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NOVEL LIGANDS FOR THE PURIFICATION OF CATECHOL-*O*-METHYL TRANSFERASE BY AFFINITY CHROMATOGRAPHY

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Abstract—Affinity chromatography materials were synthesised by reaction of catechol, guaiacol and pyrogallol with agarose gels having pendant diazotised aromatic amines. The use of these materials in the purification of catechol-*O*-methyl transferase (COMT) after ammonium sulphate precipitation and gel chromatography on Sephadex G75 resulted in a 1400-fold increase in specific activity compared to that of the rat liver homogenate. The purified enzyme was shown to be homogeneous by disc gel electrophoresis. The nature of the binding reaction between COMT and these immobilised ligands was examined by measuring their affinities under various conditions of ionic strength and bulk hydrogen ion concentration. Borate ions were shown to specifically elute the enzyme. The results in this paper are consistent with previous proposals regarding the mechanism of COMT.

Catechol-*O*-methyl transferase (COMT, E.C. 2.1.1.6) catalyses the methylation of a wide range of catechol substrates, mainly on the meta hydroxyl, using *S*-adenosyl-L-methionine (SAM) as the methyl donor. It is predominantly a soluble enzyme [1] and has been shown to be magnesium-dependent [2], affecting the catalysis of an important reaction in catecholamine catabolism, forming *O*-methylated catecholamines which have a physiological activity only 0.1% of that of the active substrates [3]. The purification of COMT from many sources has proved time-consuming and of limited success [4, 5], despite the extensive use of many protective agents, some of which, e.g. mercaptoethanol, may interfere with catalysis [6]. Final yields have always proved disappointing. The best conventional purification scheme utilises ammonium sulphate fractionation, gel chromatography, hydroxyapatite treatment and ion-exchange chromatography.

Affinity chromatography should enable an essentially complete purification to be achieved in fewer steps than the conventional scheme and should provide additional information concerning enzyme–substrate, –product and –inhibitor interactions. Two methods have been published for the purification of COMT by affinity chromatography. Creveling *et al.* [7] reported the direct attachment of dopamine to cyanogen bromide-activated Sepharose 4B. In our hands this method was complicated by the rapid oxidation of dopamine to give a pink product. The oxidised dopamine attached to agarose irreversible bound COMT in a reaction similar to that reported by Cuatrecasas *et al.* [8]. Borchardt *et al.* [9] have reported the synthesis of a ligand (3,4-dimethoxy-5-hydroxyphenylethylamine) attached to agarose by a long aliphatic chain (30 Å). This column has not been used in a large scale preparation and requires a series of synthetic steps, producing a material of ambiguous nature.

The aim of this study was to develop a simple and efficient affinity column purification procedure for COMT and to investigate the nature of substrate binding by studying the interaction of COMT with the affinity column.

MATERIALS AND METHODS

Preparation of COMT. The livers from fifteen, 3-month-old (250 g), male Wistar rats were homogenised in a Potter–Elvehjem homogeniser together with 250 ml 0.01 M pH 8.0 phosphate buffer containing 1 mM dithiothreitol (DTT), 1 mM mercaptoethanol and 0.135 M potassium chloride. The homogenate was pretreated by the method of Axelrod and Tomchick [10] as developed by Assicot and Bohuon [4]. The protein which precipitated between 30–50% saturated ammonium sulphate was redissolved in 0.01 M pH 8.0 phosphate buffer containing 1 mM magnesium chloride, 1 mM dithiothreitol and 1 mM mercaptoethanol, and applied to either a Sephadex G25 column or a Sephadex G75 for further purification.

Assay of COMT. COMT activity measured at pH 8.0 in 0.01 M phosphate buffer by the catecholphthalein method [11] which has been shown by other workers [12] to be suitable for assaying the enzyme during preparative procedures. A change of 9.95 absorbance units at 595 nm represents 1 μmole dimethylated catecholphthalein, in an assay volume of 1.6 ml ($E_{595nm} = 25900\ cm^{-1}\ M^{-1}$).

Protein determination. Protein concentrations above 0.5 mg/ml were determined by the method of Gornall *et al.* [13] and at lower concentrations by the method of Mejbaum-Katzenellenbogen and Dobryszycka [14].

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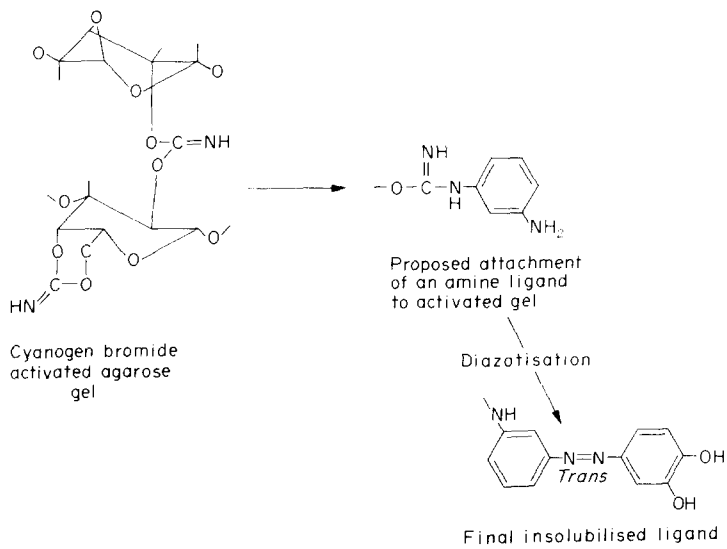

Scheme 1. Synthesis of catechol-azo-*m*-phenylene diamine Sepharose 4B.

Electrophoresis. Polyacrylamide gel electrophoresis was carried out according to the method of Davis [15]. Gels were fixed, stained with Coomassie Blue and destained as described by Weber and Osborn [16].

Preparation of affinity chromatography materials (see Scheme 1). One hundred ml washed Sepharose 4B was suspended in 150 ml 1 M sodium carbonate and activated with 20 g cyanogen bromide dissolved in 20 ml methyl cyanide. *Meta*-phenylene diamine (10 g) was reacted with the activated gel in 0.1 M pH 9.5 bicarbonate buffer for 24 hr. The aminated Sepharose (3 μmoles ligand per 1 ml settled agarose) was washed with 1 l 1 M hydrochloric acid. The gel was resuspended in 250 ml 1 M hydrochloric acid and cooled in ice. A slight excess of sodium nitrite (1.1 mole per mole coupled amine) was slowly added as a concentrated solution with stirring, and the mixture was allowed to stand on ice for 20 min. The diazonium salt agarose cake was filtered from the mixture in a glass-sinter Buchner funnel half-filled with crushed ice, and washed with 250 ml 1 M hydrochloric acid and 250 ml 5 M urea in 1 M hydrochloric acid to remove excess nitrite. Five g catechol dissolved in 250 ml 0.1 M pH 8.0 phosphate buffer was added to the cake and the resulting slurry transferred to a beaker and allowed to stand on ice for 30 min with occasional gentle stirring. The Sepharose was filtered off and washed with 5 l water. Similarly, benzidine could be coupled to Sepharose as the spacer arm and pyrogallol or guaiacol attached as the ligand. Guaiacol was coupled in 0.1 M sodium hydroxide.

This method allows sensitive polyphenols to be immobilised without excessive oxidation, as the nitrite is destroyed by urea washes, and is similar to that utilised by Brenna *et al.* [17].

Preparation of azo compounds. 4-Nitrophenyl-azo-catechol was obtained from Ralph N. Emmanuel and was found, by thin-layer chromatography, to be at least 50 per cent oxidised. Attempts to synthesise this compound in a pure state, using the method described below resulted in a material of comparable purity to that of the commercial sample.

4-Nitrophenyl-azo-guaiacol was synthesised by diazotising 1.24 g 4-nitroaniline in 10 ml 2.5 N hydrochloric acid with 0.6 g sodium nitrite on ice and allowed to stand for 20 min. Guaiacol (1.128 g) in 10 ml 5M sodium hydroxide was added and the reaction mixture allowed to stand for 20 min. The product was extracted by acidification and extraction with 10 ml ethyl acetate. The ethyl acetate extract was then washed with 1 N hydrochloric acid and 1 N sodium carbonate. The dark red product was precipitated by rotary evaporation and recrystallised from ethyl acetate/petroleum spirit (40–60° b.p.) and the crystals washed with further petroleum.

The product was shown to be homogenous on t.l.c. plates developed in 70:29:1 chloroform ethyl acetate acetic acid and 85:15 chloroform methanol.

Effect of COMT on 4-nitrophenyl-azo-catechol. Commercial 4-nitrophenyl-azo-catechol (50 μM) was incubated with partially purified COMT for 1 hr in the presence of 0.5 mM SAM, 10 mM $MgCl_2$ and 10 mM pH 8.0 phosphate buffer. The reaction was terminated by the addition of 1 ml concentrated hydrochloric acid and the azo dye extracted with ethyl acetate. The extract was dried down in a nitrogen stream and redissolved in a few drops of methanol and applied to a silica thin-layer plate. The plates were run in the two solvents used previously and authentic samples of 4-nitrophenyl-azo-guaiacol and catechol were similarly treated as well as controls incubated in the absence of enzyme and SAM.

Sephadex and Sepharose were obtained from Parmacia Fine Chemicals, Uppsala, Sweden. All other reagents used were of the highest commercial quality.

Before use, affinity chromatography materials were treated with rat liver supernatant to block non-specific binding sites which irreversibly bind COMT. This irreversibility bound activity could not be eluted with buffer alone at pH 5.0 or buffers containing 2 M KCl, 0.01 M borate or 1 mM caffeic acid at pH 8.0. Hence all material was pretreated with rat-liver supernatant (1 liver/100 ml material, homogenised in 250 ml) before use in purification procedures. The material was then washed with 2 M potassium chloride, 1 N

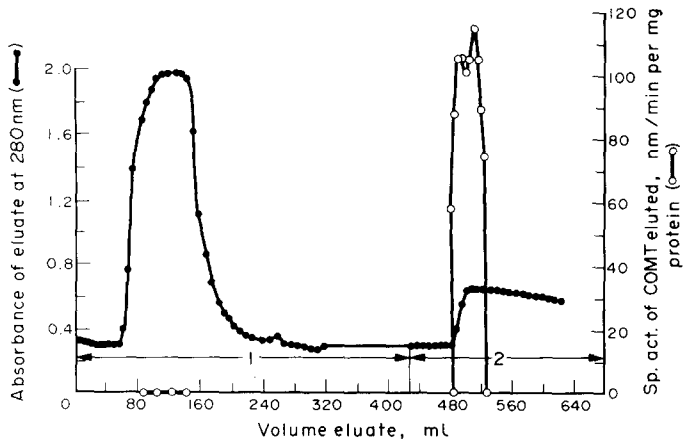

Fig. 1. Large scale purification of COMT by affinity chromatography on guaiacol-azo-benzidine Sepharose 4B. Ninety-two partially purified COMT (sp. act. = 8.46 nm/min/mg protein) was applied to a 1.6 × 30-cm (60-ml) column of guaiacol-azo-benzidine-Sepharose 4B which had been previously equilibrated with 0.01 M pH 8.0 phosphate buffer containing 1 mM $MgCl_2$, 1 mM DTT and 1 mM mercaptoethanol [1]. Excess unbound protein was eluted from the column and COMT eluted with 0.2 M pH 8.0 phosphate buffer containing 2 M KCl, 5 mM DTT, 5 mM mercaptoethanol, 1 mM $MgCl_2$ and 0.05% (w/v) BSA [2].

hydrochloric acid, 8 M urea and treated with pronase (5 mg/100 ml) overnight. After this procedure reversible binding of subsequently added COMT could be demonstrated.

RESULTS AND DISCUSSION

Preparative scale purification of COMT by affinity chromatography. The use of a guaiacol-type material was preferred to the more unstable catechol as any slight tendency to oxidation would be deterimental. The use of guaiacol-based materials may add extra specificity to the affinity chromatography technique preventing competing interaction with catecholamine binding proteins, and, since guaiacols are non-competitive inhibitors of COMT tighter interaction may occur.

COMT activity was purified by ammonium sulphate fractionation and Sephadex G75 gel chromatography to give 92 ml partially purified COMT (sp. act. = 8.46 nmole/min/mg. Protein concentration = 4.34 mg/ml). This represents a purification of 120-fold compared to rat liver homogenate and contains 40 per cent of the total activity.

The above material was supplied to a 1.6 × 30-cm (60-ml) column of guaiacol-azo-benzidine Sepharose 4B which had been previously equilibrated with 0.01 M pH 8.0 phosphate buffer containing 1 mM $MgCl_2$, 1 mM DTT and 1 mM mercaptoethanol. After excess unbound protein had been eluted from the column with this buffer, 0.2 M pH 8.0 phosphate buffer containing 2 M KCl, 5 mM DTT, 5 mM mercaptoethanol 1 mM $MgCl_2$ and 0.5% (w/v) BSA was applied.

The elution profile is given in Fig. 1. Fifty ml highly purified but very dilute COMT was eluted by the high ionic strength buffer with an average sp. act. of 96.64 nm/min/mg protein and 0.09 mg protein/ml (the BSA added as a stabilising agent [5] was eliminated in all calculations of protein concentration). This represents a purification of 1464-fold compared to rat liver homogenate and an overall yield of 8 per cent. The rest of the applied COMT activity eluted as a breakthrough peak with an average sp. act. of 4.40 nm/min/mg protein (i.e. less than that of the applied material).

This preparative method is comparatively quick and simple enabling highly purified COMT to be separated in about 40 hr. The overall yield (8 per cent as opposed to 6 per cent) and purification (1500-fold against 950) compare well with those obtained by Borchardt *et al.* [9] in a long preparation involving ammonium sulphate fractionation, desalting on Sephadex G25, hydroxyapatite absorption, affinity chromatography and Sephadex G100 chromatography. However the capacity of the column employed by Borchardt *et al.* [9] was larger perhaps due to greater steric freedom of the ligand which was coupled to the agarose by a flexible aliphatic chain.

The above highly purified COMT was subjected to polyacrylamide gel electrophoresis and two bands, staining with Coomassie blue, were observed. The denser of the two bands was identified as BSA by means of a control experiment. The more lightly stained band of higher mobility was assumed to be due to COMT as no other bands were detected. The enzyme has clearly been purified to a high degree.

Further attempts were made to obtain pure COMT by affinity chromatography without prior extensive gel filtration. COMT activity was purified by ammonium sulphate fractionation and desalted on Sephadex G25 to give 36 ml preparation (Sp. act. of COMT = 0.61 nm/min/mg protein and containing 38.2 mg protein/ml). This represented a purification of 9.2-fold compared to rat liver homogenate and contained 39 per cent of the total activity. This material applied to a 1.6 × 26.5-cm (67-ml) column of guaiacol-azo-benzidine Sepharose 4B that had been previously equilibrated with 0.01 M pH 8.0 phosphate buffer containing 1 mM $MgCl_2$, 1 mM DTT and 1 mM mercaptoethanol. After the bulk of the protein had eluted, 0.1 M pH 8.0 phosphate buffer containing

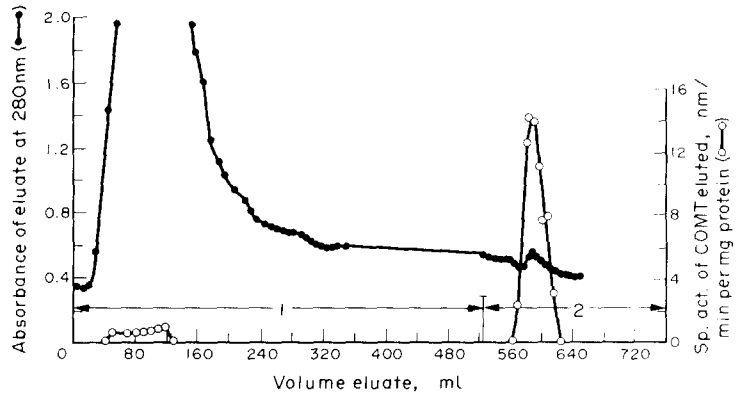

Fig. 2. Large-scale purification of COMT from a desalted 50% saturated ammonium sulphate precipitate by affinity chromatography on guaiacol-azo-benzidine Sepharose 4B. Thirty-six ml COMT preparation (sp. act. = 0.61 nm/min/mg protein) was applied to a 1.6 × 26.5-cm (67-ml) column of guaiacol-azo-benzidine-Sepharose 4B, previously equilibrated with 0.01 M pH 8.0 phosphate buffer containing 1 mM $MgCl_2$, 1 mM DTT and 1 mM mercaptoethanol and the bulk of the protein eluted with this buffer [1]. COMT was eluted with 0.1 M pH 8.0 phosphate buffer containing 5 mM DTT, 5 mM mercaptoethanol, 1 mM $MgCl_2$ and 0.05% (w/v) BSA.

5 mM DTT, 5 mM mercaptoethanol, 1 mM $MgCl_2$ and 0.05 per cent (w/v) BSA was applied.

The elution profile is given in Fig. 2. Thirty-three ml purified COMT was eluted by 0.1 M phosphate buffer with an average sp. act. of 11.28 nm/min/mg protein at a protein concentration of 0.06 mg/ml (the BSA added as a preservative was again eliminated from calculations). This represents a 171-fold purification compared to rat liver homogenate and a yield of 4 per cent. The COMT not bound by the column formed a breakthrough peak with an average sp. act. of 0.69 nm/min/mg protein.

The purification obtained was not significantly better than that of the conventional Sephadex G75 column but the 18-fold purification in this one treatment compared well to the equivalent affinity chromatography step in Borchardt's preparation [9]. This result would imply that large proteins which may include chromagranin or adrenergic receptors, discarded in Sephadex G75 chromatography, interact non-specifically with these column materials.

A high-speed supernatant was made in 0.01 M pH 8.0 phosphate buffer containing 1 mM $MgCl_2$, 1 mM DTT and 1 mM mercaptoethanol and carefully readjusted to pH 8.0. Two hundred and ten ml of this preparation was applied to a 2.5 × 24.5-cm (120-ml) column of guaiacol-azo-benzidine Sepharose 4B, previously equilibrated with the buffer used in the homogenisation. After the bulk of the protein had eluted, 0.15 M pH 8.0 phosphate buffer containing 5 mM DTT, 5 mM mercaptoethanol, 1 mM $MgCl_2$ and 0.05 per cent (w/v) BSA was applied.

The 0.15 M phosphate buffer eluted 39 ml purified COMT with a sp. act. of 3.70 nm/min/mg and 0.86 mg protein/ml, representing a 9.1-fold purification compared to the applied material, 73-fold compared to the homogenate and a 3 per cent yield. Eleven per cent of the COMT activity eluted through the column in the breakthrough peak. It is quite possible that the remaining activity was lost as a result of proteolysis on the columns during chromatography. A similar run at pH 6.5 did not result in the retention of COMT activity: all the applied activity eluted in the breakthrough peak. These results confirm the results from the small-scale experiments (see later), at low pH. The results of the successful purification are quite encouraging but prior ammonium sulphate fractionation and column chromatography do seem to be necessary to remove many interfering factors and, perhaps, proteolytic enzymes.

SMALL SCALE EXPERIMENTS

Small-scale experiments were carried out to study the nature of the interaction of COMT with the immobilised ligands. The COMT preparation used was partially purified by ammonium sulphate fractionation and Sephadex G25 chromatography to a sp. act. of 0.61 nm/min/mg protein representing a 9.2-fold purification compared to rat liver homogenate.

The results of these small-scale experiments are tabulated in Table 1.

In other experiments affinity columns were pre-equilibrated in 0.1 M pH 8.0 phosphate buffer or the enzyme was applied before desalting by Sephadex G25 chromatography. COMT activity did not bind to the columns under these conditions and eluted in a break-through peak with minimal purification by gel-filtration. Similarly it is shown in Table 1 (i), that COMT can be eluted by raising the salt concentration of the buffer used. The binding of COMT to the catechol moiety is apparently independent of the presence or absence of magnesium ions, which although necessary for catalytic activity are not apparently required for binding. This could also mean that the binding occurring during the absorption of COMT activity onto these columns is non-productive or that the active site of COMT binds Mg^{2+} so tightly that it is not removed during purification and may be the obligatory first substrate (R. Goldberg and K. F. Tipton, personal communication).

The success of the catechol affinity column would tend to support the kinetic evidence [12] for the random order binding of substrates to the enzyme mol-

Table 1. Small scale affinity chromatography results

Type of ligand*	Column dimensions (cm)	Vol. COMT preparation applied (ml)	Pre-equilibration buffer	Eluting buffer	Sp. act. of COMT eluted (mm/min/mg)	Recovery of COMT† (%)	Purification of COMT‡ (fold)
Catechol-azo-*m*-(i) phenylene diamine	1 × 18	2	0.10 M pH 8.0 P_i buffer containing 1 mM DTT and 1 mM mercaptoethanol	0.2 M pH 8.0 P_i buffer containing 1 mM DTT and 1 mM mercaptoethanol	4.70	44	71
			similar results obtaining when 1 mM $MgCl_2$ was added to the pre-equilibration buffer and 0.1 M phosphate buffer was used as eluant.				
Pyrogallol-azo (ii) benzidine	1 × 14	5	0.01 M pH 8.0 P_i buffer containing 1 mM DTT, 1 mM mercaptoethanol and 1 mM $MgCl_2$	0.01 M pH 5.5 phosphate buffer containing 1 mM DTT 1 mM mercaptoethanol and 5 mM EDTA	3.84	8	58
Catechol-azo-*m*-phenylene diamine (iii)	1 × 19	2	as above	as above except pH = 6.5	21.65	11	328
Catechol-azo-*m*-phenylene diamine (iv)	1 × 19	2	as above	0.01 M pH 8.0 borate buffer containing 1 mM DTT and 1 mM mercaptoethanol	12.85	9	195

* Strict nomenclature of the azo-affinity materials is not used as the precise nature of the ligand has not been fully determined. The system used is a 'constructional' one referring to the name of the reactants used in the syntheses.

† Compared to activity applied.

‡ Compared to rat liver homogenate.

ecule, and at least suggests that the catechol binding site is available for substrate binding even in the absence of SAM and Mg^{2+}, assuming that the binding event takes place at the site at which the catechol moiety binds in the presence of these cosubstrates and not at another site or in a non-productive mode.

The azo moiety seems to have little effect on the binding of COMT to the catechol ligand, which has also been observed in the case of the adrenergic receptor [18]. However accurate measurement of binding constants as proposed by Nichol *et al.* [19] is at present precluded by the highly labile nature of purified COMT.

The interaction between COMT and the potent inhibitor pyrogallol has been utilised in affinity chromatography (Table 1 (ii)). The capacity of this column is of the same order as those having the catechol ligands, a surprising result in view of the inhibition constants (K_i = 0.42 M for L-DOPA [20]; K_i = 8 μM for pyrogallol [21]. The ligand concentration in all the materials is approximately 3 mM, suggesting that the capacity of the columns is limited by steric factors. The length of the two 'spacers' (benzidine and phenylene diamine) studied here seem to have little effect on the column binding capacity despite the further factor involved that the essentially *para* arrangement of the benzidine-based ligands should place the phenolic moiety further away from the agarose matrix than the *meta*phenylene diamine ligands. Other workers have investigated the relationship between binding capacity and 'spacer' length [22]. However these results are not strictly comparable with those reported in this paper as the 'spacers' studied are flexible methylene chains not the aromatic, essentially rigid systems used here.

One of many attempts to raise the yield of COMT activity eluted from affinity columns, involved increasing the pH of the buffer used to pre-equilibrate the column to 8.5 as the apparent binding constants for catechol substrates decrease with increasing pH [12]. Under these conditions 84 per cent of the applied activity eluted from the catechol-azo-*m*-phenylenediamine Sepharose column in the breakthrough peak and no COMT activity was eluted by 0.1 M pH 7.0 phosphate buffer. White and Wu [5] observed dimerisation of COMT at elevated pH and consequent loss of activity. Certainly other reports [23] suggest that COMT is unstable in alkaline conditions. It may be that the effect of pH on the apparent binding constants is not a binding effect but a catalytic one, and hence COMT is not bound by the column under these conditions.

In addition to the use of high ionic strength, hydrogen ions can be used to elute COMT from an affinity column (Table 1 (ii) and (iii)). This would tend to confirm that the increase in the apparent K_m range with decreasing pH observed by Flohe and Schwabe [12] is due, at least in part, to binding effects. The dependence of the apparent K_m on a group with pK_a = 6.7 could possibly implicate the *p*-hydroxyl of the catechol substrate but the pK value is rather low [24] for such a group. The decrease in effectiveness of the enzyme substrate interaction at lower pH may be ascribed to the protonation of a group in the enzyme molecule.

COMT was eluted from the affinity column (Table 1 (iv)) by borate buffer without the requirement of increase in ionic strength or decreased pH. Borate ions form complexes with polyhydroxy compounds in alkaline media, e.g. adrenaline [25] and caffeic acid [26]. Elution by borate involves a competitive process where the borate ions can reduce enzyme-affinity ligand interaction by forming a complex with the vicinal hydroxyl groups of the ligand. This competitive displacement demonstrates that the interaction between COMT and the affinity column material is

of a genuine biospecific nature. The low apparent yield may be explained on the basis that the activity assays were sub-optimal due to reduced substrate availability as a result of complexation by residual borate ions present.

The incubation of COMT with 4-nitrophenyl-azo-catechol in the presence of SAM yielded a compound which had the same R_f on t.l.c. in two solvents as a pure preparation of 4-nitrophenyl-azo-guaiacol. However, in addition to oxidised material, another, less polar spot was observed which might correspond to the para- or dimethylated-azo compound. This strongly implies that the catechol-azo derivatives are COMT substrates and that the interaction observed between the enzyme and catechol-ligands in affinity chromatography is genuinely biospecific.

The studies reported in this paper demonstrate that the affinity columns result in retention of COMT activity by means of a genuine biospecific interaction with the column ligand groups and that the results are consistent with data from numerous studies of COMT reported by other workers.

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