excreted hydroxy compound was conjugated. The ratio of 6-hydroxylation to 7-hydroxylation was near 2:1 in all samples analyzed.

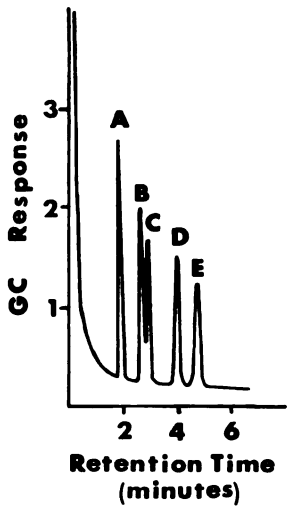


FIG. 1. *Gas chromatographic (GC) analysis of heptafluorobutyrates of butamoxane and its hydroxylated derivatives*

Curve *A*, butamoxane; *B*, 5-hydroxybutamoxane; *C*, 8-hydroxybutamoxane; *D*, 6-hydroxybutamoxane; *E*, 7-hydroxybutamoxane. Conditions were the same as described under METHODS.

Incubation of butamoxane *in vitro* with rat liver microsomes in the presence of NADPH led to the production of 6- and 7-hydroxybutamoxane in a ratio similar to that observed in the urine. The enzyme (or enzymes) that catalyzes the hydroxylation reactions is located solely in the microsomal fraction of the liver homogenates (Table 2).

TABLE 1

Urinary excretion of 6- and 7-hydroxybutamoxane in rats

Each rat received a solution of butamoxane in NaCl, 10 mg/kg intraperitoneally. Urine was analyzed before and after hydrolysis with Glusulase. In rats 1 and 2, 16.1% and 10.1%, respectively, of the excretion products were not conjugated. The metabolites were analyzed by gas chromatography as described under METHODS.

Time	6-Hydroxybutamoxane		7-Hydroxybutamoxane		Total	
	Rat 1	Rat 2	Rat 1	Rat 2	Rat 1	Rat 2
hr	*% dose*		*% dose*		*% dose*	
0–8	28.2	22.3	13.8	14.2	42.0	36.5
8–24	14.4	30.3	6.4	15.4	20.8	45.8
24–30	4.8	0.1	2.2	0	7.0	0.1
30–48	2.1	0.4	1.2	0.2	3.3	0.6
0–48	49.5	53.1	23.6	29.8	73.1	83.0

TABLE 2

Subcellular distribution of butamoxane hydroxylase activity

Subcellular fractions were prepared as described under METHODS. All incubations contained 0.5 mM substrate, 0.5 mM NADPH (as an isocitrate dehydrogenase-generating system), and 0.5 mM NADH. Incubations were performed for 30 min at 37°. Extraction and analyses were performed as described under METHODS. Each result is the average of two experiments.

Liver fraction	6-Hydroxybutamoxane	7-Hydroxybutamoxane
	μg	*μg*
10,000 × *g* supernatant	46	25
100,000 × *g* supernatant	1	2
100,000 × *g* pellet	43	25
100,000 × *g* pellet + 100,000 × *g* supernatant	42	24

The liver 100,000 × *g* supernatant fraction alone was without activity. The cofactor requirements for hydroxylation are shown in Table 3. NADPH is absolutely required. NADH, NAD^+, and $NADP^+$ are ineffective although, in the presence of NADPH, NADH and NAD^+ stimulate the reaction.

Since both positions 6 and 7 of the benzodioxane nucleus were sites of hydroxylation, either the 6- or 7-hydroxy derivative could be further hydroxylated to yield a 6,7-dihydroxycatechol. In order to assess this possibility, the method of Daly *et al.* (6) for catechol determinations was employed. This method involves incubation of the substrate in the presence of the oxidizing system *S*-adenosyl[^{14}C-methyl]-L-methionine and catechol *O*-methyltransferase. If a catechol is formed it is readily methylated in this system, and the [^{14}C]methylated product can be analyzed for radioactivity. When butamoxane or 6- or 7-hydroxybutamoxane was incubated with the rat liver 10,000 × *g* supernatant fraction, NADPH, and *S*-adenosyl[^{14}C]methionine, a new product was observed upon gas chromatographic analysis. The mass spectrum of this product was consistent with the formation of a methylated catechol derivative of butamoxane. The same product was obtained from all three substrates. The reaction was quantitated by analysis of the extracts for radioactivity. The results are shown in Table 4.

Although the fact that both 6- and 7-hydroxybutamoxane can be converted to a catechol is evidence for a consecutive hydroxylation mechanism, the possibility exists that butamoxane is converted to

TABLE 3

Cofactor requirements for butamoxane hydroxylase activity

Microsomes were prepared as described under METHODS. The substrate concentration was 0.5 mM. All cofactors were added at final concentrations of 0.5 mM. NADPH was prepared *in situ*, employing an isocitrate dehydrogenase-generating system and $NADP^+$. Incubations were performed for 30 min at 37°. Extraction and analyses were performed as described under METHODS. Each result is the average of two experiments.

Cofactor added	6-Hydroxybutamoxane	7-Hydroxybutamoxane
	μg	*μg*
NADPH	26	17
NADH	3	3
$NADP^+$	1	1
NAD^+	2	2
NADPH + NADH	40	26
NADPH + NAD^+	38	24
None	2	4

catechol via a dihydrodiol intermediate as depicted in Scheme 1, sequence 1, 2, and 3.

In order to resolve this question, incubations were performed with butamoxane under an atmosphere of $^{18}O_2$ or a mixture of $^{18}O_2$–$^{16}O_2$. If the conversion proceeded through sequence 1, 2, and 3, then under $^{18}O_2$ one of the hydroxyl groups should contain ^{18}O and the other ^{16}O. If the catechol were formed via consecutive hydroxylations (sequences 4 and 5), both hydroxyl groups should contain ^{18}O. A third possibility was the intermediate formation of an endoperoxide, as shown in sequences 6 and 7. Under $^{18}O_2$ the latter pathway would also result in 2 atoms of ^{18}O in the catechol product. In order to distinguish this pathway from that of consecutive hydroxylation an incubation was performed in which the gas phase contained a 1:1 mixture of $^{18}O_2$ and $^{16}O_2$. If the peroxide were the intermediate, the resultant catechol would contain either 2 atoms of ^{18}O or 2 atoms of ^{16}O. If consecutive hydroxylation were the pathway, 50% of the catechol molecules would have 1 atom of ^{16}O and 1 atom of ^{18}O. The results of these experiments are presented in Table 5. The mass spectra of the catechol derivative formed under $^{16}O_2$, $^{18}O_2$, and a 1:1 mixture of $^{16}O_2$ and $^{18}O_2$ are shown in Fig. 2. The pattern of incorporation of ^{18}O indicates that the phenols and the catechol are formed by direct incorporation of molecular oxygen into the butamoxane molecule. The high percentage of O_2 incorporation into the phenol indicates that the molecule is formed by direct oxidation rather than by loss of water from an intermediate dihydrodiol. This result is similar

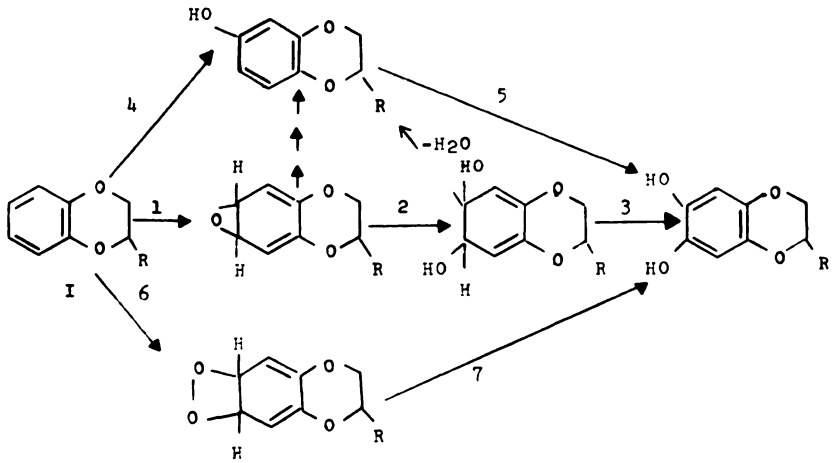
TABLE 4

Conversion of butamoxane and its monohydroxylated derivatives to 6,7-dihydroxybutamoxane by rat liver 10,000 × g supernatant fraction

Incubations were performed using 1.5 ml of a 10,000 × *g* supernatant fraction obtained from a 20% homogenate of rat liver in 0.25 M sucrose. The NADPH and substrate concentrations were each 0.5 mM. The *S*-adenosyl[^{14}C-*methyl*]methionine concentration was 0.05 mM (specific activity, 4680 dpm/nmole). The final volume was diluted to 2.0 ml with 0.05 M phosphate buffer, pH 7.4. Incubations were performed for 25 min at 37°. Extractions were performed as described under METHODS. The total amount of radioactivity in the extract was determined and the amount of product was calculated from the specific activity of the *S*-adenosyl[^{14}C]methionine.

Substrate	6,7-Dihydroxybutamoxane[a]	
	Complete system	−NADPH
	nmoles	*nmoles*
Butamoxane	13.3	
Butamoxane	14.4	0.5
6-Hydroxybutamoxane	14.4	
6-Hydroxybutamoxane	14.9	1.1
7-Hydroxybutamoxane	11.7	1
7-Hydroxybutamoxane	13.4	0.9
None	0.3	

[a] As its [^{14}C]*O-methyl* derivative (see METHODS).



SCHEME 1

TABLE 5

Incorporation of $^{18}O_2$ into products of microsomal hydroxylation

Rat liver 10,000 × *g* supernatant fraction (4 ml) prepared from a 20% homogenate of a liver from a 200-g male rat was added to 1 ml of Tris buffer (0.05 M, pH 7.4) in a 50-ml round-bottomed flask. The solution was frozen in a Dry Ice bath and then dried under high vacuum. The solution was thawed and, after the foaming ceased, refrozen. The substrate (0.5 mM) and cofactors (0.5 mM NADPH, 0.5 M NADH) were added, and the flask was re-evacuated. Then 10 ml of O_2 containing a mixture of $^{18}O_2$ and $^{16}O_2$ were added to the flasks, which were sealed and placed in a shaking bath at 37° for 45 min. The reactions were analyzed as described under METHODS.

Substrate	Isotope distribution in product			
	Gas phase	7-Hydroxybutamoxane	6-Hydroxybutamoxane	6,7-Dihydroxybutamoxane[a]
	% $^{18}O_2$	% ^{18}O	% ^{18}O	% ^{18}O
Butamoxane	94.2	89.9	89.2	87.9
Butamoxane	94.2	80.9	74.7	87.5
7-Hydroxybutamoxane	94.2			88.9
Butamoxane	50.1	48.8	51.1	26.3 ^{16}O 47.4 ^{16}O–^{18}O 26.3 ^{18}O

[a] As its *O-methyl*-^{14}C derivative (see METHODS).

to that reported by Posner *et al.* (7) for a number of aromatic hydroxylations. The incorporation of ^{18}O into the catechol clearly indicates that the reaction proceeds via consecutive hydroxylation and argues against either a dihydrodiol or endoperoxide intermediate.

The ability of liver microsomes to catalyze the hydroxylation of a wide variety of phenolic compounds was originally observed by Axelrod (8), who also postulated that this enzyme system might represent a route for the metabolism of exogenous phenolic compounds as well as an alternative pathway for the production of endogenous catecholamines (9, 10). The discovery that arene oxides are intermediates in the hydroxylation of aromatic compounds as well as in the production of dihydrodiols has suggested that this pathway may also be important for the production of catechols (11).

The production of catechols from benzenoid compounds *in vivo* has been shown for chlorobenzene (12), phenacetylurea (13), diphenylhydantoin (14), and diphenoxylate

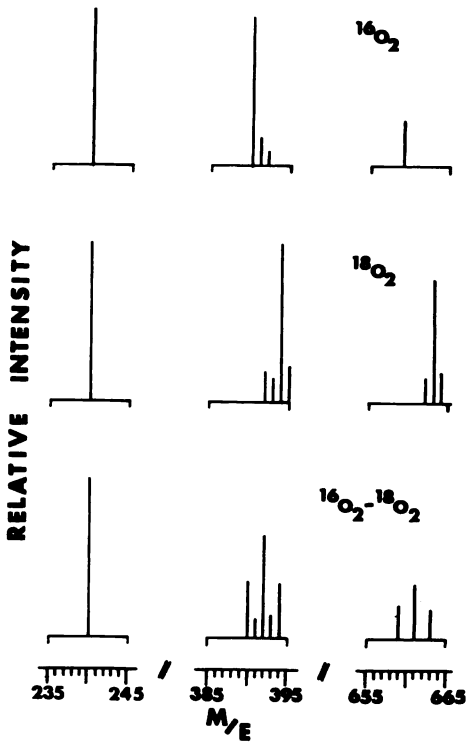


FIG. 2. *Mass spectral analysis of heptafluorobutyrates of 6/7-hydroxy-6/7-methoxybutamoxane produced in vitro under either $^{16}O_2$, $^{18}O_2$, or a 1:1 mixture of $^{16}O_2$ and $^{18}O_2$*

When the metabolite is produced under $^{16}O_2$ the parent peak is at 659 amu. The peak at 390 corresponds to the benzodioxane-containing ion produced by cleavage of the carbon-nitrogen bond. The peak at 240 amu corresponds to an ion produced by rearrangement of the side chain and does not contain the benzodioxane nucleus. When the metabolite is produced under $^{18}O_2$ the 659 peak is shifted to 663 and the 390 peak is shifted to 394. The 240 peak is unaffected by changes in the benzodioxane nucleus. When the metabolite is produced under an atmosphere of $^{18}O_2$–$^{16}O_2$ there is a mixture of ions corresponding to fragments containing 2 atoms of ^{18}O (663, 394), 1 atom of ^{18}O, 1 atom of ^{16}O (661, 392), and 2 atoms of ^{16}O (659, 390).

(15). Whether these catechol metabolites are also produced by consecutive hydroxylation or via the epoxide-dihydrodiol pathway remains to be determined.

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