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Stereochemical Course of the Transmethylation Catalyzed by Catechol *O*-Methyltransferase*

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The steric course of the methyl group transfer catalyzed by catechol *O*-methyltransferase was studied using *S*-adenosylmethionine (AdoMet) carrying a methyl group made chiral by labeling with ^1H, ^2H, and ^3H in an asymmetrical arrangement. Incubation of the two diastereomers of this substrate with catechol *O*-methyltransferase purified from rat liver and epinephrine or protocatechuic acid as acceptor gave the corresponding methylated catechols. These were degraded to convert the methoxy group in a series of stereochemically unambiguous reactions into the methyl group of acetate, which was then analyzed for its configuration. The results indicate that the transfer of the methyl group from AdoMet to either acceptor occurs in an inversion mode. The catechol *O*-methyltransferase reaction thus involves a direct transfer of the methyl group from the sulfur of AdoMet to the oxygen of the catechol in an S_N2 process, without a methylated enzyme intermediate.

Transmethylation reactions involving the transfer of the *S*-methyl group of *S*-adenosylmethionine (AdoMet) to a variety of nucleophiles as acceptors play an important role in many biological processes (1). Yet, their detailed mechanism is not well understood. Enzymes catalyzing this type of reaction can be divided roughly into three categories: (*a*) enzymes operating in "bulk" metabolic transformations, both in primary and secondary metabolism; (*b*) enzymes functioning in neuronal and neuroendocrine mechanisms, *e.g.* phenylethanolamine *N*-methyltransferase or catechol *O*-methyltransferase; and (*c*) enzymes involved in functional processing of informational biological macromolecules, *i.e.* DNA-, RNA-, and protein methylases.

In order to provide further insight into the mechanisms of enzymatic methyl group transfer, studies have recently been initiated to probe the stereochemical fate of the methyl group in such processes. Work from our laboratory (2-4) and from that of Arigoni (5) has dealt with several enzymes in the first category, and has shown that in each of these cases, the transfer of the methyl group occurs with inversion of configuration. In the present paper, we present results of a study on the stereochemical fate of the methyl group of AdoMet[1] in the transfer reaction catalyzed by catechol *O*-methyltransferase, the first example of such a study on an enzyme of the second category.

EXPERIMENTAL PROCEDURES

Materials

Organic and inorganic chemicals were purchased by Aldrich Chemical Co. and Alfa-Ventron Corp., respectively. Magnesium chloride, Trizma base, epinephrine ditartrate, DL-metanephrine-HCl, and ammonium reineckate were purchased from Sigma. Radioactive compounds were purchased from Amersham. All chemicals were reagent grade and were used without further purification.

S-Adenosylmethionine synthetase (ATP:L-methionine *S*-adenosyltransferase, EC 2.5.1.6) was isolated from frozen rabbit liver (Pel Freeze Biologicals, Rogers, Ark.) by the method of Cantoni (6). Adenosine deaminase (76 units/mg of protein, 600 units/ml) was isolated from *Aspergillus oryzae* (Sanzyme-R from Calbiochem-Behring) by the procedure of Sharpless and Wolfenden (7). Catechol *O*-methyltransferase (EC 2.1.1.6) (15 units/mg of protein, 45 units/ml) was isolated and purified by a slight modification (8) of the method of Nikodejevic *et al.* (9).

The (*methyl-S*)- and (*methyl-R*)-[*methyl*-^{14}C,$^2H_1$,$^3H_1$]methionines were synthesized by the method of Woodard *et al.* (3) and converted to AdoMet as described previously (6).

Methods

Radioactivity was measured in Aquasol with [^{14}C]- and [^3H]toluene as an internal standard in a Beckman LS-7000 liquid scintillation counter.

An Altex model 322 MP liquid chromatograph was used to monitor progress of the enzymatic reaction. A Partisil-10 ODS-2 15% C_{18} reverse phase column (4.6 × 250 mm) was employed for the separations. Chromatographic conditions were as follows: mobile phase, 1 M KPO_4 buffer, pH 3.3; flow rate, 0.55 ml/min; uv detector (254 nm wavelength monitor) set at 0.32 absorbance units full scale sensitivity. Retention times (in minutes) were as follows: AdoMet, 15.0; AdoHcy, 30.0; InoHcy, 40.0; 3-methoxy-4-hydroxybenzoic acid, 117.0; metanephrine, 24.

The chirality of the methyl group of acetate was determined by the method of Cornforth *et al.* (10) and Arigoni *et al.* (11) using a previously described procedure (4).

Enzyme Incubations—The following reagents were incubated at 37°C for 1 h: 250 μl of $MgCl_2$/Tris·HCl buffer, pH 7.9 (1 volume of 48 mM $MgCl_2$, 3 volumes of 1.334 mM Tris·HCl), 250 μl of 50 mM epinephrine; 500 μl of adenosine deaminase; 500 μl of catechol *O*-methyltransferase; 250 μl of AdoMet from *R*-acetate (2.6 μmol/ml, 1.72 μCi/ml, $^3H/^{14}C$ = 3.09), and 750 μl of H_2O. After the incubation, the mixture was frozen, lyophilized, and extracted with methanol or chloroform to give the chirally labeled metanephrine. The same procedure was used for the incubation of the AdoMet from *R*-acetate with 250 μl of 10 mM protocatechuic acid (3,4-dihydroxybenzoic acid), except that the product was extracted with chloroform. The incubation mixtures using the AdoMet from *S*-acetate were as follows: 460

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[1] The abbreviations used are: AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; InoHcy, *S*-inosylhomocysteine; COMT, catechol *O*-methyltransferase; Ad, adenosine deaminase.

μl of $MgCl_2$/Tris·HCl buffer; 460 μl of 50 mM epinephrine or 10 mM protocatechuic acid; 920 μl of adenosine deaminase; 920 μl of catechol *O*-methyltransferase; 460 μl of AdoMet (9.32 μmol/ml, 6.34 μCi/ml, $^3H/^{14}C = 2.35$) and 1380 μl of H_2O.

Degradation—The methylated catechols were diluted with 25 μmol of cold material in 100 μl of H_2O containing 2 μl of methanol, and 0.2 mmol of $Ce(NH_4)_2(NO_3)_6$ (12) was added to the mixture which was magnetically stirred in a 25-ml round bottom flask. After 10 min at room temperature, 1.5 ml of glycol was added to the mixture. The reaction flask was connected via a vacuum U-tube to a trapping flask containing 1.5 ml of cyclopentane and 1 μl of methanol. The reaction flask was heated at 80°C for 5 days while the second flask was cooled to −80°C to trap the chiral methanol. The cyclopentane was removed from the trapping flask while still at −80°C. The aqueous layer was extracted with 3 × 0.5 ml benzene and the extract was added to the cyclopentane solution. Then, 300 μmol of NaH and 100 μmol of benzenesulfonyl chloride was added to the latter and the reaction was stirred at room temperature for 30 min. Excess methanol (5 to 10 μl) was then added to convert the sulfonyl chloride to the methyl ester. The reaction mixture was filtered, dried over Na_2SO_4, and evaporated to dryness. The yield was 5 to 15%, based on radioactivity.

The chiral methylbenzenesulfonates were converted to acetates by displacement with cyanide, oxidation of the acetonitrile to acetamide, and diazotation of the latter, using the same procedures described previously for the conversion of chiral methylditosylimines to acetate (3).

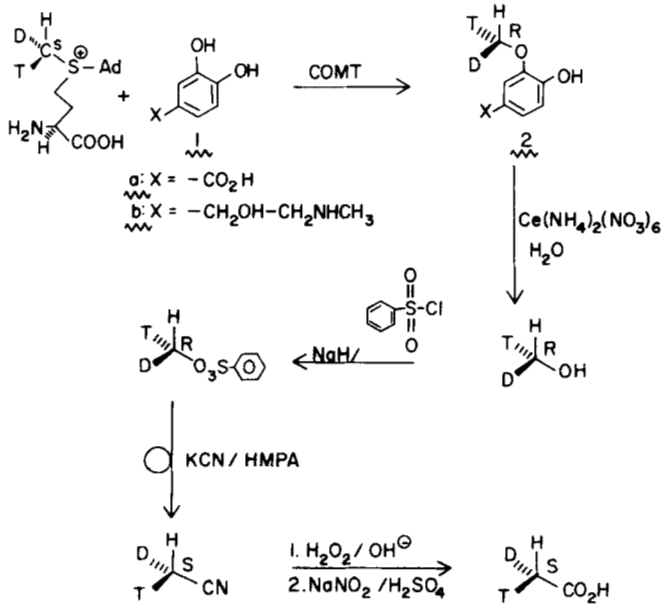
RESULTS

Catechol *O*-methyltransferase catalyzes the reaction shown at the top of Scheme 1. While epinephrine (Scheme 1, **1b**) is the primary physiological substrate, the enzyme can also methylate other catechols, *e.g.* protocatechuic acid (Scheme 1, **1a**). In the present study, we determined the stereochemical fate of the methyl group of AdoMet in the catechol *O*-methyltransferase-catalyzed transfer to epinephrine to produce methanephrine (Scheme 1, **2b**) and to protocatechuic acid to give 3-methoxy-4-hydroxybenzoic acid (Scheme 1, **2a**). The latter reaction was of interest because a number of kinetic studies on this enzyme have been carried out using protocatechuic acid as substrate. To unravel the cryptic stereochemistry (13) of these methyl transfer reactions, we used the chiral methyl group methodology (see Ref. 4), *i.e.* the methyl group being transferred was made chiral by virtue of isotopic substitutions of 1 hydrogen each by deuterium and tritium.

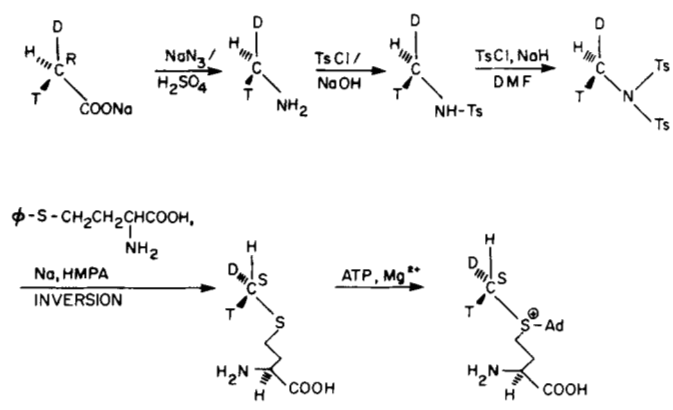
A study of this kind involves three distinct tasks. These are the synthesis of a stereospecifically labeled substrate of known absolute configuration, conversion of this substrate into the enzyme reaction product, followed finally by degradation and analysis of the latter to determine the configuration of the stereospecifically labeled center in the product. The synthesis of AdoMet carrying a chiral methyl group started from chiral sodium [2-$^2H_1$,$^3H_1$]acetate, prepared enzymatically from phosphoglyceric acid labeled stereospecifically at C-3 with deuterium and/or tritium (2–4), and involved the reaction sequence shown in Scheme 2. Chemical conversion of acetate into methionine in a 15 to 25% yield by our previously published procedures (2–4) was followed by enzymatic activation of methionine as described by Cantoni (6) to give AdoMet in a 24 to 30% yield (based on methionine). It will be noted that the conversion of acetate into AdoMet involves one inversion of configuration of the methyl group; hence the AdoMet from *R*-[2-^{14}C,$^2H_1$,$^3H_1$]acetate will carry a chiral methyl group of *S* configuration and that from *S*-acetate will have a methyl group of *R* configuration. No significant change in the $^3H/^{14}C$ ratios was observed throughout this reaction sequence.

Incubations with the two stereoisomers of chirally labeled AdoMet were carried out using catechol *O*-methyltransferase purified from rat liver. Limitations of the yield of conversion of AdoMet due to the well known product inhibition by AdoHcy (8, 14) were overcome by including adenosine deaminase in the assay mixture (8). High pressure liquid chromatographic assay of the reaction mixtures at the end of the incubation period indicated the presence of only two radioactive components, AdoMet and **2b** or **2a** (Scheme 1), in a ratio of 1.8:8.2 in the case of **2b**, and 2.4:7.6 in the case of **2a**. The reaction products were extracted from the lyophilized reaction mixtures with methanol or chloroform and diluted with small amounts of carrier material for degradation.

The third task involved conversion of the *O*-methyl group of the enzyme reaction products in a sequence of stereochemically unambiguous reactions into the methyl group of acetate for subsequent chirality analysis. This was accomplished by the reaction sequence shown in Scheme 1. The crucial step is the oxidation of **2a** or **2b** with the Ce^{4+} ion to give methanol. This reaction is known to proceed with cleavage of the bond between the oxygen and the aromatic carbon (12); hence, the methanol will have the same configuration as the methoxy group in **2a** or **2b**. A control experiment showed that [$^{14}CH_3$]AdoMet under those conditions does not give any methanol; thus, even if any unreacted AdoMet should have been carried along in the extraction, this would not alter the results. The reaction requires water; however, in view of the need for anhydrous conditions in the next step and the difficulty of recovering traces of methanol from large volumes of water, the reaction was conducted in the presence of a limited amount of water, followed by addition of glycol to aid in the separation of methanol and water by slow distillation. The



SCHEME 1



SCHEME 2

TABLE I

$^3H/^{14}C$ ratios, F values, and configurations of substrate and product methyl groups in the COMT reaction

Compound	$^3H/^{14}C$ ratio (total μCi ^3H)	*F* value found (theoretical)	Methyl group configuration	$^3H/^{14}C$ ratio (total μCi ^3H)	*F* value found (theoretical)	Methyl group configuration
Starting [2-^{14}C,$^2H_1$,$^3H_1$]acetate	2.6 (2 × 43.3)	28	*S*	3.1 (2 × 49.1)	68	*R*
[*methyl*-^{14}C,$^2H_1$,$^3H_1$]Methionine	2.3 (2 × 10.5)		*R*	3.2 (2 × 7.36)		*S*
[*methyl*-^{14}C,$^2H_1$,$^3H_1$]AdoMet	2.4 (2 × 3.17)		*R*	3.1 (2 × 1.72)		*S*
Metanephrine (Scheme I, **2b**)	(2.56)			(1.4)		
Acetate from metanephrine	2.3 (3.5×10^{-2})	68 (72)[a]	*R*	3.0 (1.9×10^{-2})	39 (32)[a]	*S*
Metanephrine *O*-methyl group			*S*			*R*
3-Methoxy-4-hydroxybenzoic acid (Scheme I, **2a**)	(2.25)			(1.3)		
Acetate from 3-methoxy-4-hydroxybenzoic acid	2.4 (5.2×10^{-2})	67 (72)[a]	*R*	2.2 (7.2×10^{-3})	44 (32)[a]	*S*
3-Methoxy-4-hydroxybenzoic acid methyl group			*S*			*R*

[a] Theoretical *F* values of the acetate, if no racemization occurred throughout the entire reaction sequence.

methanol was then converted into its benzenesulfonate under nonhydrolytic conditions. The methylbenzenesulfonates were subjected to cyanide displacement to give acetonitrile with inversion of configuration at the methyl group. The conversion of acetonitrile into acetate by alkaline hydrogen peroxide oxidation followed by diazotation of the resulting acetamide (3, 15) proceeds in almost quantitative yield and avoids the risk of racemization by α-hydrogen exchange inherent in the hydrolytic conversion. Although the yield of methylbenzenesulfonate from **2a** and **2b** was rather poor, only 2 to 5%, the amount of radioactive acetate obtained from the degradations, between 1.6×10^4 and 1.1×10^5 dpm of tritium, was quite adequate for configurational analysis.

The chirality of the acetate samples from these degradations was determined by the method of Cornforth *et al.* (10) and Lüthy *et al.* (11), using essentially Eggerer's procedure (10; see Ref. 4). This method involves conversion of acetate into acetylcoenzyme A, which is condensed with glyoxylate in a reaction catalyzed by malate synthase. Due to a kinetic deuterium isotope effect in the latter reaction, the resulting malate will show an asymmetrical distribution of tritium between the two hydrogens of the methylene group if the methyl group of the acetate is chiral. This tritium distribution can be determined by incubation with fumarase, which stereospecifically equilibrates the *pro-R* hydrogen at C-3 of L-malate with solvent protons. Calibration of the system has shown that malate derived from acetate of *R* configuration retains more than 50% of its tritium in the fumarase reaction, whereas malate from *S* acetate shows less than 50% tritium retention (10, 11). The percentage of tritium retention in the fumarase reaction of this assay is referred to as the *F* value (see Ref. 4); configurationally pure *R*-acetate shows an *F* value of 79, whereas pure *S*-acetate gives an *F* value of 21 (16).

The results from these experiments are summarized in Table I. The starting acetate samples used for the synthesis of methionine and AdoMet were of about 60 to 75% chiral purity. It is known that partial racemization due to proton exchange is an inherent problem in the pyruvate kinase reaction, which was used to generate the chiral methyl group of the acetate (4). In the subsequent conversions, the methyl group suffers a further decrease in the configurational purity, as is evident from the *F* values of the acetate samples from the degradation. We have observed this decrease in chiral purity in all of our studies on transmethylations (2–4) and attribute it, for the most part, to a partial racemization in one of the steps of the methionine synthesis, most likely the Schmidt reaction converting acetate into methylamine. As evidenced by the low $^3H/^{14}C$ ratio of the acetate obtained from the 3-methoxy-4-hydroxybenzoic acid from methionine of *S* configuration, the methyl group in this case may have undergone some additional racemization during the degradation. However, this decrease in chiral purity does not obscure the results of this study. As is evident from Schemes 1 and 2, both the synthesis of AdoMet from acetate and the conversion of the methoxy group of **2a** and **2b** into acetate each involve one inversion of configuration at the methyl group. Thus, the starting acetate and that derived from the degradation will have the same configuration if the enzymatic methyl group transfer proceeds in a retention mode; they will have opposite configurations if it proceeds in an inversion mode. In all four analyses, the acetate derived from the degradation of the enzyme reaction product has the opposite configuration as the starting acetate. Hence, the transfer of the methyl group of AdoMet to either substrate catalyzed by catechol *O*-methyltransferase occurs in an inversion mode, as shown in Scheme 1.

DISCUSSION

Recent isotope effect studies by Hegazi *et al.* (17) have shown that the transfer of the methyl group in the catechol *O*-methyltransferase reaction, or more precisely every transfer of the methyl group in the overall process, occurs through a tight, symmetrical S_N2 transition state. Therefore, no matter how many methylated species are involved in the overall process, every single transfer of the methyl group in this reaction must occur with inversion of configuration. The finding, in the present study, that the transfer of the methyl group of AdoMet to the catechol oxygen catalyzed by catechol *O*-methyltransferase proceeds with net inversion of configuration therefore indicates that the overall process involves an uneven number of transfers of the methyl group, most likely a single transfer.

The kinetic mechanism of catechol *O*-methyltransferase has been a matter of some controversy. Studies by Flohe and Schwabe (18, 19) and by Coward *et al.* (8) strongly support a random Bi Bi mechanism. On the other hand, inhibition studies with tropolones and 8-hydroxyquinolines by Borchardt (20), using protocatechuic acid as a substrate, indicate

a ping-pong mechanism, with AdoMet binding to the enzyme first. Such a mechanism would involve a methylated enzyme as an intermediate and would thus require two transfers of the methyl group, one from AdoMet to a nucleophilic site on the enzyme and a second from there to the catechol oxygen. The stereochemical result of net inversion of the methyl group configuration in the transfer, seen both with epinephrine and with protocatechuic acid as substrate, clearly rules out such a ping-pong mechanism, unless one wants to make the extremely unlikely assumption that it involves not only one, but in succession, two methylated enzyme intermediates. Our results are best compatible with a random Bi Bi mechanism, involving a direct bimolecular transfer of the methyl group from the sulfur of AdoMet to the oxygen of the catechol, in which precise alignment of the two reactants (21) and compression of the S_N2-like transition state (17) are major factors contributing to the rate enhancement brought about by the enzyme.

Finally, the stereochemical results reported here for catechol *O*-methyltransferase indicate that this enzyme, a member of the second class of methyltransferases, which function in neuronal and neuroendocrine processes, conforms to the pattern seen with enzymes of the first category, which are involved in bulk metabolic transformations, *i.e.* *C*-, *N*-, *O*-, and *S*-methyltransferases involved in the biosynthesis of the antibiotic indolmycin (2, 3), the iridoid loganin (4, 5), or vitamin B_{12} (4, 5), and in the transfer of the methyl group of AdoMet to homocysteine (4, 5). So far, without exception, all transfers of an sp^3 carbon catalyzed by methyltransferases which have been examined have been found to proceed with inversion of configuration at the migrating carbon, and all transmethylations from AdoMet to nucleophilic carbon, nitrogen, oxygen, and sulfur atoms studied to date appear to involve a direct transfer of the methyl group from the donor to the acceptor substrate. It will be of interest to examine whether this uniform pattern extends to further examples and particularly, whether it also holds for members of the third category of methyltransferases, the enzymes involved in the processing and modification of informational biological macromolecules.

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REFERENCES

1. Usdin, E., Borchardt, R. T., and Creveling, C. R. (Editors) (1979) *Developments in Neuroscience*, Vol. 5, Elsevier-North Holland, New York
2. Mascaro, L., Jr., Hörhammer, R., Eisenstein, S., Sellers, L. K., Mascaro, K., and Floss, H. G. (1977) *J. Am. Chem. Soc.* **99,** 273–274
3. Woodard, R. W., Mascaro, L., Hörhammer, R., Eisenstein, S., and Floss, H. G. (1980) *J. Am. Chem. Soc.*, in press
4. Floss, H. G., and Tsai, M.-D. (1979) *Adv. Enzymol.* **50,** 243–302
5. Arigoni, D., lectures presented at (*a*) International Symposium on Stereochemistry, Kingston, Ontario, June 27 to July 2, 1976; and (*b*) Central-Great Lakes Regional Meeting of the American Chemical Society, Indianapolis, Indiana, May 24 to 26, 1978
6. Cantoni, G. L. (1957) *Biochem. Prep.* **5,** 58–61
7. Sharpless, T. K., and Wolfdenden, R. (1967) *Methods Enzymol.* **12A,** 126–131
8. Coward, J. K., Slisz, E. P., and Wu, F. Y.-H. (1973) *Biochemistry* **12,** 2291–2297
9. Nikodejevic, B., Senoh, S., Daly, J. W., and Creveling, C. R. (1970) *J. Pharmacol. Exp. Ther.* **174,** 83–93
10. Cornforth, J. W., Redmond, J. W., Eggerer, H., Buckel, W., and Gutschow, C. (1970) *Eur. J. Biochem.* **14,** 1–13
11. Lüthy, J., Rétey, J., and Arigoni, D. (1969) *Nature (Lond.)* **221,** 1213–1215
12. Jacob, P., Callery, P. S., Shulgin, A. T., and Castagnoli, N. (1976) *J. Org. Chem.* **41,** 3627–3629
13. Hanson, K. R., and Rose, I. A. (1975) *Accts. Chem. Res.* **8,** 1–10
14. Coward, J. K., D'Urso-Scott, M., and Sweet, W. D. (1972) *Biochem. Pharmacol.* **21,** 1200–1203
15. Arigoni, D., Lynen, F., and Rétey, J. (1966) *Helv. Chim. Acta* **49,** 311–316
16. Lenz, H., and Eggerer, H. (1976) *Eur. J. Biochem.* **65,** 237–246
17. Hegazi, M. F., Borchardt, R. T., and Schowen, R. L. (1979) *J. Am. Chem. Soc.* **101,** 4359–4365
18. Flohe, L., and Schwabe, K.-P. (1970) *Biochim. Biophys. Acta* **220,** 469–476
19. Flohe, L., and Schwabe, K.-P. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* **353,** 463–475
20. Borchardt, R. T. (1973) *J. Med. Chem.* **16,** 377–382; 382–387
21. Lok, R., and Coward, J. K. (1976) *Bioorg. Chem.* **5,** 169–175