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The metabolism of dopamine, *NN*-dialkylated dopamines and derivatives of the dopamine agonist 2-amino-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN) by catechol-*O*-methyltransferase

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A variety of dopamine derivatives and analogues were investigated to assess their potential to act as catechol-*O*-methyltransferase (COMT) substrates using purified, homogeneous pig liver enzyme. This enabled accurate kinetic constants to be determined as opposed to previous in-vivo studies (Rollema et al 1980; Horn et al 1981; Costall et al 1982; Feenstra et al 1983). 2-Amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (A-6,7-DTN) proved to be a far better substrate (K_m = 0·082 mM; V_{max} = 300 mU mg^{-1} protein) than its 5,6-dihydroxy isomer (K_m = 2·60 mM; V_{max} = 113·9 mU mg^{-1} protein). This result supports evidence suggesting that differences in brain concentration of these isomers are due to their differential susceptibility to *O*-methylation by COMT (Rollema et al 1980). A similar result was obtained with a series of *NN*-di-n-alkyl substituted ADTN derivatives: the same pattern of preferential *O*-methylation of A-6,7-DTN derivatives over the corresponding A-5,6-DTN isomers was observed. However, increasing the length of the alkyl chain increased the susceptibility of both isomers to metabolism by COMT as shown by a decline in K_m. An homologous series of *NN*-di-n-alkylated dopamines showed a similar trend implying that more hydrophobic compounds are better COMT substrates.

Catechol-*O*-methyltransferase (COMT, E.C. 2.1.1.6) catalyses the methylation of a wide range of catechol substrates whilst showing a subtle specificity for the nature of the side chain attached to the dihydroxyphenyl ring (Hagan et al 1980; Gordon-smith et al 1982). The enzyme uses *S*-adenosyl-L-methionine (AdoMet) as the methyl donor in the presence of magnesium ions (see Guldberg 1979, for review). COMT is found in almost all mammalian tissues including brain (Alberici et al 1965) but liver is the richest source (Axelrod 1966). This study uses COMT purified from pig liver which has been demonstrated previously to have similar properties to the brain form by the criteria of kinetic measurements, molecular weight determinations and cross-reactivity with specific antiserum (Gulliver & Tipton 1979).

Semi-rigid analogues of dopamine have been synthesized: 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (A-6,7-DTN), corresponding to the β-rotamer of the extended (*trans*) form of dopamine, and 2-amino-5,6-dihydroxy-1,2,3,4-tetrahydronaphthalene (A-5,6-DTN) corresponding to the α-rotameric form (see Woodruff 1982, for review). Both dihydroxy isomers of ADTN are accepted to be substrates for COMT from the evidence of in-vivo inhibition studies (Rollema et al 1980; Horn et al 1981; Costall et al 1982): A-6,7-DTN is both more potent at the dopamine receptor in-vitro (Woodruff 1982) and apparently more susceptible to *O*-methylation than A-5,6-DTN (Rollema et al 1980; Costall et al 1982). The present study was intended to investigate the kinetics of COMT with these substrates in-vitro using purified enzyme in the adenosine deaminase-coupled spectrophotometric assay which prevents an accumulation of the inhibitory product, *S*-adenosyl-L-homocysteine (Schlenk & Zydek-Cwick 1968) and hence allows accurate kinetic constants to be determined.

NN-Di-n-alkyl substituted ADTNs have been prepared which may affect both *O*-methylation and brain penetration (Costall et al 1982; Feenstra et al 1983). The effect of dialkylation on the rate of *O*-methylation of dopamine derivatives was also investigated since previous studies from our labora-

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tories have indicated that an increase in hydrophobicity improves a compound's ability to act as a COMT substrate.

MATERIALS AND METHODS

All reagents were of analytical quality (BDH, Poole, UK) and were dissolved in glass distilled water. Dopamine, phenylmethane sulphonyl fluoride and dithiothreitol were obtained from Sigma, Poole, UK, *S*-adenosyl-L-methionine sulphate *p*-toluenesulphonate was the gift of Dr G. Stramentinoli, BioResearch, Milan, Italy. In addition to the compounds synthesized at the University of Groningen (all ADTN and *NN*-di-n-alkylated dopamine analogues; Horn et al 1978), a sample of TL99 (*NN*-dimethylamino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide; see Martin et al 1981) was donated by Dr D. E. McClure, Merck, Sharp and Dohme Laboratories, West Point, PA, USA.

All ADTN analogues were the hydrochloride salts and were dissolved in 10 mM acetic acid to which was added 1% (v/v) mercaptoethanol. All pH measurements were carried out at 20 °C.

Catechol-*O*-methyltransferase was purified from deep-frozen pig-liver as described by Gulliver & Tipton (1978a) except that the buffers used were modified as in Hagan et al (1980). During purification COMT activity was assessed by the direct-extraction radiochemical method of Zürcher & Da Prada (1982) using catechol as the methyl acceptor and methyl-tritiated AdoMet (0·497 Ci ^3H per mol: Amersham, UK) as the methyl donor in 0·08 M potassium phosphate buffer, pH 7·60. The coupling enzyme adenosine deaminase (E.C. 3.5.4.4) was partially purified from Takadiastase (Koch-Light, Colnbrook, UK) using the method of Sharpless & Wolfenden (1967) modified to use 150 cm^3 bed-volume column of Sephadex G25 equilibrated and run in 0.01 M potassium acetate buffer, pH 5.3. Fractions were assayed for adenosine deaminase activity by the spectrophotometric method described by these authors.

One unit (U) of COMT activity in the assay of Zürcher & Da Prada (1982) represents the formation of 1 mmol of product in 1 min at 37 °C. The specific activity of the final (affinity chromatography) fraction was thereby found to be 2172 mU mg^{-1} protein. This represents a purification of 513-fold as compared to the initial homogenate. Polyacrylamide gel electrophoresis in the presence of SDS using the system of Laemmli (1970) with a 16% main gel and a 5% stacking gel showed only one protein was present (see also Gulliver & Tipton 1978a) and this, taken with the high specific activity, implies homogeneity of the enzyme preparation.

The coupled spectrophotometric assay for COMT of Coward & Wu (1973) was utilized in the kinetic studies, as modified in Gulliver & Tipton (1978b). The assay contained 1·6 mM magnesium chloride, 0·64 IU adenosine deaminase, 0·20 M triethanolamine hydrochloride buffer pH 7.20, 0·456 mM *S*-adenosyl-L-methionine, catechol substrate and 10 μl (22·7 mU) purified COMT in a final volume of 500 μl. The pH and magnesium concentration were both optimal (Gulliver & Tipton 1978b). Initial rates of decrease in absorbance at 265 nm were measured at 37 °C in a Beckman Model 35 spectrophotometer.

Rate determinations at each substrate concentration were replicated at least eight times and the median used in kinetic analysis. The concentration range of the varied substrates was as wide as possible, limited by excessive absorbance at high concentrations and very low rates at low concentrations. The apparent kinetic parameters K_m and V_{max} were determined by the direct linear plot (Eisenthal & Cornish-Bowden 1974; Cornish-Bowden & Eisenthal 1978), confidence limits of 95% were obtained (Cornish-Bowden & Eisenthal 1974) and the mean and standard error from three sets of estimations are given in 'Results'.

Protein determinations above 1 mg ml^{-1} were determined by the biuret method (Gornall et al 1949) and, at lower concentrations, by the method of Mejbaum-Katzenellenbogen & Dobryszycka (1959) using standard curves prepared to bovine serum albumin (Sigma, Poole, UK).

Statistical assessment was carried out by the analysis of variance method.

RESULTS

The apparent kinetic constants, derived by the method of Eisenthal & Cornish-Bowden (1974) are given in Tables 1 and 2 which also show the range of concentrations of the varied substrates employed. The apparent first-order rate constant (V_{max}/K_m) is proportional to the rate of *O*-methylation at low substrate concentrations.

Table 1 shows results for the ADTN isomers. A-6,7-DTN (K_m = 0·082 mM; V_{max} = 300·0 mU mg^{-1} protein; V_{max}/K_m = 3·66 × 10^3) is a far better substrate for COMT, by a factor of 83-fold in terms of apparent first order rate constant, than its 5,6-dihydroxy isomer (K_m = 2·6 mM; V_{max} = 113·9 mU mg^{-1} protein; V_{max}/K_m = 44). Similarly, *NN*-dimethyl-A-6,7-DTN (TL99) (K_m = 0·051 mM;

Table 1. Apparent kinetic constants of pig-liver catechol-*O*-methyltransferase for 2-amino-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN) derivatives*.

Varied substrate	Concentration range of varied substrate (mM)	Apparent K_m (mM) ± s.e. of mean	Apparent V_{max} (mU mg^{-1} protein) ± s.e. of mean	V_{max}/K_m (× 10^3) ± s.e. of mean
6,7-Dihydroxy-ADTN	0·05–2·00	0·082 ± 0·01	300·0 ± 6·3	3·66 ± 0·45
NN-Dimethyl-6,7-dihydroxy-ADTN (TL99)	0·024–0·096	0·051 ± 0·005	494·4 ± 10·3	9·79 ± 1·21
NN-Dipropyl-6,7-dihydroxy-ADTN	0·01–0·10	0·033 ± 0·004	727·8 ± 15·2	22·05 ± 2·72
5,6-Dihydroxy-ADTN	0·28–6·00	2·60 ± 0·28	113·9 ± 2·4	0·044 ± 0·005
NN-Dimethyl-5,6-dihydroxy-ADTN	1·20–3·60	2·10 ± 0·22	281·9 ± 5·9	0·134 ± 0·016
NN-Dipropyl-5,6-dihydroxy-ADTN	0·02–2·00	0·66 ± 0·07	350·0 ± 7·3	0·530 ± 0·065

* Determined using the assay of Coward & Wu (1973) as modified in Gulliver & Tipton (1978a). Assay carried out in the presence of 1·6 mM magnesium chloride, 0·456 mM *S*-adenosyl-L-methionine and 0·20 M triethanolamine hydrochloride buffer, pH 7·20 at 37 °C.

V_{max} = 494·4 mU mg^{-1} protein; V_{max}/K_m = 9·79 × 10^3) is a superior substrate to *NN*-dimethyl-A-5,6-DTN (K_m = 2·10 mM; V_{max} = 281·9 mU mg^{-1} protein; V_{max}/K_m = 134) and *NN*-dipropyl-A-6,7-DTN (K_m = 0·033 mM; V_{max} = 727·8 mU mg^{-1} protein; V_{max}/K_m = 22·05 × 10^3) is a better substrate for COMT than *NN*-dipropyl-A-5,6-DTN (K_m = 0·66 mM; V_{max} = 350·0 mU mg^{-1} protein; V_{max}/K_m = 530). All these differences in stereoselectivity are at the 1% level of significance shown by analysis of variance.

However, another trend is present within these data: *NN*-di-n-alkylation of either isomer increases the avidity with which the substrate is *O*-methylated and, furthermore, the longer alkyl chain gives rise to a better substrate than the primary amine, by both decreasing the K_m and increasing the V_{max} significantly at the 1% level of probability.

This phenomenon is also demonstrated by an homologous series of *NN*-di-n-alkyl-substituted dopamines, as shown in Table 2. There is a statistically significant ($P < 5\%$) decrease in K_m with increasing alkyl chain length but this effect reaches a limit at *NN*-dipropyl dopamine (K_m = 0·042 mM V_{max} = 488·9 mU mg^{-1} protein) and *NN*-dibutyl dopamine (K_m = 0·042 mM; V_{max} = 516·7 mU mg^{-1} protein) which did not differ significantly in their kinetic constants. *NN*-Dibutyl dopamine showed a consistent deviation from Michaelis-Menten kinetics of the substrate inhibition type observed previously at high concentrations of substrate (Hagan et al 1980; Gordonsmith et al 1982). V_{max} significantly ($P < 1\%$) increases upon *NN*-di-n-alkylation of dopamine (V_{max} dopamine = 350 mU mg^{-1} protein; V_{max} *NN*-dimethyldopamine = 538·9 mU mg^{-1} protein) but unlike the ADTN isomers, longer alkyl substitution causes no significant increase in V_{max}. In each series studied, *NN*-di-n-methylation significantly

Table 2. Apparent kinetic constants of pig-liver catechol-*O*-methyltransferase for dopamine and *NN*-dialkyl-substituted dopamines*.

Varied Substrate	Concn range of varied substrate (mM)	Apparent K_m (mM) ± s.e. of mean	Apparent V_{max} (mU mg^{-1} protein) ± s.e. of mean	Apparent first order rate constant V_{max}/K_m (× 10^3) ± s.e. of mean
Dopamine	0·2–1·0	0·43 ± 0·05	350·0 ± 7·3	0·81 ± 0·10
NN-Dimethyl dopamine	0·05–0·50	0·15 ± 0·02	538·9 ± 11·3	3·59 ± 0·44
NN-Diethyl dopamine	0·05–0·50	0·11 ± 0·02	588·9 ± 12·3	5·35 ± 0·66
NN-Dipropyl dopamine	0·01–0·20	0·042 ± 0·005	488·9 ± 10·2	11·64 ± 1·43
NN-Dibutyl dopamine†	0·03–0·12	0·042 ± 0·005	516·7 ± 10·8	12·30 ± 1·52

* Determined using the assay of Coward & Wu (1973) as modified in Gulliver & Tipton (1978a). Assay carried out in the presence of 1·6 mM magnesium chloride, 0·456 mM *S*-adenosyl-L-methionine and 0·20 M triethanolamine hydrochloride buffer, pH 7·20, at 37 °C.

† Consistent deviation from Michaelis-Menten kinetics of the substrate-inhibition type observed with this substrate at high concentrations.

improved the apparent first order rate constant of the primary amine.

DISCUSSION

The most intriguing results of this study were those obtained with derivatives of ADTN. The dopamine molecule is a flexible structure and can adopt a number of different conformations. In the extended (*trans*) conformation it can reach two rotameric extremes, α and β. ADTNs contain the dopamine skeleton in a fixed, semi-rigid structure. A-6,7-DTN corresponds to the β-rotamer of dopamine and has been predicted to be the more active isomer in the central nervous system (see Rollema et al 1980; Woodruff 1982; Law et al 1982). Greater behavioural potency has, however, been demonstrated for the 5,6-dihydroxy isomer in mice (Costall et al 1982). The difficulty in ascertaining the relative potency of the two isomers lies with the difference in their rate of biotransformation; for instance, after equal doses, the 5,6-dihydroxy isomer reaches brain concentrations several times greater than the 6,7-dihydroxy isomer (Rollema et al 1980; Horn et al 1981). Inhibition of COMT has been shown to abolish this apparent difference (Rollema et al 1980; Horn et al 1981; Costall et al 1982).

The results presented here confirm the in-vivo inference that A-5,6-DTN is a markedly inferior substrate for COMT than the 6,7-dihydroxy isomer. The disparity between the biochemical and behavioural potencies of A-6,7-DTN can thus be explained by the rapid inactivation, through *O*-methylation, of the compound in-vivo. COMT thus shows a marked stereoselectivity toward A-6,7-DTNs: the β-rotameric form of dopamine appears to be the preferred conformation when acting as a COMT substrate just as it may be the preferred conformation at the dopamine receptor (Woodruff 1982). These results also indicate that increasing penetration of the brain by *NN*-dialkyl substitution of ADTNs may also have the effect of making them better substrates for COMT.

The results obtained with the homologous series of *NN*-dialkyl substituted dopamines show that the longer alkyl chain confers upon a compound a greater ability to act as a COMT substrate. This confirms previous studies demonstrating the sensitivity of COMT to the nature of the side-chain of its catechol substrate (Hagan et al 1980; Gordonsmith et al 1982) and the general preference of the enzyme for hydrophobic substrates (Raxworthy & Gulliver 1982). *NN*-Dibutyl dopamine appears to be the limit of this hydrophobicity effect and introduces a new factor, substrate inhibition.

In conclusion, COMT has been shown to demonstrate stereoselectivity for the 6,7- compared to the 5,6-dihydroxy isomer of ADTN. This trend was continued throughout the pharmacologically interesting *NN*- dialkyl-substituted series of both ADTN isomers. The enzyme's preference for hydrophobic substrates was also apparent within the above series and within a series of *NN*-dialkylated dopamines: the avidity of *O*-methylation tended to increase for substrates with longer alkyl chain substituents.

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