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SUBSTRATE STEREOSPECIFICITY AND SELECTIVITY OF CATECHOL-*O*-METHYLTRANSFERASE FOR DOPA, DOPA DERIVATIVES AND α-SUBSTITUTED CATECHOLAMINES

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Abstract—The substrate specificity of highly purified pig liver catechol-*O*-methyltransferase has been investigated kinetically. This enzyme shows stereospecificity towards the naturally occurring L-isomer of 3,4-dihydroxyphenylalanine (DOPA) which has a higher affinity and maximal velocity as a substrate than the D-form. We have confirmed the implication of the *in vivo* study of Ito *et al.* [1], that methylation of 5-*S*-L-cysteinyl-L-DOPA is catalysed extremely slowly by catechol-*O*-methyltransferase, despite the comparatively high affinity of the enzyme for the substrate. Salbutamol is not a substrate for the enzyme and DL-*threo*-3,4-dihydroxyphenylserine (DOPS) is such a poor substrate that accurate kinetic analysis proved impossible. Alpha-substitution of DOPA, noradrenaline and isoprenaline causes a decrease in the affinity of catechol-*O*-methyltransferase for these compounds. However, the 'suicide' inhibitors of aromatic-L-amino acid decarboxylase (DOPA decarboxylase), fluoro- and difluoro-α-methyl DOPA are more superior catechol-*O*-methyltransferase substrates than α-methyl DOPA, presumably because the electron-withdrawing effect of the presence of fluorine in their structure overcomes the steric influence of the α-methyl group. A DOPA decarboxylase inhibitor in clinical use, benserazide, is, however, a much superior catechol-*O*-methyltransferase substrate and may have the therapeutic advantage of decreasing methylation of L-DOPA [2]. α-Methyl dopamine has a lower K_m and higher V_{max} than the parent compound.

Catechol-*O*-methyltransferase (COMT, EC 2.1.1.6) shows an extremely wide specificity towards catechol substrates including all the catecholamines and their acid [3], alcohol [4] and aldehyde metabolites [5]. Methylation can thus occur at any stage in catecholamine catabolism [6]. Other COMT substrates include apomorphine [7], 2-hydroxy-17β-oestradiol [8], isoprenaline [9], nitrocatechol [10] and *bis*-catecholphthalein [11]. The reaction utilises *S*-adenosyl-L-methionine as the methyl donor [12] in the presence of magnesium ions. The properties of the enzyme have been extensively reviewed [13, 14].

COMT is found in almost all mammalian tissues, including brain [15] but liver is the richest source [16] and it is highly purified enzyme from this organ which was used in this investigation.

The purpose of this study was to ascertain whether COMT shows any degree of kinetic specificity and to investigate the structural requirements of a COMT substrate; for although COMT catalyses the methylation of a wide range of catechol substrates (see above), it does so at greatly varying rates and with an extremely wide range of affinities (see e.g. [2]).

The dopamine deficiency in the central nervous system occurring in Parkinson's disease can be overcome to some extent by administering L-DOPA. 3,4-Dihydroxyphenylserine has been used to increase brain noradrenaline [17]. The effectiveness of these compounds may be increased by co-administration of DOPA decarboxylase (aromatic-L-amino acid decarboxylase, EC 4.1.1.28) inhibitors. The two inhibitors in current clinical use have been shown to be COMT substrates [2] but recently α-fluoromethyl DOPA derivatives have been developed as DOPA decarboxylase inhibitors. Alpha-difluoromethyl DOPA has been implied to be a substrate of COMT and Lo *et al.* [18] have shown α-methyl DOPA levels to be dependent on COMT activity. However, another derivative of DOPA, 5-*S*-L-cysteinyl-L-DOPA, which may have a selective toxicity to tumour cells [19] has been postulated to be a poor COMT substrate from *in vivo* studies of its metabolism [1].

Our study was undertaken to assess *O*-methylation as a potential route of biotransformation of several drugs in the process of development. During the course of the investigation, α-substituted catecholamines were also investigated as COMT substrates, since this feature in their structure seemed to influence the enzyme's action.

MATERIALS AND METHODS

All reagents were of analytical quality (BDH, Poole, UK) and were dissolved in glass distilled water. All pH measurements are relative to 20°.

Catechol-*O*-methyltransferase was purified from deep frozen pig liver by homogenisation, ammonium sulphate precipitation from the supernatant, chromatography of the redissolved precipitate on Sephadex G75 and affinity chromatography on 2,6-dimethoxyphenol - azo - phenyl - methylene - anilino -

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agarose as in [20] but with modified buffer [2]. See Table 1 for the results of this purification.

During purification, COMT activity was assayed by the direct-extraction radiochemical method [21] using 3,4-dihydroxyphenylacetic acid as the methyl acceptor and methyl-tritiated *S*-adenosyl-L-methionine (Radiochemical Centre, Amersham, U.K.) as the methyl donor in 0.20 M triethanolamine hydrochloride buffer pH 7.20. One unit (U) of activity represents the formation of one micromole of product in one minute at 37°.

Adenosine deaminase was partially purified from Takadiastase (Koch-Light, Colnbrook, U.K.) using the method of Sharpless and Wolfenden [22] up to and including the dialysis stage, modified to use 150 g Takadiastase and dissolving the final ethanol precipitate in 75 ml cold water.

The coupled spectrophotometric assay of Coward and Wu [23] was utilised in the kinetic studies, as modified in [21]. The assay contained 1.6 mM magnesium chloride, 0.64 U adenosine deaminase, 0.20 M triethanolamine hydrochloride buffer pH 7.20, 0.456 mM *S*-adenosyl-L-methionine, catechol substrate and 5–25 μl (4.3–21.5 mU) purified COMT, in a final volume of 500 μl. Higher COMT concentrations cause derivations from linearity of response in the assay. The pH and magnesium concentrations are both optimal [20, 21]. Initial rates of decrease in absorbance at 265 nm were measured at 37° in a Beckman Model 35 spectrophotomer.

Determinations were replicated at least eight times and the median used in kinetic analysis. As wide as possible a concentration range of the different varied catechol substrates was used, limited by excessive absorption at high concentrations. The apparent kinetic constants, K_m and V_{max}, were determined by the direct linear plot [24] and the mean and standard error of the mean of the kinetic constants derived from three sets of estimations are given in the results.

Protein concentrations, above 1 mg/ml, were determined by the biuret method [25] and, at lower concentrations, by the method of Mejbaum-Katzenellenbogen and Dobryszycka [26] using standard curves prepared to bovine serum albumin (Sigma, Poole, U.K.).

L-DOPA, D-DOPA, DL-DOPA, dopamine, DL-*threo*-3,4-dihydroxyphenylserine, (−)noradrenaline hydrogen tartrate, (−)adrenaline hydrogen tartrate and 3,4-dihydroxyphenylacetic acid were obtained from Sigma (Poole, U.K.). Isoprenaline sulphate was obtained from Ward, Blinkinsop & Co., Widnes, U.K.

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RESULTS AND DISCUSSION

The purification of pig liver catechol-*O*-methyltransferase used in this study is summarised in Table 1. The specific activity of COMT in the initial homogenate is extremely high which accounts for the comparatively low absolute degree of purification. However the specific activity of the pooled affinity chromatography fractions of this preparation is also higher than that obtained previously [2, 20] implying that the enzyme is indeed homogenous. These discrepancies are almost certainly due to the inherent biological variation in agricultural animals.

Table 1. Purification of pig-liver catechol-*O*-methyltransferase*

Stage	Volume (ml)	Activity (mU/ml)	Total activity (U)	Protein (mg/ml)	Specific activity (mU/mg)	Overall yield (%)	Overall purification (-fold)
Homogenate	2000	0.41	814.63	270.73	1.50	100	1
Redissolved 55% saturating ammonium sulphate precipitate	135	0.91	122.18	121.00	7.48	15.00	4.97
Pooled Sephadex G75 column fractions	450	0.26	118.93	4.75	55.628	14.60	36.98
Pooled affinity column fractions	66	0.86	56.82	0.626	1375.18	6.98	914.05

* See Ref. 20. Pig liver (1322 g) was homogenised in 1200 ml 0.01 M triethanolamine hydrochloride buffer pH 8.00 containing 0.13 M potassium chloride, 10 mM mercaptoethanol, 3 mM dithiothreitol, 0.2 mM phenylmethanesulphonyl fluoride, 2.6 mM magnesium chloride and 1 mM EDTA.

The catechol-*O*-methyltransferase assay contained in a volume of 500 μl: 1.6 mM magnesium chloride, 3.0 mM 3,4-dihydroxyphenylacetic acid, 0.9 mM *S*-adenosylmethionine (0.5002 Ci ^{3}H per mole), 0.64 U adenosine deaminase, 0.20 M triethanolamine hydrochloride buffer pH 7.20 and 5–100 μl appropriate fraction. 15 min incubation at 37°.

Table 2. Apparent kinetic constants of pig-liver catechol-*O*-methyltransferase for DOPA stereoisomers, cysteinyl DOPA, 3,4-dihydroxyphenylacetic acid and adrenaline*

Varied substrate	Concentration range of varied substrate (mM)	Apparent K_m (mM) ± S.E. of mean	Apparent V_{max} (mU/mg protein) ± S.E. of mean	V_{max}/K_m ± S.E. of mean
L-DOPA†	0.72–4.30	1.70 ± 0.015	291.9 ± 4.8	171.7 ± 3.2
D-DOPA	0.72–4.31	2.05 ± 0.017	194.6 ± 3.2	94.93 ± 1.5
DL-DOPA†	0.72–3.60	1.86 ± 0.016	252.5 ± 4.1	135.8 ± 1.7
5-*S*-L-Cysteinyl† L-DOPA	0.49–2.43	0.74 ± 0.006	72.3 ± 1.2	97.7 ± 1.6
3,4-Dihydroxy-phenylacetic acid	0.36–1.44	0.68 ± 0.006	778.3 ± 12.7	1144.6 ± 18.7
(−) Adrenaline†	0.18–0.96	0.51 ± 0.004	590.9 ± 9.6	1158.6 ± 18.8

* Determined by the assay of Coward and Wu [23] as modified by Gulliver and Tipton [21] in the presence of 1.6 mM magnesium chloride, 0.456 mM *S*-adenosyl-L-methionine and 0.2M triethanolamine buffer, pH 7.20, at 37°.
† Consistent deviation from Michaelis–Menten kinetics observed with this substrate at high concentrations.

The most novel results obtained in this investigation are expressed in Table 2. The kinetic constants for L-DOPA agree well with previous studies [2], but the wider concentration range used has enabled us to show deviations from Michaelis–Menten kinetics of the substrate inhibition type. The kinetic constants, and apparent first order rate constant (V_{max}/K_m) for D-DOPA imply that the non-physiological isomer is an inferior substrate. The racemic mixture, as might be expected, shows an activity as a COMT substrate intermediate between that of the resolved isomers. This stereospecificity of COMT for L-DOPA has not been noted before, although indications of a stereoselectivity for the natural *laevo* isomers of noradrenaline and adrenaline have been obtained by Creveling *et al.* [27] working with a less highly purified enzyme activity and an assay without the addition of adenosine deaminase to prevent deviations from linear reaction time course [21]. This stereoselectivity of a major enzyme in DOPA catabolism [28] may explain why L-DOPA causes fewer side-effects in the therapy of Parkinson's disease than the racemic mixture; the D-isomer is biotransformed much more slowly.

We have confirmed the *in vivo* experiments of Ito *et al.* [1] (see Table 2). 5-*S*-L-Cysteinyl-L-DOPA is indeed a poor COMT substrate but, unusually, it has quite a low K_m together with a tiny V_{max} implying tight binding to the enzyme—certainly better than L-DOPA—but that COMT is less capable of catalysing the methylation of 5-*S*-L-cysteinyl-L-DOPA. The other results in Table 2 are incorporated for comparison. The kinetic constants for 3,4-dihydroxyphenylacetic acid are very similar to those obtained previously (K_m = 0.64 mM; V_{max} = 705.6 mU/mg protein [20]; K_m = 0.68 mM; V_{max} = 778.3 mU/mg protein [this study]). However, this is at variance with the results reported in Hagan *et al.* (K_m = 1.46 mM; V_{max} = 1320.0 mU/mg protein [2]). These discrepancies are probably due to inherent biological variation in agricultural animals.

Table 3 contains results which extend and confirm our previous studies on the *O*-methylation of DOPA decarboxylase inhibitors by COMT [2]. The results for the clinically-used inhibitors, benserazide and carbidopa, are very similar to those obtained previously. DL-α-MethylDOPA is an inferior substrate to the parent compound, DL-DOPA, as previously

Table 3. Apparent kinetic constants of pig-liver COMT for DOPA decarboxylase inhibitors*

Varied substrate	Concentration range (mM)	Apparent K_m (mM) ± S.E. of mean	Apparent V_{max} (mU/mg protein) ± S.E. of mean	V_{max}/K_m ± S.E. of mean
Benserazide	0.12–1.20	0.20 ± 0.002	815.3 ± 13.3	4076.5 ± 57
Carbidopa†	0.60–4.80	2.25 ± 0.019	248.3 ± 4.1	110.4 ± 2.0
L-DOPA†	0.72–4.30	1.70 ± 0.015	241.9 ± 4.1	171.7 ± 3.2
DL-DOPA†	0.72–3.60	1.86 ± 0.016	252.5 ± 4.1	135.8 ± 1.7
DL-α-MethylDOPA	1.08–5.40	3.35 ± 0.028	206.2 ± 3.4	61.6 ± 1.2
S-α-Fluoromethyl DOPA†	0.37–3.70	1.92 ± 0.016	326.60 ± 5.3	170.1 ± 2.5
DL-α-Difluoromethyl DOPA†	0.28–2.10	1.74 ± 0.015	393.8 ± 6.4	226.3 ± 4.6

* Determined by the assay of Coward and Wu [23] as modified by Gulliver and Tipton [21] in the presence of 1.6 mM magnesium chloride, 0.456 mM *S*-adenosyl-L-methionine and 0.20 M triethanolamine hydrochloride buffer at 37*.
† Consistent deviation from Michaelis–Menten kinetics observed with this substrate at high concentrations.

Table 4. Apparent kinetic constants of pig-liver COMT for various catechols and their α-substituted derivatives*

Varied substrate	Concentration range (mM)	Apparent K_m (mM) ± S.E. of mean	Apparent V_{max} (mU/mg protein) ± S.E. of mean	V_{max}/K_m ± S.E. of mean
DL-DOPA†	0.72–3.60	1.86 ± 0.016	252.5 ± 4.1	135.8 ± 1.7
DL-α-MethylDOPA	1.08–5.40	3.35 ± 0.028	206.2 ± 3.4	61.6 ± 1.2
Dopamine	0.36–1.80	0.75 ± 0.006	157.5 ± 2.6	210.0 ± 3.5
α-Methyldopamine†	0.20–1.50	0.56 ± 0.005	727.3 ± 12.3	1298.75 ± 22.0
(−) Noradrenaline†	0.12–1.20	0.52 ± 0.004	342.8 ± 5.6	659.2 ± 10.8
(±) α-Methylnoradrenaline	0.36–3.00	1.09 ± 0.012	639.3 ± 11.8	586.5 ± 10.9
(±) α-Ethylnoradrenaline	0.60–5.00	2.43 ± 0.021	686.0 ± 12.3	282.3 ± 5.1
(±) Isoprenaline	0.12–0.60	0.37 ± 0.003	474.8 ± 7.8	1283.2 ± 21.1
(±) α-Ethylisoprenaline	0.50–5.00	1.50 ± 0.014	608.0 ± 10.9	405.3 ± 7.3

* Determined by the method of Coward and Wu [23] as modified by Gulliver and Tipton [21] in the presence of 1.6 mM magnesium chloride, 0.456 mM *S*-adenosyl-L-methionine and 0.20 M triethanolamine buffer, pH 7.20, at 37°.
† Consistent deviation from Michaelis–Menten kinetics observed with this substrate at high concentrations.

shown [2]. However, the presence of fluorine in the α-methyl substituent causes a decrease in K_m, an increase in V_{max} and an increase in the apparent first order rate constant (V_{max}/K_m). This effect is partially disguised by the mono-fluoro derivative being a pure stereoisomer and the di-fluoro compound being racemic. The general conclusion can be made that α-methylation of DOPA produces an inferior COMT substrate that this is overcome, to a large extent, by the huge electron-withdrawing effect of fluorine, which, when substituted on the catechol ring, can cause an enormous alteration to the proportion of para methylation by the enzyme [29]. Furthermore, these results imply that the α-fluoromethyl derivatives would not have as large an effect on the methylation of L-DOPA *in vivo* as benserazide which competes for COMT with DOPA and causes a large demand on the restricted methyl donor pool. This may mean that these compounds will have no clinical advantages over benserazide.

The absolute requirement for a COMT substrate to have a vicinial dihydroxyphenyl structure is illustrated by our attempts to detect methylation of salbutamol catalysed by COMT. Even at salbutamol concentrations as high as 10 mM, methylation was not detected. More surprisingly, DL-*threo*-dihydroxyphenylserine was an extremely poor COMT substrate giving methylation rates so low that kinetic analysis proved impossible using this assay technique. At a concentration of 2 mM DL-*threo*-dihydroxyphenylserine, a rate of only 46.4 mU/mg protein was observed. This illustrates why this compound can be used successfully to elevate brain noradrenaline [17]: catabolism via *O*-methylation is effectively non-existent.

The effect of α-substituents was further investigated and the results are given in Table 4. The effect of α-substitution on β-hydroxylated catecholamines differs from its effect on DOPA, in that although the K_m is raised in the same way, V_{max} is increased. However, the overall effect shown by V_{max}/K_m, is that α-substituted β-hydroxylated catecholamines are inferior substrates with a lower apparent first order rate constant than the parent compounds. This may imply that α-ethylisoprenaline would have a longer lasting effect in asthma than isoprenaline since *O*-methylation is the major catabolic route of these drugs [9]. Indeed, this specificity is similar to that of monoamine oxidase in that α-substituted amines are not substrates for that enzyme [30]. However α-methyl dopamine, lacking the β-hydroxyl group, seems to fall into a different category since it is a better substrate than the parent compound, having a lower K_m and a high V_{max}. It also differs in showing, at high substrate concentrations, deviations of the substrate inhibition type from Michaelis–Menten kinetics.

The general conclusion from these studies is that

Table 5. Comparison of techniques for analysing enzyme kinetic results

Substrate	Analysis by direct linear plot (see ref. 24). K_m	V_{max}	Number of runs*	Number of substrate concns	Analysis by the method of Atkins and Nimmo [31] K_m	V_{max}	Number of runs
(±) α-Methyl noradrenaline	1·09	639.3	6	10	1.13	671.7	5
(±) α-Ethyl noradrenaline	2.43	686.0	7	11	2.52	703.2	7
(±) α-Ethyl isoprenaline	1.50	608.0	4	11	1.67	658.0	4

* Kinetic analysis by the non-parametric method of Cornish-Bowden and Eisenthal [32] using data from which results given in text were derived. All constants are within the 95% confidence limits.

COMT has an ability to differentiate between different side chains on the catechol nucleus, despite the enzyme's wide general specificity in being able to catalyse the methylation of catechol alkaloids [7], oestrogens [8], drugs [2, 9] and synthetic dyes [10, 11] as outlined above. To some extent, this confirms the work of Creveling *et al.* [27] who showed that *para*-methylation was dependent on the charge on the side chain but it appears that a more subtle form of specificity has been detected here. On the whole, the natural isomer of the catechol is the preferred substrate and biosynthetic precursors of the catecholamines are poor substrates.

Further remarks may be made with regard to the suggestion of Atkins and Nimmo [31] that kinetic constants may be best determined by analysing results obtained at two substrate concentrations, one extremely low, the other extremely high. Throughout these studies certain substrates showed deviations from Michaelis–Menten kinetics of the substrate-inhibition type at high concentrations thus precluding any fitting of kinetic constants by this method. However, the kinetic constants of three substrates which showed no such deviation were analysed by the two concentration method, and the results are surprisingly good when used to fit the data obtained from a large number of concentrations (see Table 5). This technique may offer a way of estimating preliminary kinetic constants in order to determine the correct range of substrate concentration for accurate analysis.

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