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STUDIES ON CATECHOL O-METHYLTRANSFERASE

Thesis presented

by

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TABLE OF CONTENTS

I. REVIEW OF THE LITERATURE CONCERNING CATECHOL O-METHYLTRANSFERASE

1. Role of the Enzyme.

In 1958 it was found that a supernatant fraction from rat liver was capable of catalyzing the methylation of catechols in the presence of biological methyl donors. The catechol acids, protocatechuic acid, 3,4-dihydroxyphenylacetic acid, caffeic acid, and 3,4-dihydroxymandelic acid were found to form the corresponding 3-methoxy derivatives when incubated in the presence of magnesium, methionine, reduced glutathione and ATP (1). The purification of an enzyme from rat liver capable of transferring the labile methyl group from S-adenosyl-L-methionine (S-AMe) to epinephrine (E) was also described (2). Since the enzyme required magnesium and was capable of catalyzing the methylation of catechol acids, its identity to the catechol acid methylating fraction is apparent. The enzyme was called catechol O-methyltransferase and it has been given the number 2.1.1.6 by the Enzyme Commission.

The above experiments were initiated in order to explain at the enzyme level the urinary excretion of 3-methoxy-4-hydroxyphenolic acids which had been observed by several workers (3, 4, 5). Metabolic studies soon made it apparent that the catabolism of catecholamines was primarily due to the action of two enzymes: the recently discovered catechol O-methyltransferase (COMT), and the long known enzyme monoamine oxidase

(MAO) (6). The relative role of these two enzymes in the inactivation of catecholamines then became the object of many studies. It was postulated that MAO was responsible for inactivation of catecholamines in tissues and COMT for the degradation of circulating amines (7). This idea was supported by the finding that iproniazid, a MAO inhibitor, increased the amount of norepinephrine (NE) in tissues, whereas no rise in tissue NE could be shown following the administration of the COMT inhibitor pyrogallol (8). However, it is important to distinguish between the metabolism of the catecholamines NE and E. E is derived almost exclusively from adrenal medullary secretion in man (44), whereas NE is secreted at sympathetic nerve endings (45). Since the liver contains both MAO and COMT in large amounts, the metabolism of E in the circulation is attributed to these enzymes in this organ (9). The situation with NE is more complex, but the assignment of relative roles to the enzymes for the metabolism of both catecholamines has certain common difficulties.

Most metabolic experiments have been done with dl E and dl NE. Recently it has become apparent that there is a significant difference between the uptake of the d and l isomers of both E and NE in isolated granules (11). The measurement of clearance of amines by cat liver (12) has shown that pyrogallol reduced the clearance of lE but did not reduce the much smaller clearance of dE. A similar effect was noted with l and d NE. Since COMT is not stereospecific (2) it was postulated that this effect might be due to a difference of uptake for d and l. If the

possibility of different rates of uptake for stereoisomers is considered, results of experiments following the injection of dl compounds become difficult to interpret.

A further continuing problem in assessing the relative roles of COMT and MAO in catecholamine metabolism is the lack of understanding of uptake mechanisms. It is now thought that the mechanism of uptake of NE at nerve endings consists of two amine concentrating components; namely, amine transport through the cell membrane (cell membrane pump), and uptake into specific intraneuronal sites (13). Each component can be selectively blocked by certain drugs. Reserpine is thought to release the amine from storage sites into the cell where it can be metabolized by MAO. Tyramine or amphetamine release NE from the cell into the circulation as NE where it is metabolized by COMT or inactivated by reuptake. Hence, in considering amine metabolism one must consider at least three pools of NE - that in circulation, that which is free in the cell, and that which is stored in the cell. This concept may be represented as shown in Diagram I (46).

However, in canine heart the role of MAO is questionable. Perfusion of isolated canine heart with 7-H^3-NE resulted in extraction of 74% of the NE by the myocardium (10) which was then slowly released from storage sites. The bulk of the released NE was metabolized before it reached the blood and the formation of NM accounted for 40-60% of this metabolism.

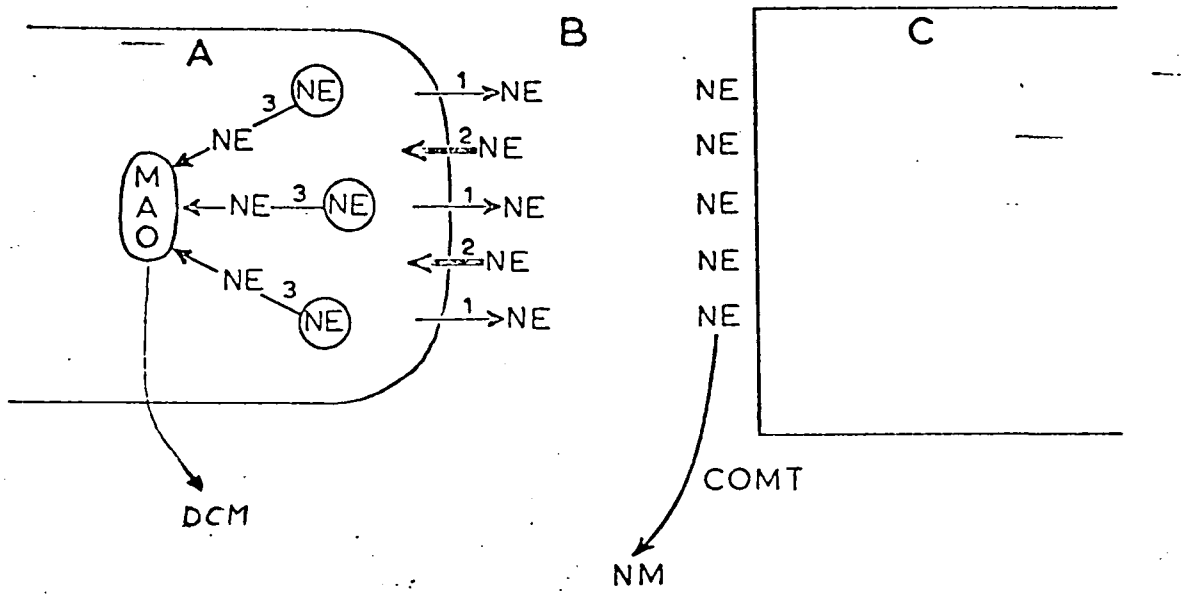


DIAGRAM I Schematic representation of (A) a noradrenergic nerve ending, (B) synaptic cleft, and (C) receptor.

NE - norepinephrine; NM - normetanephrine; DCM - deaminated catecholamine; COMT - catechol O-methyltransferase; MAO - monoamine-oxidase (within a mitochondrion).

1. Discharge of NE into synaptic cleft and onto a receptor.
2. Reuptake of NE from synaptic cleft.
3. Intracellular release of NE from storage granules, and onto mitochondrial MAO.

The activity which remained in the stores was largely (85%) unmetabolized NE. This finding indicates that there is very little MAO activity in the preparation. The injection of tyramine resulted in the release of some of this bound NE. However, the finding that the NE released in this fraction was not metabolized to the same degree as the spontaneously released NE suggests that the ability to O-methylate is limited. Similar results were obtained in isolated rat heart (14). These experiments indicate that all the reactions represented in Diagram I are not operative in all tissues capable of metabolizing NE. The metabolism of NE is further complicated by the finding of species differences (16). The metabolism of NE by homogenates of rat and human heart was inhibited by a MAO inhibitor, but not by a COMT inhibitor, nor was it stimulated by L-Dopa. The metabolism by cat heart homogenate, on the other hand, was stimulated by L-Dopa and not affected by MAO inhibitors.

There is a large amount of interest in solving the remaining outstanding problems of NE metabolism because of the relationship of drugs which affect NE to behaviour (46). These drugs which cause a depletion or inactivation of NE produce sedation or depression, while those which potentiate or increase brain NE are associated with behavioral stimulation. These observations have led to the catecholamine hypothesis of affective disorders which holds that depression is associated with a deficiency of NE at adrenergic receptor sites in the brain, whereas elation may be associated with an excess of this amine. In evaluating this hypothesis it should be remembered that most of the concepts of NE metabolism and

uptake are derived from work on peripheral nerves, and that the catechol amine hypothesis applies to levels of NE in the brain. Though it appears that similar concepts are helpful in explaining the action of drugs on NE levels in the brain (48), the observations of tissue and species differences in NE metabolism make it apparent that mechanisms in peripheral tissue cannot be assumed to be directly related to mechanisms in the brain. Attempts have been made to clarify the roles of COMT and MAO in the brain by the location of these activities in specific fractions. Mild fractionation of rat brain homogenates indicated that COMT occurred mainly in the nerve ending fraction, whereas MAO was in the mitochondrial fraction (17). However, the extreme solubility of COMT made this localization questionable.

The injection of E has also shown that this amine, too, is distributed between metabolically active and inactive pools. In isolated perfused rat heart 95% of bound tritium at any time after injection with H^3-E was unmetabolized (14). The unbound H^3-E was metabolized by COMT. Hence, the activity of MAO in the metabolism of E in rat heart is very limited.

The injection of DL-epinephrine-2-C^{14} into women patients (15) and collection of urine has shown that the main excretion of E is in the 2-5 and 5-10 minute periods following injection. However, trace amounts occur in urine up to 24 hours after injection, indicating that while some of the injected epinephrine was immediately metabolized, some was

bound and stored and subsequently released. Metanephrine and 3,4-dihydroxymandelic acid were the immediate products (2-10 minutes) with 3-methoxy-4-hydroxymandelic acid becoming more important after 20 minutes, at which time it accounted for 50% of the radioactivity in urine. Conjugation as sulfates became more important with increasing time. These experiments show that both MAO and COMT are active in E metabolism in humans. The presence of metabolically inactive stores is shown by the release of small amounts of unmetabolized E for up to 24 hours. However, the relative roles of uptake and metabolism cannot be surmised from experiments of this type since the distribution of amines from exogenously administered pools is not necessarily identical with the distribution of endogenously formed amines (47).

Attempts have been made to assess the role of COMT in various diseases. No difference was observed in the levels of the enzyme between patients with vitiligo and normal patients (21). Portal cirrhosis does not affect COMT activity (9). Although the level of methyl donor is high in leukemic white blood cells, no increased COMT activity was observed (22). Therefore no relationship between levels of the enzyme and disease has, as yet, been shown.

It may be concluded that the role of COMT in the inactivation of free catecholamines is well established. However, lack of understanding of mechanisms of uptake and storage make it difficult to assess accurately

relative roles in overall catecholamine inactivation. Representative reactions of circulating epinephrine metabolism are shown in Diagram 2.

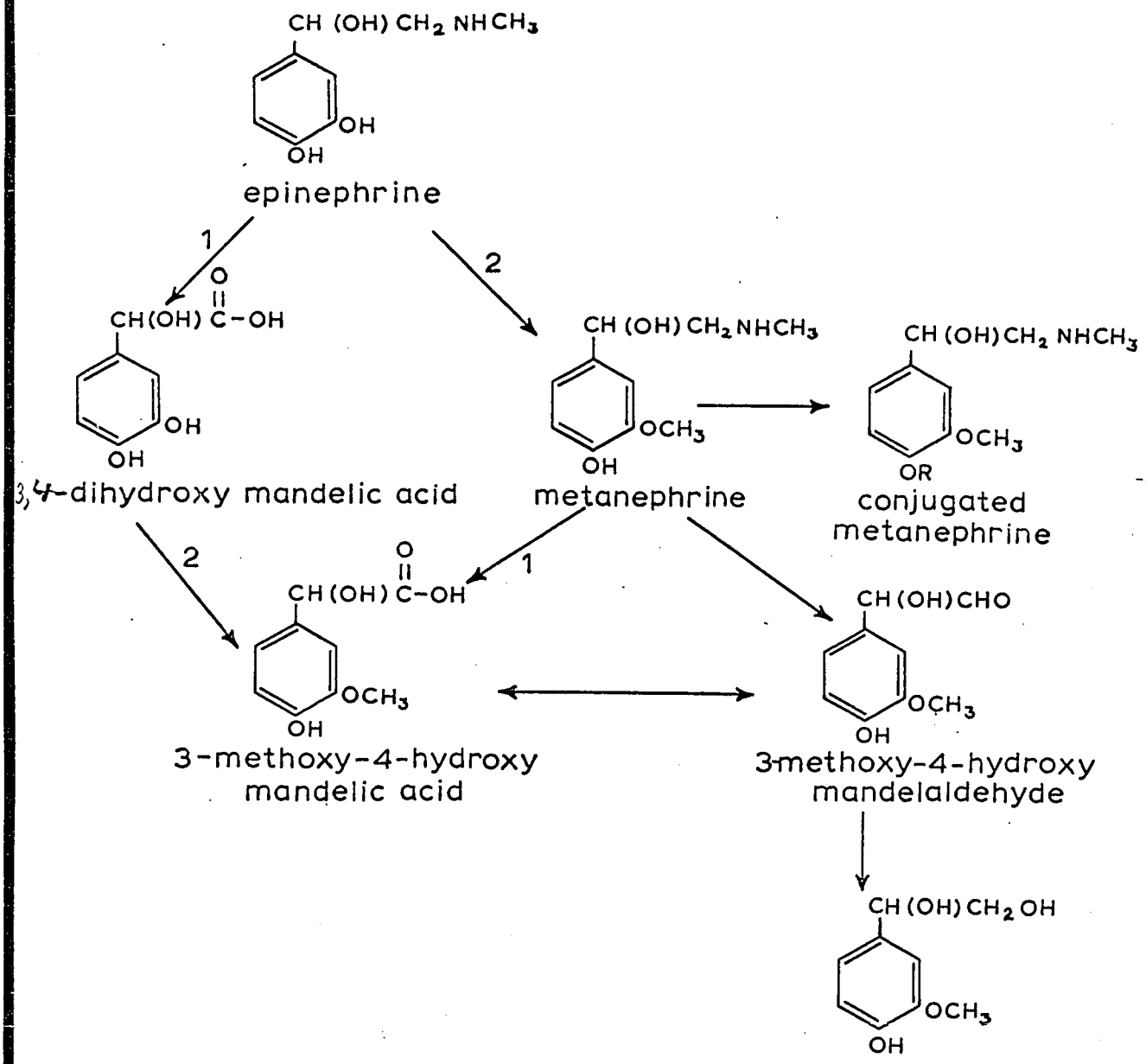


DIAGRAM II.

Metabolism of circulating adrenalin.

1.- monoamine oxidase activity.

2.- catechol O-methyltransferase activity.

2. Distribution and variety of O-methyltransferases.

COMT is widely distributed among tissues and species (19). It is most abundant in liver, but is found in other organs and in peripheral nerves. Of the mammalian livers examined those of monkeys were highest in activity and those of rabbits were lowest. Radioactive methylated derivatives of NE have been found in frog excreta following the injection of C^{14}-NE (20) indicating that catecholamine metabolism in this species is similar to that in mammals. COMT has been found in the skin of man, rats, mice and frogs (21) and it has been found in adipose tissue (19).

In plants, an enzyme capable of methylating catechols has been found in apple cambial tissue (22) and in pumpkin grass (23). A methyltransferase which methylated the 4-hydroxy group of norbelladine has been located in flowering bulbs of Narine bowdenii (24). In microorganisms, a polyphenol O-methyltransferase has been isolated from streptomyces rimosus (25).

Two enzymes in the rat capable of catalyzing O-methylation and differing in properties from COMT have recently been discovered. An enzyme that O-methylates 3,5-diiodo-4-hydroxybenzoic acid has been described (26). This enzyme differs from COMT in that it does not require a divalent cation for maximum activity as well as in substrate affinity, for in no case has COMT been shown to methylate a monohydroxy phenol. An enzyme in the microsomal fraction of rat liver which differs

from COMT in its pH optimum and response to cold stress and benapyrene (27) has been described. It appears to be present in much smaller amount than the soluble O-methyltransferase.

From the foregoing it is apparent that the ability to catalyze the O-methylation of catechols is widely distributed throughout nature. Though the prime function of O-methylation in mammals appears to be the inactivation of catecholamines, the discovery of an iodophenol O-methyltransferase indicates that O-methylation may be important in other metabolic routes.

3. Specifity and properties of the enzyme.

(a) Substrates.

Catechol O-methyltransferase from rat liver requires a substrate that is a derivative of benzene with adjacent hydroxy groups. Monophenolic compounds are not methylated by the enzyme (1, 2). A large variety of catechol derivatives have been tested (1), and all have proven to be substrates. Catechol itself proved to be a better substrate than either catecholamines or catechol acids. The relative lack of steric effects is apparent since the substitution of a bulky t-butyl group at the 5 position in 3,4-dihydroxyacetophenone does not prevent enzymatic O-methylation (29).

Trihydroxy compounds have been tested and found to be substrates. 3,4,5-trihydroxyphenylethylamine is O-methylated primarily in the 4 position (30). Gallic acid (3,4,5-trihydroxybenzoic acid) is methylated in the 4 position (31). With pyrogallol, methylation occurred only at the middle hydroxyl group. Therefore, it appears that in compounds containing 3 adjacent hydroxy groups methylation occurs only at the middle group where one methylation will suffice to abolish adjacent hydroxy groups. The observations on pyrogallol methylation are in contrast to a previously reported result in which it was claimed that the primary product of enzymatic O-methylation of pyrogallol was 1-hydroxy-2,3-dimethoxy benzene (60). The possibility of two different methyltransferases cannot be

excluded, since these two reports used enzyme preparations of different purity.

The methylation of norbelladine by rat liver COMT has been reported to result in about 80% 3-O-methyl product (59).

3,4-dihydroxy compounds are methylated in either the 3 or 4 position depending on the nature of the side chain. The in vitro O-methylation of caffeic acid (3,4-dihydroxycinnamic acid) using liver slices (32) led to the formation of both 3-methoxy-4-hydroxycinnamic acid (ferulic acid) and 3-hydroxy-4-methoxycinnamic acid (isoferulic acid). On the other hand, the O-methylation of protocatechuic acid and 3,4-dihydroxyphenylacetic acid resulted in primarily the methylation of the 3 hydroxy group (32). These results indicate that increasing the length of the side chain increases the amount of 4-O-methylation.

The in vitro O-methylation of other catechol derivatives with the supernatant fraction from rat liver (33) also showed that the extent of 3- and 4-O-methylation depended on the nature of the side chain. 3,4-dihydroxyacetophenone gave 54% 3-O-methylation and 46% 4-O-methylation whereas dopamine and 3,4-dihydroxyphenylmethylcarbinol were 3-O-methylated to a much greater extent. Adrenalone, arterenone and epinephrine were preferentially 3-O-methylated in vitro, but significant amounts of 4-O-methylation were detected (34). In all cases 4-O-methylation was less in vivo than in vitro, and for epinephrine which exhibited 12% 4-O-methyl-

ation in vitro no 4-O-methylation could be shown in vivo. Only in the case of 3,4-dihydroxyacetophenone could the extent of 4-and 3-O-methylation in vitro be rationalised on the basis of resonance structures. 4-O-methyl derivatives were shown to be more rapidly demethylated than 3-O-methyl derivatives in vivo and this finding was used to explain the discrepancy between the extent of 4-O-methylation in vitro and in vivo. It may, however, be concluded that the rationalisation of the extent of 3-and 4-O-methylation awaits further characterization and understanding of the mechanisms of the enzymes involved in both methylation and demethylation.

(b) Inhibitors.

The search for inhibitors of COMT has resulted in the discovery of two classes of compounds which are inhibitors and are not catechols. Tropolones have been shown to inhibit COMT from rat liver (35) and the inhibition has been shown to be competitive (36). 4-isopropyltropolone has been shown to inhibit O-methylation in vivo (51). Tropolones also inhibit COMT from mouse brain in vitro (37) and in vivo inhibit the activity of the enzyme in mouse peripheral organs, but not in brain (38). On both mouse brain (37) and rat liver preparations (35), 4-methyltropolone proved to be the most potent of the tropolones tested.

Iodophenols (39, 40) and thyroid hormone analogues (41) are inhibitors of COMT from rat liver in vitro. The addition of an alanine

side chain to either the one ring or the two ring iodophenols abolished the inhibition (36), demonstrating that it is unlikely that thyroxine inhibits COMT in vivo. Concentrations of iodophenol one hundred times in excess of the substrate concentration failed to inhibit COMT prepared from mouse brain (37). This result suggests that there are catechol methylating enzymes of different properties in mouse brain and rat liver.

The injection of thyroxine has been found to result in a 45% decrease in the methylating ability of livers of male rats (62) and a decreased urinary excretion of metanephrine. These results indicated that in vivo thyroxine has an effect on COMT.

The effect of vitamin B_{12} on COMT activity both in vitro and in vivo has been examined (61). Although this cofactor has been found to be important in some methylating reactions, no effect of B_{12} was observed on COMT activity either in vitro or in vivo.

Drugs that block adrenergic receptors, such as dibenzyline, phentolamine, dichloroisoproterenol, and pronethalol, do not inhibit the enzyme (47). Lithium salts do not affect the activity of COMT. Hence, the effects of drugs used in the treatment of affective disorders do not appear to be at the level of COMT activity.

Other than tropolones and iodophenols, all the inhibitors of COMT that have been discovered have been catechol derivatives. Pyrogallol in

equimolar concentrations to that of epinephrine inhibits the rat liver enzyme about 50% (28). It has been shown to be a substrate (31) and the type of inhibition has been found to be competitive (42), but it has been claimed that the inhibition is partly non-competitive (52). The testing of a series of α-substituted 3,4-dihydroxyphenylacetamides as COMT inhibitors indicated that α-propyl-3,4-dihydroxyphenylacetamide was the most potent (43). 4-tropolonacetamide also proved to be a potent inhibitor of an enzyme preparation from rat liver. A wide variety of substituted catechols, pyrogallols, 5-dihydroxyflavonoids, and tropolones have been tested as inhibitors of mouse brain COMT (37). Esters of gallic acid, D-catechin, 4-methylesculetin, 3,4-dihydroxymandelamide, 8-hydroxyquinoline, and 4-methyltropolone proved to be the best inhibitors.

Adnamine, an acid degradation product of epinephrine, and diadrenaline ether have been found to be inhibitors of COMT from rat liver (50). Adnamine has been implicated as a depression causing metabolite and it is claimed that this may be due to COMT inhibition. These experiments, however, were carried out in a system which required the enzymatic generation of S-AMe from ATP and methionine. Since two enzymatic processes are involved in these studies, the results should be interpreted with caution.

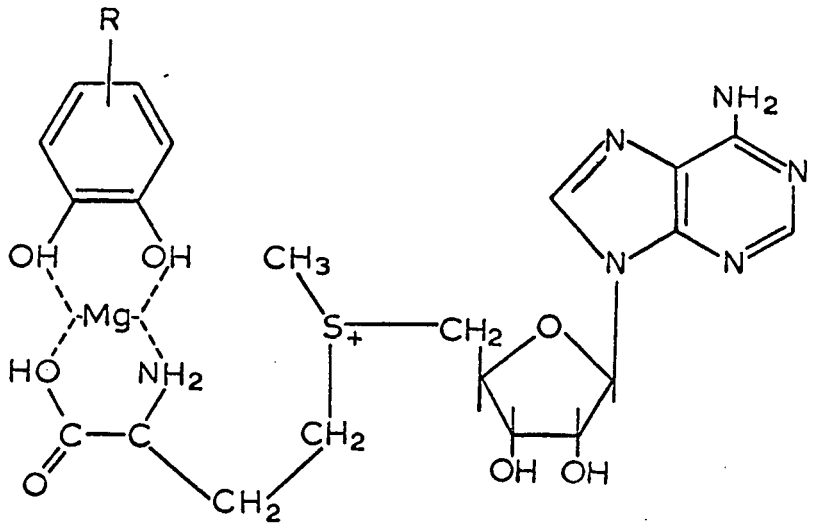
4. Mechanism of Action.

It has been found using rat liver COMT that 3×10^{-5}M p-chloromercuribenzoate and 2×10^{-3}M iodoacetic acid inhibit the enzyme activity about 50% when the substrate concentration is 3×10^{-4}M (2). It would therefore appear that sulfhydryl groups are necessary for enzyme activity. However, the conditions under which the inhibitions were determined are not described and interaction of p-chloromercuribenzoate with the substrate cannot be dismissed.

Metal ions have been found to be necessary for maximum activity. Magnesium or a number of other divalent cations could be used (2). 30 fold purified enzyme to which no metal was added exhibited about 50% of the activity detected when metal was added (53) indicating that the preparation contained bound metal. This activity could be abolished by the addition of 0.1 mM of EDTA.

The role of the metal was postutated to be in the binding of substrate and methyl donor to each other (33,53). This hypothetical complex is shown in Diagram 3. The electrophilic methyl group of S-AMe is thought to be transferred to the hydroxyl group of the substrate by a nucleophilic displacement reaction.

This hypothetical model has been used to explain the inhibitory action of tropolones (54). Tropolones were said to specifically eliminate magnesium and S-AMe from the enzyme because of their ability to chelate



Hypothetical role of magnesium in the O-methylation of catechols

DIAGRAM 3.

magnesium. The possibility that magnesium chelation could lead to enzyme inhibition was tested using the known magnesium chelating compound kojic acid (36). Under conditions similar to those in which tropolone inhibits, kojic acid has no effect. It is therefore apparent that magnesium chelation does not eliminate magnesium and S-AMe from the enzyme. This evidence, together with the finding that tropolones are competitive inhibitors, casts doubt on the validity of the proposed mechanism.

Generally, it can be said of biological transmethylation reactions that they proceed from onium poles (55). The onium center places a partial positive charge on the adjacent carbon atoms making them susceptible to nucleophilic attack by nucleophilic sites on the acceptor molecules. This results in the displacement of the sulfur or nitrogen onium atom with its remaining substituents from the methyl carbon atom. A bond is then formed between the methyl group and the attacking reagent. The onium poles supply the driving force for transmethylation reactions, since the free energy of hydrolysis of a sulphonium compound at physiological pH is of the order of the free energy of hydrolysis of the terminal phosphate of ATP.

Although COMT requires a divalent cation for maximum activity, metals do not appear to be required for the majority of methyltransferases. The only other methyltransferase reported to have a metal requirement is microbial homocysteine transmethylase (50).

Only a few studies have been made of the mechanism of enzymatic methylation. In the case of thetin-homocysteine methylpherase it appears that methyl transfer occurs directly when one or both substrates are bound to the enzyme (57). No evidence of a methylated enzymic intermediate could be obtained. Studies on the enzymic methylation of histamine (58) also gave no evidence of a methylated enzyme intermediate.

5. Purification Attempts.

OCT has been reported to have been 30 fold purified (2). The purification consisted of a pH 5 precipitation of the supernatant portion of rat liver. The supernatant of the pH 5 treatment was fractionated with ammonium sulfate. The enzyme was found to be precipitated in highest specific activity in the 30-50% ammonium sulfate fraction. Adsorption to and elution from calcium phosphate gel after dialysis resulted in a further purification. The enzyme was found to be adsorbed on calcium phosphate from 0.02M acetate buffer, pH 5.0. It was eluted with 0.02M phosphate buffer, pH 6.9. The purification is summarized in Table I. About 30 fold purification with a yield of 15 to 30% was claimed.

Attempts to repeat this procedure (36) did not result in comparable results. No comparable purification was obtained in the pH 5 precipitation. The specific activity of the 30-50% ammonium sulfate fraction was about four times as great as that of the supernatant compared to the 7 fold purification to this stage which was previously reported. 0.02M phosphate buffer pH 6.4 instead of 6.9 was found to give the best elution from calcium phosphate gel. The overall purification again was about 30 fold, but the yield was low. It was also established in conjunction with this work that the assay procedure used in the previous purification procedure employed an activity measurement determined at a time when product formation did not increase linearly with time. This failing has been noted by others (8,61).

TABLE I

PROCEDURE	TOTAL UNITS	SPECIFIC ACTIVITY
Soluble fraction	132	0.014
pH 5.0 supernatant	121	0.027
30-50% ammonium sulfate precipitate	93	0.080
Calcium phosphate adsorption and elution	38	0.420

Axelrod's purification of COMT

Activity is calculated as nmoles of metanephrine formed per 30 minutes of incubation

II INTRODUCTION

It is apparent from the foregoing review that many unanswered problems remain regarding the enzyme catechol O-methyltransferase. The action of the enzyme may be explored at two levels; namely, in vivo and in vitro. There are criticisms of both approaches. An in vivo approach can be criticized as having too many variables and hence leaving results open to several interpretations. In vitro results, on the other hand, are obtained in a situation which has little in common with that in the organism and therefore may not reflect the conditions in the intact animal. On the view that reasoning from a simple system to a more complex one is less dangerous than trying to ascribe an observation to one effect when many might alternately be considered, the in vitro approach has been adopted in the following work.

The present study is an attempt to characterise rat liver catechol O-methyltransferase through purification, investigation of substrates and inhibitions, and examination of some physico-chemical properties. It is hoped that these studies will help answer the questions of how and to what extent the enzyme functions in the metabolism of the catecholamines.

III. EXPERIMENTAL METHODS AND RESULTS

1. Purification Studies.

a) Assay procedure:

Previously reported purification studies of COMT from rat liver (2,36) had not used the recently developed, but widely used, techniques of molecular sieve chromatography and ion exchange chromatography on substituted celluloses. Preliminary experiments indicated that the main drawback in the use of column chromatography techniques was the lack of a rapid, sensitive assay for the enzyme. The fluorometric assay (28) involves two extractions and much consequent manipulation. Assays involving the use of H^3-S (28) or S-AMe-Me-C^{14} (63), while more sensitive than the fluorometric assay, also require extractions and are quite time consuming. It was therefore thought that it would be worthwhile to spend some time in a search for a more convenient and rapid assay for the enzyme.

It was felt that the indicator pyrocatecholphthalein (CP) should be a substrate for the enzyme and that its O-methylated product might have different indicator properties. Accordingly, it was decided to test this compound in a catechol methylating system from rat liver.

Preliminary experiments indicated that CP was insoluble in water. It proved to be soluble in alcohol giving a colourless solution and was

also soluble in basic solution. 10 mg could be dissolved in 50 ml of 0.5M phosphate buffer, pH 8.0, with slight heating. The colour of the basic solution was blue. After standing overnight at room temperature the solution became brown, so in initial experiments only freshly prepared CP solution was used. The incubation mixture consisted of the following:

0.2 ml of 0.1M $MgCl_2$

0.4 ml of 2.5×10^{-3}M S-AMe

0.5 ml of 5.7×10^{-4}M CP in 0.5M phosphate buffer, pH 8.0

0.9 ml of enzyme.

Blanks were prepared by omitting S-AMe. The enzyme used consisted of a 30-50% ammonium sulfate fraction from rat liver which had been dialyzed for 4 hours against water. Incubations were carried out in a water bath at 37°C and were stopped by the addition of 1.0 ml of 0.25M borate buffer at pH 10.0.

The addition of borate after 10 minutes of incubation changed the colour of samples, whereas the colour of blank solutions was unaffected. Blank solutions were brown due to the coloured enzyme solution. The incubation of CP in the presence of S-AMe and the addition of borate imparted a bluish tinge to the brown solutions. Addition of S-AMe to blanks after the addition of borate did not result in a change in colour, nor was a colour change observed when incubations were carried out using

boiled enzyme.

The absorption maximum for colour development was determined using a Bausch and Lomb Spectronic 505. Figure 1 shows the visible spectra obtained for samples incubated for 5 minutes, 10 minutes and 20 minutes. The samples were centrifuged at 1,500 rpm for 5 minutes after the addition of borate and read against blanks to which no methyl donor had been added. The maximum absorbance was found to be at about 595 mμ.

Figure 2 shows that the absorbance at this wavelength is directly proportional to both time of incubation and quantity of enzyme. Therefore, it appeared that the colour development observed upon the addition of borate buffer at pH 10 to CP incubated in the presence of S-Ado and COMT was due to an enzymatically formed product. The colour was very stable, no difference being noted in the absorption values after one day. Whether several day old or fresh CP solution was used did not affect the observed absorption.

That the enzyme responsible for this reaction was identical with COMT was demonstrated by following the purification of the enzyme by both a modification of a fluorometric assay and by using the absorbance at 595 mμ after the addition of borate to measure activity. In the case of the fluorometric assay, the specific activity was calculated as μg metanephrine produced per 2½ minutes of incubation per milligram of protein. When CP was used as substrate specific activity was calculated as absorbance

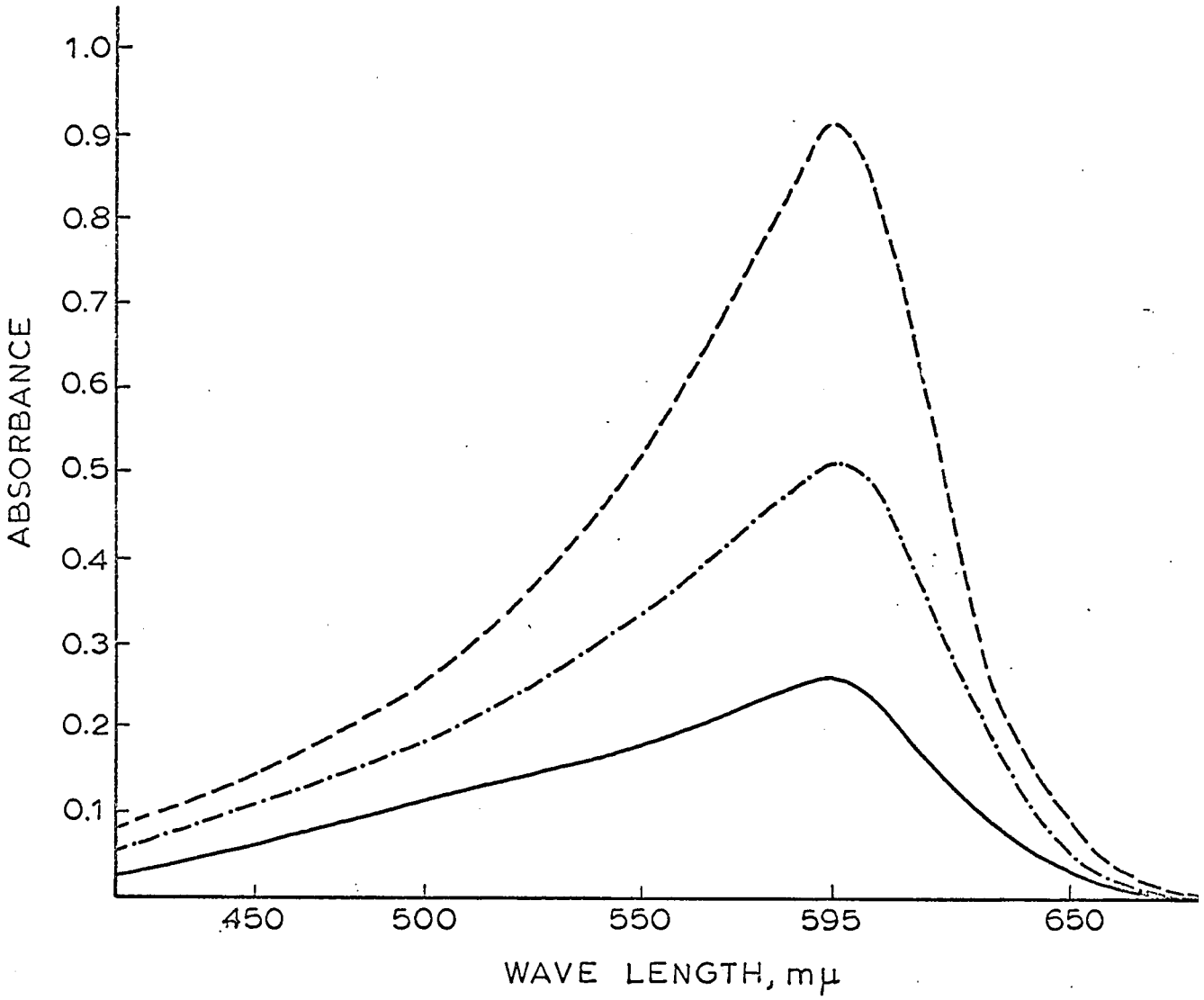


FIGURE 1.

Visible spectra obtained when pyrocatecholphthalein (CP) is incubated in the presence of COMT. Incubation for: ___, 5 minutes; .-.-.-; 10 minutes; —— 20 minutes.

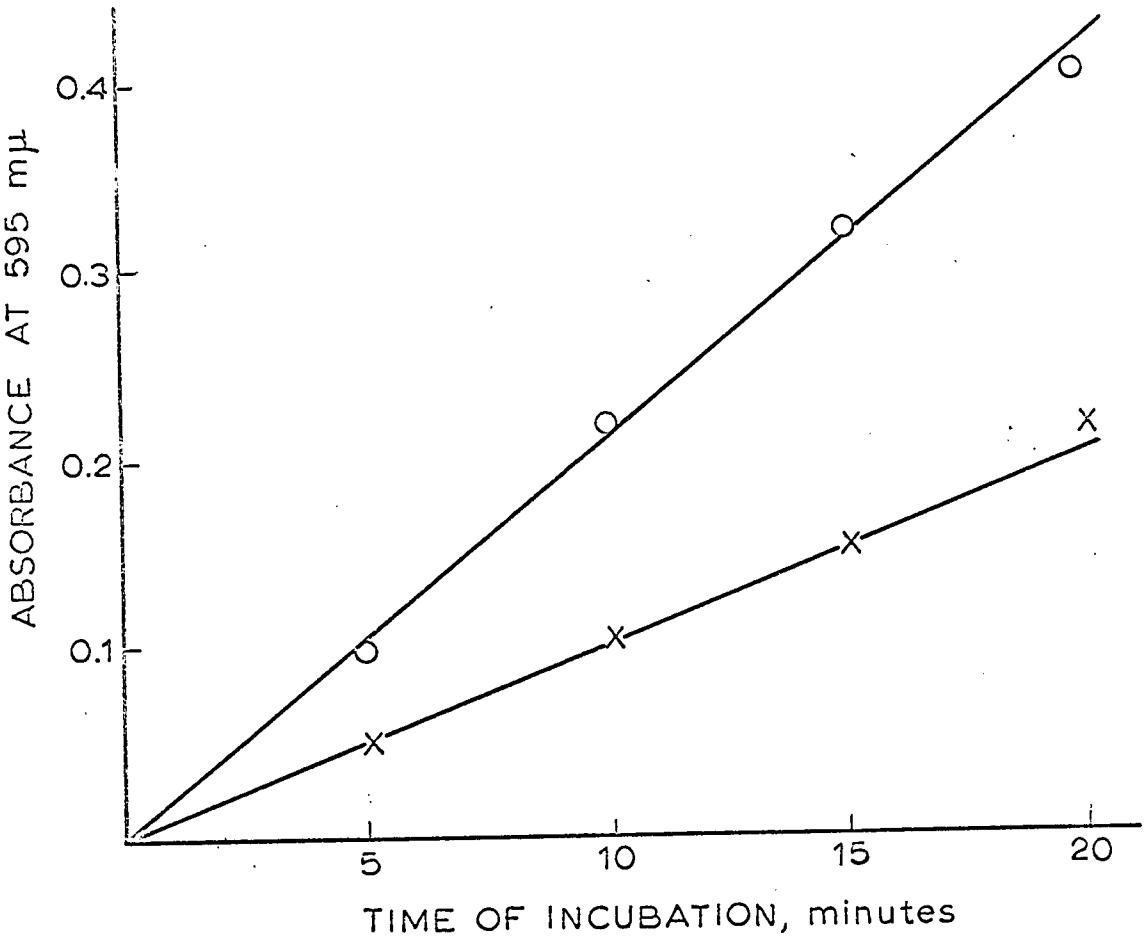


Figure 2.

The effect of incubation time and of enzyme concentration on the absorbance at 595 mμ. Milliliters of enzyme solution: x, 0.4; o, 0.8.

at 595 mμ per 10 minutes incubation per milligram of protein. The fluorometric assay used was essentially that of Axelrod (2) except that substrate and S-AMe concentrations were increased to 10^{-3}M and 5×10^{-4}M in a volume of 2 ml and the time of incubation was decreased to 2½ minutes. Under these conditions a linear relationship between product formation and enzyme concentration was obtained.

The following conditions were adopted for the standard CP assay:

0.2 ml of 0.1M $MgCl_2$

0.6 ml of 5.7×10^{-4}M CP in 0.5M phosphate buffer, pH 8.0

0.4 ml of 2.5×10^{-3}M S-AMe

0.8 ml of enzyme solution.

Blanks were prepared by the addition of 0.4 ml of water instead of S-AMe. Incubations were carried out in centrifuge tubes at 37° for 10 minutes. After stopping the reaction by the addition of 1.0 ml of 0.25M borate buffer pH 10.0, the tubes were left 10 minutes to allow a precipitate which formed to settle. The tubes were then centrifuged for 5 minutes at 1500 rpm. The supernatant was transferred into optical cuvettes and absorbance at 595 mμ was read in a Bausch and Lomb Spectronic 20. Under these conditions absorbance was found to increase linearly with time of incubation and concentration of enzyme.

Protein measurements were carried out using either a microbiuret method (64) or using an ultra violet (UV) absorption method in which

protein concentration is calculated as $1.55 D_{280} - 0.76 D_{260}$ (65) where D_{280} is the optical density of the protein solution at 280 mμ in a 1 cm light path and D_{260} is the optical density at 260 mμ.

The purification procedure used was similar to that described by Axelrod (2) except that a Waring Blendor was used to homogenize the livers. The tissue was prepared for centrifugation by homogenizing at ½ maximal speed for 90 seconds. The calcium phosphate gel treatment was also modified. Negative adsorption was carried out after dialysing the 30-50% ammonium sulfate fraction against 0.02M phosphate buffer pH 6.4 for 5 hours. Calcium phosphate gel (25 mg solids/ml) was used in ratio of 2 mg phosphate per mg of protein. The specific activities and purification achieved by each step as measured by both assay procedures are shown in Table 2. It can be seen that only slight differences in specific activities result from estimation of protein by the different methods. Since the UV adsorption method gives comparable values to micro-biuret protein and its use is much more convenient, it was used for all protein determinations throughout the subsequent work. Table 2 also shows that there is no difference in the enrichment of enzyme as measured by both assay methods during approximately 10 fold purification. This indicated that activity as measured by the CP assay was due to the enzyme which O-methylates epinephrine.

Further evidence that the CP assay was measuring activity due to

TABLE 2

Procedure	Specific activity (Biuret protein)		Purification		Specific activity (UV absorption protein)		Purification	
	F	CP	F	CP	F	CP	F	CP
Supernatant from 40,000 xg centrifugation	0.62	0.01	1	1	0.50	0.008	1	1
30-50% ammonium sulfate precipitate	1.50	0.028	2.5	2.8	1.80	0.034	3.5	4.2
Supernatant of calcium phosphate gel treatment	4.8	0.078	7.7	7.8	5.1	0.102	12	12.7

A comparison of the increase in specific activity obtained during a purification of COMT using the CP assay and a fluorescence assay (F) to measure COMT activity. The conditions of the assays and the definitions of units are given in the text

COMT was obtained by using two inhibitors of the enzymes. Tropolones have been found to be potent inhibitors of COMT when epinephrine was used as substrate (35). The ability of the tropolone β-thujaplicin to inhibit the activity as measured by the CP assay was tested using a final concentration of the tropolone of 10^{-4}M. The tropolone was added dissolved in alcohol and control values were determined by the addition of alcohol. The conditions of assay were the following:

0.2 ml of 0.1M $MgCl_2$

0.6 ml of 5.7×10^{-4}M CP in 0.5M phosphate buffer, pH 8.0

0.4 ml of 2.5×10^{-3}M S-AMe

0.1 ml inhibitor in alcohol, or 0.1 ml of alcohol for controls

0.7 ml enzyme

Reactions were carried out for 5 and 10 minutes. After these times of incubation, samples to which inhibitor had been added exhibited about 25% of the activity of controls.

The effect of the COMT inhibitor 3,5-diiodo-4-hydroxybenzoic acid (DIHBA) (36) was examined in a similar fashion. 10^{-4}M DIHBA was found to have very slight inhibitor action. However, samples to which DIHBA was added in a final concentration of 10^{-3}M exhibited about 70% of the activity of controls. It should be noted that β-thujaplicin is a much more potent inhibitor of the CP reaction than is DIHBA. In contrast, when epinephrine was used as the substrate tropolones and DIHBA exhibited

about the same inhibitory capacity (36). However, the fact that the CP reaction was inhibited by both a tropolone and DIMBA was taken as further evidence that the CP assay was measuring activity due to CONT.

To ascertain what was happening when CP and S-AMe were incubated in the presence of CONT, experiments were carried out using S-AMe-methyl-C^{14} as the methyl donor. The S-AMe-methyl-C^{14} was diluted with carrier S-AMe in the following way. Ampules of 10μC containing 0.101 mg of S-AMe were diluted to 10 mls with water and 13.1 mg of non-radioactive S-AMe was added. Incubation mixtures were prepared as before, but with a 2 fold increase in volume. The reactions were stopped by the addition of 0.4 ml of concentrated HCl. Blanks were obtained by the addition of S-AMe after the addition of HCl. After centrifugation at 2000 rpm for 5 minutes the supernatant was extracted with ethylacetate until aliquots from the aqueous phase no longer turned blue in the presence of base. The ethylacetate extracts were pooled and evaporated. The residue was dissolved in 0.2 ml of methanol and thin-layer chromatography was carried out with benzene: methanol: acetic acid (45:8:4) as solvent according to a procedure described for the separation of phenols (66). Spots were located by spraying with either tetrazotised benzidine (66) or 2.5 N NaOH.

Blank incubations showed only one spot after spraying with tetrazotised benzidine or 2.5 N NaOH and this spot was due to CP. It was coloured red-brown by tetrazotised benzidine and blue by 2.5 N NaOH and

had an R_f of 0.25. Incubations carried out in the presence of the complete system showed the spot of R_f 0.25 and in addition new spots at R_f 0.40 and R_f 0.60. These spots were pink in colour when tetraazotized benzidine was used as the spray. When sprayed with 2.5% NaOH both spots were dark blue in appearance.

That these new spots were due to the methylation of CP was apparent when the spots were scraped and examined for radioactivity by liquid scintillation counting. A procedure utilising thixotropic gel powder in toluene to suspend the particles was adopted (67). The scrapings were suspended in 15 ml of 4% (w/w) thixotropic gel powder in toluene scintillation solution containing 5g PPO and 0.3 g dimethyl POPOP per liter of toluene. Lanes of the chromatograms were divided into $1cm^2$ sections and these sections were scraped and counted. Only in the areas corresponding to the new spots were large numbers of counts detected.

Figure 3 shows the results obtained when incubations were carried out for various times, then extracted, separated and counted as described. 50µl aliquots were applied to the chromatograms and the results were corrected for blanks which were always very low. Though the radioactivity associated with each product spot increased with increasing time of incubation, neither increased linearly with time of incubation. However, the sum of the radioactivity in both spots exhibits a linear relationship to time of incubation. These results would be compatible with the reaction

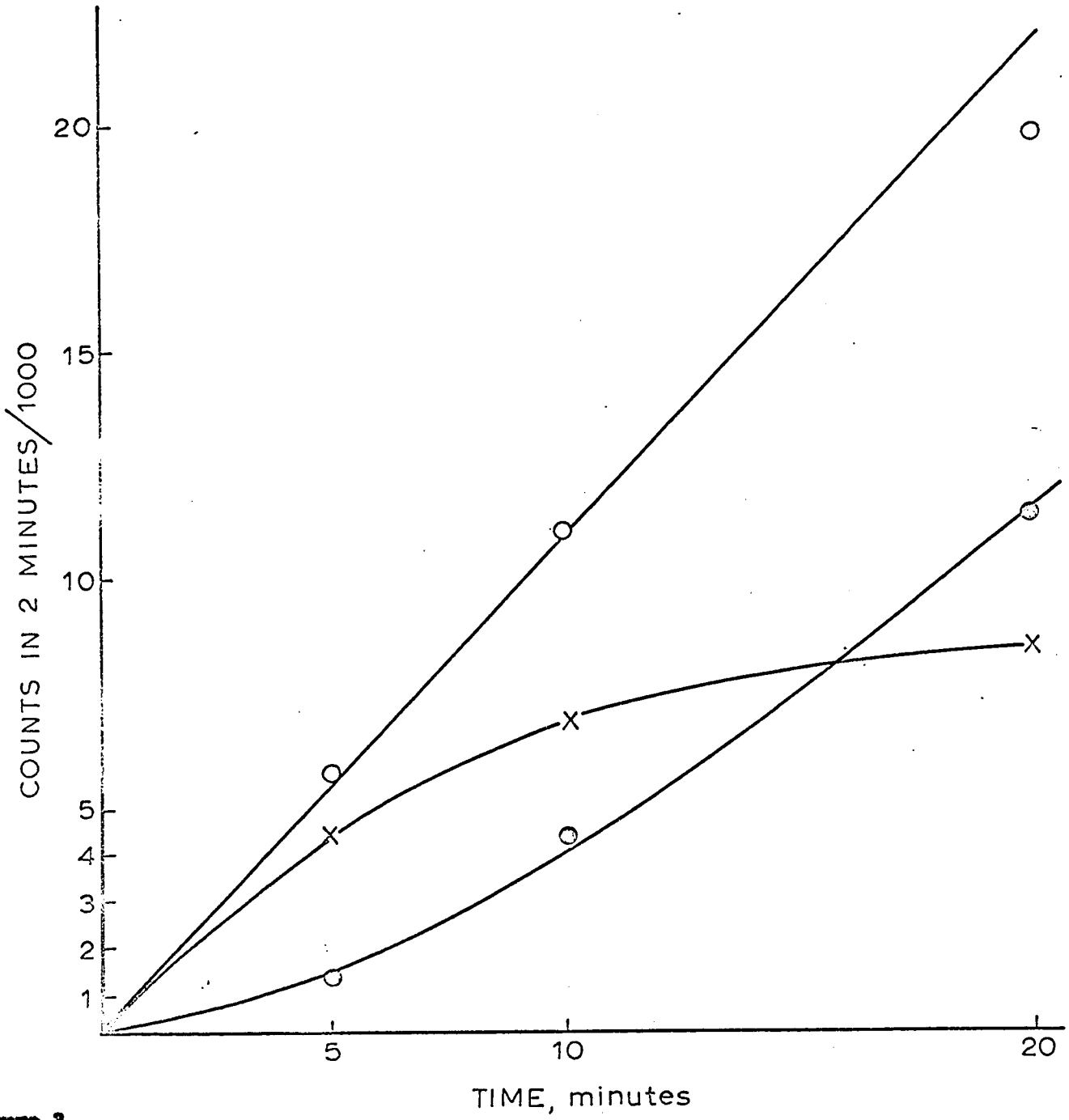


Figure 3.

The rate of C^{14} incorporation into the two products of the enzymatic methylation when 3-AMe-methyl-C^{14} was used as the methyl donor. Product: X, Rf 0.35-0.50; O, Rf 0.55-0.65; o, total radioactivity in both spots.

sequence shown in Figure 4.

In order to determine whether guaiacolphthalein was a product of the reaction, this compound was synthesized. Synthesis of guaiacolphthalein by an old procedure (69) yeilded a product that was not pure in the chromatographic system. A recent modification of this procedure was therefore adopted (68), but this also yielded a product which was impure in the chromatographic system. Both syntheses involved the condensation of pthalic anhydride and guaiacol and differ only in the procedure for the isolation of product. In both cases two spots could be located after chromatography when 2.5 N NaOH was used as spray. A spot at R_f about 0.60 appeared to be present in largest amount.

To obtain chromatographically pure guaiacolphthalein, preparative thin layer chromatography utilizing plates with a 1 mm thick layer of silica gel G was attempted. The sample was disolved in methanol and applied as a band along the origin. After development, the area of the chromatogram 5.5 to 6.5 cm from the origin was scraped and the compound was extracted from the scrapings with methanol. When rechromatographed this extract gave only one spot, migrating with an R_f of 0.60.

Larger amounts of chromatographically pure compound were prepared using a column of silica gel. The column was equilibrated with benzene: methanol: acetic acid (45:8:4) and the sample was applied in this solvent. The sample was eluted with the same solvent under 10 psi nitrogen pressure

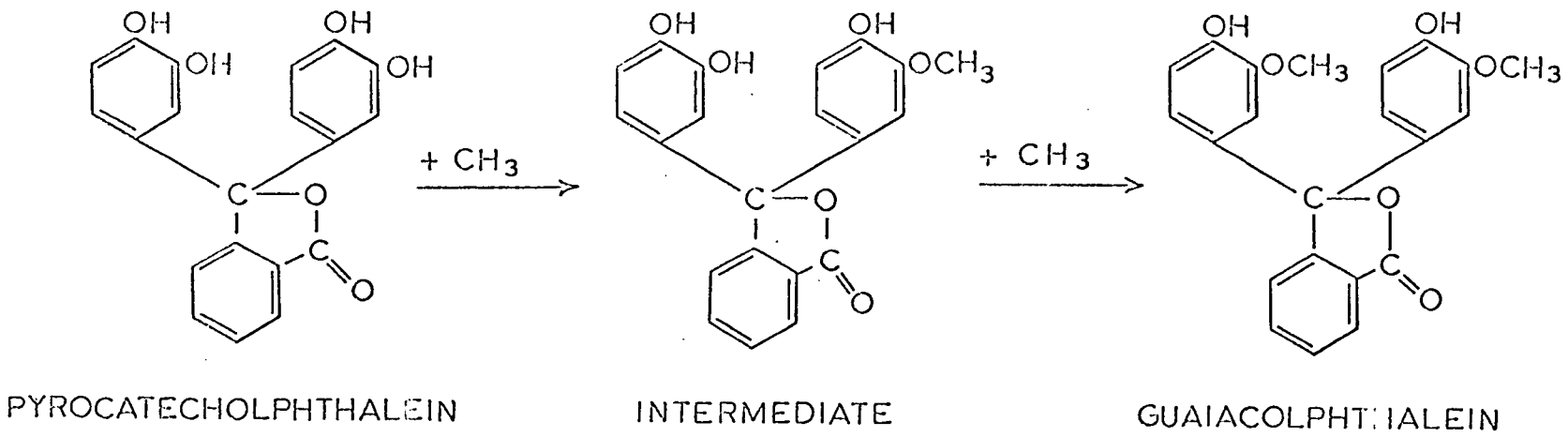


Figure 4. The postulated course of the enzymatic methylation.

to maintain a reasonable flow rate. 10 ml fractions were collected. Early samples contained only the compound which migrated with an R_f of 0.60.

The chromatographically pure compound was scanned for absorption at visible wavelengths on a Bausch and Lomb Spectronic 505. 1.5 mg of compound was dissolved in 0.1 ml of alcohol. 0.5 ml 1N NaOH and 39.4 ml of water were added and the solution was scanned against a blank to which no compound had been added. The absorbance maximum was between 591 and 597 mμ, which agrees exactly with the absorbance peak for the colour developed in the enzyme assay and well with a reported value for guaiacolphthalein of 586-592 under identical conditions (70).

To determine whether the chromatographically pure compound could account for the colour development observed in the assay for CONT the following experiment was performed. 2 mg of the compound was dissolved in 0.1 ml of alcohol and 4.9 ml of phosphate buffer pH 8.0 was added. Similarly, 2 mg of CP was dissolved and diluted with phosphate. Proportions of each solution to make up 0.6 ml were added to mixtures containing 0.2 ml $MgCl_2$, 0.4 ml water, 0.8 ml enzyme solution, and 1.0 ml of 0.25 M borate buffer pH 10.0. Optical density was read at 595 mμ against a mixture to which only CP had been added. The results are seen in Figure 5. It is apparent that the purified compound can account for the increase of optical density at 595 mμ observed under the conditions of the assay.

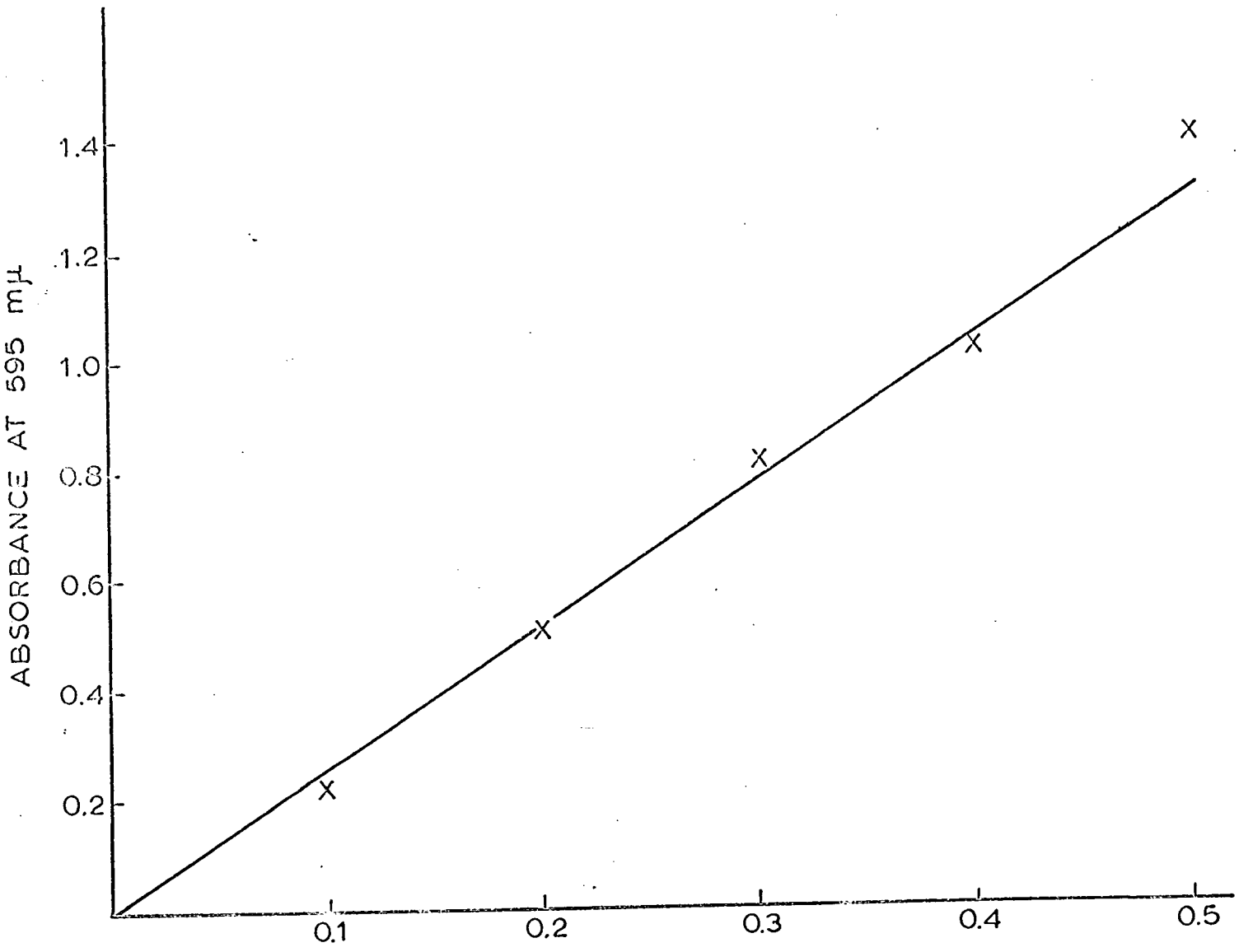


Figure 5. The effect of the addition of chromatographically pure synthetic compound to incubation mixtures.

In addition, the colour was found to be stable for at least one day. By decreasing the amount of CP added, substituting instead phosphate buffer, it was found that the amount of CP present had no effect on the absorbance at 595 mu in the condition of the assay.

The infra-red spectrum of the purified compound was scanned on a Unicam SP200. 3 mg of compound was mixed with 100 mg of KBr and pressed into a disc. The spectrum of CP was also scanned for comparative purposes. The spectra of the two compounds were very similar. However, the purified compound had absorption peaks at 2830 cm^{-1} and 1050 cm^{-1} which were not present in the CP spectrum. These peaks are due to methyl ethers (71).

The visible and infra-red spectra as well as the chromatographic properties of the purified compound were strong evidence that the compound was guaiacolphthalein.

That this compound was a product of the enzymatic O-methylation of CP was shown by several findings. The visible spectrum of the compound in basic conditions was identical to that observed in the CP assay. In both cases the absorption maximum was in the range of 591-557 mu. The R_f of the compound (0.60) corresponded exactly to one of the spots observed when extracts of the CP assay were chromatographed. The behavior of the compound in the conditions of the assay showed that it possessed the necessary properties to produce the colour observed.

It was therefore concluded that in the presence of S-AMe and COMT,

CP is enzymatically O-methylated to form guaiacolphthalein. This compound, unlike CP, is coloured when made basic by borate buffer. This difference in properties may arise from the ability of borate to complex polyhydroxide compounds (72). Since CP is a polyhydroxide compound, it would be expected to form a complex with borate. The formation of this complex may prevent the proton and electron shifts necessary for colour formation. Guaiacolphthalein, which does not have adjacent hydroxy groups, would not be expected to form a complex with borate and therefore could be coloured by basic borate buffer.

In conclusion, it is seen that the O-methylation of CP furnishes a rapid quantitative assay for COMT. The use of this assay procedure made feasible the application of column techniques to the purification of the enzyme, for only by the use of such a method could the analysis of the large numbers of fractions that these techniques necessitate be accomplished. The following work describes attempts to apply these techniques to the purification of the enzyme.

b) Molecular sieve chromatography.

Of the recently developed techniques that are used in the purification of enzymes, molecular sieve chromatography or gel filtration is, perhaps, the mildest. The recent literature contains many reports of good purifications with little loss in activity. It was therefore decided to attempt to apply this technique to the purification of COMT.

Two types of gels capable of separating molecules on the basis of molecular weight are available. A series of dextrans are marketed by Pharmacia Fine Chemicals under the trademark of Sephadex. Bio-Rad Laboratories sells a series of polyacrylamide gels with the trade name Bio-Gel. Since preliminary experiments with the Sephadexes had proved unsuccessful (36), the Bio-Gel series was tested first.

The 30-50% ammonium sulfate fraction was dialyzed against 0.01M phosphate buffer, pH 6.0, for six hours. Enzyme was applied in and eluted with this buffer because it was planned to pass the eluted enzyme through DEAE cellulose and preliminary experiments had indicated that the enzyme was retained on DEAE cellulose under these buffer conditions.

The Bio-Gels P-10 with an exclusion limit of molecular weight of 10,000, P-60 with an exclusion limit of molecular weight of 60,000, and P-100 with an exclusion limit of molecular weight of 100,000 were allowed to swell in an excess of the buffer overnight. Columns 1 cm x 30 cm were

prepared by pouring suspensions of the swollen gels into columns half filled with buffer. Buffer was allowed to drip out of the bottom of the columns and the volumes were replenished by the addition of gel suspensions until the desired bed height had been reached. Columns were prepared and run in a cold room at 4°C.

2 ml portions of enzyme solution were applied to the settled columns. After the enzyme had entered into the columns, phosphate buffer was used to elute. 3 ml fractions were collected and protein and activity measurements made. Only with the Bio-Gel P-60 column did the enzyme activity appear to be separated from the bulk of the protein. In this case, it appeared that the enzyme was significantly retarded by the gel, whereas most of the protein present was not.

Larger P-60 columns were prepared. On a 2.5 cm x 45 cm column it was found that the 30-50% ammonium sulfate fraction from 120 ml of pH 5 supernatant could be applied dissolved to a total volume of 15 ml in 0.01M phosphate buffer pH 8.0. Five ml fractions were collected at a flow rate of about 50 ml per hour.

Under these conditions enzyme began to come off about 25 ml after the first protein and activity continued to be eluted up to 45 ml. The bulk of the activity was in the volume between 30 and 40 ml after the first protein. When this fraction was assayed it was found to be about forty fold purified compared to the original supernatant and it contained

about two-thirds of the activity originally placed on the column.

The 30-50% ammonium sulfate fraction was applied without preliminary dialysis and it was found that a single run on P-60 resulted in both purification and desalting. On a 2.5 cm x 45 cm column with 15 ml of the 30-50% ammonium sulfate fraction applied, ammonium did not begin to appear in the eluate until 20 ml after the last of the enzyme had been eluted. Ammonium was detected by the addition of 0.2 ml of eluate to 0.5 ml of Nessler's Reagent. In the presence of ammonium the clear Nessler's Reagent formed a bright orange precipitate. Elution was carried out until all the ammonium had been removed from the column. Another batch of enzyme was then applied and comparable results were obtained.

Since the 30-50% ammonium sulfate precipitate was about five fold purified over the supernatant and the P-60 eluate was about forty fold purified, it is apparent that passage through Bio-Gel P-60 results in about 8 fold purification of the enzyme with little loss in activity. The first fractions of enzyme eluted did not have as high a specific activity as subsequent fractions. The purification of these fractions could not be increased by another passage through the column. Since they represented only about one quarter of the applied activity, they were discarded.

In subsequent analysis of fractions of effluent from columns, the CP assay was modified slightly to facilitate more rapid location of activity.

The following quantities of reagents were mixed in a 10 ml test tube:

0.2 ml of 5.7×10^{-4}M CP in 0.5M phosphate buffer, pH 8.0

0.1 ml of 0.1M $MgCl_2$

0.1 ml of 2.5×10^{-3}M S-AMe

0.2 ml aliquot of the fraction to be tested.

Incubations were carried out at 37° for 10 minutes. Reactions were stopped by the addition of 0.5 ml of 0.25M borate buffer, pH 10.0. A deep blue colour on the addition of borate indicated the presence of the enzyme in the fraction tested. Fractions containing no activity were not coloured by the addition of borate. The fractions giving the deepest colour were pooled. Protein estimation and quantitative enzyme assay either by the standard CP assay or the modified fluorometric assay were carried out on the pooled fractions of high activity. Since the enzyme in the effluent of P-60 columns was considerably more concentrated than in the supernatant fraction, the volume of enzyme added in both the CP assay and the fluorometric assay was decreased from 0.8 ml to 0.2 ml. 0.6 ml of water was added to make up for the volume decrease in the incubation mixtures.

To test the effect of pH on the separation, P-60 columns were equilibrated and eluted with 0.01M phosphate pH 6.7 and 0.01M acetate pH 5.0. Separations carried out under these conditions were identical to those obtained when 0.01M phosphate buffer pH 8.0 was used. Therefore the separation appeared to be independent of pH. However, decreasing the molarity of the eluting buffer ten fold was deleterious to the enzyme. Although in this case too it was apparent that the enzyme was retarded

relative to most of the protein, the activity which was recovered was very low.

Sephadex G-75, the dextran gel of comparable exclusion limit to Bio-Gel P-60, was tested. Previous attempts to use this gel in the purification of COMT had been unsuccessful (36). Very slow flow rates were encountered. This problem was claimed to be overcome by the manufacturers by making the gels in the form of beads, so this new bead form was used. Columns were prepared in an identical manner to the Bio-Gel P-60 columns. Although the flow rate was adequate and reasonable purification was obtained, the resolution was not as great as with Bio-Gel P-60 and more dilution of the activity occurred. For these reasons, Bio-Gel P-60 was used in routine preparations of the enzyme.

The P-60 eluate containing the enzyme activity was colourless and the enzyme was quite stable at this stage since very little loss of activity was encountered on several days storage at 4°. The preparation could be kept indefinitely after lyophilization with no loss in activity.

c) Ion-exchange chromatography on substituted celluloses.

Purification of enzymes on substituted celluloses has become very common in the last ten years. Although a number of cellulose ion exchangers are available, two are much more frequently used than others. Diethylaminoethylcellulose (DEAE) is the most widely used anion exchanger and carboxymethylcellulose (CM) is the most frequently used cation exchanger. Accordingly, the ability of these two exchangers to effect a purification of COMT was examined.

A previous attempt to use DEAE to purify COMT had been unsuccessful (36). However, another report showed that by the use of DEAE, iodophenol O-methyltransferase activity could be separated from catechol O-methyltransferase activity (26). COMT was eluted from the column using a phosphate gradient at pH 8.0, but a high degree of purification was not obtained. These results, however, suggested that DEAE chromatography using phosphate for elution offered possibilities for purification and so these conditions were adopted in preliminary attempts to purify COMT. Either the 30-50% ammonium sulfate fraction after passage through Sephadex G-25 in 0.01M phosphate, pH 8.0, or eluted activity from P-60 columns were applied to columns of DEAE. The DEAE used was high capacity Cellex D obtained from Bio-Rad Laboratories. Early experiments utilised the technique recommended by the manufacturers for the preparation of the adsorbent. This consisted of stirring the adsorbent in about a 50 x (W/v) excess of starting buffer, leaving for about 15 minutes and decanting the

supernatant. The procedure was then repeated. The resulting precipitate was resuspended in a small volume of buffer and the column poured.

Columns were equilibrated with 0.01M phosphate buffer, pH 8.0, and enzyme was applied in this buffer. The enzyme was retained at this molarity. Elution was carried out using either a gradient or a stepwise increase in buffer molarity. Gradients were prepared by placing a given volume of 0.01M buffer, pH 8.0, in a container having a side arm at the bottom. A separating funnel containing an equal volume of 0.2M phosphate pH 8.0 was fitted on top of the lower container using a rubber stopper. Mixing was carried out in the lower chamber by means of a magnetic stirrer. Stepwise elution was carried out by applying buffers of increasing molarity as soon as all of the preceeding buffer had sunk into the column.

Preliminary attempts at purification utilized columns containing either 5 or 10 g dry weight of exchanger. Enzyme preparations which had been passed through either G-25 or P-60 were applied. The quantity of enzyme applied corresponded to that which was obtained in 50 ml of supernatant. Although some purification was noted with both enzyme preparations and both types of elution, yields were low and it appeared that the enzyme was quite unstable when eluted. The stepwise elution gave a more concentrated enzyme solution with a slightly better yield of activity and for this reason further attempts were made using the stepwise elution procedure.

Smaller columns corresponding to 1 g dry weight of exchanger were prepared. It was found that when the same amount of enzyme activity that had been applied to larger columns was placed on the small columns all of the activity was retained. It could not be eluted by 0.01M phosphate, pH 8.0, or 0.02M phosphate, pH 8.0. When 0.06M phosphate was applied, the enzyme was eluted in a quite concentrated solution. The yield was about 30% of the original activity. When enzyme which had been passed through Bio-Gel P-60 was applied a further four fold purification was obtained. However, the eluted enzyme again was very unstable. Attempts to preserve the activity by the addition of cysteine or mercaptoethanol were unsuccessful. Also attempts to use a gradient elution on the small column were unsuccessful.

An examination of the procedure used to prepare the exchanger revealed that after the recommended procedure had been carried out the pH of the effluent was higher than that of the equilibrating buffer. A new wash procedure was devised which led to equilibration of the exchanger with the eluting buffer. This consisted of suspending the DEAE in a hundred times excess (W/v) of 0.5M primary sodium phosphate, leaving 15 minutes to settle, and pouring off the cloudy supernatant. The residue was filtered on a scintered glass funnel using vacuum, then resuspended in 0.5M primary sodium phosphate and treated as before. After filtration, the exchanger was washed with 0.01M phosphate pH 8.0, and then suspended in a hundred times excess of this buffer and treated as before. Three

treatments with this buffer resulted in equilibration as measured by the pH of the filtrate. After equilibration had been achieved the DEAE was suspended in a small volume of buffer and the column was poured.

When columns were prepared in this manner and a stepwise elution was used, about 50% of the activity placed on the column could be recovered. When P-60 eluate enzyme corresponding to 120 ml of supernatant was applied to columns made from 2 g dry weight of DEAE, a further four fold purification was achieved. The stepwise elution consisted of passing through a volume of 0.01M phosphate buffer equal to the volume of enzyme applied. Then twice the volume of 0.02M phosphate, pH 8.0, was applied. 0.06M phosphate was then applied to elute the enzyme. The eluted activity could be preserved indefinitely if this preparation was immediately lyophilized.

CM-cellulose was used to examine if a cation exchanger could be of value in the purification of the enzyme. Samples were prepared for application by passage through P-60 equilibrated with appropriate buffers. The CM-cellulose was prepared by the procedure recommended by the manufacturers which was identical to that recommended for the preparation of DEAE. Columns equilibrated with 0.01M acetate buffer pH 5.0 and 0.01M phosphate pH 6.7 were prepared. In both cases the enzyme activity and most of the protein present were not retained. Therefore, under these conditions CM-cellulose was of no value in the purification of the enzyme.

Attempts to purify the enzyme using columns of hydroxylapatite proved unsuccessful. Fractionation of the enzyme using ethanol also yielded no worthwhile purification.

d) Summary of the purification procedure.

For routine preparation of the enzyme about 65 g of rat liver were homogenised in 4 volumes of ice cold isotonic KCl in a Waring Blendor at ½ maximum speed for 45 seconds. All operations were carried out at 4° C. The supernatant after centrifuging at 40,000x g for ½ hour was made to pH 5.0, as determined with a pH meter, by the addition of 1M acetic acid. After 10 minutes this preparation was centrifuged for 10 minutes at 10,000 g. Ammonium sulfate was added to the supernatant with stirring until 30% saturation was reached. After 30 minutes stirring the solution was centrifuged at 10,000x g for 10 minutes. The supernatant was made to 50% saturation with ammonium sulfate and stirred and centrifuged as before. The precipitate of this treatment contained the enzyme. At this stage the enzyme could be stored for months in the deep freeze without loss of activity.

The ammonium sulfate precipitate corresponding to 120 ml of supernatant was dissolved to a volume of 15 ml in 0.01M phosphate buffer, pH 8.0, and passed through a 2.5 x 45 cm P-60 column equilibrated with this buffer at a flow rate of 50 ml per hour. Two such columns could conveniently be run at once. The fractions of the P-60 eluate containing the maximum activity were pooled and placed on a DEAE column made from 2 g dry weight of DEAE and equilibrated with 0.01M phosphate, pH 8.0. The enzyme was eluted by a stepwise increase in the molarity of phosphate

buffer as described previously. The results of a typical purification are summarized in Table 3.

Purifications were usually about 200 fold as compared to the supernatant, with a yield of about 10% of the initial activity. The enzyme could be stored for months after lyophilization without significant loss of activity.

TABLE 3

Procedure	Volume (ml)	Activity (unit/ml)		Total units		Protein mg/ml	Specific activity		Yield		Purification	
		CP	F	CP	F		CP	F	CP	F	CP	F
Supernatant	200	0.47	46.9	94.0	9380	37.0	0.0125	1.25	100%	100%	1	1
30-50% ammonium sulfate fractionation of pH 5 supernatant, followed by passage through BioGel P-30	37.5	0.65	72.4	24.4	2715	1.5	0.433	48	25%	29%	34	38
DEAE eluate	42	0.17	23.4	7.14	983	0.10	1.7	234	8%	10%	135	187

Summary of the purification of CONT

CP activity was measured as optical de sity at 595 mμ per 10 minutes of incubation and activity as measured by the fluorescence assay (F) is expressed as μg metanephrine formed per 5 minutes in incubatio

The conditions of the assays are described in the text

e) Polyacrylamide gel electrophoresis.

In order to determine the extent of purification achieved, polyacrylamide gel electrophoresis was carried out on the enzyme preparations. A Canalco Model 12 apparatus was used. Gels were prepared using the formulations given by Canalco for the standard 7% gel. The stacking gel was prepared from the following Stock Solutions:

(B) 1N HCl 48 ml
Tris 5.98 g
Temed 0.46 ml
Water to make 100 ml

(D) Acrylamide 10 g
Bis 2.5 g
Water to make 100 ml

(E) Riboflavin
4 mg in 100 ml water

(F) Sucrose 40 g
Water to make 100 ml

The gel was formed by mixing 1 part of Stock Solution B, 1 part of Stock Solution D, 1 part of Stock Solution E, 1 part water and 4 parts Stock Solution F.

The separating gel was prepared from the following Stock Solutions:

(A) 1N HCl 48 ml
Tris 36.3 g
Temed 0.23 ml
Water to make 100 ml

(C) Acrylamide 28 g
Bis 0.735 g
Water to make 100 ml

(G) Catalyst
Ammonium persulfate 0.14 g
Water to make 100 ml

The gel was formed by mixing 1 part Stock Solution A, 1 part Stock Solution C, and 2 parts Stock Solution G immediately before using. A recommended procedure in which samples were added to the gel column just prior to electrophoresis was adopted. The gel columns consisted of two separately polymerized gels, the stacking gel and the separating gel. They were prepared in glass tubing 5 mm id x 64 mm in the following way. One end of the glass tubing was plugged with a rubber stopper and 200 ul

Note: 2-amino-2-hydroxymethyl-1-3-propandiol tris
N,N,N',N'Tetramethylethylenediamine temed
N,N'-methylenebisacrylamide bis

of 40% sucrose was added to the column by means of a syringe. 200µl of stacking gel was layered on top of the sucrose and overlayered with water to prevent curvature of the gel due to the meniscus. Polymerization was carried out by exposing the gel to a fluorescent light for thirty minutes. The columns were then filled with separating gel. Polymerization was complete in about 30 minutes. At the end of this time, the rubber stoppers were removed and the sucrose was poured out. Columns were connected to the upper bath by means of rubber adapters, so that the stacking gel was on top. 250 ml of a cold buffer containing 3 g tris and 14.4 g glycine per liter was added to the top bath which contained the cathode and 500 ml of the same buffer was added to the bottom bath containing the anode. One half ml of the tracking dye, bromphenol blue, was added to the top bath and the assembly was placed in the cold room. Lyophilised enzyme preparations were dissolved in 40% sucrose and layered into the space on top of the stacking gel in the columns. A current of three ma per column was applied and the electrophoresis was carried out until the tracking dye had migrated to the end of the column. This required about one hour. The current was then shut off and the gels were removed from the columns. Staining was carried out using aniline black at a concentration of one gram of stain per 200 ml of 7% acetic acid. Destaining was carried out electrophoretically with a current of 12.5 ma per tube. Acetic acid was placed in the top and bottom baths instead of the tris glycine buffer when destaining.

It was found that under the above conditions an application of 100

μg of protein in 100μl of sucrose gave the best results upon staining as evidenced by the appearance of sharp, distinct bands in the destained samples. It was also found that the buffer in the baths should be changed after every run.

When lyophilized P-60 eluate was subjected to polyacrylamide gel electrophoresis, at least 13 distinct bands were apparent. Treatment with NEM reduced the number of bands visible to 8. In both preparations there were three bands which were much darker than the others. These bands were quite close together migrating between 2.5 cm and 3.0 cm into the separating gel when the tracking dye was allowed to migrate 5 cm.

Attempts were made to locate the enzyme activity in the gels. After electrophoresis the gels were removed, and placed in test tubes containing 1.5 ml of 5.7×10^{-4} M CP in phosphate, 0.3 ml of 0.1M $MgCl_2$ and 0.2 ml of 2×10^{-2} M 5-MSa. Blanks were prepared by the substitution of water for 5-MSa. Incubations were carried out at 37° for 15 minutes. The incubation mixture was then removed and the gels were washed once with water. The water was removed and 2.0 ml of 0.25M borate buffer pH 10.0 was added.

Before the addition of borate, the gels were dark blue. The addition of borate caused a gradual decolorization starting from the exterior of the gels and progressing toward the inside. Samples incubated in the absence of 5-MSa were completely decolorized within 45 minutes. When incubations were carried out in the presence of 5-MSa, bands were apparent on the addition of borate. These were a different shade of blue than the entire

gel and did not become decolorized as borate penetrated the gel. When 2 mg of lyophilized P-60 eluate was applied in 100 µl of sucrose, two dark blue bands were apparent when the enzyme assay procedure was carried out. These bands were quite close together and were located in a region between 2.5 and 3.0 cm into the separating gel when the tracking dye had been allowed to migrate 5 cm. The assay of enzyme preparations which had been passed through DEAE also showed the presence of two bands occuring in the same region as before. For both preparations the bands which appeared reached a maximum intensity in about twenty minutes. They became more diffuse with increasing time until after three hours they were no longer visible.

It was apparent that the activity bands were in the region of greatest protein concentration as judged by the staining procedure. This finding indicates that most of the protein in the purified preparations has about the same electrophoretic mobility as the enzyme in the gel system used. The reason for the two bands of activity that were observed is not known. Possibly the application of preparative procedures of polyacrylamide gel electrophoresis may furnish an explanation.

f) Estimation of the molecular weight of COMT using Sephadex G-100.

It has been reported that using Sephadex G-100 a linear correlation can be obtained between the ratio of the elution volume, V, of a protein on a given column to the void volume, V_o, of the column and the logarithm of the molecular weight of the protein (79). An equation has been derived which allows the calculation of molecular weight from data obtained by gel filtration (80). It was shown that the molecular weight as calculated using the equation

$$M^{\frac{1}{3}} = \frac{C^{\frac{1}{3}}}{g}\left(1 + g - \left(\frac{V}{V_o}\right)^{\frac{1}{3}}\right)$$

where $M^{\frac{1}{3}}$ is the cube root of the molecular weight, $C^{\frac{1}{3}}$ and g are constants, V is the elution volume, and V_o is the void volume, agreed very well with values determined for a variety of proteins by physico-chemical methods. Only with enzymes capable of binding carbohydrates were serious discrepancies noted. It was therefore decided that an estimation of the molecular weight of COMT based on its elution volume on Sephadex G-100 would be of value.

Since it has been found that ion exchange may occur on Sephadex when low ionic strength buffers are used (81), 0.01M acetate buffer, pH 6.0, containing 0.1M NaCl, which had been shown to be of sufficient ionic strength to eliminate this effect (79), was used. Sephadex G-100 (lot no. TO 2946, particle size 40-120μ) was allowed to swell in an excess of this buffer for three days. A column 1.5 cm x 70 cm was then poured in the cold room. A final operating pressure of 15 cm was used. Buffer was run

through the column until a constant flow rate of 3 ml/hr was obtained. 2 ml fractions were collected. The void volume of the column determined using 1 ml of a 0.1% solution of Blue Dextran 2000, a substance of high molecular weight which is completely excluded by Sephadex G-100, according to the manufacturers of Sephadex, was found to be 36 mls. In order to calculate the constants g and $G^{\frac{1}{3}}$ two proteins of known molecular weight were chromatographed. 3x crystallized α-chymotrypsin of molecular weight 22,500 (82) gave a $^{V}/_{V_0}$ of 2.10 and 2x crystallised pepsin of molecular weight 35,500 (79) gave a $^{V}/_{V_0}$ of 1.60. 5 mg of sample was applied in 1 ml of a 0.1% solution of Blue Dextran 2000 and the elution volumes were determined using $O.D._{280}$ to estimate protein in the eluate. From these ratios the value of g was calculated to be 0.95 and $G^{\frac{1}{3}}$ was calculated to be 40.0. To estimate the molecular weight of CCMT, 5 mg of the lyophilised Bio-Gel P-60 eluate was applied to the column. CCMT was located in the eluate using both $O.D._{280}$ and a CP activity measurement. The protein and activity peaks of the eluate were symmetrical and the centres of the peaks corresponded exactly. Three separate determinations of $^{V}/_{V_0}$ were made for the CCMT. Very similar values were obtained in each experiment. The average value was 1.79. Using this value of $^{V}/_{V_0}$ and values of g and $G^{\frac{1}{3}}$ of 0.95 and 40.0 respectively, the molecular weight of CCMT was calculated to be 29,000.

2. Specificity Studies.

a) Inhibitory potencies and substrate values.

An investigation of the requirements necessary for the binding of a compound to the active centre of COMT was undertaken in the hope that some information on the nature of the active centre and the mechanism of action of the enzyme might be obtained. It was decided that the examination of a variety of catechol analogues both as inhibitors and as substrates would be the best way of obtaining information of this kind.

Inhibition studies were carried out using the CP assay to measure activity. Complete kinetic analysis was not attempted because of the complexity of the CP reaction. Incubation mixtures consisted of the following:

0.2 ml of 0.1M $MgCl_2$

0.4 ml of 0.5M phosphate buffer, pH 8.0

0.4 ml of 2.5×10^{-3}M S-AMe

0.1 ml of substrate in alcohol

0.1 ml of inhibitors in alcohol, or 0.1 ml of alcohol for the control.

0.6 ml of water

0.2 ml of enzyme (P-60 eluate)

Final substrate concentrations of 1.5×10^{-4}M and 3.0×10^{-4}M were used. Inhibitors were present in a final concentration of 1.5×10^{-4}M.

These inhibition studies showed that only compounds with adjacent

hydroxy groups were capable of inhibiting the CP reaction. 4-chloro-3-hydroxytoluene, O-aminophenol, and O-nitrophenol had no effect on the reaction, demonstrating that neither chloro, amino, nor nitro groups could be substituted for the second hydroxyl group. Adjacent sulfhydryl groups could not be substituted for adjacent hydroxyl groups as 3,4-dimercaptotoluene had no inhibitory action.

All catechols tested exhibited some degree of inhibition. Variation of both the nature and position of the side chain indicated that both of these factors were important in determining the degree of inhibition. Table 4 summarizes the results of this work. A comparison of the inhibition observed in the presence of 4-nitrocatechol, 4-chlorocatechol, and 4-hydroxycatechol shows that the electronic nature of the group in the four position has an effect on the degree of inhibition. The presence of the electron attracting nitro groups produces the most marked inhibition. This is compatible with a mechanism in which a negatively charged catechol derivative is formed as an intermediate (33). The presence of an electron attracting group would stabilize such an intermediate.

Increasing the size of the alkyl side chain at position 4 appears to increase the inhibition since both 4-tertoctylcatechol and 4-isopropylcatechol are better inhibitors than 4-methylcatechol. Insufficient data is available to attempt to rationalize this observation. The shifting of the bulky isopropyl group from the four position to the three position decreases the inhibition. This result suggests that it is possible

TABLE 4

INHIBITORY ABILITY OF CATECHOL DERIVATIVES

COMPOUND	CONCENTRATION OF SUBSTRATE	CONCENTRATION OF INHIBITOR	% INHIBITIO (10 MINUTES
4-hydroxycatechol	1.5×10^{-4}M	1.5×10^{-4}M	49
(1,2,4-trihydroxy benzene)	3.0×10^{-4}M	1.5×10^{-4}M	41
4-chlorocatechol	1.5×10^{-4}M	1.5×10^{-4}M	42
	3.0×10^{-4}M	1.5×10^{-4}M	30
4-nitrocatechol	1.5×10^{-4}M	1.5×10^{-4}M	86
	3.0×10^{-4}M	1.5×10^{-4}M	67
4- methylcatechol	1.5×10^{-4}M	1.5×10^{-4}M	40
	3.0×10^{-4}M	1.5×10^{-4}M	33
3-methylcatechol	1.5×10^{-4}M	1.5×10^{-4}M	36
	3.0×10^{-4}M	1.5×10^{-4}M	35
4-iso-propylcatechol	1.5×10^{-4}M	1.5×10^{-4}M	55
	3.0×10^{-4}M	1.5×10^{-4}M	52
3- iso-propylcatechol	1.5×10^{-4}M	1.5×10^{-4}M	40
	3.0×10^{-4}M	1.5×10^{-4}M	30
4-tert-octylcatechol	1.5×10^{-4}M	1.5×10^{-4}M	55
	3.0×10^{-4}M	1.5×10^{-4}M	52
3,4-dihydroxybenzaldehyde	1.5×10^{-4}M	1.5×10^{-4}M	47
	3.0×10^{-4}M	1.5×10^{-4}M	29
purpurogallin	1.5×10^{-4}M	1.5×10^{-4}M	82
	3.0×10^{-4}M	1.5×10^{-4}M	67
4-methyldaphenetine	1.5×10^{-4}M	1.5×10^{-4}M	82
	3.0×10^{-4}M	1.5×10^{-4}M	67

sterically to hinder the binding of a potential substrate to the enzyme. The position of the less bulky methyl groups appears to have no effect on the inhibition as 3- and 4-methylcatechol exhibit about the same degree of inhibition.

Both double ring compounds tested proved to be potent inhibitors.

It was reasonable to assume that the observed inhibitions were due to competition for the active site of the enzyme, the inhibitors themselves being substrates; however, it was decided that further evidence was required for this assumption. Accordingly, a series of compounds were tested as substrates for the enzyme in the following way. S-AMe-methyl-C^{14} was used as the methyl donor diluted with carrier S-AMe. To an ampule of 10 uC S-AMe-methyl-C^{14} containing 0.101 mg of S-AMe, 13.1 mg of cold S-AMe was added. The volume was made to 10 ml with water. Incubation mixtures contained 0.2 ml of 0.5M phosphate buffer, pH 8.0, 0.2 ml of 0.1M $MgCl_2$, and 0.4 ml of the above radioactive S-AMe solution. Potential substrates were added in 0.2 ml of alcohol so that the final concentration was 1×10^{-3}M. 0.5 ml of P-60 eluate was used as the source of enzyme. Reactions were stopped by the addition of 1.0 ml of 10% sulfosalicylic acid. Blanks were prepared by the addition of the methyl donor after the addition of sulfosalicylic acid. The acidified incubation mixtures were filtered, the filter having been previously wetted with acid. 1 ml of acid was used to wash the residue. The filtrate was then extracted 3 times with 5 ml ethyl acetate. The extracts were

pooled and a 0.5 ml aliquot was pipetted into a scintillation vial containing 9.5 ml of a scintillation mixture, consisting of 0.4% PPO dissolved in a mixture of 50% ethanol and 50% toluene. Samples were counted for two minutes in a Nuclear Chicago Mark I Scintillation Counter.

An examination of the time course of the methylation of 4-methylcatechol and 4-tertoctylcatechol revealed that the reactions were complete within 30 minutes. In both cases the reactions were about 80% completed in five minutes and about 90% completed in 10 minutes. All compounds tested were evaluated as substrates relative to 4-methylcatechol as one. Values were determined for both 5 and 10 minute incubation times. Identical relative values were obtained for both times. Table 5 summarizes these results. It is apparent that all catechols are substrates. Table 4 has shown that all catechols are inhibitors. But a comparison of values obtained for some compounds as inhibitors and as substrates indicates that a good substrate is not necessarily a good inhibitor. 4-nitrocatechol appeared to be the most potent inhibitor of the catechols tested, yet it is a poor substrate for the enzyme. Similarly, 4-hydroxycatechol is a better inhibitor than its substrate value would indicate. 4-chlorocatechol is a weaker inhibitor than the above two, yet it is a much better substrate.

The substrate values for 4-methyl and 4-isopropylcatechol are quite close although the isopropylcatechol appears to be a better inhibitor. 4-tertoctylcatechol is very close to 4-isopropylcatechol in its inhibitory potency, but is not as good a substrate. 3-isopropylcatechol is a weaker

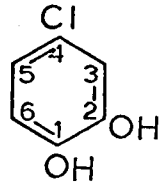
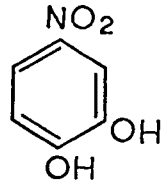
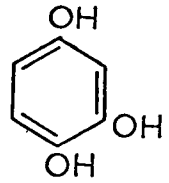
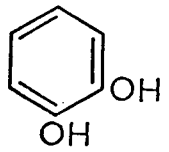
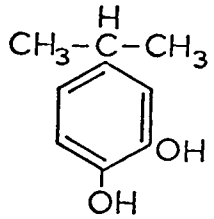
COMPOUND	STRUCTURE	SUBSTRATE VALUE RELATIVE TO 4 METHYLCATECHOL
4-chlorocatechol		1.7
4-nitrocatechol		0.5
4-hydroxycatechol		0.2
catechol		2.8
4-isopropylcatechol		1.1

Table 5.

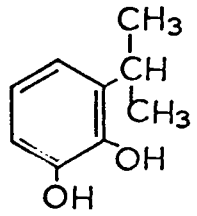
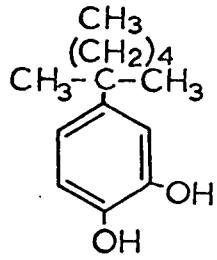
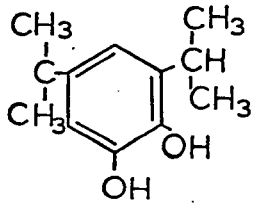
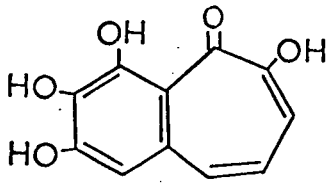
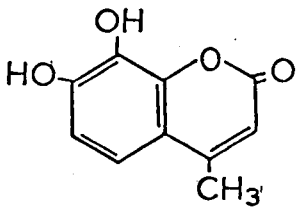
COMPOUND	STRUCTURE	SUBSTRATE VALUE RELATIVE TO 4 METHYLCATECHOL
3-isopropylcatechol		0.2
4-tertoctylcatechol		0.5
3,5-diisopropylcatechol		0.1
Purpurogallin		0.4
4-methyldaphnetine		1.5

Table 5.

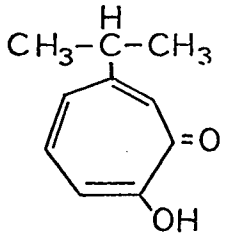
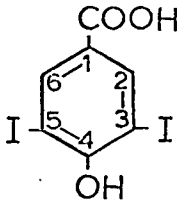
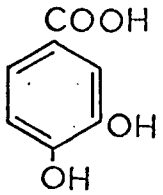
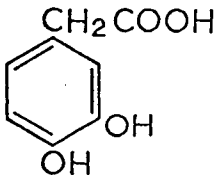
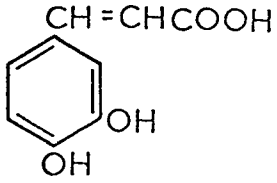
COMPOUND	STRUCTURE	SUBSTRATE VALUE RELATIVE TO 4 METHYLCATECHOL
β thujaplicin		0.0
3,5-diiodo-4-hydroxybenzoic acid		0.0
3,4-dihydroxybenzoic acid		2.5
3,4-dihydroxyphenylacetic acid		2.5
3,4-dihydroxycinnamic acid		2.7

Table 5.

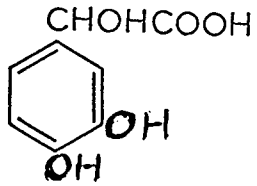
COMPOUND	STRUCTURE	SUBSTRATE VALUE RELATIVE TO 4-METHYLCATECHOL
3,4-dihydroxymandelic acid		1.9

Table 5.

inhibitor than 4-isopropylcatechol and is a much poorer substrate. Thus, with the alkyl catechols there is no clear relationship between substrate value and inhibitory potency, although it is apparent that a poor substrate (4-tertoctylcatechol) can be a good inhibitor.

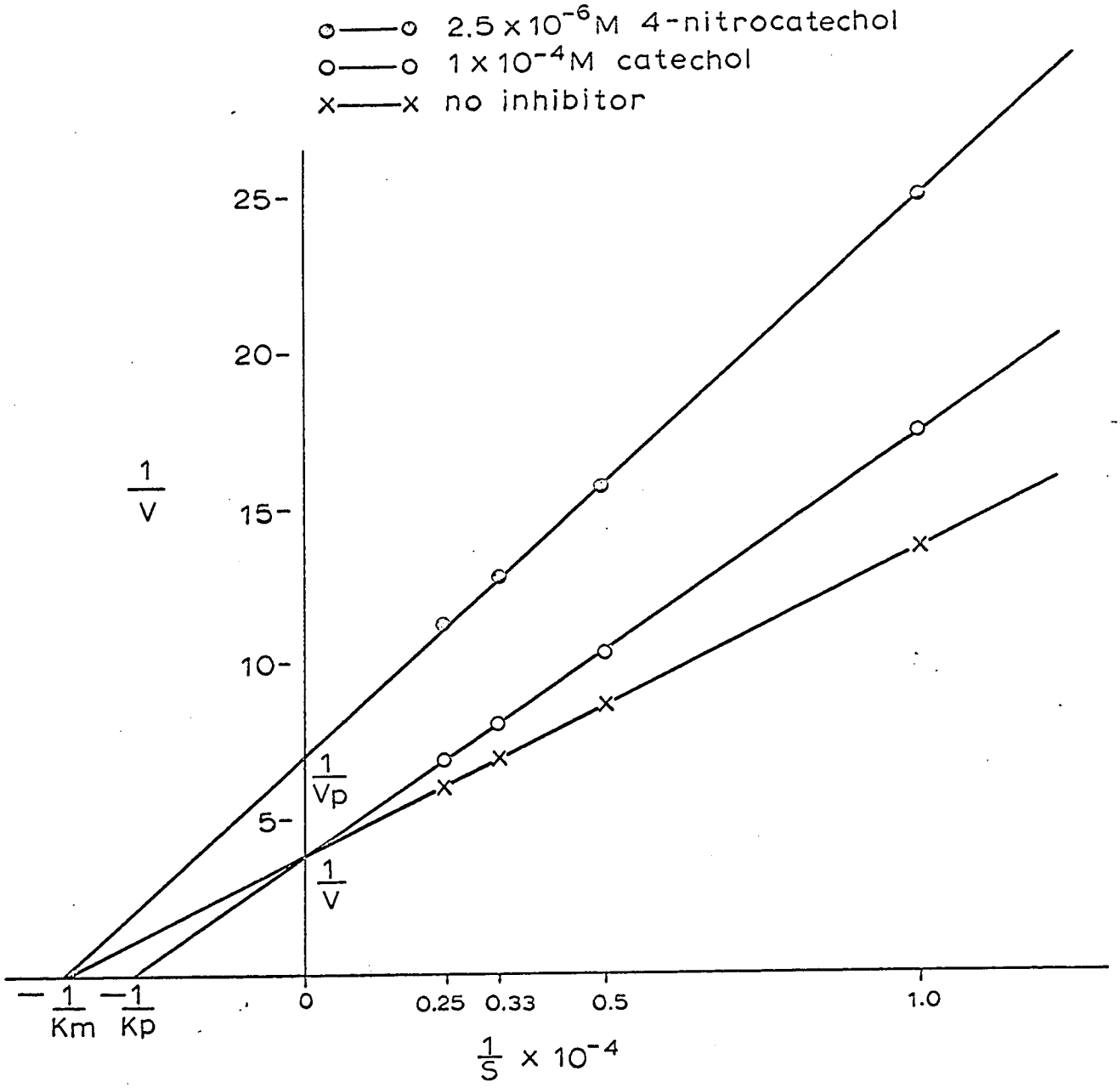
The methylation of purpurogallin and 4-methyldaphnetine shows that even double ring compounds can be substrates. The good substrate value for 4-methyldaphnetine is quite surprising, although the nature of the ring system is such that very little steric hindrance of the hydroxyls would be expected. Purpurogallin is a much better inhibitor than its substrate value would indicate. It is not surprising that it is a good inhibitor since it is composed of two potent COMT inhibitors, tropolone and pyrogallol.

It is apparent that catechol itself was the best substrate of the compounds tested. The catechol acids were also very good substrates. This would indicate that the enzyme prefers a substrate with no substitution, but if there is a substituent, a carboxyl group is preferred. Chain length appears to have little effect on the ability of a compound to be a substrate. Hence, 4-methyl and 4-isopropylcatechol are equivalent substrates. Similarly, no difference is apparent between 3,4-dihydroxybenzoic acid and 3,4-dihydroxyphenylacetic acid. However, this may be true only within limits as 4-tertoctylcatechol is not as good a substrate as 4-methyl and 4-isopropylcatechol.

The position of a bulky side chain does appear to be important as 3-isopropylcatechol is not as good a substrate as 4-isopropylcatechol. The small amount of O-methylation of 1,5-diisopropylcatechol indicates that both the extent and position of bulky groups are of importance in determining whether a compound will be a good substrate.

Since 4-nitrocatechol was the most potent inhibitor tested and yet proved to be a poor substrate, it was decided to examine this inhibition using kinetic techniques. At an S-AMe concentration of 5×10^{-4}M, velocities were determined at concentrations of epinephrine of 1×10^{-4}M, 2×10^{-4}M, 3×10^{-4}M and 4×10^{-4}M in the presence and absence of the inhibitor by the fluorescence assay. As well as the inhibition obtained in the presence of 4-nitrocatechol, the inhibition in the presence of catechol was obtained for comparative purposes. Figure 6 is a Lineweaver and Burk plot of this data. Velocity was calculated as μg metanephrine produced per minute of incubation.

From the curves it can be seen that 4-nitrocatechol behaves as a potent non-competitive inhibitor whereas catechol is a competitive inhibitor. Inhibitor constants (K_i values) can be calculated from this data (73). For non-competitive inhibition the intercepts with the vertical axis give $1/V$ and $1/V_p$ where V and V_p are the maximum velocities in the absence and presence of the inhibitor and:



Lineweaver and Burk plots of inhibition by catechol and 4-nitrocatechol

Figure 6

$$K_i = \frac{i}{\frac{V}{V_p} - 1}$$

where i is the concentration of inhibitor used.

In the competitive case the intersection of the lines with the horizontal axis gives $\frac{-1}{K_m}$ in the absence of inhibitor and $\frac{-1}{K_p}$ in the presence of inhibition and $K_i = \frac{i}{\frac{K_p}{K_m} - 1}$

From the data in Figure 6, K_m for epinephrine was calculated to be 2.8×10^{-4}M which agrees quite well with an estimate obtained under similar conditions (36). The K_i for 4-nitrocatechol was calculated to be 3.0×10^{-6}M indicating that this compound is a more potent inhibitor than either DIMBA or the tropolones (36). A K_i of 2.3×10^{-4}M was obtained for catechol.

Catechol is an excellent substrate for the enzyme as seen in Table 5. It is therefore to be expected that it would be bound at the substrate site in a reversible manner and hence exhibit competitive inhibition. The Lineweaver and Burk plot shows that this expectation is indeed what is observed.

The case with 4-nitrocatechol is more complex. Even though it is a poor substrate, the fact that O-methylation occurs shows that it can be

bound to the substrate site. However, in non-competitive inhibition the substrate and the inhibitor are bound at different sites on the enzyme (74). Since 4-nitrocatechol is both a substrate and a non-competitive inhibitor, it appears that it is bound at both sites. The effect of the nitro group on the acid dissociation constant of the phenolic group may explain how 4-nitrocatechol may act as both an inhibitor and as a substrate. For example, phenol has a K_a of 1.3×10^{-10} whereas 4-nitrophenol has a K_a of 6.5×10^{-8} (75), due to a strong election withdrawing effect of the nitro group. It is therefore possible at pH 8.0 to have a large proportion of the hydroxyls of 4-nitrocatechol dissociated to form phenoxide ions. Since catechol with a K_a of 3.3×10^{-10} is an excellent substrate at pH 8.0, it is reasonable to assume that the enzyme requires the hydroxyl form for binding to the substrate site. Thus, the non ionic form of 4-nitrocatechol may be bound to the substrate site resulting in a small amount of product formation. However, in presence of excess substrate (epinephrine) this competition for the substrate site is of no importance. The ionic form, on the other hand, may be bound at another site on the enzyme, and non-competitive inhibition may result.

b) Summary of the results of specificity studies.

All the catechols tested were both inhibitors and substrates. Catechol proved to be the best substrate of the compounds tested whereas 4-nitrocatechol was the best inhibitor. Inhibition by catechols, therefore, appeared to be due not only to binding to the substrate site, since the best substrate was not the best inhibitor. The non-competitive character of the inhibition by 4-nitrocatechol lends support to this idea.

Variation in the electronic nature of the substituent at the four position showed that both the ability of a compound to be a substrate and its inhibitory potency were affected by the electronic nature of this substituent. Since catechol was the best substrate, it appears that O-methylation is favoured by the lack of any electronic effects. The inhibition observed in the presence of 4-nitrocatechol indicates that an electron withdrawing substituent promotes inhibition.

Steric effects of compounds in the four position appear to be of little importance in determining either the extent of O-methylation or the inhibitory potency. However, the presence of a bulky group in the 3-position decreases both the extent of O-methylation and the inhibitory potency. Thus, steric hindrance would appear to occur only when a bulky group is present next to the hydroxyl groups.

3. Extent of 3- and 4-O-methylation.

a) Position of O-methylation of 3,4-dihydroxyphenylalanine.

It has been reported that both 3- and 4-O-methylation of a variety of catechols occur in the presence of rat liver COMT (32, 33). The extent of O-methylation in these positions was different with different catechols. It was found that 4-O-methylation was less in vivo than in vitro for all compounds tested (34). No satisfactory explanation of these results has been made.

Reports on the position of O-methylation of pyrogallol are conflicting. It has been claimed that the primary product of enzymatic O-methylation of pyrogallol is 1-hydroxy-2,3-dimethoxy benzene (60). However, another report states that O-methylation only occurred at the 2 position (31). It should be noted that different preparations of rat liver enzyme were used by the different workers.

Since the previous experiments were all carried out with quite crude preparations, the possibility remained that more than one enzyme with COMT activity was present. The examination of this possibility was considered a prerequisite to any attempt to postulate a mechanism of O-methylation. It was decided that evidence in this regard could be best achieved by examination of the extent of O-methylation in the presence of crude and purified enzyme. If one enzyme was responsible for both 3- and 4-O-methylation, no change in the ratio of 3- to 4-O-methylation should be noted on

purification. Conversely, a change in the ratio would constitute evidence for more than one COMT activity.

3,4-dihydroxyphenylalanine (DOPA) was chosen as a substrate as it was believed that this compound and its O-methylated products 3-methoxy-4-hydroxyphenylalanine and 3-hydroxy-4-methoxyphenylalanine could be separated on a Beckman Model 120 B Amino Acid analyzer. DOPA and 3-methoxy-4-hydroxyphenylalanine were commercially available, but 3-hydroxy-4-methoxyphenylalanine had to be synthesized. This was done by the condensation of hydantoin with isovanillin to give 3-hydroxy-4-methoxybenzalhydantoin (76). The benzalhydantoin was reduced over sodium amalgan to give 3-hydroxy-4-methoxybenzylhydantoin, which was then hydrolyzed to give 3-hydroxy-4-methoxyphenylalanine (77). The infra-red spectra of both intermediates and of the final product were identical with the reported spectra of these compounds (76). The infra-red spectrum of the final product had peaks at 3340, 3120, 2600, 2040, 1612, 1551, 1527 and 1509 cm^{-1} which corresponds exactly to the reported spectrum of 3-hydroxy-4-methoxyphenylalanine.

Thin layer chromatography of the final product on cellulose using either formic or butanol, acetic acid, water (60:15:25) as solvent showed the compound to be chromatographically pure. Only one spot was observed and it was both ultra-violet absorbing and ninhydrin positive. In both solvents it migrated identically to the commercial 3-methoxy-4-hydroxyphenylalanine. It was therefore concluded that this compound was the desired product.

Concentrations of 1 μM/ml of DOPA and 3-methoxy-4-hydroxyphenylalanine, and a concentration of 0.5 μM/ml of 3-hydroxy-4-methoxyphenylalanine were used to determine the Amino Acid Analyzer Constants. Using the standard column equilibrated with buffer at pH 3.28 and eluting with buffer at pH 4.25 gave elution times of 80 minutes, 95 minutes and 104 minutes for DOPA, 3-methoxy-4-hydroxyphenylalanine, and 3-hydroxy-4-methoxyphenylalanine respectively. Constants were calculated using the formula $C = \frac{W \times H}{c}$ where C is the constant, W is the width of the peak corresponding to the compound, H is its height, and c is the concentration in μ moles of compound applied to the column. Values of C for DOPA, 3-methoxy-4-hydroxyphenylalanine and 3-hydroxy-4-methoxyphenylalanine were found to be 19.5, 20.4 and 20.6 respectively. From these values unknown concentrations can be calculated from the product of W and H.

Experiments to determine the position of O-methylation of DOPA utilized an incubation mixture in which DOPA was dissolved to a final concentration of 4×10^{-3}M in a mixture of 0.4 ml of 0.5M phosphate buffer, pH 8.0, 0.2 ml of 0.1M $MgCl_2$, and 0.4 ml of 2.5×10^{-3}M S-AMe. Blanks of three types were used. In some cases water was added instead of S-AMe. In other cases no substrate was added. And some blanks were prepared by stopping the reaction immediately after the addition of enzyme. Either the supernatant or the P-60 eluate was used as the source of the enzyme. Incubations were carried out at 37° and were stopped by the addition of 100 mg of sulfosalicylic acid. Then the tubes were centrifuged, and the

supernatant was filtered and 1 ml of the filtrate was applied to the Analyser column equilibrated with buffer at pH 3.28. Elution was carried out using buffer of pH 4.25.

Experiments with the supernatant fraction presented a problem since it was apparent from the chromatograms that the preparation was highly contaminated with amino acids. When blanks were prepared by stopping the reaction immediately after the addition of enzyme, peaks were apparent having the same elution times as the products. Whether methyl donor was present or absent had no effect on these peaks.

By running standards it was found that the elution time of 3-methoxy-4-hydroxyphenylalanine was only 2 minutes different from that of tyrosine. Similarly 3-hydroxy-4-methoxyphenylalanine had an elution time very close to that of phenylalanine. Therefore, the presence of other amino acids could account for the peaks observed at the elution times of the products after zero time of incubation and their presence would therefore interfere with attempted measurements of product formation.

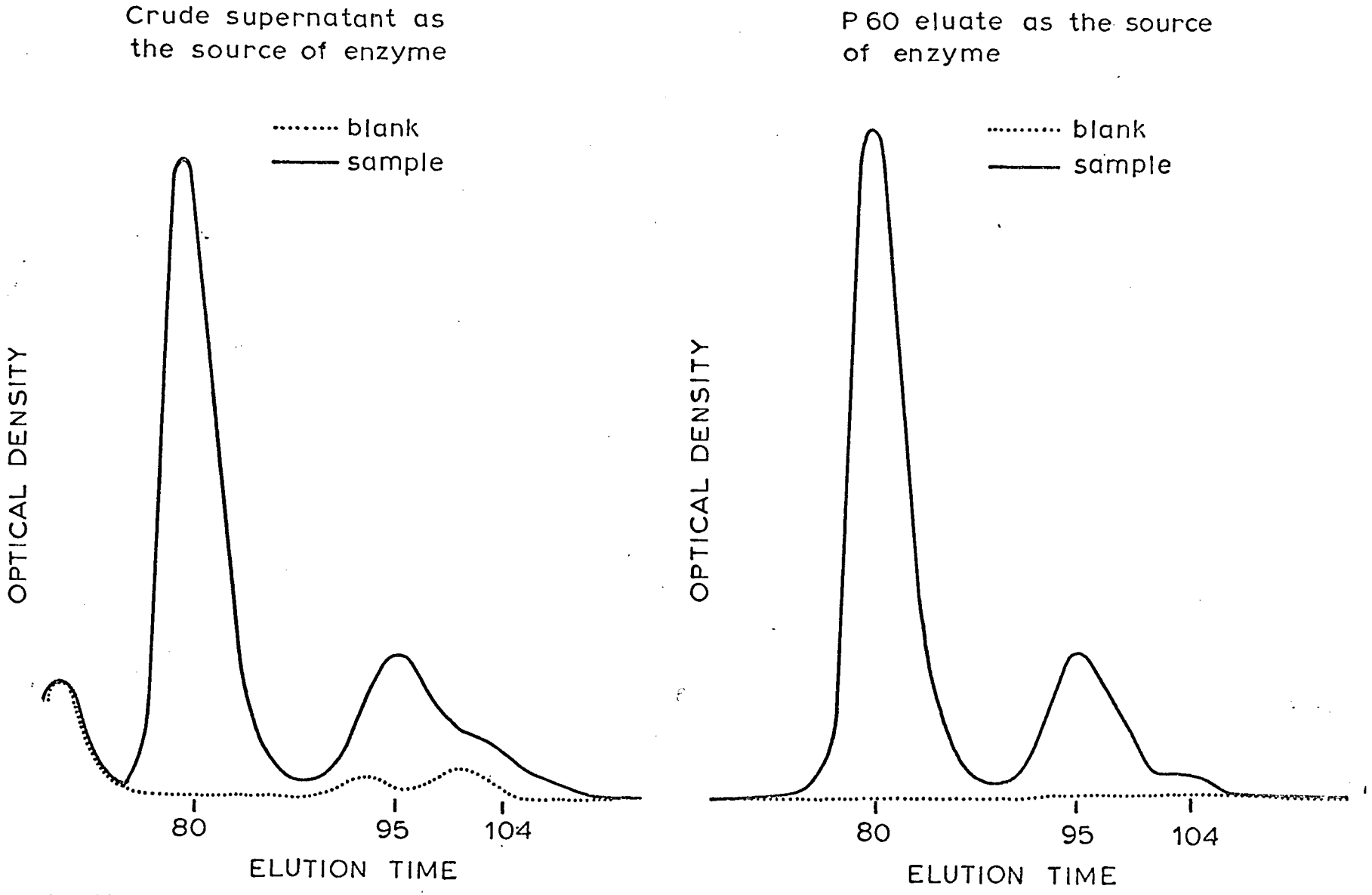
In order to rid the preparation of free amino acids the supernatant was lyophilized. The lyophilizate was dissolved in water to a concentration of 200 mg/ml and passed through a column of G-25 Sephadex. The first protein fractions were collected and used as the enzyme preparation. Chromatograms of incubations carried out with this preparation showed that the amount of free amino acids had been considerably decreased, but

that contamination still was present. These peaks due to neither substrate nor product increased with increasing time of incubation indicating that the preparation contained some system capable of hydrolyzing protein. Therefore, with this preparation the product peaks had to be corrected for response due to tyrosine and phenylalanine.

When P-60 eluate was used as the enzyme preparation, these problems were not encountered. Blanks showed no peaks at the elution times of the enzyme products.

Figure 7 shows pertinent regions from chromatograms of one hour incubations using both enzyme preparations. In this case blanks were prepared by the omission of DOPA. It is apparent from the figure that in both cases the largest product by far was 3-methoxy-4-hydroxyphenylalanine. The amount of 3-hydroxy-4-methoxyphenylalanine formed in the presence of either enzyme preparation was very small. This, together with the peaks in the blank in the region of product peaks when the supernatant was used, made a quantitative assessment of the relative amounts from this sort of data very difficult.

Using a split stream on the Amino Acid Analyzer, it is possible to divide the effluent from the columns into two fractions. One fraction of the effluent is reacted with ninhydrin and the colour is read and recorded while another fraction is passed directly into a fraction collector. It was decided to use this device and to use S-Adenosyl-methyl C^{14} as the methyl



Elution time
80 minutes = DOPA
95 minutes = 3 methoxy-4-hydroxyphenylalanine
104 minutes = 3 hydroxy-4-methoxyphenylalanine

Figure 7. Chromatograms from the Amino Acid Analyzer showing the separation of DOPA and its O-methylated derivatives

donor. By counting the radio-activity in the peaks corresponding to products, it was hoped that a quantitative assessment of the relative quantities in each peak would be obtained.

Incubation mixtures were identical to those previously used except that S-AMe-methyl C^{14} diluted with carrier S-AMe was used as the methyl donor. Fractions were collected every two minutes using a fraction collector. 0.5 ml aliquots of the fractions were placed in scintillation vials containing 10 mls of a scintillation mixture consisting of 0.4% PPO in 50% toluene and 50% ethanol and counted for two minutes.

It has been found that in the split stream operation a peak passes through the fraction collector 12 minutes before its presence is shown on the recorder. This is because the recording of peaks requires that the effluent must pass through much additional tubing. Thus, radioactive peaks corresponding to the two products were maximal in the two minute fractions from 82 to 84 minutes and 90 to 92 minutes. When the supernatant was used as the source of the enzyme the radioactivity in the second peak was 6% of that in the first peak. When the P-60 eluate was used as the source of the enzyme the radioactivity in the second peak was 5% of that in the first peak. DOPA is therefore about 95% 3-O-methylated in vitro and it does not appear that this percentage is changed by purifying the enzyme.

b) Position of O-methylation of 3,4-dihydroxycinnamic acid.

Since DOPA was 4-O-methylated to such a small extent, it was decided to examine the O-methylation of another compound with the two enzyme preparations. 3,4-dihydroxycinnamic acid (caffeic acid) had been reported to be O-methylated in both the 3 and 4 positions, giving ferulic acid and isoferulic acid respectively, by rat liver slices (32). However, the separation of products was not very good and no quantitative estimation of the relative amounts of 3- and 4-O-methylation was attempted.

Thin layer chromatography on polyamide which separates compounds on the basis of reversible hydrogen bond formation has been reported to be of value in the separation of isomeric phenols (78). It was therefore decided to examine whether this support could be used to separate ferulic acid and isoferulic acid.

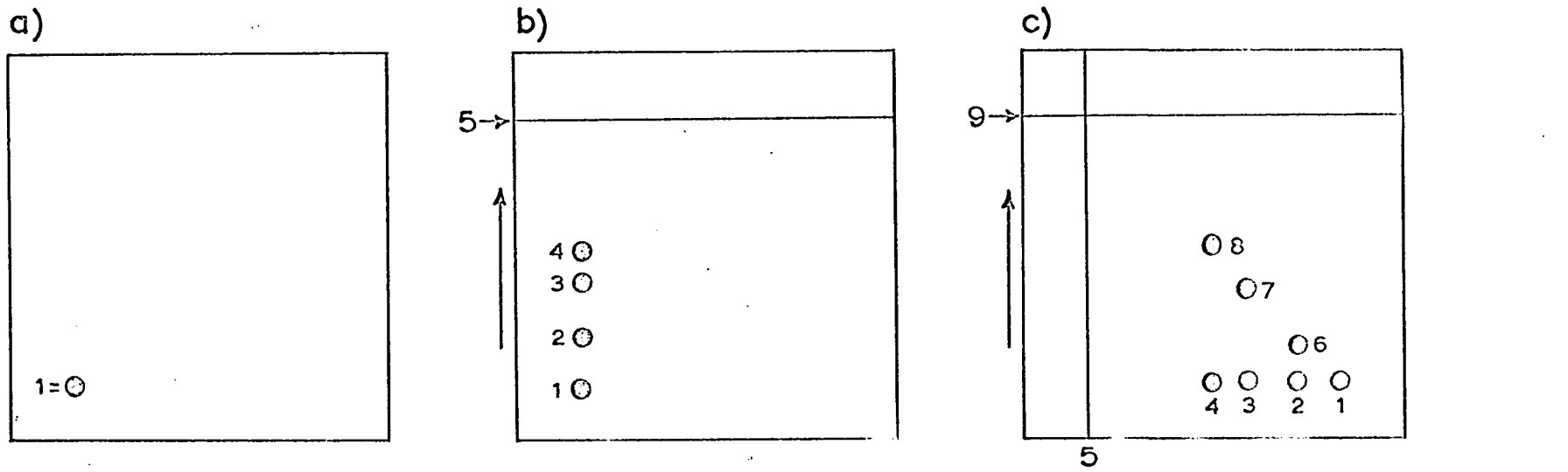
Polyamide for thin-layer chromatography was purchased from Alupharm Chemicals. An eluotropic series of solvents increasing in elutive power was examined. In order of increasing elution power these were water, ethanol, methanol, acetone, and sodium hydroxide solution. Of these, acetone gave the best separation of ferulic and isoferulic acid. In this solvent ferulic acid had an R_f of 0.54 and isoferulic acid had an R_f of 0.43. Spots could be located either by spraying with tetrazotized benzidine or by observation under UV light. Ferulic acid was coloured brown by tetrazotised benzidine and appeared blue under UV light. Isoferulic acid was coloured red by the spray and appeared white under UV light.

The following procedure was adopted to examine the position of O-methylation in the presence of the enzyme preparations. Caffeic acid was added in 0.2 ml of alcohol to a final concentration of 3×10^{-3}M to a mixture of 0.2 ml of 0.1M $MgCl_2$, 0.4 ml of 0.5M of phosphate buffer, pH 8.0, and 0.4 ml of 2.5×10^{-3}M S-AMe (S-AMe-methyl-C^{14}). Blanks were prepared by the omission of caffeic acid, 0.2 ml of alcohol being added instead. Reactions were initiated by the addition of weighed amounts of lyophilized enzyme preparations and stopped by the addition of 1 ml of 10% sulphosalicylic acid. The stopped reactions were filtered and the filtrate was extracted three times with five ml portions of ethylacetate. The pooled extracts were washed once with water and then evaporated to dryness under vacuum. The residue was dissolved in 0.2 ml of ethyl acetate and 3 ml were applied to the chromatograms.

Since chromatography on thin layers of polyamide results in spots that are quite diffuse, chromatograms were run in two directions, the solvent being allowed to migrate 15 cms from the origin in both directions. Figure 8 represents the procedure. It can be seen that the two directional run results in a clear separation of ferulic acid and isoferulic acid.

In order to determine the amount of radioactivity in the two enzymatic products, the spots were located and marked under UV light. The chromatogram was sprayed lightly with water to make the layer more matted and thus easier to scrape, and was then divided into cm^2 sections and scraped.

THIN LAYER CHROMATOGRAPHY OF ISOFERULIC ACID AND FERULIC ACID ON POLYAMIDE



1= Origin of application

1=Origin of application
2= Substrate (caffeic acid)
3= Isoferulic acid
4= Ferulic acid
5= Solvent front

1,2,3,4- Origins after one direction
5- First solvent front
6- Substrate after 2nd direction
7- Isoferulic after 2nd direction
8- Ferulic after 2nd direction
9- 2nd Solvent front

Figure 8

The scrapings were placed in vials containing 9.5 ml. of a scintillation mixture consisting of 0.4% PPO dissolved in 50% ethanol, 50% toluene and counted for 2 minutes. The areas corresponding to the products contained by far the largest amount of radioactivity, although some counts were located between the spots. When blanks were spotted, a standard mixture of ferulic and isoferulic acids was spotted at the same location and after development the regions corresponding to these compounds were scraped and counted. No radioactivity was located in these regions.

When the supernatant was used as the source of the enzyme, it was found that isoferulic acid accounted for 28% of the radioactivity of the products and ferulic acid accounted for 72%. This result is the average of three separate incubations and chromatograms in which the percentage of the radioactivity in the spot corresponding to isoferulic acid was 28%, 25% and 32%. Incubations were carried out for 30 minutes and 3 μl of the redissolved residue of the ethyl acetate extracts were spotted.

The results obtained under identical conditions when P-60 eluate was used as the source of enzyme showed that isoferulic acid accounted for 28%, 32% and 30% of the radioactivity in three separate experiments. The average value of the percentage of product radioactivity due to isoferulic acid was therefore 30% and that due to ferulic acid was 70% when P-60 eluate was used.

e) Summary of the O-methylation of DOPA and caffeic acid.

Since the extent of O-methylation in a particular position is not altered by purifying COMT about forty fold when either DOPA or caffeic acid was used as substrate, it is highly unlikely that O-methylation in the 4 position is catalysed by a different enzyme than the enzyme which O-methylates in the 3 position. It therefore appears that COMT is capable of O-methylating catechols in either the 3 or 4 position.

In agreement with other reports (32, 33) it has been found that the nature of the side chain determines the extent of O-methylation in each position. However, the suggestion that increasing 4-O-methylation results from increasing the chain length of the side chain (32) is not supported by these experiments. Much more 4-O-methylation was observed when caffeic acid was used as substrate than when DOPA was used as substrate. Since the chain length of these compounds is the same, it appears that the nature of the side chain is much more important than its length in determining the extent of 4-O-methylation.

4. Examination of the mechanism of the COMT catalyzed reaction.

Many enzyme catalyzed transfer reactions are thought to involve transfer of the labile group from the donor molecule to the enzyme prior to transfer to the acceptor (83). This type of mechanism may be represented as follows:-

$$(A - B) + E = A + (B - E)$$

$$D + (B - E) = B - (D + E)$$

In terms of the COMT catalyzed reaction a mechanism of this sort may be formulated as:

1) S-AMe + E = S + E-Me

2) E-Me + C = E + Me - C

where S-AMe is the methyl donor, S-adenosylmethionine; S is its demethylated product, S-adenosylhomocysteine; E is the enzyme; C is a catechol; and Me-C is an O-methylated catechol. If isotope exchange were observed between S-AMe and S in the absence of a catechol, it would constitute strong evidence for reaction 1 and therefore indicate that the mechanism involving the methylated enzyme as an intermediate was correct.

Isotope exchange in the presence of the enzyme would be demonstrated if by the incubation of radioactive adenosylhomocysteine in the presence of cold adenosylmethionine, radioactive adenosylmethionine were formed. Experiments to examine this possibility were therefore initiated.

Adenosylhomocysteine (S) was prepared from adenosine and homocysteine using homocysteine condensing enzyme from rat liver (84). Rat liver was homogenised in a Waring Blendor at ½ maximum speed for 35 seconds. The homogenates were stored at -20°C until used. The incubation mixture consisted of 1.5 m moles of adenosine, 6.0 m moles of DL-homocysteine (free base), and 1.0 m mole of phosphate buffer pH 6.5 in a volume of 100 ml (85). The flask containing this mixture was flushed with nitrogen for 10 minutes to produce anaerobic conditions. 5 ml of the enzyme preparation was then added and the flask was sealed. Incubation was carried out for 3 hrs. at 37°C. 0.025 ml of thiodiglycol was then added and the mixture was placed in boiling water for 5 minutes. After cooling in an ice bath, the precipitate was removed by centrifugation at 9000 x g for 20 minutes.

Purification of the product was attempted on columns of Dowex 1-HCO_3 at room temperature. It had been reported that pure S could be prepared by the use of this type of column and that an ion exchanger could be used to separate quantitatively S and S-AMe (86). Dowex 1-HCO_3 columns were prepared by washing the columns with an amount of 4N NaCl equal to 15 times the column volume. The columns were then washed with 0.5N $NaHCO_3$ until the eluate was free of chloride (no precipitate with Ag NO_3).

100 ml of the deproteinised incubation mixture was applied to a column 4 cm x 5 cm and 15 ml fractions were collected. Elution was carried

out using distilled water until the $O.D._{260}$ of the eluate decreased to 0.1. This took about 3 liters of water. 500 mls of 0.01M NaCl was passed through the column and the $O.D._{260}$ of the eluate decreased below 0.05. 0.10M NaCl was then used to eluate the compound. After about 1 liter of 0.10M NaCl had been passed through the column, the $O.D._{260}$ of the eluate increased to greater than 2.0. All the fractions with an $O.D._{260}$ of greater than 1.0 were pooled. The concentration of S present was calculated using a value of E_{260} of 15,400. To the pooled eluate 1.25 ml of concentrated H_2SO_4 per liter was added and the S was precipitated using 1 ml of 20% phosphotungstic acid per μmole of product. After leaving the mixture in the cold overnight most of the supernatant was removed by decantation. The remaining amount was removed by centrifuging at 1500 rpm for 10 minutes. The precipitate was washed once with 10 volumes of ice cold water.

Organic solvents have been used to decompose the phosphotungstate (85, 86, 87). It was recommended that the phosphotungstate precipitate from the Dowex 1-HCO_3 columns be dissolved in a 3:1 mixture of acetone and 1N H_2SO_4 and then extracted with 4 volumes of a 1:1 mixture of isoamyl alcohol and ether (86). When this procedure was attempted no separation of the mixture into an organic phase and an aqueous phase occurred after prolonged centrifugation. It was therefore decided to adopt an earlier procedure in which the phosphotungstate was dissolved in a 1:1 mixture of acetone and 1N H_2SO_4 (87) and extracted as before. In this case the layers

were easily separated by centrifugation. The upper organic phase was discarded. The extraction was repeated 4 x with 2 volumes of the organic solvent, discarding the organic phase each time. Traces of isoamyl alcohol were removed from the aqueous phase by four extractions with 4 volumes of ether. Traces of ether were removed by a stream of nitrogen. The solution was neutralised using a freshly prepared saturated solution of barium carbonate. The precipitate of barium sulfate which formed was removed by centrifugation. The supernatant was then expected to contain the purified compound.

Chromatography of the supernatant on a thin-layer of cellulose with a solvent consisting of n-butanol: acetic acid: water (60:15:25) revealed only one ninhydrin positive, UV absorbing spot. This spot had an R_f of 0.35.

No standard compound was available so it was impossible to demonstrate directly whether the purified compound was adenosylhomocysteine. It was thought that indirect evidence would be obtained from the effect of the compound on the COMT catalysed O-methylation of CP. Since S is a product of the reaction, it would be expected that when an excess was present the O-methylation of CP would be inhibited. Neither adenosine, nor homocysteine alone or together inhibited COMT. Nor did the homocysteine condensing enzyme preparation from rat liver. However, the deproteinised supernatant from the enzymatic preparation of S was found to contain a potent inhibitor. When 0.4 ml of this solution was added to an incubation mixture containing 0.4 ml of 2.5×10^{-3}M S-AMe, 0.2 ml of $MgCl_2$, 0.6 ml

of 5.7×10^{-4}M CP in 0.5 M phosphate buffer, pH 8.0, and 0.4 ml of the P-60 eluate COMT preparation, about 85% inhibition was observed as compared to an identical preparation in which water was added instead of the S containing solution. It therefore appeared that S was capable of inhibiting the O-methylation of CP.

When the S preparation which had been purified by passage through Dowex 1-HCO_3^- was examined as an inhibitor of COMT it was found to possess no inhibitory properties. This indicated that the purified compound was either a product formed by the enzymatic synthesis that was devoid of inhibitory ability, the observed inhibition by the unpurified preparation being due to another compound, or that in the purification procedure some chemical modification occurred making the compound inactive as an inhibitor.

Chromatography of the deproteinized incubation mixture in butanol, acetic acid, water revealed that only one UV absorbing, ninhydrin positive spot was present. This occurred at R_f 0.42. Thus, this compound did not have the same R_f as the UV absorbing, ninhydrin spot observed after purification by passage through Dowex 1-HCO_3^-. It therefore appeared that passage through the ion exchanger chemically modified the enzymatic product. It was therefore decided to use another ion exchange procedure to purify the enzymatically formed S. It had been found that S could be purified on Dowex 50-H^+ (85). If 0.25 ml of thiodiglycol per liter was added to the eluting solutions and if the column was run in the cold room, it was found that oxidation of S was prevented.

Subsequent attempts to purify S, therefore, were carried out under these conditions. A 5 cm x 10 cm column of Dowex 50-H^+ was used. 100 mls of the deproteinized preparation was applied to the column. Elution was carried out using 1N H_2SO_4 until the $O.D._{260}$ of the effluent decreased to 0.025. This required 4 liters of 1N H_2SO_4. 1 liter of 3N H_2SO_4 was then applied to remove traces of adenine. S was eluted using 3 liters of 6N H_2SO_4. The first liter of 6N H_2SO_4 eluate was discarded. The second and third liters were pooled and treated with phosphotungstate and organic solvents as before. The purified compound was chromatographed. On chromatography one UV absorbing, ninhydrin positive spot was observed. The R_f of the spot, 0.41, was the same as that observed for the UV absorbing, ninhydrin positive spot of the deproteinized incubation mixture. The solution of the purified compound also proved to be capable of inhibiting [illegible]. It was therefore concluded that by using a column of Dowex 50-H^+, a purified preparation of S could be obtained. The claim that pure S could be obtained by passage of the incubation mixture through Dowex 1-[illegible] (36) appears to be false. This treatment results in the formation of a product of S which has a lower R_f than S, and which does not possess any [illegible] inhibitory ability. This product may be formed by the oxidation of S. Even if oxidation occurs, the ability of a column to separate S-Ade and S would be of use in the detection of isotope exchange. It was therefore decided to synthesize radioactive S. To do this 10μc of adenosine-8-C^{14} was added to the incubation mixture. The S formed was purified by passage through Dowex 50-H^+.

Incubation mixtures consisting of the following were prepared:

0.2 ml of 0.1 M $MgCl_2$

0.2 ml of 0.5 M phosphate buffer pH 8.0

0.8 ml of 2.5×10^{-3} S-AMe

1.0 ml of CORT (P-60 eluate), or water for the blank

0.8 ml of 1.7×10^{-3}M S (made in the presence of 10μ 6 of adenosine-8-Cl^4)

The sample and the blank were preincubated for 3 minutes before the addition of S, then incubated a further 20 minutes at 37°C. At the end of this time both preparations were placed on identical 1 x 10 cm columns of Dowex 50-Na^+. This form of ion exchanger had been shown to be of use in the separation of S and S-AMe (86). S is eluted by 0.1 N NaCl whereas S-AMe is not. In order to elute this compound 6N H_2SO_4 must be used. Accordingly, 0.1 N NaCl was used to elute the S from the columns. 10 ml fractions were collected. 0.5 ml aliquots from these fractions were placed in scintillation vials containing 9.5 mls of a 1:1 mixture of ethanol and toluene which contained 0.4% PPO. The vials were then counted for 1 minute. Elution of the columns with 0.1 N NaCl produced sharp radioactive peaks in both columns which declined from about 5000 counts per minute in the first tube to about 100 counts per minute by tube 8. 6N H_2SO_4 was then used to elute the remainder of the activity on the column. If radioactive S-AMe had been formed from radioactive S in the presence of the enzyme, a peak of radioactivity would be expected in the 6N H_2SO_4 eluate of the column to which the complete system had been added. No such

peak would be expected in the blank. The examination of the 6N H_2SO_4 eluate of both the complete system and the blank showed that in both cases no significant amount of radioactivity was eluted. It therefore appeared that isotope exchange does not occur in the COMT catalysed reaction. In view of this it was unlikely that a methylated enzyme is formed as an intermediate.

Attempts to demonstrate a methylated enzyme intermediate in other transmethylation reactions have proved unsuccessful. For thetin - homocysteine methylpherase, the evidence was strongly against this mechanism (57). Isotope exchange could not be demonstrated, nor could a methylated protein intermediate be isolated.

Incubation of H^3-adenosylhomocysteine in the presence of S-AMe and histamine N-methyltransferase showed that no label was incorporated into the S-AMe (56). It was concluded that the methyl group transfer from S-AMe to histamine occurred without a methylated enzyme as an intermediate.

It therefore appears that COMT, like other methyltransferases, catalyses the transfer of the methyl group directly from the donor, S-AMe, to the acceptor molecule.

5. Attempted isolation of intermediates.

Although the results of the experiments with radioactive S were evidence against the formation of a methylated enzyme intermediate, it was still possible that such an intermediate might occur. If the methylation reaction is written:

$$E + S\text{-}AMe \rightleftharpoons E\text{-}Me + S$$

it is obvious that the occurrence of a methylated intermediate will be detected by isotope exchange only if the reaction is reversible. Since it was still possible that in the absence of an exchange reaction that a methylated enzyme could occur, it was decided that further evidence in this regard would be of value.

In the absence of a catechol it is reasonable to expect COMT and S-AMe to react in one of the following ways:

1) $E + S\text{-}AMe \rightleftharpoons E\text{-}Me + S$

2) $E + S\text{-}AMe \rightleftharpoons E\text{-}S\text{-}AMe$

If one of these complexes were formed and if it were sufficiently stable to resist breakdown in the cold, then incubation of the enzyme in the presence of S-AMe (S-AMe-methyl-C^{14}) with no catechol present and passage of the incubated mixture through a column of Sephadex G-25 in the cold should result in the isolation of radioactivity incorporated into the protein fraction. To determine whether label could be incorporated into the protein, 1.5 ml of COMT (P-60 eluate containing about 4 mg protein) was incubated in the presence of 0.1 ml $MgCl_2$, 0.3 ml of 0.5 M

phosphate buffer, pH 8.0, and 0.6 ml of 2.5×10^{-5}M S-AMe (S-AMe-methyl-C^{14}) for 10 minutes. A blank was prepared by the substitution of a 1.5 ml of water for the P-60 eluate. Both preparations were passed through 2.5 x 20 cm columns of G-25 Sephadex using 0.01M phosphate buffer, pH 8.0, to elute. 3 ml fractions were collected. 0.5 ml aliquots from the fractions were added to scintillation vials containing 9.5 mls of 0.4% PPO in a 1:1 solution of ethanol and toluene. Vials were counted for two minutes. In the case of the incubated enzyme preparation, a radioactive peak was found associated with the enzyme in the eluate. Although the radioactivity in the peak was only a small amount compared to the amount detected in the peak corresponding to the methyl donor, it was sufficiently above the background to indicate that the methyl donor was in some way becoming associated with the enzyme. In the water blank no radioactive peak was eluted by the same volume.

In order to determine whether this association of the methyl donor with the enzyme was specific, the experiment was carried and using 4 mg of bovine serum albumin instead of the COMT preparation. In this case a radioactive peak associated with the protein peak also was observed. When a portion of the P-60 eluate having no COMT activity was incubated, radioactivity again became associated with the protein. It therefore appears that non-specific association of S-AMe with protein occurs. How this may be accomplished cannot be surmised at this time, but the fact that it does occur must be considered in any studies of this sort. The label appears

to be quite strongly associated with the protein as the supernatant after treatment of the protein-bound radioactivity fraction with sulfosalicylic acid contained no radioactivity.

These findings, it should be noted, do not agree with reports of specific binding of S-AMe by methionie activating enzyme (88,89). It was claimed that when methionine activating enzyme from bakers yeast was incubated in the presence of S-AMe-methyl-C^{14} that radioactivity remained associated with the protein after passage through G-25 Sephadex. Such an effect was not observed when bovine serum albumin, ovalbumin, and pepsin were used.

It is very difficult to reconcile these findings with those observed when the binding of S-AMe to COMT was examined. It has been reported recently that an enzyme system from mouse tumour is capable of transferring the methyl group of S-AMe to ovalbumin (90). However, this report sheds little light on the contradictory findings on the binding of S-AMe to protein, as it is unlikely that methylating enzyme would be present in one preparation and not in another.

A similar experiment was done with epinephrine-C^{14}. Again on passage through G-25 Sephadex it was found that counts were associated with the protein peaks when either a COMT preparation ar albumin were used. It therefore appears that in the case of epinephrine, too, a certain amount of non-specific binding to protein occurs. This binding

was not examined in enough detail to allow an explanation.

Since the binding of both S-Ado and epinephrine, when measured in this way, exhibits no specificity for COMT, the value of such methods in the isolation of intermediates is doubtful, and because of the binding to blanks no more attempts to isolate intermediates were made.

IV DISCUSSION AND CONCLUSIONS

The understanding of an enzyme catalyzed reaction requires a knowledge of the structure of the transition state and the nature of the intermediates of the reaction. In order for this knowledge to be obtained the following objectives must be achieved (91) : (1) the pathway for the enzyme catalyzed reaction must be elucidated; (2) the amino acids involved in bond breaking and bond making reactions must be identified; (3) the arrangement of these amino acids in space must be determined; and (4) the velocity of the enzyme reaction must be explained by assigning specific catalytic roles to the groups involved.

Two approaches to the study of the pathway of the enzyme catalyzed reaction are possible. A kinetic approach can give information concerning the sequence of the reaction. Although different pathways lead to different kinetic equations, it is difficult to determine reaction sequences unequivocally by kinetic studies alone, for several different patterns may lead to kinetics which cannot readily be distinguished (92). Therefore a second approach consisting of the isolation of intermediates of the reaction is necessary in order to elucidate the pathway.

A pure enzyme preparation is not necessary for useful kinetic studies, but unambiguous results regarding the nature of the active center can only be obtained with such a preparation. Therefore, a necessary prerequisite to the understanding of an enzyme catalyzed reaction is the preparation of pure enzyme.

The purification of an enzyme is greatly facilitated by the availability of a good source of the enzyme and of a good assay for enzyme activity. When the foregoing work on catechol O-methyltransferase catalyzed reaction was initiated only thirty fold purification of the enzyme had been reported (2). The failure to purify the enzyme further may be attributed in part to the lack of a rapid, convenient assay for enzyme activity. The assay procedures which were available consisted of; (a) a flourometric estimation of metanephrine formed from the enzymatic O-methylation of epinephrine (2); (b) the estimation of metanephrine-H^3 formed from epinephrine-H^3 (93); and (c) the estimation of radioactive vanillic acid formed from S-adenosyl-L-methionine-methyl C^{14} and 3,4-dihydroxybenzoic acid (94). All of these techniques require extraction and subsequent manipulation. It was therefore decided that a search for a more convenient assay would be of value. It was found that the use of the acid-base indicator pyrocatecholphthalein as a substrate for the enzyme provided such an assay. Whereas pyrocatecholphthalein could not be colored by basic borate buffer solution, its O-methylated derivative guaiacolphthalein exhibited a blue color in this solution. The incubation of pyrocatecholphthalein in the presence of Mg^{++}, S-adenosyl-L-methionine, and a catechol O-methyltransferase preparation from rat liver resulted in the formation of guaiacolphthalein. The guaiacolphthalein so formed produced a blue color when basic borate buffer was added to the incubated mixture. The absorbance due to this color at its absorbance

maximum of 595 mμ was found to increase in a linear fashion with both quantity of enzyme and time of incubation when pyrocatecholphthalein was incubated as described.

The use of pyrocatecholphthalein as a substrate provided a rapid quantitative estimation of enzyme activity which was therefore suitable for application to purification studies.

Conditions for the application of the techniques of gel filtration and ion exchange chromatography on substituted celluloses to the purification of the enzyme were examined. Bio-Gel P-60, a polyacrylamide gel with an exclusion limit of 60,000, was found to retard the enzyme. When an enzyme fraction precipitated between 30 and 50% saturation by ammonium sulfate was passed through a column of Bio-Gel P-60, the enzyme was retained relative to most of the protein. Most of the applied activity was recovered and the recovered activity had a specific activity about eight times higher than the applied enzyme preparation. This degree of purification on Bio-Gel P-60 indicates that catechol O-methyltransferase has a low molecular weight relative to most of the proteins in crude preparations from rat liver.

Further purification was obtained using diethylaminoethyl cellulose. When the enzyme was applied to a column of this adsorbent in 0.01M phosphate buffer, pH 8.0, the activity was retained on the column. The activity could be eluted by 0.06M phosphate buffer, pH 8.0. The passage of an enzyme preparation which had been passed through a Bio-Gel P-60 column through such a column resulted in a further four fold purification of the enzyme. However, approximately one half of the activity placed

on such a column was lost and the eluted enzyme proved to be very unstable. The overall purification obtained was approximately two hundred fold. The instability of the purified enzyme prevented further attempts at purification. The reasons for the loss of activity which occured on passage through the ion exchanger and the instability of the resulting eluate are not known. Possibly the charges on the exchanger cause some irreversable structural change in the enzyme or either neccessary protecting proteins or cofactors are separated from the enzyme.

Although the objective of a purified enzyme was not reached, it was decided that useful information pertaining to the enzyme catalyzed reaction could still be obtained. To this end experiments designed to characterize the enzyme by an examination of its physical properties and an exploration of the pathway of the reaction it catalyzed were carried out.

The electrophoretic mobility of the enzyme on polyacrylamide gel was examined using a modification of the method of Ornstein and Davis (95). When an enzyme preparation which had been purified by passage through Bio-Gel P-60 was subjected to electrophoresis two activity bands, as measured by a modification of the pyrocatecholphthalein assay, and at least thirteen protein bands were located. Only eight protein bands were located in the two hundred fold purified preparation and again there were two activity bands. The multiple protein bands indicated that the enzyme preparation of highest specific activity still contained contaminating proteins. The activity bands migrated farther than the majority of the protein bands. The separation achieved by polyacrylamide

gel electrophoresis is on the basis of both charge and molecular size. Since the protein preparations placed on the gels were quite uniform with respect to molecular size, having been resolved on Bio-Gel P-60, the greater migration of the enzyme as compared to that of most of the other proeins in the preparation may indicate that at the running pH of the gels (pH 9.5) the enzyme may have a large net negative charge. The reason for the two activity bands is unknown. The existence of isozymes is possible, but it also is possible that some alteration in the enzyme occurs on purification giving two electrophoretically distinct catalytic species. The number of bands in crude preparations could not be ascertained as the enzyme assay procedure was not sensitive enough to detect the maximum amount of activity which could be applied. Only three milligrams of protein could be resolved on the polyacrylamide gel columns and in crude preparations this amount of protein does not give enough activity to be detected by the assay.

The molecular weight of catechol O-methyltransferase was estimated by gel filtration on Sephadex G-100 to be 29,000. The validity of this figure depends on the assumption that no adsorption occurs, and that the molecular weight of the enzyme bears a typical relationship to the cube of its radius (80). That no adsorption occurs cannot be said with certainty. However, the behavior of the enzyme on Bio-Gel P-60 is compatible with the molecular weight determined on Sephadex G-100. The cases in which adsorption has been observed have been with enzymes which have carbohydrate substrates. It is highly unlikely that catechol O-methyltransferase binds

carbohydrates. For these reasons it is reasonable to assume that the enzyme is not adsorbed on Sephadex G-100. The assumption that the enzyme is a protein of 'typical' shape can only be shown to be valid by a comparison of the value obtained for the molecular weight by gel filtration with values obtained by physico-chemical techniques. Since serious discrepancies between the molecular weight as determined by the behavior of proteins on Sephadex G-100 and values determined by physico-chemical means are rare (80), it is therefore highly likely that the value of the molecular weight of catechol O-methyltransferase as estimated by its behavior on Sephadex G-100 is representative of the true molecular weight of the enzyme.

An exhaustive kinetic study of the enzyme has not been carried out. The lack of a suitable assay prevented such a study. The flourometric assay (2) was used in some cases, but the time consuming manipulations involved in this procedure precluded its application to all the substrates studied. Although the pyrocatecholphthalein assay proved to be valuable in purification and electrophoretic studies of the enzyme, the complexity of the reaction prevented its application to kinetic studies. It has been shown that at least two products are formed on the enzymatic O-methylation of pyrocatecholphthalein (Figure 3). Thus the use of this compound as a substrate further complicates an already complex two substrate, metal activated reaction.

The majority of two substrate reactions which have been investigated appear to take place via one of the following mechanisms (96): (1) an ordered mechanism in which only one substrate can form a

binary complex in each direction; (2) a random mechanism in which binary complexes of both substrates with the enzyme as well as ternary complexes are possible; and (3) a ping pong mechanism in which the enzyme reacts with each substrate via a binary complex, and no ternary enzyme-substrate complexes are formed. In order to distinguish between alternative mechanisms of two substrate reactions data of exceptional accuracy and obtained under a wide variety of conditions are necessary (97). It is obvious that such data can be obtained only when an assay that is extremely accurate and convenient is available. Since no such assay for catechol O-methyltransferase was available, no exhaustive kinetic studies were attempted.

However it was decided that a survey of a variety of compounds both as inhibators and as substrates would be of value. Although such studies could not be expected to reveal the mechanism of the reaction, it was expected that some information regarding the conditions necessary for binding to the enzyme would be obtained.

If a compound were a substrate for catechol O-methyltransferase, it would be expected that its presence would inhibit the enzymatic O-methylation of pyrocatecholphthalein because of competition for the substrate binding site of the enzyme. The measurement of the ability of a wide variety of compounds to inhibit the O-methylation of pyrocatecholphthalein could be made with a minimum of difficulty. Hence the examination of the ability of a compound to inhibit the O-methylation of pyrocatecholphthalein provided a convenient method to screen potential substrates of catechol O-methyltransferase.

All the catechols examined were found to inhibit the O-methylation of pyrocatecholphthalein. However, since both iodophenols (36) and tropolones (35) had been found to inhibit the enzyme competitively, but were not substrates, the fact that all catechols which were tested inhibited the enzyme could not be taken as conclusive evidence that they were substrates. On the other hand, the inability of the phenols and the dimercaptotoluene which were tested to inhibit the enzyme is conclusive evidence that these compounds do not interact with the substrate binding site of the enzyme.

Direct evidence that the catechols were substrates was obtained by measuring the incorporation of radioactivity into ethylacetate extracts of incubations of the catechols when S-adenosyl-L-methionine-methyl C^{14} was used as the methyl donor. All the catechols examined by this method were shown to be substrates.

It was apparent from the inhibitory potencies and substrate values of the various catechols that both the inhibation caused by a compound and the extent of O-methylation of the compound were dependent on the nature of the group substituted on the catechol. For both cases the electronic nature of the substituted group appeared to be of more importance than its size. No correlation between good substrates and good inhibitors could be made. The inhibitions observed were under conditions where one enzyme was catalyzing two reactions simultaneously. In such a case the second substrate is expected to behave as a competitive inhibitor so far as the reaction with the first substrate is concerned (98). In competitive inhibition the kinetic parameter K_m is altered, but the parameter V_{max} is unchanged. Thus in a case of strict competitive inhibation one would expect only an effect on binding of the substrate to the enzyme. The greater the affinity of the enzyme for the inhibitor,

the more the K_m for the substrate will be increased. If the second substrate (the inhibitor) is a good substrate for the enzyme, it is to be expected that the enzyme has a high affinity for it. Therefore it would be expected that a good substrate would be a better inhibitor of the enzyme than a poor substrate if the inhibition were strictly competitive.

Since it was apparent from an examination of the values obtained for the various catechols that a good substrate was not necessarily a good inhibitor, it was decided that there could be a non-competitive aspect to some of the inhibitions observed in the presence of catechols. This possibility was examined by a kinetic study of the inhibitions observed in the presence of 4-nitrocatechol and catechol. It was shown that 4-nitrocatechol was an extremely potent non-competitive inhibitor of the enzyme. Non-competitive inhibition can be interpreted as being due to the binding of the inhibitor at a site other than the substrate site where it effects the reactivity of the enzyme without effecting the binding of the substrate (99). The non-competitive character of the inhibition by 4-nitrocatechol, therefore, indicates that a catechol can inhibit the enzyme by effects other than a competition for the substrate binding site. The lack of correlation between inhibitory potencies and substrate values may, therefore, be due to the enzyme having sites differing in affinities for the various catechols. Insufficent data have been obtained to treat the further complications in the interpretation of the results which arise if the possibility of allosteric inhibition is considered.

No evidence for the existence of a methylated enzyme intermediate was obtained. However, if an ordered reaction mechanism in which the substrate must be bound prior to interaction of the methyl donor with the enzyme to give a methylated enzyme intermediate occurs, the existence of a methylated enzyme will not be detected by an exchange reaction. If either a ping pong mechanism or a random mechanism occurs, one would expect to detect the presence of a methylated enzyme intermediate by an exchange reaction. One cannot visualize a ping pong mechanism in which the catechol forms a product prior to interaction of the methyl donor with the enzyme. However, a ping pong mechanism in which methyl donor interacts with the enzyme to form a product prior to the binding of the substrate is possible. If such a mechanism occured, the existence of a methylated enzyme intermediate would be detected by an exchange reaction.

The occurence of a random mechanism would be expected to lead to isotope exchange if a methylated enzyme were formed since the interaction of the methyl donor with the enzyme would be independent of the substrate. Thus, the failure to demonstrate isotope exchange between S-adenosyl-L-methionine and S-adenosyl-L-homocysteine is evidence that the enzyme catalyzed O-methylation of catechols does not proceed via a random mechanism or a ping pong mechanism involving a methylated enzyme intermediate.

It may be concluded that no complete picture of the catechol O-methyltransferase catalyzed reaction can be formulated from the preceding work. However, it is hoped that that the examination of the

many necessary preliminary considerations in the understanding of the enzyme catalyzed O-methylation of catechols that it presents will be of value in future attempts to understand the enzyme, catechol O-methyltransferase.

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