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FACTORS AFFECTING THE ENZYMATIC FORMATION OF O-METHYLATED DIHYDROXY DERIVATIVES

JOSEPH K. INSCOE, JOHN DALY and JULIUS AXELROD

Laboratory of Clinical Science, National Institute of Mental Health; and Laboratory of Chemistry, National Institute of Arthritis and Metabolic Diseases; National Institutes of Health, Bethesda, Md., U.S.A.

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Abstract—The effect of 3,4-benzpyrene and cold stress on the enzymatic formation of O-methylated dihydroxy compounds from monohydroxy compounds in rats was investigated. Enzyme activity was increased in treated rats only when monohydroxy N-acetyl derivatives (N-acetylserotonin, N-acetyltyramine, and N-acetyl-*p*-aminophenol) were used as subtrates. The effect of cold stress and 3,4-benzpyrene was shown to be on the hydroxylating enzyme in the microsomes. Pretreatment with phenobarbital increased, while serotonin decreased, the formation of the O-methylated dihydroxy metabolite of N-acetylserotonin. An enzyme was found in liver microsomes that could O-methylate catechols and that differed from soluble catechol O-methyltransferase in its response to 3,4-benzpyrene and cold stress treatment as well as in pH optimum.

AN ENZYME system has been described, that catalyzes the formation of catechol amines from monophenolic amines.[1, 2] This enzyme system, localized in the microsomal fraction of rabbit liver and requiring reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor, provides an alternative minor pathway for catecholamine formation.[3] The microsomal system also converts N-acetylserotonin to the dihydroxy derivative.[4] Since catechols and dihydroxy indoles are unstable, the microsomal preparations were incubated in the presence of the catechol O-methylating enzyme[5] and methyl-^{14}C-S-adenosylmethionine. As soon as the catechol is formed, it is immediately O-methylated by catechol-O-methyltransferase, an enzyme that can attack catechols but not monophenols. Thus a stable radioactive O-Methylated derivative is formed which can be readily measured.

The induction of hydroxylating enzymes in rat liver microsomes has been demonstrated with drugs, benzpyrene,[6] and cold stress.[7] This paper will show that the hydrocarbon, 3,4-benzpyrene, and cold stress in some cases increase the ability of rat liver microsomes to form O-methylated dihydroxy derivatives. Other studies of the enzymatic hydroxylation of phenols and monohydroxyindoles, including the isolation and identification of the O-methylated products, are described elsewhere.[4, 8]

Previous work had shown that catechol-O-methyltransferase is highly localized in the soluble fraction of the cell.[5] In a study of the subcellular distribution of catechol-forming enzyme by rabbit liver it was found that a methyl-^{14}C product was obtained with certain substrates (e.g. N-acetyltyramine) when incubation was carried out with the microsomal fraction as the only source of enzyme. Since this indicated that the microsomal as well as the soluble fraction could O-methylate catecholamines, the

O-methylation of *l*-epinephrine by microsomes and the induction of this enzyme by benzpyrene and cold stress was also studied.

MATERIALS AND METHODS

Sprague-Dawley male rats weighing 150–200 g were killed and their livers homogenized in 4 volumes of ice-cold isotonic KCl. A microsomal and soluble fraction was obtained from the supernatant suspension after centrifuging at 8,000 *g* for 10 min. Microsomes were separated from the soluble fraction by centrifuging at 105,000 *g* for 45 min. The microsomes were washed, resedimented, and resuspended in the original volume of isotonic KCl before using.

For induction studies a group of four rats was kept at 3° for 9 days, and 5 mg of 3,4-benzpyrene in 0·5 ml sesame oil was injected i.p. on the first, fourth, and seventh days. A control group of four rats kept at room temperature was injected with sesame oil in the same manner as the treated group.

In another experiment six groups of rats, four animals per group, were treated as follows: sodium phenobarbital in saline, 100 mg/kg, was injected i.p. for 7 days; prednisolone, 1 mg/kg in aqueous suspension was given s.c. for 7 days; 3,4-benzpyrene, 5 mg in 0·5 ml sesame oil, was given i.p. initially and the treatment repeated on the third day; serotonin creatinine sulfate, 12 mg/kg, was given i.p. in saline for 7 days. Rats were kept at 3° for 9 days; control rats were untreated and kept at room temperature.

All rats were killed on the second day following the last treatment with the exception of cold-stressed rats, which were killed on the day of removal from the cold.

For the enzymatic formation of O-methylated catechols, incubations were carried out at 37° in a 15-ml glass-stoppered centrifuge tube with tissue from 20 mg of liver (8,000 *g* preparation), 100 μg substrate, 200 μliter 0·5 M phosphate buffer at pH 7·9, 5 μmoles of $MgCl_2$, 0·6 μmole NADP, 1·5 μmoles glucose-6-phosphate, and 3·7 mμmoles methyl-^{14}C-S-adenosylmethionine (10,000 cpm) in a total volume of 0·8 ml.

After incubation, 0·5 ml of 0·5 M borate buffer at pH 10·0 was added to the mixture and the samples shaken for 10 min with 6 ml of a toluene: isoamyl alcohol mixture (3:2). When phenol was used as a substrate the samples were acidified with 0·5 ml of 0·1 N HCl before extracting. A 4-ml aliquot of the extract was transferred to a vial containing 2 ml ethanol and 10 ml phosphor and the radioactivity measured in a liquid scintillation spectrometer.

For the O-methylation of *l*-epinephrine the microsomes were sedimented and washed four times with isotonic KCl. Microsomes from 20 mg liver were incubated for 1 hr at 37° with 100 μg *l*-epinephrine, 200 μliter 0·5 M phosphate buffer at pH 7·0, 5 μmoles $MgCl_2$, and 3·7 mμmoles methyl-^{14}C-S-adenosylmethionine (10,000 cpm) in a total volume of 0·6 ml. 'Blank' samples were incubated concurrently by omitting the substrate. After incubation, 0·5 ml borate buffer (pH 10) and 6 ml toluene:isoamyl alcohol (3:2) were added, and the radioactivity was extracted and measured as described above.

The radioactive O-methylated dihydroxy compounds formed enzymatically from tyramine, N-acetyltyramine, synephrine, neosynephrine, *p*-octopamine, N-acetylserotonin, phenol, and estradiol were identified by paper chromatography in at least two solvent systems.[4, 8]

Methyl-^{14}C-S-adenosylmethionine was prepared enzymatically from ^{14}C-methionine.[9]

RESULTS

The effect of 3,4-benzpyrene and cold stress on enzymatic formation of O-methylated dihydroxy compounds

The combined treatment of 3,4-benzpyrene and cold stress was used in an attempt to stimulate enzymatic formation of O-methylated dihydroxy compounds when monophenols and hydroxyindoles were used as substrates. This treatment has been shown to increase acetanilide hydroxylation 11-fold.[10] The enzyme preparation from rat liver contained microsomes for catechol formation, and the soluble fraction for O-methylation and for the generation of NADPH from NADP and glucose-6-phosphate. Several monophenols and hydroxyindoles were incubated with the above enzyme preparations obtained from livers of untreated and benzpyrene-treated rats that were kept in the cold (Table 1). With the cold-stressed rats treated with benzpyrene,

TABLE 1. ENZYMATIC FORMATION OF O-METHYLATED DIHYDROXY DERIVATIVES

Substrate	Enzyme activity	
	Control	Benzpyrene and cold-stress-treated
	(mμmoles O-methylated project formed/g liver)	
Tyramine	10·7 ± 2·1	8·4 ± 3·0
N-Acetyltyramine	31·2 ± 4·1	53·6 ± 6·5*
Synephrine	7·2 ± 1·8	7·4 ± 1·5
Neosynephrine	9·2 ± 1·7	5·4 ± 1·5
p-Octopamine	8·4 ± 1·3	8·5 ± 1·8
Tryptamine	0	0
N-Acetyltryptamine	0	0
5-Hydroxytryptamine (serotonin)	0	0
5-Hydroxy-N-acetyltryptamine (N-acetylserotonin)	3·1 ± 0·6	34·7 ± 4·8*
4-Hydroxy-N-acetyltryptamine	0	0
Phenol	20·0 ± 2·3	26·6 ± 3·1
p-Aminophenol	0·9 ± 0·1	0·6 ± 0·2
N-Acetyl-*p*-aminophenol	9·1 ± 1·5	27·0 ± 2·8*
Acetanilide	0	0
Estradiol	31·5 ± 3·3	25·3 ± 3·4
Stilbestrol	47·0 ± 2·8	45·4 ± 3·2

*$P < 0.001$.

Treatment and enzyme assay were carried out as described in Methods. The mean value ± SE of the mean is given.

there was a 2- to 10-fold increase in enzyme activity with certain substrates which contained a monohydroxy and an N-acetyl group, although these compounds were otherwise unrelated in structure. The metabolic products were not identified after benzpyrene and cold stress although they have been before this treatment.[8]

The increase in enzyme activity could reside in the microsomal fraction or soluble supernatant fraction. The subcellular localization of the activated enzyme was examined in the following experiment. Soluble supernatant fractions obtained from control and treated rats were cross-mixed with microsomes from control and treated

rats. These four mixtures were then incubated with N-acetylserotonin. From the results shown in Table 2 it is apparent that the increased enzyme activity occurred only in the microsomal fraction.

It is most likely that the increase in enzyme activity is mainly due to the hydroxylating enzyme, since benzpyrene–cold stress treatment causes a 10-fold increase in the

TABLE 2. ENZYMATIC HYDROXYLATION AND O-METHYLATION OF N-ACETYLSEROTONIN IN BENZPYRENE AND COLD-STRESS-TREATED RATS: LOCALIZATION OF THE ACTIVATED ENZYME

Subcellular fraction	Enzyme activity (mμmoles O-methylated product formed/g liver)
Untreated soluble fraction + untreated microsomes	2·8
Untreated soluble fraction + treated microsomes	23·7
Treated soluble fraction + untreated microsomes	1·7
Treated soluble fraction + treated microsomes	24·5

Treatment and enzyme assay are described in Methods.

N-acetylserotonin metabolite and only a 50 per cent rise in microsomal O-methylation. Furthermore, this increase is not due to catechol-O-methyl transferase in the soluble fraction, since this enzyme is in 100-fold excess (see Table 5). There is also the possibility that benzpyrene–cold stress treatment might induce the formation of a new metabolic reaction (e.g. O-methylation of the monophenol or by the addition of a hydroxy group which is not *ortho* to the monohydroxy group). The formation of such metabolites could involve a fundamental change in the substrate specificity of catechol-O-methyl transferase which seems to be an unlikely possibility.

The effect of various treatments on formation of O-methylated dihydroxy derivatives from monohydroxy compounds

The combination of 3,4-benzpyrene and cold stress was used in the initial studies reported here because it produced greater stimulatory effects on hydroxylation than did other treatments used.[10] Other compounds such as phenobarbital[11] and prednisolone which have been shown to affect microsomal enzymes, and serotonin which is a potent inhibitor for the hydroxylation of N-acetylserotonin *in vitro*,[4] were examined for their ability to affect the formation of O-methylated dihydroxy derivatives from monohydroxy compounds (Table 3).

Again a stimulatory effect by 3,4-benzpyrene was found only with substrates having an N-acetyl group. The increase in N-acetyltyramine hydroxylation was not as great as that observed in combination with cold stress. No significant increase in activity due to cold stress was found except with N-acetylserotonin as the substrate. Serotonin inhibited enzyme activity only when N-acetylserotonin or phenol was used as a substrate. A significant increase in enzyme activity after phenobarbital treatment was found only with N-acetylserotonin as the substrate. No effect was observed with prednisolone treatment.

TABLE 3. EFFECT OF VARIOUS TREATMENTS ON FORMATION OF O-METHYLATED DIHYDROXY DERIVATIVES IN RAT LIVER WITH DIFFERENT SUBSTRATES

Treatment	Substrate					
	N-acetyl-serotonin	Tyramine	N-acetyl-tyramine	Phenol	N-acetyl-*p*-aminophenol	Estradiol
Control	3·5±0·2	10·5±2·1	46·4±4·1	37·2±3·8	8·7±0·9	43·5±2·7
Phenobarbital	5·3±0·3*	14·9±2·1	55·1±8·0	40·3±2·9	8·7±0·5	44·2±4·6
Prednisolone	3·5±0·2	14·9±2·8	45·2±3·1	28·8±3·1	7·8±0·4	47·4±4·1
Benzpyrene	28·0±3·6†	12·2±2·3	58·2±3·7‡	37·1±3·5	21·8±1·8†	50·6±5·5
Serotonin	1·8±0·2*	7·9±1·1	38·9±5·0	24·9±3·0	7·8±0·3	42·1±2·6
Cold stress	7·9±1·4*	8·7±1·5	50·4±4·1	31·7±4·1	10·5±1·9	40·2±4·3

* $P < 0.01$.
† $P < 0.001$.
‡ $P < 0.05$.
Treatment and enzyme assay were carried out as described in the text. The results are expressed in mμmoles O-methylated product formed per gram liver ± SE of the mean.

Enzymatic O-methylation in microsomes

Subcellular distribution studies of the formation of O-methylated dihydroxy derivaties from monophenols showed that O-methylation could occur in the microsome in the absence of the soluble fraction. These observations suggested the presence of an O-methylating enzyme in the microsomes. Microsomes obtained from rat and rabbit liver were washed four times to remove any traces of soluble fraction and were incubated with *l*-epinephrine or N-acetyltyramine and ^{14}C-S-adenosylmethionine. Both rat and rabbit microsomes O-methylated *l*-epinephrine and N-acetyltyramine (Table 4). The radioactive metabolite formed from epinephrine by liver microsomes was chromatographed in butanol:acetic acid:water (4:1:1) and isopropanol:ammonia:water (8:1:1) and was found to have the same *RF* as authentic metanephrine. The rabbit microsomes formed a greater amount of the O-methylated product from

TABLE 4. FORMATION OF O-METHYLATED CATECHOLS BY LIVER MICROSOMES

Species	Substrate	
	N-Acetyltyramine	*l*-Epinephrine
Rat	16·2	16·6
Rabbit	33·2	12·8

Enzyme assay was carried out as described in the text. Results are given as the mean (2 rabbits, 4 rats) in mμmoles O-methylated product per gram liver.

N-acetyltryamine. This species difference is presumably due to enzymatic hydroxylation rather than O-methylation. The hydroxylating enzyme is greater in the rabbit,[4] while the microsomal O-methylating enzyme is about the same in both species (Table 4).

The relative amounts of O-methylation of *l*-epinephrine by rat and rabbit liver microsomes distinguish the microsomal catechol-O-methyltransferase from soluble catechol-O-methyl transferases which have been shown to be 50 times greater in rat than in rabbit.[5] Also, the pH optimum for microsomal O-methylation was 7·0 compared to 7·9 for the soluble enzyme.[5]

TABLE 5. THE EFFECT OF 3,4-BENZPYRENE AND COLD STRESS ON THE O-METHYLATION OF *l*-EPINEPHRINE BY RAT LIVER MICROSOMAL AND SOLUBLE FRACTIONS

Treatment	Microsomal	Soluble
	Metanephrine formed (mμmoles/g)	Metanephrine formed (mμmoles/g)
Control	22·7 ± 1·0	2,530 ± 170
Benzpyrene	32·6 ± 2·7*	2,520 ± 90
Cold stress	34·6 ± 2·6†	2,360 ± 180
Benzpyrene + cold stress	35·3 ± 1·6†	2,700 ± 130

* $P < 0.01$.
† $P < 0.001$.
Treatment and assay are described in the text. Results are expressed as mμmoles O-methylated epinephrine (metanephrine) formed per hour per gram ± S.E. of the mean. Microsomes equivalent to 20 mg liver or soluble fraction equivlaent to 0·2 mg liver were incubated with 5 μmoles $MgCl_2$, 3·7 μμmoles ^{14}C S-adenosylmethionine and 200 μliters 0·5 M, pH 7·0 (for microsomes), or 0·5 M, pH 7·9 (for soluble fraction), in a final volume of 0·6 ml.

Rats were treated with 3,4-benzpyrene, cold stress, and a combination of the two to determine whether the activity of the microsomal O-methylating enzyme could be increased. All three treatments gave approximately 50 percent increase when epinephrine was used as a substrate (Table 5). No significant increase was produced in the soluble O-methylating enzyme from the same rats.

DISCUSSION

There are many monohydroxy compounds that can form O-methylated catechols by enzymes in the microsomal and soluble fractions. Treatment of rats with benzpyrene and cold stress can increase the enzymatic formation of O-methylated catechols only when the substrate contains an N-acetyl monohydroxy group. Since acetylation decreases the polarity, this may indicate an increased permeability to these compounds into the microsomal membrane, thus making the enzyme sites more accessible. Such a mechanism for increased microsomal enzyme activity has been suggested by Gelboin and Sokoloff.[12] An alternative explanation for the increase in metabolism is the induction of these enzymes. Convincing evidence for induction of microsomal enzymes by hydrocarbons[13] and phenobarbital[14] treatment has recently been reported. The observation that enzyme activity is increased by some substrates and not by others would suggest that more than one enzyme may be involved in the hydroxylation reaction. Evidence of the presence of more than a single enzyme for catechol formation in the microsomes has been reported.[4]

A surprising finding was the ability of microsomes to carry out the O-methylation reaction. The microsomal O-methylating enzyme has a lower pH optimum (pH 7·0

compared to 7·9). Enzyme activity in rat and rabbit microsomes are approximately equal, whereas soluble catechol-O-methyltransferase is 50 times greater in the rat than in the rabbit. Treatment with cold stress, benzpyrene, or a combination of the two increases the activity of the microsomal enzymes but has no effect on soluble catechol-O-methyltransferase. Recently, Tomita *et al.*[15] described another microsomal O-methylating enzyme which appears to be different from the microsomal enzyme described here with respect to its subcellular distribution.

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