On the Mechanism of Oxidative Cleavage of Aryl-Alkyl Ethers by Liver Microsomes

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(Received May 3, 1965)

SUMMARY

Studies with $^{18}O_2$ and $H_2{}^{18}O$ have shown that oxidative O-demethylation of *p*-methoxyacetanilide by liver microsomes involves the cleavage of the oxygen methyl bond with no incorporation of oxygen into the phenolic product. They indicate that the intact methoxy group is not displaced by a hydroxyl group. Furthermore, *p*-methoxyphenylalanine was not converted to tyrosine by liver phenylalanine hydroxylase. These findings indicate that the microsomal cleavage of aryl-alkyl ethers is not catalyzed by an aromatic hydroxylase.

INTRODUCTION

Liver microsomes are capable of dealkylating aryl-alkyl ethers (1) in the presence of TPNH, oxygen, and magnesium ions. *p*-Methoxyacetanilide (methacetin), for instance, can be cleaved by microsomes with the formation of *p*-hydroxyacetanilide and formaldehyde. This reaction can be viewed as occurring by a displacement of the methoxy group by molecular oxygen or by cleavage of the oxygen-methyl bond (Fig. 1). In the upper reaction (Fig. 1), the methoxyl group is replaced by a hydroxyl moiety, resulting in the incorporation of a new oxygen atom. This displacement type of reaction would be analogous to the known hydroxylation reactions catalyzed by microsomes in the presence of molecular oxygen and TPNH (2, 3). The present experiments were initiated to determine the mode of cleavage of the methoxyl moiety during the O-dealkylation of methoxyacetanilide, catalyzed by liver microsomes. *p*-Methoxyphenylalanine was also tested for its ability to serve as substrate in a similar reaction using rat liver phenylalanine hydroxylase.

MATERIALS AND METHODS

Livers from unfasted male New Zealand rabbits were quickly removed and chilled in isotonic KCl. They were homogenized for 1 min in 2 vol of 0.05 M potassium phosphate buffer pH 7.4 in a Potter-Elvehjem mixer. Microsomes were obtained by differential centrifugation, washed two times with isotonic saline, and stored at —10°. In some experiments a 9000 *g* fraction (microsome-rich) was used. In the experiments carried out with $^{18}O_2$, large-scale incubations were made using sealed, side-arm flasks as described by Hayaishi (4). Oxygen-18 gas, obtained by electrolysis of $H_2{}^{18}O$, was mixed with nitrogen in a ratio of 3 to 7 and introduced into the 500 ml flasks after elimination of the oxygen contained in the incubation mixtures. Samples of air in the flasks were taken at the beginning and the end of each incubation to determine the actual concentration of $^{18}O_2$ during the course of the reaction. The incubations contained microsomes from 32 g of liver suspended in isotonic KCl, 0.1 M potassium phosphate buffer pH 8.2, 16×10^{-2} M glucose, 2×10^{-2} M TPN, glucose

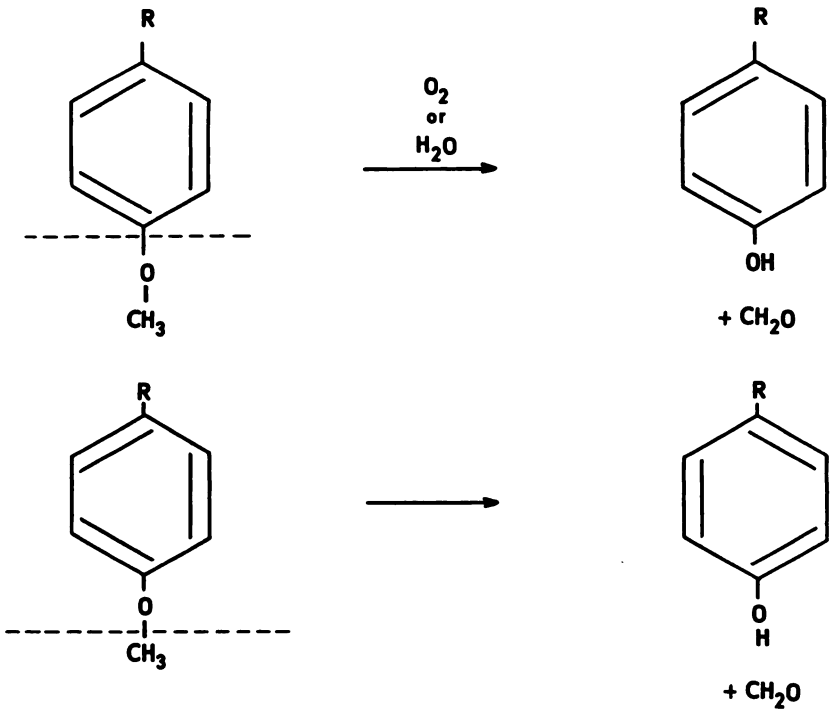

FIG. 1. *Possible mechanisms of oxidative cleavage of aryl-alkyl ethers*

dehydrogenase, 4×10^{-3} M $MgCl_2$ and 1.2×10^{-3} M *p*-methoxyacetanilide or 2×10^{-3} M acetanilide in a final volume of 50 ml. The flasks were shaken at 37° for 6 hr.

For the experiments with $H_2{}^{18}O$, a 9000 *g* supernatant fraction was used, corresponding to 7 g of fresh liver. The incubations also contained 0.1 M Tris buffer pH 8.4, 2×10^{-3} M TPN, 1×10^{-3} M glucose 6-phosphate, glucose 6-phosphate dehydrogenase, 1×10^{-2} M nicotinamide, 5×10^{-3} M $MgCl_2$, and 1×10^{-3} M *p*-methoxyacetanilide in a final volume of 50 ml. The incubations were performed for 12 hr with shaking at 37°.

At the end of the incubation 5 ml of 20% zinc sulfate was added, followed by 1 *N* NaOH until neutrality was reached. The precipitated proteins were removed by centrifugation and washed twice with 30 ml of water. The supernatant fractions were pooled, and an aliquot was assayed colorimetrically for *p*-hydroxyacetanilide. An amount of authentic *p*-hydroxyacetanilide equivalent to three or four times the amount found after incubation was then introduced as carrier. The combined supernatant fractions were saturated with NaCl and extracted three times with 5 vol of a mixture of peroxide-free ether and isoamyl alcohol (1.5 ml per 100 ml of ether) (5). The ether extracts were combined, the volume was reduced to one-third of the original and extracted with 30 ml of 0.1 N NaOH followed by 2 additional extractions with 10 ml of 0.1 N NaOH. The combined alkaline extracts were neutralized, saturated with NaCl, and reextracted three times with 5 vol of the same mixture of ether-isoamyl alcohol. The combined final solvent ether extract was brought to dryness, and the residue was dissolved in methanol and chromatographed on Whatman no. 1 paper (ascending using benzene, acetic acid, water 4:4:2) (6). *p*-Hydroxyacetanilide was detected and delineated on paper by its UV absorption; the corresponding area was cut out and eluted with ethanol. The ethanolic extract was dried,

and the residue was submitted to sublimation at 150°. The white powder that was obtained on sublimation was recrystallized from chloroform before being submitted to chemical and physical analysis. The material was identified as *p*-hydroxyacetanilide by its UV characteristics, infrared spectrum, and behavior on gas–liquid chromatography. It melted at 168–170°, corresponding to the synthetic compound. The recovery of the product through the purification procedure was over 90%. Samples of about 4 mg of the product were analyzed in duplicate for ^{18}O content by Analytica Corporation, New York. The colorimetric determination of *p*-hydroxyacetanilide was made by the *α*-nitroso-*β*-naphthol procedure as originally described for serotonin by Udenfriend *et al.* (7). For phenylalanine hydroxylase rat liver was homogenized in 2 vol of isotonic KCl. The supernatant fraction after centrifugation at 100,000 *g* was used as the enzyme source. D,L,*p*-Methoxyphenylalanine (3.3×10^{-3} M) or L-phenylalanine (2.0×10^{-3} M) were incubated in presence of 0.1 M phosphate buffer pH 6.8, DPN (2×10^{-3} M) and nicotinamide (3×10^{-2} M) with 2.1 ml of enzyme at 37° for 1 hr. The final volume was 3 ml. Tyrosine formation was assayed fluorometrically by the method of Waalkes and Udenfriend (8).

RESULTS

The results are summarized in Table 1. When *p*-methoxyacetanilide was converted to *p*-hydroxyacetanilide by microsomes in a medium containing 7.3 atom % excess of $^{18}O_2$, no significant amount of ^{18}O could be detected in the *p*-hydroxyacetanilide formed. As a control, the microsomal hydroxylation of acetanilide to *p*-hydroxyacetanilide was also investigated. In this case 0.7 atom % excess ^{18}O was found in the product. This corresponds to more than 90% of the expected value for the ^{18}O content if all the oxygen atoms introduced were derived from air, taking into account the dilution of any oxygen incorporated by the addition of carrier and the presence of a second atom of oxygen in the compound. These results on the hydroxylation of acetanilide confirm the previous studies by Posner *et al.* (3). When *p*-methoxyacetanilide was demethylated in the presence of $H_2{}^{18}O$, less carrier was needed and the final dilution was only 8-fold. However, it can be seen that in this case also no ^{18}O was incorporated in the resulting *p*-hydroxyacetanilide. The results with $H_2{}^{18}O$ and ^{18}O indicate that the upper reaction (Fig. 1) does not occur during enzymic O-demethylation.

TABLE 1

^{18}O incorporation during hydroxylation and O-demethylation by liver microsomes

Acetanilide, 100 μmoles, and *p*-methoxyacetanilide, 60 μmoles, were used in the ^{18}O experiment. *p*-Methoxyacetanilide, 50 μmoles were used in the $H_2{}^{18}O$ experiment. The yields of *p*-hydroxyacetanilide after enzymic reaction were 32.4, 32.5, and 40 μmoles, respectively. At the end of the incubation, 130, 130, and 120 μmoles of authentic *p*-hydroxyacetanilide were added, respectively, as carrier before starting the isolation procedures. The details of the experiments are described in the text.

Medium	Atom % excess in medium	Substrate	Atom % excess found in *p*-hydroxyacetanilide	Maximum theoretical value if ^{18}O was incorporated	% of theoretical value
$^{18}O_2$	7.3	Acetanilide	0.70	0.73	96.0
			0.69		94.0
		p-Methoxyacetanilide	0.032	0.73	4.4
			0.033		4.5
			0.025		3.4
$H_2{}^{18}O$	3.44	*p*-Methoxyacetanilide	0.020	0.43	4.7
			0.020		

When *p*-methoxyphenylalanine was incubated with the fortified rat liver preparation, no substrate-dependent formation of tyrosine was detected. The amount of enzyme which was used formed 5 μmoles of tyrosine from comparable amounts of L-phenylalanine. As little as 0.01 μmole of tyrosine formation could have been detected.

DISCUSSION

The observation that phenylalanine hydroxylase could catalyze the displacement of halogen groups by the entering hydroxyl group (9) suggested that other hydroxylases could carry out similar displacement reactions. It has recently been found that the aromatic hydroxylase of liver microsomes can convert *p*-fluoroaniline to *p*-aminophenol (10) and that proline hydroxylase from guinea pig granuloma can convert *trans*-4-fluoroproline to *trans*-4-hydroxyproline (A. Gottlieb, Y. Fujita, S. Udenfriend and B. Witkop, in preparation).

All these observations suggested that groups other than halogens could be displaced by the entering hydroxyl groups during catalysis by a hydroxylase. The observed conversion of *p*-methoxyacetanilide to *p*-hydroxyacetanilide by a liver microsomal system fortified with all the requirements for hydroxylation of acetanilide (1, 2) suggested that aromatic methoxy groups behaved like halogens. If so, then *p*-methoxyphenylalanine, like *p*-fluorophenylalanine, should have been converted to tyrosine by phenylalanine hydroxylase. This did not occur. In the case of *p*-methoxyacetanilide which was converted to the corresponding phenol by microsomal preparations, the conversion did not occur through displacement of the methoxyl group. It must be concluded that the oxidative attack was on the methyl group as shown in the lower equation of Fig. 1.

It is clear that intact methoxy groups are not displaced by hydroxyl groups during enzymatic hydroxylation of aromatic rings. One must assume then that the enzyme which conyerts *p*-methoxyacetanilide to *p*-hydroxyacetanilide is distinct from the aromatic hydroxylase of liver microsomes, even though it has the same cofactor requirements. This would indicate that there is an oxidative attack on the methyl group ultimately producing formaldehyde. The same type of mechanism is probably involved in the metabolism of other drugs which are aryl-alkyl ethers, such as codeine, papaverine, quinine, and mescaline.

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