BBA 65761

PHENOL-*O*-METHYLTRANSFERASE*

JULIUS AXELROD AND JOHN DALY
Laboratory of Clinical Science, National Institute of Mental Health and Laboratory of Chemistry, National Institute of Arthritis and Metabolic Diseases, Bethesda, Md. (U.S.A.)

(Received February 20th, 1968)

SUMMARY

An enzyme that transfers the methyl group of *S*-adenosylmethionine to phenol to form anisole is described. The enzyme is highly localized in the microsomes of the liver and lung of mammals and is also present in other tissues. A variety of simple alkyl-, methoxy- and halophenols can serve as substrates for the phenol-O-methylating enzyme. Activity of the enzyme is inhibited by sulfhydryl reagents and SKF 525. Hypophysectomy in rats increases the activity of phenol-*O*-methyltransferase in rat liver but not in lung or brain.

INTRODUCTION

In a study on the formation of O-methylated catechols from monophenols[1], it was observed that the incubation of phenol with liver microsomes and *S*-adenosyl-[*Me*-^{14}C]methionine resulted in the formation of a radioactive product, presumably anisole. Several *O*-methyltransferases (catechol-*O*-methyltransferase[2], hydroxyindole-*O*-methyltransferase[3], and diiodotyrosine-*O*-methyltransferase[4]) have previously been reported. This report will describe another *O*-methyltransferase enzyme that O-methylates a variety of monophenolic compounds.

METHODS

Measurement of enzyme activity

All preparations of tissue samples were carried out at 0–3°. Young animals of either sex were stunned and exsanguinated; the livers were immediately removed and cooled on cracked ice and homogenized with 10 vol. of isotonic KCl. After centrifugation at 9000 × *g* the supernatant solution was again centrifuged at 80 000 × *g*. The supernatant fluid was decanted and the microsomes were resuspended in 5 vol. of isotonic KCl.

Enzyme activity was measured by incubating microsomes obtained from 10 mg of liver in a 15-ml glass-stoppered centrifuge tube containing 1 mμmole *S*-adenosyl

* A preliminary report was made: *Federation Proc.*, 25 (1966) 745.

[*Me*-^{14}C]methionine (50 mμC), 2 μmoles phenol, 50 μl 0.5 M phosphate buffer (pH 7.9) in a final volume of 200 μl. After 30 min incubation at 37° 0.5 ml 0.5 M borate buffer (pH 10) was added to the incubation mixture and the [^{14}C]*O*-methylphenol (anisole) was extracted with 6 ml of toluene containing 1% isoamyl alcohol. A 4-ml aliquot of the organic phase was transferred to a vial containing 1 ml of ethanol and 10 ml of phosphor and the radioactivity measured. A correction was made for a small amount of a radioactive product formed* when the enzyme was incubated without phenol.

RESULTS

Enzymatic O-methylation of phenol by mammalian liver

A guinea pig homogenate was incubated with *S*-adenosyl[*Me*-^{14}C]methionine and phenol at 37° for 30 min. At the end of the incubation period, borate buffer (pH 10) was added and the mixture shaken with toluene containing 1% isoamyl alcohol. Considerable amounts of a radioactive product were formed that were extractable into the solvent (Table I). When phenol was omitted much less radioactivity was

TABLE I

SUBCELLULAR DISTRIBUTION OF PHENOL-*O*-METHYLTRANSFERASE

Guinea pig liver was homogenized with 10 vol. of isotonic sucrose and the various subcellular fractions separated by differential centrifugation. Fractions equivalent to 10 mg liver were incubated with or without phenol and *S*-adenosyl[*Me*-^{14}C]methionine as described in METHODS. After 30 min incubation, the mixture was extracted with toluene, 1% isoamyl alcohol and the radioactivity measured.

Subcellular fraction	*Without phenol (counts per min)*	*With phenol (counts per min)*	*Difference (counts per min)*
Whole homogenate	1620	10 510	8 890
Microsomes	450	12 580	12 130
Mitochondria	350	3 660	3 310
Nuclei	200	2 240	2 040
Supernatant	760	780	20

extracted. These results indicated the presence of an enzyme in guinea pig liver that transferred the ^{14}C-methyl group of *S*-adenosylmethionine to phenol.

The distribution of the phenol-methylating enzyme was examined in various subcellular fractions of the liver. These were separated by differential centrifugation and the various fractions were incubated with and without phenol and in the presence of *S*-adenosyl[*Me*-^{14}C]methionine. Enzyme activity was found in all subcellular fractions except the supernatant fluid. Microsomes had considerably more enzyme activity than any of the other fractions (Table 1).

The identity of the O-methylated product was examined as follows: anisole (25 mg) was added to an ether extract of a mixture of microsomes obtained from 1 g

* The product formed when the liver microsomes were incubated without substrate was identified as [*Me*-^{14}C]-phosphatidylcholine (R. O. BRADY AND J. AXELROD, unpublished observations).

of rabbit liver which had been incubated with 10 μmoles of phenol and 10 mμmoles *S*-adenosyl[*Me*-^{14}C]methionine. The radioactive product was investigated by thin-layer chromatography (silica gel-G, chloroform, petroleum–ether (1:5, by vol.) and by gas chromatography (8% carbowax 400 column at 100°). The radioactive enzymatic product had the same R_F value (0.50) and retention time (5.5 min) as authentic anisole. Additional evidence for the identity of the enzymatically formed anisole was obtained by extracting the incubation mixture with chloroform, adding 50 mg anisole and brominating with 2 equivalents of bromine to yield 2,4-dibromoanisole. At least 80% of the total radioactivity of the extract was recrystallized to constant specific activity.

Substrate specificity

A number of monophenols were examined for their ability to enzymatically accept a methyl group from *S*-adenosylmethionine. The substrates were incubated with rabbit liver microsomes and *S*-adenosyl[*Me*-^{14}C]methionine and the radioactive product was extracted with toluene containing 10% isoamyl alcohol at pH 7.4. Under these conditions, the ^{14}C-methylated products were quantitatively extracted. When phenolic acids were used as substrates, extractions were carried out at pH 4.5. All simple alkyl- and methoxyphenols examined served as substrates for the enzyme

TABLE II

SUBSTRATE SPECIFICITY OF PHENOL-*O*-METHYLTRANSFERASE

Microsomes obtained from 50 mg rabbit liver were incubated with: substrate 0.5 μmole; phosphate buffer, (pH 7.9), 200 μmoles; and *S*-adenosyl[*Me*-^{14}C]methionine (50 μC) 1 mμmole, in a final volume of 0.6 ml.

Substrate	*Enzyme activity (mμmoles product formed per g)*	*Substrate*	*Enzyme activity (mμmoles product formed per g)*
Phenol	16	Isovanillin	<5
p-Chlorophenol	25	*p*-Hydroxybenzoic acid	0
m-Chlorophenol	23	Estradiol	<5
o-Chlorophenol	10	Levorphanol	<5
2,6-Dichlorophenol	0	Phenazocine	0
m-Bromophenol	13	Morphine	0
m-Nitrophenol	7	3-Methoxy-4-hydroxyphenethylamine	0
p-Methylphenol	26	4-Methoxy-3-hydroxyphenethylamine	0
m-Methylphenol	13	Tyramine	0
o-Methylphenol	12	α-Methyltyramine	0
p-Ethylphenol	18	*m*-Tyramine	0
p-Hydroxyacetanilide	9	Hordenine	0
p-Hydroxyacetanilide	11*	3,5-Dimethoxy-4-hydroxyphenethylamine	0
4-Hydroxyacetophenone	<5	*N*-Acetyltyramine	0
4-hydroxy-3,5-diiodobenzoic acid	0	*N*-Acetylserotonin	0
o-Methoxyphenol	11	*p*-Hydroxyphenethanol	<5
m-Methoxyphenol	16	*p*-Hydroxyphenylglycol	0
p-Methoxyphenol	6		

* *S*-adenosyl[*ethyl*-^{14}C]ethionine used as the methyl donor. Guinea pig microsomes were the source of enzyme.

(Table II). Monohalophenols were also O-methylated, but 2,6-dichlorophenol was inactive. *p*-Hydroxyacetanilide was a good substrate and the enzyme was capable of forming the analgesic, acetophenetidin, when *S*-adenosylethionine was the alkyl donor. When phenolic amines or related compounds were used as substrates, no O-methylated products were detected.

The products formed from the methylation of *p*-chlorophenols and *p*-hydroxyacetanilide were identified as follows: an ether extract of guinea pig liver microsomes was obtained after microsomes from 1 g of liver were incubated with 50 μmoles of *p*-chlorophenol and 5 μmoles of nonradioactive *S*-adenosylmethionine. The ether extract was concentrated under nitrogen to a small volume and then injected into the LKB 9000 gas chromatograph—mass spectrometer. A small peak was observed which had the same retention time as *p*-chloroanisole (6.5 min on 15% carbowax 1000, 155°). The metabolite was positively identified as *p*-chloroanisole by its mass spectrum.

For the identification of *p*-methoxyacetanilide, guinea pig microsomes from 1 g liver were incubated with 10 μmoles of *p*-hydroxyacetanilide and 10 mμmoles *S*-adenosyl[*Me*-^{14}C]methionine. The radioactive product extracted into toluene 10% isoamyl alcohol was found to cochromatograph with *p*-methoxyacetanilide in thin-layer chromatographic systems (silica gel-G, benzene–methanol–acetic acid (90:8:4, by vol.) R_F 0.58; chloroform–methanol (4:1, by vol.), R_F 0.82).

Properties of the O-methylating enzyme

In phosphate and Tris buffer the O-methylating enzyme had a pH optimum from 7.6 to 8.3. The time course of the enzyme reaction is shown in Fig. 1. After 30 min of

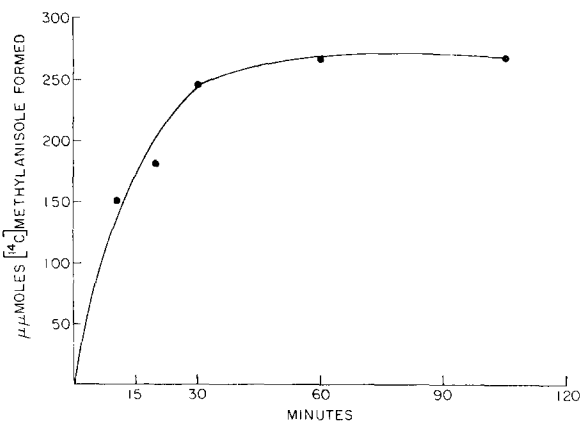

Fig. 1. Rate of O-methylation of phenol. Microsomes obtained from 10 mg guinea pig were incubated with 100 μg phenol and *S*-adenosyl[*Me*-^{14}C]methionine as described in METHODS.

incubation most of the enzyme activity was lost. SKF 525, (β-diethylaminoethyl diphenylpropylacetate), a compound that can inhibit enzymes present in microsomes[5] also inhibited the phenol-O-methylating enzyme (Table III). Both *p*-chloromercuribenzoate and *N*-ethylmaleimide effectively blocked enzyme activity indicating the necessity for an SH group. Tropolone, a compound that inhibits catechol-*O*-methyltransferase[6], had no influence on the enzyme. Neither did metal binding agents such as α,α′-dipyridyl, 8-hydroxyquinoline, nor EDTA.

TABLE III

ENZYME INHIBITORS

Microsomes obtained from 20 mg guinea pig liver were incubated with *S*-adenosyl[*Me*-^{14}C]-methionine (1 mμmole), phenol ($2 \cdot 10^{-3}$ M), 50 μl phosphate buffer (pH 7.9) in a final volume of 250 μl. Results are expressed as % inhibition of formation of [^{14}C]anisole.

Compound added	*Inhibition (%)*	*Compound added*	*Inhibition (%)*
p-Chloromercuribenzoate ($1 \cdot 10^{-5}$ M)	85	α,α′-Dipyridyl ($5 \cdot 10^{-4}$ M)	0
N-Ethylmaleimide ($1 \cdot 10^{-4}$ M)	90	8-Hydroxyquinoline ($1 \cdot 10^{-3}$ M)	0
SKF 525 ($5 \cdot 10^{-4}$ M)	56	Tropolone ($5 \cdot 10^{-4}$ M)	0
Mg^{2+} ($1 \cdot 10^{-2}$ M)	61	EDTA ($1 \cdot 10^{-3}$ M)	0

Tissue and species distribution of phenol-O-methylation enzyme

The ability to O-methylate phenol was examined in the particulate fraction of several guinea pig tissues (Table IV). Of all tissues studied, liver and lung had the highest enzyme activity. Smaller amounts of activity were present in kidney, spleen, adrenal gland, gut, and testes. Little or no enzyme could be found in the brain, muscle, or heart.

TABLE IV

TISSUE DISTRIBUTION OF PHENOL-*O*-METHYLTRANSFERASE

Male guinea pig tissues were homogenized in 10 vol. of isotonic KCl and centrifuged at 80 000 × *g*. The particulate fraction equivalent to 20 mg tissue was incubated with 200 μg phenol, 1 mμmole *S*-adenosyl[*Me*-^{14}C]methionine, 50 μl phosphate buffer 0.5 M (pH 7.9) in a final volume of 200 μl. After incubation for 30 min [^{14}C]anisole was extracted into a mixture of toluene and 1 % isoamyl alcohol. Enzymic activity is expressed as mμmoles anisole formed per g tissue.

Tissue	*Enzyme activity*	*Tissue*	*Enzyme activity*
Liver	31.0	Gut	1.3
Lung	34.0	Testes	1.5
Kidney	15.2	Brain	0.8
Spleen	6.9	Muscle	0.8
Adrenal gland	4.1	Heart	0.0

TABLE V

SPECIES DIFFERENCES IN PHENOL-*O*-METHYLTRANSFERASE ACTIVITY

Microsomes obtained from 10 mg liver were incubated with 200 μg phenol *S*-adenosyl[*Me*-^{14}C]-methionine (1 mμmole), 50 μl buffer 0.5 M (pH 7.9) in a final volume of 250 μl. After 30 min incubation the reaction mixture was assayed for [^{14}C]anisole. Results are expressed as mμmoles anisole formed per g liver.

Species	*Enzyme activity*
Guinea pig	48.5
Mouse	27.0
Rabbit	15.2
Rat	9.9

Phenol-*O*-methyltransferase activity was measured in the microsomes of the liver of a number of mammalian species (Table V). Enzyme activity was present in all species examined and was highest in the guinea pig liver and lowest in the rat.

The availability of hypophysectomized rats in our laboratory provided an opportunity to examine the activity of phenol-*O*-methyltransferase in several tissues (Table VI). There was a marked increase in enzyme activity in the liver of rats without pituitary glands, but enzyme activity in lung and brain was unchanged. There also

TABLE VI

EFFECT OF HYPOPHYSECTOMY ON PHENOL-*O*-METHYLTRANSFERASE

Sprague–Dawley rats were hypophysectomized for 30 days. Tissues were removed, homogenized, and assayed for phenol-*O*-methyltransferase as described in METHODS. Each group contained 6 rats. Results are expressed as mμmole [^{14}C]anisoles formed per g tissue ± S.E.

Tissue	*Control*	*Hypophysectomized*
Liver	13.8 ± 0.8	35.0 ± 1.2*
Lung	38.1 ± 7.6	47.9 ± 4
Brain	4.1 ± 0.4	2.9 ± 0.4

* $P < 0.001$.

appeared to be differences in the relative distribution of phenol-*O*-methyltransferase in tissues of rats and guinea pig. In guinea pig, enzyme activity in lung and liver was about the same, while in the rat, enzyme activity in the liver was about one-third that of the lung. Considerable amounts of phenol-*O*-methyltransferase activity were found in the rat brain while little or no activity was present in guinea pig brain.

DISCUSSION

The phenol-*O*-methyltransferase described in this communication is different from other *O*-methyltransferase enzymes with respect to substrate specificity, subcellular and tissue distribution. Catechol-*O*-methyltransferase cannot O-methylate monophenols[2]; hydroxyindole-*O*-methyltransferase is found only in the mammalian pineal gland and methylates 5-hydroxyindoles[3]. Diiodotyrosine-*O*-methyltransferase is present in the soluble fraction of the cell[4] and the substrate for this enzyme is not O-methylated by phenol-*O*-methyltransferase.

The phenol-*O*-methyltransferase has a limited specificity. Simple alkyl-, methoxy-, and halophenols are relatively good substrates for the enzyme, whereas phenolic amines and related compounds are not. The monophenol, 3-methoxy-4-hydroxyphenethylamine, a normally occurring metabolite of dopamine does not form 3,4-dimethoxyphenylethylamine, nor could the formation of *p*-methoxyphenylethylamine from tyramine be detected. The O-methylation of monophenols *in vivo* is yet to be found. Thus the physiological significance of this enzyme remains to be established.

REFERENCES

1 J. AXELROD, J. K. INSCOE AND J. DALY, *J. Pharmacol.*, 149 (1965) 16.
2 J. AXELROD AND R. TOMCHICK, *J. Biol. Chem.*, 233 (1958) 702.
3 J. AXELROD AND H. WEISSBACH, *J. Biol. Chem.*, 236 (1961) 211.
4 E. TOMITA, C. MOCHA AND H. A. LARDY, *J. Biol. Chem.*, 239 (1964) 1202.
5 J. R. COOPER, J. AXELROD AND B. B. BRODIE, *J. Pharmacol.*, 112 (1954) 55.
6 B. BELLEAU AND J. BURBA, *J. Med. Chem.*, 6 (1963) 755.
7 J. AXELROD, S. SENOH, AND B. WITKOP, *J. Biol. Chem.*, 233 (1958) 697.

Biochim. Biophys. Acta, 159 (1968) 472–478