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Kinetic Studies on Catechol *O*-Methyltransferase. Product Inhibition and the Nature of the Catechol Binding Site†

James K. Coward,* Edwin P. Slisz, and Felicia Ying-Hsiueh Wu

ABSTRACT: The potent inhibition of catechol *O*-methyltransferase by one of the products, *S*-adenosylhomocysteine, has necessitated the development of two new assay techniques for monitoring this enzyme-catalyzed reaction. Using these techniques, initial velocity and product inhibition studies have been carried out. The results of these studies are in accord with a mechanism involving random binding of substrates and products to the enzyme. These data, together with data from the literature, suggest a mechanism of enzyme catalysis which involves two modes of catechol binding and participation of the adjacent hydroxyl group in enzyme-catalyzed methylation of catechols.

The metabolic fate of (nor)epinephrine has been extensively studied (Axelrod, 1971) and the important role of the enzyme catechol *O*-methyltransferase (EC 2.1.1.6) in the inactivation of catecholamines is now well established. The enzyme has been isolated and purified by several groups of workers (Nikodejevic *et al.*, 1970; Assicot and Bohuon, 1970; Flohe and Schwabe, 1970; Ball *et al.*, 1971) and some of its physical and chemical properties have been described. In order to get some insight into the mechanism of biological transmethylation processes, we have carried out kinetic studies on several nonenzymic (Coward and Sweet, 1971) and enzyme-catalyzed methyl transfer reactions. Initial kinetic studies on the reaction catalyzed by the catechol methylase revealed a strong inhibition by one of the products, AdoHcy[1] (Coward *et al.*, 1972). This type of inhibition has been observed in the methylation of tRNA (Hurwitz *et al.*, 1964; Kjellin-Straby, 1969; Pegg, 1971), homocysteine (Shapiro *et al.*, 1965), histamine and *N*-acetylserotonin (Zappia *et al.*, 1969), fatty acids (Akamatsu and Law, 1970), and biogenic amines (Deguchi and Barchas, 1971). This is of considerable interest in regulation of methylation (Deguchi and Barchas, 1971) and in the design of methylase inhibitors (Coward and Sweet, 1972; Coward and Slisz, 1973). However, our initial kinetic results showed that because of the strong product inhibition, reliable kinetic data would be difficult to obtain

† From the Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510. *Received September 21, 1972.* This research was supported by funds from the Public Health Service, Grant No. MH-18,038 and CA-10,748.

[1] Abbreviations used are: AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; TTO, toluene–Triton X-100–Omnifluor scintillation fluid; *p*-Cl-HgBzO, *p*-chloromercuribenzoate.

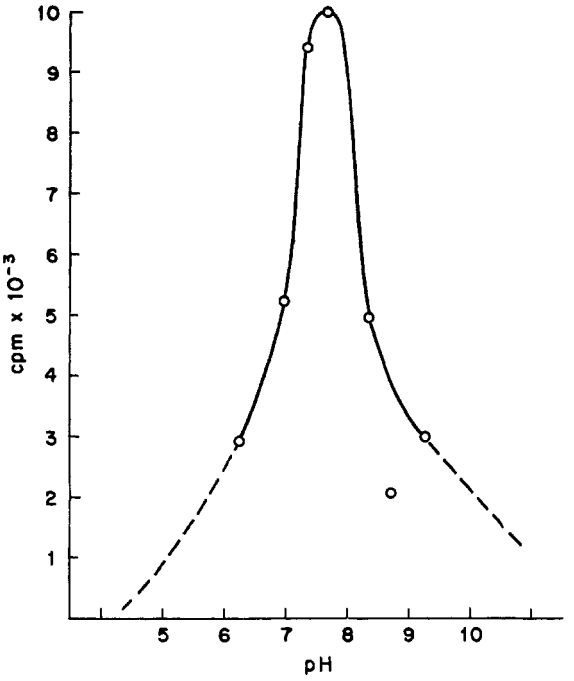


FIGURE 1: pH dependence of the catechol methylase reaction: epinephrine = 2.0 mM; AdoMet = 1.06 mM; $MgCl_2$ = 1.2 mM. Buffers (0.1 M) employed were phosphate, pH 5.9–6.9; Tris, pH 7.5–8.5; and borate, pH 9.0–10.0.

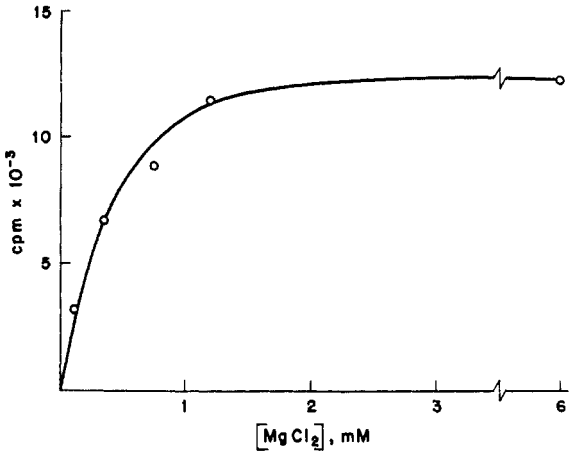


FIGURE 2: Magnesium concentration dependence of the catechol methylase reaction. Conditions are as given for Figure 1, except pH 7.9.

using the standard radiochemical or fluorescence techniques. With the aid of two new techniques which eliminate the inhibitory effect of AdoHcy on the reaction rate, we have carried out initial velocity and product inhibition studies (Cleland, 1970) on the reaction catalyzed by the catechol methylase. The results of these studies and their mechanistic significance are the subject of this paper.

Materials and Methods

Rat liver catechol methylase was isolated and purified by the method of Nikodejevic *et al.* (1970) with minor modifications. Adenosine deaminase was prepared from Taka-diastase (Calbiochem) according to the procedure of Sharpless and Wolfenden (1967); the enzyme used for the spectral assays was purified 200-fold by ethanol fractionation. The protein concentrations of both enzymes were determined by ultraviolet absorbance at 280 and 260 nm (Layne, 1957). *S*-Adenosylmethionine and *S*-adenosylhomocysteine were purchased from Boehringer Mannheim. L-Epinephrine bitartrate, D,L-metanephrine hydrochloride, and 3,4-dihydroxybenzoic acid were from Sigma. D,L-Epinephrine-7-*t* (8–18 Ci/mmol) and Omnifluor were purchased from New England Nuclear Corp. Paranephrine hydrochloride was prepared by a synthetic route originally devised by Dr. Sidney Archer of the Sterling-Winthrop Research Institute (S. Archer, 1972, personal communication). This route involved conversion of 3-acetoxy-4-methoxyacetophenone (Schwarz and Capek, 1952) to the corresponding ω-chloro ketone, followed by reaction with *N*-methylbenzylamine. The resulting *N*-methyl-*N*-benzylaminomethylacetophenone was reduced catalytically using 10% Pd/C catalyst in methanol. Work-up gave crude paranephrine, which was converted to the hydrochloride and recrystallized from ethanol–ether: mp 225–228° dec. *Anal.* Calcd for $C_{10}H_{15}NO_3 \cdot HCl$: C, 51.4; H, 6.84; N, 5.98. Found: C, 51.75; H, 6.11; N, 5.81. All other materials were reagent grade. Stock solutions of AdoMet, epinephrine, metanephrine, and paranephrine were freshly prepared and made up in 1 mM hydrochloric acid in order to minimize decomposition. The concentrations of substrates and products in the stock solutions were determined spectrophotometrically. Scintillation fluid (TTO) was prepared as described by Patterson and Greene (1965) and contained purified Triton X-100 (1 l.), toluene (2 l.), and Omnifluor (5.5 g). All assays were run at 37°.

Assay Procedures. The continuous spectral assay of this transmethylation reaction is based on the known ability of "nonspecific" adenosine deaminase to deaminate adenosine 5′-thioethers while lacking the ability to deaminate the corresponding sulfonium compounds (Schlenk *et al.*, 1971). The conditions employed are such that a low steady state concentration of AdoHcy is rapidly achieved, and transmethylation by the catechol transferase is the rate limiting step in the coupled assay (McClure, 1969). Details of this assay procedure will be published elsewhere (Coward and Wu, 1973). The radiochemical assay is based on the adsorption on alumina of epinephrine in preference to metanephrine (Anton and Sayre, 1962). The conditions described below are such that the per cent conversion of the saturating substrate to product is always less than 5%, and in most cases below 3%. The final volume for each assay was 0.5 ml, and except for experiments where the pH or Mg^{2+} concentration was varied, the final concentration of buffer (Tris, pH 7.9) and $MgCl_2$ were 0.1 M and 1.2 mM, respectively. In a typical assay, a premixed solution of buffer and $MgCl_2$ (100 μl) in a culture tube was placed in ice. To this solution was added 100 μl each of epinephrine (containing up to 25 μCi/ml of epinephrine-7-*t*) and *S*-adenosylmethionine from stock solutions of known concentration. If product inhibition was being studied, a 100-μl aliquot of AdoHcy, metanephrine, or paranephrine solution was added to the tube at this point. For noninhibited experiments, water (100 μl) was added instead of the inhibitor solution. The tube was placed in a water bath and preincubated at 37° for 30 sec, after which the catechol methylase (100 μl, *ca.* 5–10 mg/ml) was added and the resulting solution was incubated at 37° for 3 min. The reaction was quenched by addition of 1.0 g of alumina and 1.0 M Tris buffer, pH 8.4 (2.0 ml). The mixture was shaken for 1 min and then centrifuged for 5 min. An aliquot (1.0 ml) of the supernatant was removed, added to 10 ml of TTO scintillation fluid, and counted on a Packard scintillation counter. For controls,

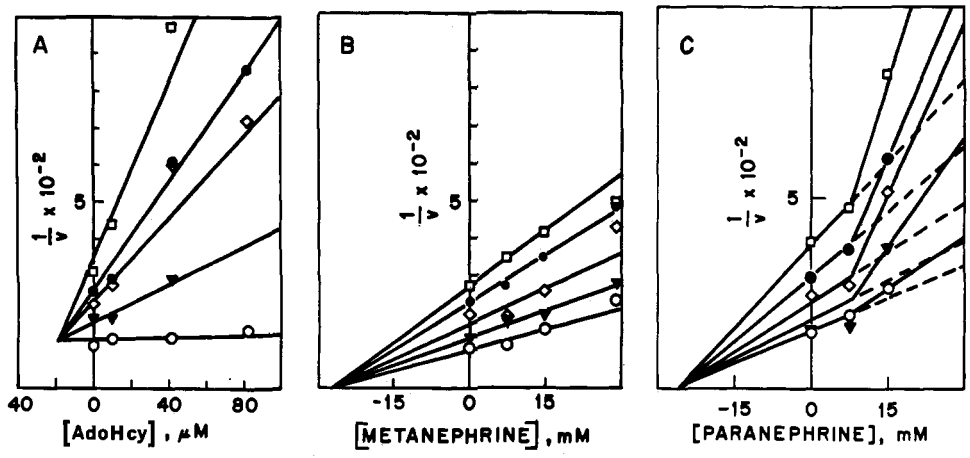


FIGURE 3: Dixon plots of product inhibition at 1.18 mM (○), 0.31 mM (▼), 0.31 mM (◇), 0.14 mM (●), and 0.06 mM (□) AdoMet. Epinephrine = 2.0 mM.

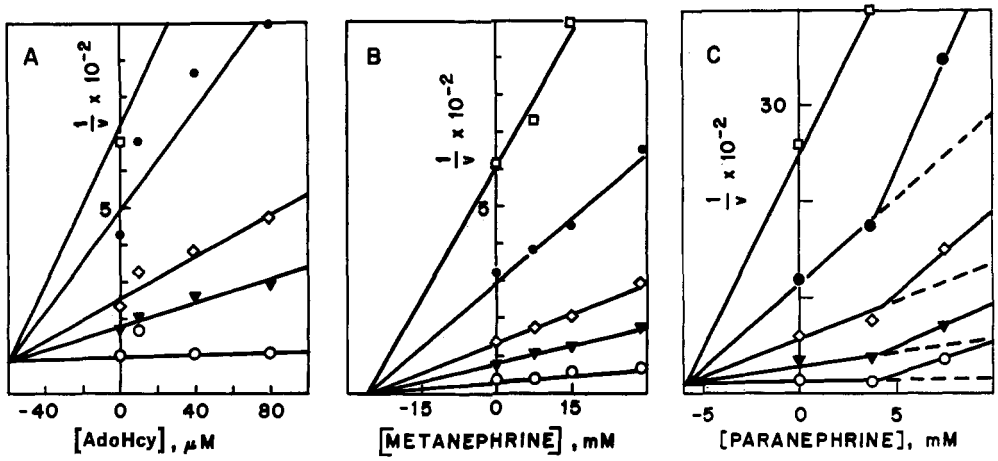


FIGURE 4: Dixon plots of product inhibition at 2.0 mM (○), 0.40 mM (▼), 0.20 mM (◇), 0.08 mM (●), and 0.04 mM (□) epinephrine. AdoMet = 1.06 mM in A and B, 1.15 mM in C.

water (100 μl) was substituted for the catechol methylase. Duplicate enzyme reactions and at least one control were run at each concentration of variable substrate, with and without inhibitor. The amount of methylated product formed was calculated by subtracting the control value from the value obtained in the presence of the catechol methylase, and converting the resulting cpm value to μmole by use of standard solutions of known concentration. All velocities are given in units of micromoles of product formed per minute per milligram of protein.

Results

The effect of variations in pH and Mg^{2+} concentration for the enzyme-catalyzed transmethylation are shown in Figures 1 and 2, respectively. The pH profile is seen to have a very sharp optimum around pH 7.9 and the maximum rate of methylation occurs at *ca.* 2 mM $MgCl_2$. These data agree well with optimal conditions found with several other catechol methylase assay techniques (Creveling and Daly, 1971). The sharp pH optimum observed is presumably due to the known effects of pH on Mg binding to the catechol methylase (Flohe and Schwabe, 1972). A similar sharp pH–rate profile has been reported by Connett and Kirschner (1970) for the reaction catalyzed by phenethanolamine *N*-methyltransferase. The optimum pH of 7.9 was used in all subsequent experiments. Variations in the Mg concentration in the presence of saturating amounts of epinephrine and AdoMet gave results such as those depicted in Figure 2; the apparent K_m derived from these data is *ca.* 0.3 mM. Nearly maximal rates of methylation were obtained at Mg concentrations of 1.2 mM, which was used in all other experiments. Initial velocity experiments, carried out using the coupled spectral assay, gave the type of kinetic data expected of a saturable enzyme-catalyzed reaction. Owing to technical problems involving the high absorbance of AdoMet at 265 nm, large spectrophotometer slit widths were observed at concentrations of AdoMet greater than *ca.* 0.5 mM in the reference cell. This problem was not encountered with epinephrine as the nonvaried substrate, since AdoMet was present only in the sample cell in those experiments. Therefore, the data obtained from the spectral assay with AdoMet as the nonvaried substrate were not useful for ascertaining K_m for epinephrine, since the concentrations of AdoMet present were not saturating. However, the data obtained with epinephrine as the nonvaried substrate led to reliable values of K_m for AdoMet. Product inhibition experiments were carried out using the radiochemical assay. Plots of these data according to the method of Dixon (1953) (Figures 3 and 4) are in accord with the type of inhibition deduced from Lineweaver–Burk plots (not shown) (Lineweaver and

TABLE I: Summary of Kinetic Data from Initial Velocity and Product Inhibition Studies of Catechol *O*-Methyltransferase.[a]

Saturating Substrate[b]	Variable Substrate	Product Inhibitor	K_m (mM)[c]	Type of Inhibn[d]	K_i
AdoMet (1.06)	Epinephrine		0.570 ± 0.150 (5)		
Epinephrine (2.0)	AdoMet		0.105 ± 0.011 (2)[e]		
Epinephrine (2.0)	AdoMet		0.117 ± 0.010 (3)[f]		
AdoMet (1.06)	Epinephrine	AdoHcy		C	60 μM
Epinephrine (2.0)	AdoMet	AdoHcy		C	15 μM
AdoMet (1.06)	Epinephrine	Metanephrine		NC	26 mM
Epinephrine (2.0)	AdoMet	Metanephrine		NC	27 mM
AdoMet (1.15)	Epinephrine	Paranephrine		C	6 mM
Epinephrine (2.0)	AdoMet	Paranephrine		NC	25 mM

[a] Assays were carried out as described in text at pH 7.9 in the presence of 1.2 mM $MgCl_2$. [b] Concentration (mM) given in parentheses. [c] Values are averages plus and minus one standard deviation. Number of determinations given in parentheses. [d] C = competitive; NC = noncompetitive. [e] Spectral assay. [f] Radiochemical assay.

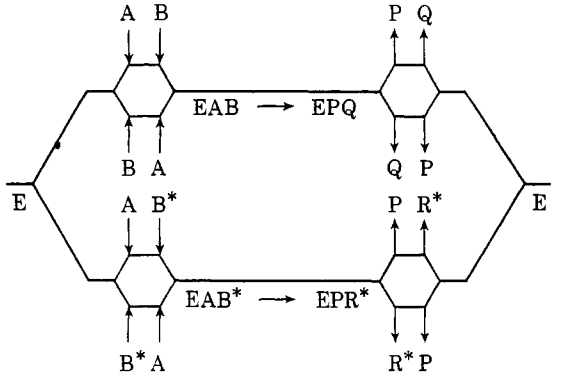
Burk, 1934). Kinetic constants obtained from these experiments are summarized in Table I, together with the type of inhibition observed. These results indicate a random mechanism involving two modes of catechol binding and formation of two dead-end complexes (Scheme I).

Discussion

Enzyme-catalyzed transmethylation reactions are usually monitored by noncontinuous radiochemical assays (Creveling and Daly, 1971), in which the labeled product is extracted into an organic solvent for counting. In order to avoid interference by product inhibition, means of removing the AdoHcy as it is formed or keeping its formation low were devised. The first alternative was achieved by use of the continuous spectral assay. This spectral assay has been used for the initial velocity studies described herein, but could not be used for product inhibition studies. For the latter, we chose to use a radiochemical assay in which the maximum amount of radioactive product was obtained from the reaction mixture under conditions of low conversion to products. This was accomplished by avoiding extraction into organic solvents and using an aliquot of the aqueous reaction mixture directly. By this method we were able to obtain data which could be directly converted to micromoles of product formed, without any assumptions about per cent product extracted. The success in eliminating the inhibitory effect of AdoHcy in the radiochemical assays is demonstrated by the similarity in the value of K_m obtained for AdoMet (Table I) using either the radiochemical assay or the spectral assay in which AdoHcy is removed as formed. The known instability of highly purified catechol methylase and the presence of thiols as stabilizers (Assicot and Bohuon, 1970; Flohe and Schwabe, 1970; Ball *et al.*, 1971) present operational problems in a kinetic investigation. Flohe and Schwabe (1970, 1972) have carried out extensive initial velocity studies using the catechol methylase preparation stabilized with 2-mercaptoethanol. It is known that rat liver preparations will effect the transmethylation reaction from AdoMet to 2-mercaptoethanol (Rosenthal *et al.*, 1965). Therefore, we have utilized a catechol methylase preparation which has been partially purified by the method of Nikodejevic *et al.* (1971), and to which no thiols have been added as stabilizing antoxidants. This preparation is stable for several months if stored at −20°. On warming to 4°, the enzyme becomes less stable and should be used within 72 hr.

Initial velocity studies of the catechol methylase reaction were independently carried out by Flohe and Schwabe (1970). The data from these studies gave rise to double-reciprocal plots which intersected on the negative x axis. The plots showed a lack of effect of either substrate on K_m of the appropriate cosubstrate, and therefore the catechol methylase reaction was classified as random bi-bi (Cleland, 1970). However, this is not a valid conclusion from initial velocity data alone (Cleland, 1970; Mahler and Cordes, 1966); initial velocity studies can only distinguish between sequential and nonsequential mechanisms. Therefore, it is imperative to investigate inhibition by the products in order to distinguish between ordered and random binding processes. We have confirmed the nonparallel nature of double-reciprocal plots based on initial velocity studies and thus a sequential mechanism is indicated. There is no evidence for a Ping-Pong mechanism, such as proposed for histamine *N*-methyltransferase (Thithapandha, 1971), or the thiaminase reaction (Lienhard, 1971). More recently, Flohe and Schwabe (1972) have extended their initial velocity studies over a wide range of pH, and investigated the effects of Mg^{2+} on the rate of reaction and the binding of epinephrine and AdoMet. We have maintained the concentra-

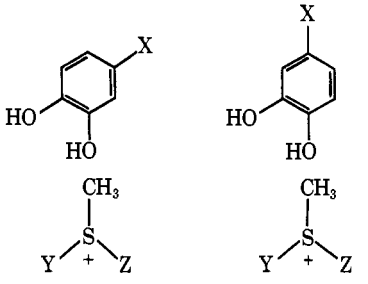
SCHEME I[a]



[a] The dead-end complexes are: EA + Q* ⇌ EAQ* and EA + R ⇌ EAR; A = AdoMet; B = epinephrine; P = AdoHcy; Q = metanephrine; and R = paranephrine.

tion of Mg^{2+} (1.2 mM) and pH of the reaction mixture (pH 7.9) at near maximum values (Figures 1 and 2). Therefore, the extensive studies of Flohe and Schwabe (1972) on pH and Mg^{2+} effects are complementary to the work presented here.

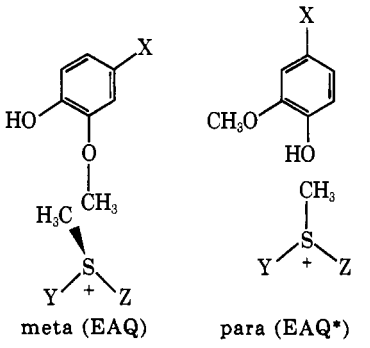
Product inhibition studies of the catechol methylase reaction are complicated because the enzyme catalyzes methylation at both meta and para position of numerous catechols (Creveling *et al.*, 1970, 1972; Frere and Verly, 1971). We have therefore assumed two modes of catechol binding, dependent upon whether the catechol is methylated in the meta or para position (Creveling *et al.*, 1970, 1972). This requires branching of the binding paths to form the reactive ternary complexes (Scheme I). Our observations on product inhibition are compatible with the mechanism put forward in Scheme I, if one assumes these two modes of catechol binding, which can be described as the "meta" and "para" binding modes. The juxtapositioning of reacting groups in these complexes is depicted in **1**. (The letters refer to the complexes shown



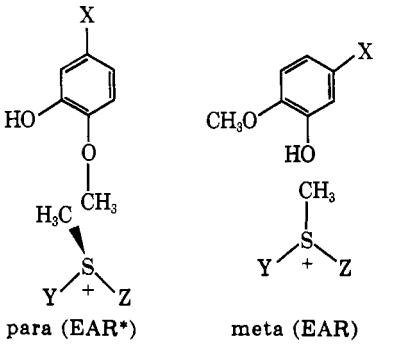
1

in Scheme I). The fact that we observe competitive inhibition by AdoHcy against either AdoMet or epinephrine as the variable substrate (Figures 3A and 4A) indicates that the inhibitor (AdoHcy) is competing with both substrates for the same form of the enzyme, *i.e.*, free enzyme or enzyme complexed with the saturating cosubstrate: The competitive inhibition of the catechol methylase by paranephrine against epinephrine as the variable substrate (Figure 4C) can be interpreted in a similar manner. However, the fact that metanephrine and paranephrine both inhibit the enzyme in a noncompetitive manner against AdoMet as the variable substrate (Figure 3B,C) indicates that a dead-end complex forms between AdoMet and the methylated product.

This type of product inhibition pattern is usually interpreted in terms of a random bi-bi mechanism involving formation of a dead-end complex (Cleland, 1970). The more common dead-end complex between substrate and product lacking the transferred group, *i.e.*, CH_3 in the present case, apparently is not formed since noncompetitive kinetics are not observed in the inhibition of the catechol methylase by AdoHcy against varied levels of epinephrine (Figure 4A). Formation of a dead-end complex between the product and substrate containing the transferred group is not generally observed because of the thermodynamic instability of such a crowded complex (Cleland, 1970). In the catechol methylase reaction, however, it is possible to avoid formation of such an unstable complex by simply rotating the metanephrine molecule from the meta to the para binding mode as shown in **2**. The opposite rotation accomplishes the same result for paranephrine (**3**). The complexes resulting from the interaction of the catechol methylase, AdoMet, and the "rotated" product would be dead-end complexes, since it is known that the



2

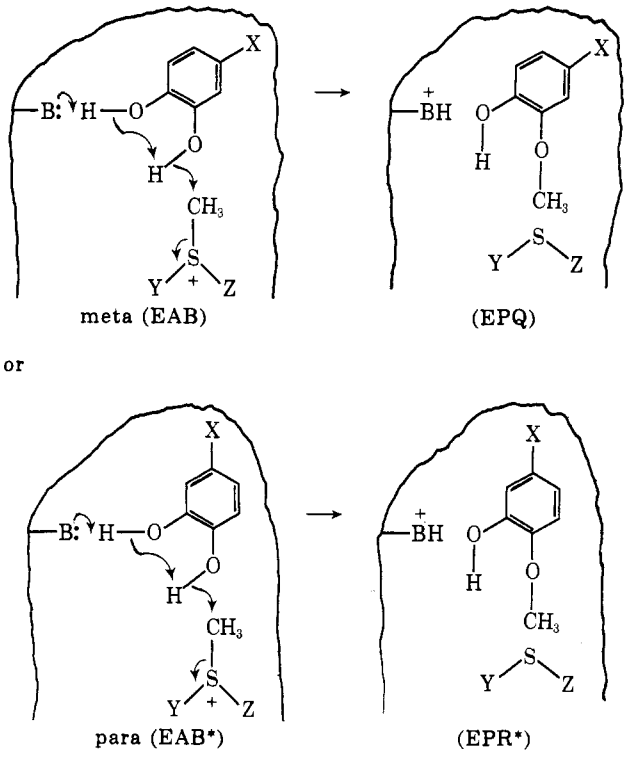


3

catechol methylase does not catalyze the formation of dimethoxy derivatives of catechols (Frere and Verly, 1971).

The noncompetitive inhibition of the catechol methylase by metanephrine *vs.* epinephrine (Figure 4B) as the variable substrate indicates that metanephrine and epinephrine do not compete for the same enzyme form. Binding of metanephrine in the presence of AdoMet requires rotation of the molecule to give EAQ* as shown in **2**, where the side chain, X, is not binding in its "normal" position; *i.e.*, as in EAB (**1**). Under these conditions, the enzyme···AdoMet complex may be forced to undergo certain conformational changes prior to binding metanephrine in order to accommodate metanephrine in the EAQ* complex. This results in a form of the enzyme which differs from the one leading to EAB, and thus noncompetitive kinetics are observed with metanephrine *vs.* epinephrine. In contrast, paranephrine binds as EAR, with the X group in the "normal" position. Therefore no changes in enzyme conformation are required and competitive kinetics are observed with paranephrine *vs.* epinephrine (Figure 4C). The data in Table I show that there is a large difference in the K_i values for the various enzyme–inhibitor complexes, the most obvious being the difference of *ca.* 10^3 in the K_i values for AdoHcy when compared to either of the methylated products. The K_i values obtained for metanephrine acting as a competitive inhibitor against either AdoMet or epinephrine are identical within experimental error. This is in agreement with the suggestion made above, in that the noncompetitive kinetic pattern observed for the inhibition by metanephrine *vs.* epinephrine results from formation of the EAQ* complex. This complex is involved in the noncompetitive inhibition of the catechol methylase by metanephrine *vs.* AdoMet (Figure 3B). The K_i value for paranephrine acting as a noncompetitive inhibitor against AdoMet is close to that obtained for metanephrine (Table I), as would be expected if paranephrine forms a dead-end complex, EAR, similar to the one formed by metanephrine (EAQ*). However, unlike EAQ*, EAR does not involve enzyme conformational changes in order to orient

SCHEME II



the X side chain from its "normal" position. Thus, the enzyme form to which paranephrine binds in the presence of saturating levels of Ado-Met is the same as that to which epinephrine binds, and therefore competitive kinetics are observed (Figure 4C). The fact that little or no distortion of the enzyme is involved in the binding of paranephrine results in a considerably lower K_i value of 6 mM (Table I). The K_i values for paranephrine are derived from extrapolation of the linear portion of biphasic Dixon plots (Figures 3C and 4C). The reason for the deviation from linearity at higher concentrations of paranephrine is not readily apparent at this time, but is probably indicative of a more complex, perhaps quadratic, rate equation. It should be noted that the K_i values reported for paranephrine are known with much less certainty than similar kinetic constants obtained for AdoHcy and metanephrine due to this nonlinearity in the Dixon plots.

The data discussed above are indicative of a catechol methylase active site in which the catechol can bind in two different modes, and the complexes which are formed between the methylated products and AdoMet–enzyme cannot be further methylated. The present work clearly establishes that the catechol methylase reaction involves random addition of the substrates to the enzymes. Although these data are consistent with a random release of products from the ternary EPQ complex, unambiguous proof of this facet of Scheme I has not been possible in the present work. Isotope exchange (Plowman, 1972) which is usually employed to distinguish between random and ordered release of products cannot be utilized with an irreversible reaction such as that catalyzed by the catechol methylase. Solvent isotope effects (k_{H_2O}/k_{D_2O}) in the enzyme-catalyzed transmethylation, in addition to reactions of appropriate model compounds, would be expected if the mechanism of Scheme II is operative for these reactions. It is obvious that such a mechanism precludes further methylation of the monomethylated catechol, since replacing a proton in the catechol OH by a methyl group in the product prevents general-base catalysis in EAQ* and EAR, and therefore these complexes are dead ends. General-base catalysis of nucleophilic attack at sp^3 carbon has received little attention in the chemical literature (Jencks, 1969; Bender, 1971). The cases which have been well studied (Swain *et al.*, 1965; Koskikallio, 1967) involve alcohols with pK_a's considerably higher than catechols. It has been known for some time that the catechol methylase has a reactive sulfhydryl group which can be alkylated by iodoacetate, *p*-Cl-HgBzO (Axelrod and Tomchick, 1959), or *N*-ethylmaleimide (J. K. Coward and B. S., Brown, unpublished data) with concomitant loss of enzyme activity. Recently, Lutz *et al.* (1972) have shown that a meta SH analog of epinephrine irreversibly inhibits catechol methylase by forming a disulfide bond with an active-site SH group. Whether this SH group is functioning as B: in Scheme II remains to be established.

The potent inhibition of the catechol methylase by AdoHcy occurs at levels which are present in plasma and tissue (Salvatore *et al.*, 1971). Utilization of this inhibition by AdoHcy to control biological transmethylation would be a means of studying the significance of these pathways in cellular events. The further metabolism of AdoHcy to noninhibitory products appears to play a role in the regulation of transmethylation (Deguchi and Barchas, 1971; Chung and Law, 1964). However, this degradation of AdoHcy limits its pharmacologic use *in vivo*. The synthesis of stable analogs of AdoHcy as a means of probing the AdoHcy binding site and as possible drugs for *in vivo* regulation of transmethylation has been described in previous papers from this laboratory (Coward and Sweet, 1972; Coward and Slisz, 1973) and is the subject of continuing investigations.

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Some Properties of Hepatic Reduced Nicotinamide Adenine Dinucleotide Phosphate–Cytochrome *c* Reductase†

Takashi Iyanagi‡ and H. S. Mason*

ABSTRACT: A purified NADPH–cytochrome *c* reductase prepared by trypsin solubilization and chromatography from rabbit liver microsomes had the following properties: (1) it contained two flavines per single polypeptide of mol wt 6.8×10^4 (sodium dodecyl sulfate gel electrophoresis) or 7.9×10^4 (sedimentation equilibrium); (2) one flavine was FAD, the other, FMN, approximately equimolar; (3) upon reduction by NADPH in the presence of O_2, an O_2-stable neutral semiquinone containing one flavine free radical per two flavines formed; the other flavine appeared to be fully oxidized; (4) excess NADPH in the absence of O_2 only partially reduced the flavine semiquinone, and the NADPH-residual free radical had properties similar to those of the O_2-stable semiquinone; and (5) the NADPH-reduced enzyme autoxidized by both one-electron equivalent and two-electron equivalent mechanisms. A free radical similar to the O_2-stable flavoprotein semiquinone appeared in whole microsomes when reduced by NADPH in the presence of O_2.

Liver NADPH–cytochrome *c* reductase (NADPH: cytochrome *c* oxidoreductase, EC 1.6.99.2) was first isolated from beef liver acetone powder by Horecker (1950) and was subsequently purified from hog and beef liver microsomes by Williams and Kamin (1962), Phillips and Langdon (1962) and Baggot and Langdon (1970), from rabbit liver microsomes by Nishibayashi *et al.* (1963), and from rat liver microsomes by Omura and Takesue (1970). These various investigators used either trypsin or a lipase to solubilize the enzyme, and reported that it contained one to two molecules of FAD per molecule of enzyme and was free of heavy metal. The enzyme catalyzed one-electron transfer such as a typical one-electron reduction of quinones (Iyanagi and Yamazaki, 1969), cytochrome *c*, and ferricyanide (Masters *et al.*, 1965a,b). The reduction mechanism was clarified by Masters *et al.* (1965a,b), who concluded from spectrophotometric and

† From the Department of Biochemistry, University of Oregon Medical School, Portland, Oregon 97201. *Received March 2, 1973.* This study was supported by grants from the American Cancer Society (BK-1C) and the National Institutes of Health (AM 0718) which we acknowledge with thanks.

‡ On leave from the Department of Biophysics, Research Institute of Applied Electricity, Hokkaido University, Sapporo, Japan.