Properties of Catechol *O*-Methyltransferases from Brain and Liver of Rat and Human

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Kinetic and electrophoretic properties of catechol *O*-methyltransferases (EC 2.1.1.6) from brain and liver were studied. The enzyme of either rat or human tissues exhibited a single molecular form when subjected to electrophoresis at pH 7.9. At pH 9 a second, apparently oxidized, form was detected. Isoelectric-focusing experiments also indicated only one enzyme form, which was identical from extracts of brain and liver of each species ($pI = 5.2$ for rat, 5.5 for human). Similarities between brain and liver catechol *O*-methyltransferase of a given species were also demonstrated by kinetic parameters, *meta/para* ratios of products, and inhibitor potencies. Human catechol *O*-methyltransferase exhibited lower K_m values than did the rat enzyme for *S*-adenosyl-L-methionine, dopamine and dihydroxybenzoic acid. Adrenochrome inhibited both rat and human enzyme. It was concluded (1) that only a single enzyme form could be demonstrated in the physiological pH region; (2) that catechol *O*-methyltransferase of brain could not be distinguished from the liver enzyme of the same species; and (3) that species differences exist between the enzymes of rat and human tissues.

Catechol *O*-methyltransferase (EC 2.1.1.6), which catalyses the *O*-methylation of catecholamines in the brain, is also found in peripheral tissues such as the liver, kidneys and erythrocytes. Because liver is a rich source of catechol *O*-methyltransferase, the enzyme of rat liver has been extensively purified and studied by various workers (Axelrod & Tomchick, 1958; Anderson & D'Iorio, 1968; Nikodejevic *et al.*, 1970; Assicot & Bohuon, 1970; Flohe & Schwabe, 1970, 1972; Frère & Verly, 1971; Creveling *et al.*, 1972; Coward *et al.*, 1973). Little attention has been directed to the enzyme from tissues other than liver or from species other than the rat, although the purification and some kinetic properties of the enzyme of human placenta (Gugler *et al.*, 1970) and of human liver (Ball *et al.*, 1971, 1972) have been reported.

Since catechol *O*-methyltransferase is capable of catalysing the *O*-methylation of almost any catechol, and because of its wide distribution throughout the central nervous system and peripheral tissues (Axelrod & Tomchick, 1958), it was decided to determine whether catechol *O*-methyltransferase may exist as a set of isoenzymes or other multiple forms that might be distributed differently in various tissues. The existence of such forms could be relevant to the suggestions of various authors (Himwich *et al.*, 1967; Brown *et al.*, 1965; Price, 1972; Barrass *et al.*, 1972) that aberrations in human *O*-methylating enzymes

may be causative factors in illnesses such as schizophrenia or Parkinsonism. The purpose of the present study was therefore to compare the kinetic and electrophoretic properties of catechol *O*-methyltransferase from brain and liver of both rat and human. Some of the work with the rat enzyme has been reported in preliminary form (White & Wu, 1973).

Experimental

Extract preparations

Sprague-Dawley male rats were killed by decapitation, and brains and livers were quickly removed. Human autopsy tissue was obtained approx. 5 h after death from a 5-month-old male (crib death) and a 54-year-old male (alcohol poisoning), and approx. 2 h after death from a 64-year-old female (renal failure). All tissues were homogenized at 0°C in 2.5 vol. (w/v) of 0.1 M-KCl containing 0.1 mM-dithiothreitol. The following purification steps were carried out at 0–4°C. The crude homogenates were centrifuged at 14000g for 30 min and then at 100000g for 60 min (Nikodejevic *et al.*, 1970). Supernatants were dialysed against 400 vol. of 1 mM-sodium phosphate–0.1 mM-dithiothreitol, pH 7.0. The pH of the dialysed extract was slowly adjusted to 5.3 with 1 M-acetic acid, and the solution was centrifuged at 14000g for 30 min. After neutralization of the supernatant to pH 6.8, saturated $(NH_4)_2SO_4$ was slowly added. The fraction precipitating between 30

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and 55% saturation with $(\text{NH}_4)_2\text{SO}_4$ contained most of the liver enzyme, and that precipitating between 45 and 65% saturation contained most of the brain enzyme. The $(\text{NH}_4)_2\text{SO}_4$ pellets were collected by centrifugation at 14000g for 30min, resuspended in 3mM-Tris-HCl-0.1mM-dithiothreitol, pH7.0, and eluted through a Sephadex G-100 column (0.9cm $\times$ 30cm) by using the same buffer. Further purification could be accomplished on an ion-exchange column containing DEAE-Sephadex. With an increasing linear salt gradient (100ml of 3–500mM-Tris-HCl buffer containing 0.1mM-dithiothreitol, pH7) the enzyme of rat liver was eluted in a single sharp peak at 200mM-Tris-HCl. Catechol *O*-methyltransferase at this stage was very unstable, but could be stabilized by adding bovine serum albumin (final concentration 0.05%, w/v). However, since the kinetic properties of the enzyme were not changed by the ion-exchange procedure, and because of the possibility that isoenzyme forms might be lost during ion exchange, the comparative studies of brain and liver enzymes were performed with extracts after the gel-filtration step. To study the localization of brain catechol *O*-methyltransferase, synaptosomal and mitochondrial fractions were separated on a discontinuous Ficoll gradient, by the procedure of Abdel-Latif (1966) as modified by Autilio *et al.* (1968). Protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Enzyme assay

For typical assays, components in a total volume of 0.41 ml were as follows: Tris-HCl, pH7.9, 50mM; *S*-adenosyl-L-[Me- ^{14}C]methionine, 0.1mM; catechol substrate [either dopamine (3,4-dihydroxyphenethylamine) or 3,4-dihydroxybenzoic acid, freshly prepared in O_2 -free water and protected from light], 1mM; MgCl_2 , 1mM; dithiothreitol, 0.5mM; EDTA, 0.1mM; and extract (1–100 μg of protein). The reaction was started by adding the substrate *S*-adenosyl-L-[Me- ^{14}C]methionine (obtained from New England Nuclear Corp., Boston, Mass., U.S.A.); 50–55mCi/mmol, more than 97% radiochemically pure, diluted to a specific radioactivity of either 0.3 or 2.4mCi/mmol with unlabelled *S*-adenosyl-L-methionine (P-L Biochemicals, Milwaukee, Wis., U.S.A.). This substrate was found to be of sufficient purity, since enzyme activities were the same whether or not *S*-adenosyl-L-methionine was further purified on an ion-exchange column by the method of Shapiro & Ehninger (1966). Incubation was at 37°C in a N_2 atmosphere for 30min, except in some kinetic studies described below. Blank assays, containing extract but no catechol substrate, were incubated at the same time. After incubation, reaction mixtures containing amine substrates were made alkaline with 0.5ml of 0.5M-sodium borate, pH10, and those in

which acidic catechols were present were treated with 0.1ml of 1M-HCl. *O*-Methylated products were extracted, by the procedure of Axelrod (1962), into 10ml of a toluene-3-methylbutan-1-ol mixture (3:2, v/v, for amine substrates and 7:3, v/v, for acid substrates). A 5ml portion of the organic solvent extract was added to 15ml of scintillation fluor [4g of Omnifluor (New England Nuclear Corp.) per litre of toluene-Triton X-100, 2:1, v/v] and counted at approx. 70% efficiency in a Packard Tri-Carb Scintillation Spectrometer (Downers Grove, Ill., U.S.A.).

Electrophoretic techniques

Electrophoresis was performed at 4°C in an Ortec (Oak Ridge, Tenn., U.S.A.) electrophoresis chamber, with a pulsed-power supply. Experiments were performed at pH7, 7.9 and 9. Polyacrylamide-gel slabs (7%), prepared with 0.35% *NN'*-methylenebisacrylamide and ammonium persulphate polymerization catalyst in 0.375M-Tris-0.035M-citrate buffer, which contained dithiothreitol (0.1–1mM), were pre-electrophoresed at low voltage overnight, with the pulsed-power supply set at 75 pulses/s and 80V, before addition of enzyme samples. After sample application, gels were electrophoresed, with 0.065M-Tris-0.02M-sodium borate as the tank solvent, for 3.5h, by following the instructions in the Ortec Electrophoresis Manual. Maximum voltage was 300V with pulsed-power set at 200 pulses/s and 1.0 μF . Standard proteins (bovine serum albumin and haemoglobin) were electrophoresed in adjacent wells of the same gel slab. Immediately after the run was terminated, gels were cut into lengthwise columns, and sections from cathode to anode were sliced horizontally into either 1 or 2mm fractions. Each slice was assayed for catechol *O*-methyltransferase activity, by using the radiometric method described above. Appropriate sections of the slab were stained with Amido Black to locate standard proteins and also to detect protein bands in enzyme extracts.

Isoelectric focusing

Polyacrylamide-gel slabs were prepared with 5% acrylamide, 0.25% *NN'*-methylenebisacrylamide, 2.4% (w/v) Ampholine (LKB Produkter, Bromma, Sweden) and 0.1mM-dithiothreitol. When short-range pH gradients were prepared (pH range 4–6 or 5–8), an additional 0.2% of Ampholine (pH range 3–10) was included to improve linearity of the gradient. Gels were prefocused overnight at 4°C, at 100V, 0.5 μF and 5–10mA. This allowed the pH gradient to form before enzyme samples were applied. Portions of extract in 20% (w/v) sucrose (usually 50 μl) were placed in sample wells at the top of the gel, and current was applied (150V, 95 pulses/s, 0.5 μF , 10mA initially) for an additional 3 or 4h. The cathodic solvent was 0.15M-ethanolamine, and the

anodic solvent was 0.1 M-HCl (Ortec Manual AN32). Sections of the gel slab were sliced into 1 or 2 mm fractions, and each slice was assayed for enzyme activity. The pH gradient was determined simultaneously by running a series of standard proteins (bovine albumin, haemoglobin, chymotrypsin and chymotrypsinogen) in adjacent wells of the same gel slab. After electrofocusing, portions of gels containing the standards were stained with Bromophenol Blue by the method of Awdeh (1969). With short-range pH gradients a lengthwise column of gel was sliced after electrofocusing into 5 mm portions, each portion was eluted with 2 ml of glass-distilled water, and the pH of the solution was determined with a Fisher microelectrode.

Kinetic studies

For determination of the K_m values for *S*-adenosyl-L-methionine, the concentration of this substrate was varied from 1 μ M to 0.1 mM (10 mCi/mmol) with a catechol concentration of 1 mM, and the incubation time was decreased to 5 min to ensure that substrate consumption remained below 10% at the lowest concentrations. K_m values for catechols were determined by varying catechol concentrations from 0.05 to 1 mM, with *S*-adenosyl-L-methionine concentration held constant at 0.1 mM. Kinetic parameters were extrapolated from plots of reciprocal of velocity against reciprocal of molar concentration of the variable substrate. Other workers have shown that with catechol *O*-methyltransferase of rat liver (Flohe & Schwabe, 1970) and human liver (Ball *et al.*, 1972) the K_m for each substrate is essentially unaffected by the concentration of the other substrate.

Radioautography

For determination of molar ratios of *m*-methoxy to *p*-methoxy products, incubation times were increased to 1 h, under N_2 ; *S*-adenosyl-L-methionine specific radioactivity was 2 mCi/mmol (0.1 mM) and dithiothreitol concentration was 1 mM. After incubation, 10 μ g of each of the appropriate unlabelled *m*-, *p*-, or di-methoxy products was added to the reaction mixtures to serve as carrier and internal standard during chromatography. After adjustment of pH, as described above, the mixtures were extracted with 6 ml of ethyl acetate (Creveling *et al.*, 1972).

A portion (3 ml) of each ethyl acetate extract was evaporated to dryness at room temperature under vacuum, and a 0.5 ml sample was counted for radioactivity by liquid-scintillation techniques to measure the total amount of product. The dried residue was resuspended in 25 μ l of ethyl acetate, and then spotted at the origin of a silica-gel t.l.c. plate (Eastman Chromagram sheets; Rochester, N.Y., U.S.A.) and chromatographed in a Brinkmann (Westbury, N.Y., U.S.A.) sandwich chamber in two dimensions. For products of dopamine *O*-methylation, chromato-

grams were developed twice in the first dimension with butan-1-ol-acetic acid-ethanol (7:2:2, by vol.) and once in the second dimension with chloroform-triethylamine (5:1, v/v). For products of dihydroxybenzoic acid, chromatograms were developed with benzene-methanol-acetic acid (45:8:4, by vol.) in both dimensions. After drying, the chromatograms were placed in direct contact with medical X-ray film (20 cm $\times$ 25 cm; Kodak No-Screen) for 1 week. The positions of [14 C]methylated products were compared with positions of the added unlabelled standard compounds on the chromatogram, as detected by u.v. light or appropriate stains [ninhydrin or Gibbs reagent as used by Creveling *et al.* (1972)]. Areas corresponding to radioactive products were scraped from the chromatogram and counted for radioactivity in scintillation fluor. Incubation mixtures from blank assays, containing extract and *S*-adenosyl-L-[Me - 14 C]methionine, but no catechol, were chromatographed in the same way. The *meta/para* ratio was expressed as the ratio of d.p.m. obtained for the two isomeric products on a given chromatogram.

Post-mortem effects on catechol O-methyltransferase

Sprague-Dawley rats were killed by cervical dislocation. Brains and livers were removed either immediately or after 2 h, 6 h or 23 h at 5°C. Crude homogenates were prepared in 0.9% NaCl and were dialysed against 1 mM-dithiothreitol-1 mM-sodium phosphate, pH 7.0, before assay of enzyme activity.

Gel filtration

Pharmacia columns (0.9 cm $\times$ 30 cm) were prepared at 4°C with Sephadex G-100 and equilibrated with 3 mM-Tris-HCl buffer-dithiothreitol (0.1 or 1 mM), pH 7.0, or with 65 mM-Tris-20 mM-sodium borate-dithiothreitol (0.1 or 1 mM), pH 9.0. Extract (0.1 ml) was applied to the top of the column, and 10-drop fractions were collected from the bottom. A 0.1 ml portion of each fraction was assayed for enzyme activity. Molecular weights were estimated from standard plots of log molecular weight versus V_e/V_0 (Determann, 1968). Molecular-weight standards were bovine serum albumin (67 000), ovalbumin (45 000), chymotrypsinogen A (25 000) and cytochrome *c* (13 000).

Results

Enzyme yields

After the gel-filtration step in the purification procedure, the specific activity of rat catechol *O*-methyltransferase was increased more than 100-fold over the crude homogenate. Activities with *S*-adenosyl-L-methionine and 3,4-dihydroxybenzoic acid as substrates were about 66 and 20 nmol/min per mg of protein for rat liver and brain respectively. Further purification by ion exchange on a DEAE-

Table 1. *Kinetic properties of catechol O-methyltransferase*

K_m values were determined with enzyme after gel filtration as described in the Experimental section. V_{max} was extrapolated from intercepts of reciprocal plots ($1/v$ versus $1/s$). Ratios of V_{max} obtained with 0.1 mM-*S*-adenosyl-L-[Me- 14 C]methionine and extrapolated to saturating concentrations of either 3,4-dihydroxybenzoic acid or dopamine as the second substrate are given below. Yields in crude homogenates were measured with 0.1 mM-*S*-adenosyl-L-[Me- 14 C]methionine and 1 mM-dihydroxybenzoic acid as substrates. Other assay conditions are as described in the text. The two sets of values given for human enzyme were obtained with enzyme from a 5-month-old male and a 54-year-old male respectively.

	Rat		Human	
	Brain	Liver	Brain	Liver
K_m (μ M- <i>S</i> -adenosyl-L-methionine)	6.2	6.2	— 4.8	3.1 3.3
K_m (mM-dopamine)	0.15	0.11	0.083 0.085	0.083 0.091
K_m (mM-3,4-dihydroxybenzoic acid)	0.36	0.40	0.0094 0.013	0.0098 0.015
Relative V_{max} (3,4-dihydroxybenzoic acid/dopamine)	1.1	1.1	1.6	1.2
Yield from tissue (nmol/min per g wet wt.)	23	101	1.4 1.2	6.7 13.2

Sephadex column yielded rat liver fractions with specific activities of 200–700 nmol/min per mg. However, at this high purity the enzyme was very unstable, and as other workers have found (Assicot & Bohuon, 1970; Ball *et al.*, 1971), dithiothreitol was no longer effective in preventing loss of activity during storage. The addition of bovine albumin (0.05%) did stabilize the activity for at least 3 months during storage at -20°C .

Catechol *O*-methyltransferase was found in much lower concentrations in human brain and liver than in corresponding tissues of the rat (Table 1), although the ratios of brain/liver tissue yields were about 0.1–0.2 for both species. The concentration of enzyme found in both brain and liver of the 64-year-old female subject was about 10-fold lower than that found in the tissues of the 5-month-old and 54-year-old males. Also the human enzyme appeared to be more labile than the rat enzyme during purification. Therefore human enzyme from the male subjects was obtained with apparent purification factors of only 46 (liver) and 23 (brain). These preparations (human male) were used in the kinetic and electrophoretic experiments.

Because of the low yields of human enzyme, post-mortem effects on enzyme activity were investigated in rats. These experiments showed that catechol *O*-methyltransferase activities in dialysed crude homogenates of rat brain and liver remained unchanged for at least 6 h after death. At 24 h there was a decrease of only 11% in enzyme activity.

Subcellular localization

About 30% of the total enzyme was present in the initial pellet (14000g) when rat brains were homogenized (at solvent/tissue wt. ratio of 2.5) in 0.9%

KCl–0.1 mM-dithiothreitol. Two-thirds of this activity was solubilized by a single wash with 3 mM-Tris–HCl, pH 7.4. Nearly all of the solubilized enzyme remained in the supernatant of a 100000g centrifugation. Less than 1% of the total enzyme was found in the microsomal fraction. Rat brain synaptosomes prepared on a discontinuous Ficoll gradient contained only 0.7% of total enzyme, and the mitochondrial fraction of the same gradient contained 0.3%.

Kinetic parameters

Table 1 summarizes K_m values obtained with enzyme of rat and human tissues. An optimal concentration of Mg^{2+} (1 mM) was present in all assays. The brain and liver enzymes of each species exhibited similar K_m values for the substrates *S*-adenosyl-L-methionine, dopamine and 3,4-dihydroxybenzoic acid. However, K_m values with human enzyme were significantly lower than those found with the rat enzyme. Maximum velocities obtained with 3,4-dihydroxybenzoic acid as the catechol substrate were greater than with dopamine for all extracts studied.

Values in Table 1 were obtained with extracts purified through the gel-filtration step. Further purification of rat enzyme by ion exchange did not significantly influence K_m values. However, cruder preparations [after $(\text{NH}_4)_2\text{SO}_4$ fractionation and before gel filtration] yielded higher apparent K_m values.

Inhibitors

Species differences in the response of human and rat catechol *O*-methyltransferase to inhibitors were observed, although the brain and liver enzymes of each species gave similar results. Table 2 lists inhibitor

Table 2. *Inhibition of catechol O-methyltransferase*

I_{50} values (inhibitor concentrations causing 50% inhibition) were determined with substrates *S*-adenosyl-L-methionine at 0.1 mM and dopamine at its K_m concentration for each extract, as given in Table 1.

Inhibitor	I_{50} (mM)			
	Rat		Human	
	Brain	Liver	Brain	Liver
Apomorphine	0.1	0.1	1.4	1.4
3,4-Dimethoxy-5-hydroxybenzoic acid	0.1	0.1	0.08	0.08
Adrenochrome	not done	0.04	0.02	0.02

Table 3. *Meta/para ratios of catechol O-methyltransferase products*

Ratios of *m*-methoxy/*p*-methoxy products were determined by two-dimensional chromatography of reaction products after incubations at pH 7.9, followed by exposure of chromatograms to X-ray film. Details are given in the Experimental section.

Substrate	Rat		Human	
	Brain	Liver	Brain	Liver
Dopamine	2.3	2.1	6.2	5.1
3,4-Dihydroxybenzoic acid	4.7	5.0	5.1	4.3

concentrations which produced 50% inhibition of each extract. Although the human enzymes appeared to have a lower affinity for apomorphine, 3,4-dimethoxy-5-hydroxybenzoic acid (Nikodejevic *et al.*, 1970) and adrenochrome were more effective as inhibitors of the human enzyme.

Meta/para ratios

Table 3 summarizes the *meta/para* ratios found after radioautography of reaction products from incubations under conditions described in the Experimental section. These values were 10–20% lower when the dithiothreitol concentration was decreased from 1.0 to 0.1 mM or when the pH was raised from 7.8 to 9.

With either dopamine or 3,4-dihydroxybenzoic acid as the catechol substrate, no significant amount of dimethoxy product was observed. When isovanillic acid (either Sigma, St. Louis, Mo., U.S.A., or Gallard-Schlesinger, Carle Place, N.Y., U.S.A.) was used as substrate, small amounts of *m*- and *p*-products were detected chromatographically, indicating that the commercially available compound contained approx. 2% of a catechol impurity.

An unidentified radioactive product was observed on X-ray films, after radioautography, when *S*-adenosyl-L-[Me- 14 C]methionine and 3,4-dihydroxybenzoic acid were used as the substrates for the human enzymes. This product was more prominent with human brain extracts, representing as much as 5% of the total radioactivity recovered from the t.l.c. plate and measured by scintillation counting, whereas

with human liver extracts only 0.6% of the total radioactivity occurred in this position. This product had lower chromatographic mobilities in both dimensions on the chromatograms than any expected methylated product. It was not found in blanks or when dopamine was the catechol substrate.

Molecular weights

Gel filtration on Sephadex G-100 at pH 7.0 gave relatively crude estimates of molecular weight. Catechol *O*-methyltransferase of rat brain or liver was eluted in the same fraction as was chymotrypsinogen and therefore was estimated to have mol.wt. 25000 ± 3000 . Human enzyme may be slightly smaller than the rat enzyme, since it was eluted just after chymotrypsinogen.

Isoelectric focusing

To detect possible multiple forms of catechol *O*-methyltransferase having different net surface charges, isoelectric focusing was performed on 5% polyacrylamide-gel slabs, with pH range 3–10. Fig. 1 shows that only one activity peak was observed in the electrofocusing patterns with either liver or brain enzyme of rat. When the same extracts were focused on a short-range (pH 4–6) gradient, each extract still exhibited only one enzyme form (Fig. 2) with a pI value of 5.2 ± 0.05 .

Similar results were obtained with rat enzyme at various stages of purification after $(\text{NH}_4)_2\text{SO}_4$ fractionation as far as DEAE-Sephadex elution. The pI value was not affected by increasing dithiothreitol

Fig. 1. Electrofocusing pattern obtained with rat brain (-----) and liver (—) catechol *O*-methyltransferase by using a pH 3–10 gradient

Polyacrylamide gel (5%) was prefocused for 17 h before addition of extracts near the cathodic end. After current was applied for an additional 3 h, the gel was sliced and assayed as detailed in the Experimental section. The left-hand ordinate represents enzyme activity. The pH gradient is indicated by the diagonal line (····) and the right-hand ordinate. Recovery of applied enzyme activity was more than 70%.

Fig. 2. Short-range (pH 4–6) electrofocusing pattern obtained with rat liver (—) and brain (-----) catechol *O*-methyltransferase on a prefocused polyacrylamide-gel slab

The arrow indicates the initial position of the enzyme. Other details are as in the legend to Fig. 1. No enzyme activity was found in any fractions other than those shown. pH gradient is indicated by ●.

concentration in the gels up to 1 mM or by including 1 mM-Mg²⁺ in the system. In addition, pI values were not changed if the enzyme was applied near the anodic end of the gradient rather than the cathodic end. However, enzyme recoveries were greater under the latter condition (over 70% of applied enzyme activity).

In a similar manner, human catechol *O*-methyltransferase was electrofocused on both long-range and short-range pH gradients. However, because the human enzymes exhibited higher pI values than rat enzymes, a short-range gradient of pH 5–8 was more suitable. A typical experiment on such a gradient is shown in Fig. 3. In repeated experiments human enzyme of brain and liver from either the 5-month-old or 54-year-old subject exhibited a single pI value of 5.5±0.1.

Portions of extract electrofocused in adjacent positions on the gel slab and stained for protein indicated that enzyme activity corresponded to a specific protein band, and that three to eight other proteins were also present in the various extracts.

Electrophoresis

Polyacrylamide-gel electrophoresis of human or rat catechol *O*-methyltransferase at pH 6.9 or 7.9 produced only one peak of enzyme activity (Fig. 4). This peak occurred in the same position when gel fractions were assayed with dopamine as the catechol substrate instead of 3,4-dihydroxybenzoic acid. At pH 7.9, human enzyme of brain or liver exhibited the same electrophoretic mobility as bovine albumin, which was used as a standard at an adjacent position on the same gel slab. Rat enzyme moved somewhat slower, with a mobility relative to bovine albumin of about 0.9. Recoveries of applied enzyme activity in different experiments varied from 50 to 70%.

To determine whether the apparently membrane-bound catechol *O*-methyltransferase could be distinguished by electrophoresis from the soluble enzyme, the washed pellet from the first centrifugation (14000g), which contained 11% of total enzyme activity, was extracted with 0.1% Tween-80–0.1 mM-dithiothreitol. This extract, after (NH₄)₂SO₄ fractionation, was subjected to electrophoresis simultaneously with a sample of soluble catechol *O*-methyltransferase at an adjacent position on the gel slab. The extract of solubilized membrane-bound enzyme exhibited a single peak of activity with the same mobility as soluble enzyme.

When electrophoresis was performed at pH 9, recoveries of applied enzyme activity were less than 10% unless dithiothreitol was incorporated into the gels and buffers. With 1 mM-dithiothreitol, enzyme recovery after electrophoresis was about 30%, and two apparent enzyme forms were detected about 4 mm apart on the gels. These two forms catalysed the *O*-methylation of both dopamine and 3,4-

Fig. 3. Short-range (pH 5-8) electrofocusing pattern of human brain (----) and liver (—) catechol O-methyltransferase

The arrow indicates the position where enzyme was introduced. Other details are as in the legend of Fig. 1.

Fig. 4. Electrophoresis of human catechol O-methyltransferase on polyacrylamide gel at pH 7.9

Human liver extract (50 μ l) was applied near the cathodic end of a 7% polyacrylamide-gel slab. Power was increased as follows: 200 V, 90 pulses/s for 5 min; 200 V, 250 pulses/s for 5 min; 300 V, 200 pulses/s for a total of 3.5 h. Gel slices (2 mm) were assayed for enzyme activity, which is represented on the ordinate by c.p.m. of O-methylated product. Total gel distance was 70 mm. No enzyme activity was found in any fractions not shown above.

dihydroxybenzoic acid with the same relative velocities. Gel-filtration experiments at pH 9 also yielded two peaks of enzyme activity, indicating that a larger, perhaps dimeric, enzyme form is generated at pH 9, although enzyme activity is rapidly lost. Dithiothreitol partially stabilized the activity, but did not prevent the appearance of the second enzyme form.

Discussion

The yield of catechol O-methyltransferase from human liver (Table 1) appeared to be lower than that found in a sample of human liver obtained during a kidney-transplant operation by Ball *et al.* (1971). However, it was similar to a value reported by Axelrod & Tomchick (1958). These latter authors observed species differences in enzyme activities of tissue homogenates which varied over a 50-fold range. The kinetic parameters reported by Ball *et al.* (1972) for their human liver preparation were obtained by using 2-hydroxyoestradiol-17 β as a substrate and therefore cannot be directly compared with values found in the present study. Properties of human brain catechol O-methyltransferase have, to our knowledge, not been previously reported.

Although it is important to realize that human enzymes were obtained from only three subjects during the present study, and that concentrations of enzyme might vary with age, sex, or other factors, there is no evidence available that would suggest that marked variations in kinetic properties of this enzyme would be found among individuals of the same species. Post-mortem effects are another consideration, and although experiments with rats indicated that such effects were insignificant over the time-period involved, the human enzyme might be more labile than the rat enzyme during the post-mortem period.

Several different K_m values for S-adenosyl-L-methionine with rat liver catechol O-methyltransferase have been reported in the literature. The value of 6 μ M (Table 1) for the rat enzyme is similar to values reported by McCaman (1965) and by Flohe & Schwabe (1970). The K_m values in Table 1 for catechol substrates are of the same magnitude as values reported by Nikodejevic *et al.* (1970) for 3,4-dihydroxybenzoic acid and for noradrenaline. Creveling *et al.* (1972) have also found that the maximum enzyme velocity with 3,4-dihydroxybenzoic acid as a substrate for rat liver enzyme was approx. 1.1 times that obtained with dopamine.

Although experimentally determined K_m values, especially for a multi-substrate enzyme, may not be a direct indication of substrate affinities, the low K_m values found for 3,4-dihydroxybenzoic acid with human enzyme may suggest that the human enzyme has a higher affinity for acidic than for basic cate-

chols. This would favour the *O*-methylation of products of monoamine oxidase metabolism, sparing relatively more of the catecholamines for re-uptake across the presynaptic membrane.

The inhibition of catechol *O*-methyltransferase by adrenochrome is of particular interest, since this compound is an oxidation product of adrenaline and since other catecholamines can form analogous oxidation products. Adrenochrome is known to cause psychotic symptoms when administered to humans, and its possible participation as a causative agent in schizophrenia has been discussed by Heacock (1971).

Although a number of workers have attempted polyacrylamide-gel electrophoresis of catechol *O*-methyltransferase, they have usually not been successful in recovering enzyme activity from the gels. However, Frère & Verly (1971) reported finding two peaks of enzyme activity after gel electrophoresis of a 20-fold purified rat liver extract at pH 8.3. Axelrod & Vesell (1970), using starch gels at pH 8.6, also found two broad peaks of enzyme activity. Anderson & D'Iorio (1968) detected two or three bands of activity after polyacrylamide-gel electrophoresis of rat liver extract at high pH, using a pyrocatecholphthalein-staining technique, which, in our hands, was relatively insensitive and non-specific.

In the present study the inclusion of dithiothreitol in gels and buffers and pre-electrophoresis of the gels before adding enzyme samples served to increase the stability of catechol *O*-methyltransferase during electrophoresis. These modifications made possible the recovery of about half of the applied enzyme activity when experiments were performed at pH 7.9 or below, conditions under which only single enzyme forms were detected. At higher pH values two peaks of enzyme activity were observed, in agreement with Frère & Verly (1971). However, recovery of enzyme activity was much lower at high pH values, and it is very likely that the second peak of activity was that of an oxidized (perhaps dimer) form of the enzyme. Gel-filtration experiments at pH 9 supported this possibility. A dimeric form of rat liver enzyme has been suggested by Assicot & Bohuon (1970), on the basis of velocity-sedimentation experiments.

Meta/para ratios obtained with 3,4-dihydroxybenzoic acid as the substrate for rat liver catechol *O*-methyltransferase (Table 3) are similar to those reported by Creveling *et al.* (1972) and Thomas *et al.* (1972). The ratios reported by Creveling *et al.* (1972) with dopamine as substrate for rat liver enzyme, using a different procedure for separation of products, were about 2-fold higher than the corresponding values in Table 3. However, as observed by others (Senoh *et al.*, 1961; Frère & Verly, 1971; Creveling *et al.*, 1972) and by ourselves, the experimentally determined *meta/para* ratios depend to a large extent on a variety of factors, including pH, Mg^{2+}

concentration, time of incubation, concentration of dithiothreitol, and techniques by which products are separated and assayed. *Meta/para* ratios of *O*-methylated products estimated *in vivo* (Daly *et al.*, 1960; Mathieu *et al.*, 1972; Price, 1972) are generally higher than those found *in vitro*, suggesting that further metabolism, especially of the *p-O*-methylated product, may occur *in vivo*.

It is possible that the oxidation state of the enzyme may influence the relative proportion of *m-/p*-methoxy products of catechol *O*-methyltransferase, since a decrease in *meta/para* ratios of enzymic products was observed as pH was increased. Other workers (Frère & Verly, 1971; Creveling *et al.*, 1972) have also observed a decreased *meta/para* ratio with increased pH, and have suggested that the effect of pH on the ionization state of the catechol substrate may influence the ratio of products. In our experiments incubations at pH 7.9 produced lower *meta/para* ratios of products if dithiothreitol concentration was decreased in the incubation medium, an observation which indicates that oxidation of the enzyme may significantly affect *meta/para* ratios.

Although catechol *O*-methyltransferase of the rat differed from the human enzyme in kinetic properties, stability, pI values, and response to some inhibitors, the enzyme of brain could not be distinguished from the liver enzyme of the same species. Therefore our results suggest that any attempt to obtain a specific inhibition of brain enzyme for therapeutic purposes cannot be based on intrinsic properties of central-nervous-system and peripheral enzymes.

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