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THE DISTRIBUTION OF CATECHOL-*O*-METHYLTRANSFERASE IN PIG LIVER AND BRAIN

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Abstract—Subcellular fractionation studies of pig liver and brain show the enzyme catechol-*O*-methyltransferase (E.C. 2.1.1.6) to be confined to the soluble phase with no appreciable amounts being associated with particulate material. The regional distribution of the enzyme in brain is relatively even. Impure preparations of the enzyme exist in a high molecular weight form and a reversibly inhibitory material may be separated from purified preparations of the enzyme by high-speed centrifugation. The presence of this material results in a non-linear dependence of initial velocity of the reaction catalysed on protein concentration.

Despite earlier evidence indicating catechol-*O*-methyltransferase (E.C.2.1.1.6) to be confined to the soluble portion of the cell [1] several workers have, more recently, found significant amounts of the enzyme to be associated with the particulate fractions obtained in subcellular fractionation studies [2–5]. Microsomal forms of the enzyme have been found in rat liver [6–8] and fish kidney [9, 10] and membrane-bound forms of the enzyme have been reported to be associated with mouse liver plasma membranes [11], and rat erythrocyte membranes [12]. These bound forms of the enzyme have been reported to differ from the soluble enzyme in antigenic properties, K_m values and pH optima [6, 7, 10]. Creveling and his co-workers [11] have, however, been unable to detect antigenic differences between the soluble and particulate forms of the enzyme from rat liver and, after dissociation of the enzyme from rat liver microsomes by the use of a detergent, the molecular weight, pH optimum and kinetic properties were similar to those of the originally soluble enzyme [8, 12].

In this paper we report the results of subcellular fractionation studies that indicate that there are no appreciable amounts of catechol-*O*-methyltransferase associated with the particulate fractions of pig liver and brain, although the enzyme is capable of associating reversibly with membrane material with modification of its activity.

MATERIALS

Ammonium sulphate (low in heavy metals, product number 27203) was obtained from British Drug Houses Ltd. Poole, Dorset, U.K. Antimycin A, NADH and *S*-adenosyl-L-methionine were obtained from Boehringer Corp. (London) Ltd, London, U.K. Anticholinesterase BW 284C51 (1:5-*bis*-(allyldimethylammonium phenyl)-pentan-3-one dibromide) was obtained from Wellcome Reagents Ltd, Beckenham, Kent, U.K. and *l*-adrenaline bitartrate was obtained from Sigma (London) Ltd, London, U.K. Triton X-100, tetraisopropyl phosphoramide and *m*-*O*-methyl-*l*-adrenaline hydrochloride were obtained from Koch-Light Ltd, Colnbrook, Bucks, U.K. Sephadex gels and blue dextran were obtained from Pharmacia Ltd, London, U.K., and Kieselguhr H nach stahl was obtained from Anderman and Co. Ltd., East Moseley, Surrey, U.K.

^{14}C-labelled *n*-hexadecane (1.859×10^4 dps) and *S*-adenosyl-L-(methyl[^{14}C]) methionine of specific activities 0.5 mCi/m-mole and 58 mCi/m-mole were obtained from The Radiochemical Centre, Amersham, Bucks, U.K.

Pancreatic chymotrypsinogen A, β-galactosidase from *E. coli* (E.C. 3.2.1.23), pig heart lactate dehyrogenase (E.C. 1.1.1.27), malate dehydrogenase (E.C. 1.1.1.37) and pyruvate kinase (E.C. 2.7.1.40) were obtained from Boehringer Corp. (London) Ltd, London, U.K. and Taka-distase was obtained from Sankyo Pharmaceutical Co. Ltd., Tokyo, Japan.

All other chemicals were obtained from British Drug Houses Ltd, Poole, Dorset, U.K.

Diaflo membranes were obtained from Amicon Corp. Inc., Lexington, Mass, U.S.A. and dialysis tubing was obtained from The Scientific Instrument Centre Ltd, London, U.K.

METHODS

Subcellular fractionation. The method used with pig brain was that described by Whittaker [13] and the procedures described by De Duve and his co-workers [14, 15] were used with pig liver. These procedures are shown diagramatically in Figs. 1 and 2. Pellets obtained from the fractionation procedures

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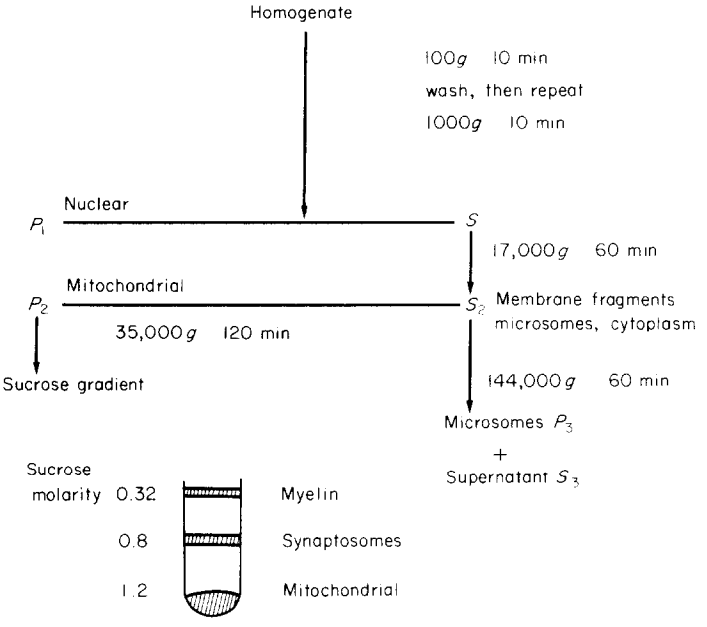

Fig. 1. Subcellular fraction of pig brain. The method used was that described Whittaker [13]. A 1:10 homogenate of brain in 0.32 M sucrose was prepared using a Dounce homogeniser. All steps were carried out at 0–4°. *S* represents the supernatant and *P* the pellet from the centrifugation steps.

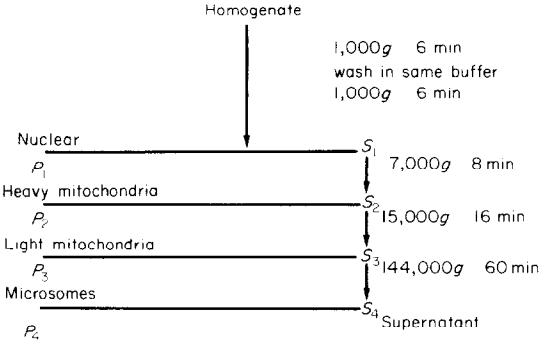

Fig. 2. Subcellular fractionation of pig liver. The method used was taken from that described by De Duve and his coworkers [14, 15]. A 1:5 homogenate of liver in 0.25 M sucrose was prepared using a Dounce homogeniser and filtered through gauze before the centrifugation steps. All operations were carried out at 0–4°. *S* represents the supernatant and *P* the pellet from the centrifugation steps.

were suspended in 0.02 M phosphate buffer, pH 7.9 and were frozen in liquid nitrogen for storage [16].

Assay methods. Catechol-*O*-methyltransferase was assayed by either using a coupled assay or a radiochemical method. The coupled assay [17, 18] employed adenosine deaminase, prepared from Takadiastase according to the method of Sharpless and Wolfenden [19] up to and including the ethanol precipitation step, to catalyse the conversion of the *S*-adenosyl homocysteine, formed by the action of catechol-*O*-methyltransferase, to *S*-inosyl methionine. The assay mixture contained, in a total volume of 2.0 ml of 20 mM phosphate buffer, pH 7.2, 0.94 mU adenosine deaminase, 5 mM $MgCl_2$, 0.2 ml saturated KCl, 0.96 mM adrenaline bitartrate and 80 μM *S*-adenosyl methionine. The reaction was followed spectrophotometrically at 265 nm, at 25°. The calculation of McClure [20] was used to ensure that the amount of adenosine deaminase present was not rate limiting.

The radiochemical assay method used was essentially that of Axelrod and Tomchick [1]. The assay mixture contained, in a total volume of 1.0 ml of 20 mM phosphate buffer pH 7.2, 10 mM $MgCl_2$, 0.1 ml saturated KCl, 200 μM ^{14}C-labelled *S*-adenosyl methionine (0.5 mCi/m-mole) 0.96 mM adrenaline and enzyme. In cases where a more sensitive assay was required the reaction volume was reduced to 0.1 ml and the *S*-adenosyl methionine was at a higher specific activity (58 mCi/m-mole); the concentrations of the other reagents were as used in the larger assay volumes. The reaction was allowed to proceed at 25° for 10 min before it was stopped by the addition of 1.0 ml of 125 mM borate buffer, pH 10.0. The product was extracted twice with 1.5 ml of toluene: isoamyl alcohol (3:2 vol:vol) and 2.0 ml of the combined organic phases were mixed with 1.0 ml of a scintillation mixture, containing 5g- 2,5-diphenyloxazole per litre of Triton: toluene (3:2 vol:vol), for liquid scintillation counting. Time-courses of the assay were used to ensure that the rate of the reaction was linear for more than 10 min in the ranges of activities used in these studies.

Acid phosphatase was assayed by the method of Appelmans *et al.* [21], aldehyde dehyrodrogenase by the method of Racker [22], antimycin-insensitive-NADH-cytochrome-*c* reductase by the method of Edelhoch *et al.* [23], β-Galactosidase by the method of Mehler *et al.* [25], monoamine oxidase by the method of Tabor *et al.* [26], lactate dehydrogenase by the method of Kornberg *et al.* [27], and succinate

dehydrogenase by the method of Slater and Bonner [28]. Pyruvate kinase was assayed by a modification of the method of Valentine and Tanaka [29]. Assays contained, in a total volume of 3.0 ml, 50 mM triethanolamine-KOH buffer pH 7.2, 100 mM KCl, 20 mM $MgCl_2$, 1.0 mM phosphoenolpyruvate, 2 mM ADP 0.1 mM NADH, 5 units of lactate dehydrogenase and the pyruvate kinase sample. NADH oxidation was followed spectrophotometrically at 340 nm, Acetylcholine esterase was assayed by the method of Ellman *et al.* [30] and butyrylcholine esterase was assayed in a similar way except that anti-cholinesterase of 0.1 mM was included in the assay mixture and butyrylthiocholine iodide (0.5 mM) was used as the substrate.

All assays were carried out at 20° and one unit of enzyme activity is defined as the amount of catalysing the production of 1 μmole of product per minute.

Protein concentration was determined using the microbiuret method [31] or the absorbance at 280 nm and 260 nm [32]. DNA was determined by the method described by Burton [33].

Catechol-*O*-methyltransferase was purified from pig liver by the method of Nikodejevic *et al.* [34].

Molecular weights were determined by gel-filtration through 1.0 × 50 cm or 2.5 × 50 cm columns of Sephadex G 100 or G 200 equilibrated at 4° in 50 mM Tris–HCl buffer or 20 mM phosphate buffer, pH 7.2, both containing 500 mM NaCl. The columns were calibrated either using marker proteins as described by Andrews [35] or by calculation [36].

Lipid material was examined after extraction with chloroform:methanol (2:1, vol:vol) [37, 38]. Twenty volumes of chloroform:methanol were mixed with the sample and it was left overnight at 4°. The lower phase was filtered through glass wool or glass-fibre paper and taken to dryness by rotary evaporation at a temperature of less than 50°. The residue was dissolved in 30 ml of the chloroform:methanol and 7.5 ml of a 0.9% sodium chloride solution was added. After vigorous shaking the phases were allowed to separate and the lower phase was taken to dryness as before. The residue was dissolved in 1 to 2 ml of chloroform and a few drops of methanol were added to recombine the phases. This material was examined by thin layer chromatography using petroleum ether:ether:acetic acid (85:15:1, vol:vol:vol) (solvent system I) or chloroform:methanol:water (65:25:3, vol:vol:vol) (solvent system II). Spots were stained for unsaturated bonds with iodine [39] or rhodamine G [40], for sugar residues with α-naphthol in methanol [41], for phosphate with the stain of Dittmer and Lester [42], and for amino groups with ninhydrin [43].

RESULTS

The distribution of the enzyme in the subcellular fractions obtained from pig liver is compared with the distributions of a number of marker enzymes, represented by the method of De Duve *et al.* [14], in Fig. 3. The results clearly indicate catechol-*O*-methyltransferase to be localised in the cytoplasmic fraction with negligible amounts being present in the particulate fractions.

Similar representations of the distribution of catechol-*O*-methyltransferase and a number of marker

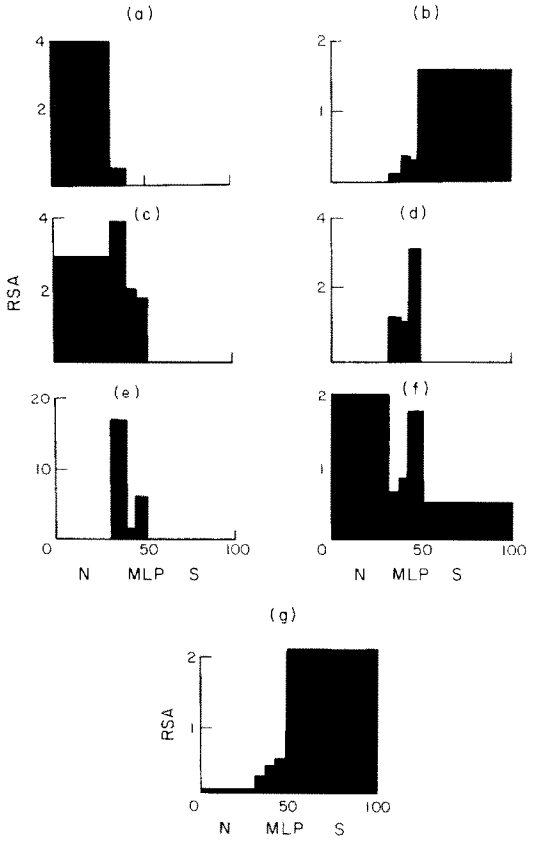

Fig. 3. The distribution of catechol-*O*-methyltransferase in subcellular fractions obtained from pig liver. The subcellular fractionation procedures and the presentation of the results used the procedures of De Duve and his co-workers [14, 15] as shown in Fig. 2. The relative specific activity (R.S.A.) in each fraction is expressed as:

$$\text{R.S.A.} = \frac{\text{Percentage recovery of enzyme (or DNA) in the fraction}}{\text{Percentage recovery of protein in the fraction}}$$

The fractions are designated as follows:

N, Nuclear; M, Heavy Mitochondrial; L, Light Mitochondrial; P, Microsomal; S, Cytoplasmic.

The components assayed were (a) DNA, (b) Lactate dehydrogenase (c) Monomaine oxidase (d) Antimycin-insensitive-NADH-cytochrome-*c* reductase, (e) Aldehyde dehydrogenase, (f) Acid phosphatase and (g) Catechol-*O*-methyltransferase.

enzymes in the subcellular fractions obtained from pig brain are shown in Fig. 4. Again the enzyme from this source appeared to be confined to the soluble phase and very little activity was found to be associated with the synaptosomal fraction. The distribution of the enzyme in various regions of brain is shown in Table 1.

The variation of the enzyme activity with the concentration of enzyme is shown in Fig. 5 with a pig liver homogenate as the enzyme source. Such curved plots were obtained whether the radiochemical assay or the coupled assay was used and also with brain homogenate as the enzyme source. When a sample of the enzyme from rat liver that had been

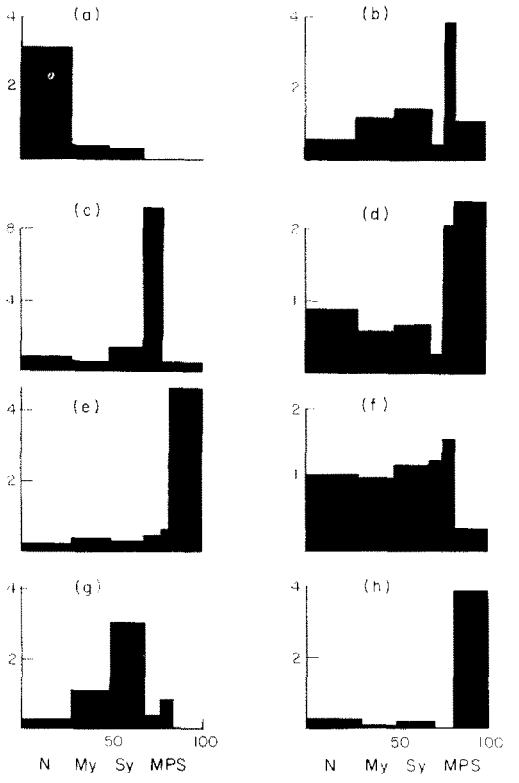

Fig. 4. The distribution of catechol-*O*-methyltransferase in subcellular fractions obtained from pig brain. The procedure of Whittaker *et al.*, [13] as shown in Fig. 1, was used for the fractionation and the results are expressed as described in Fig. 3. The fractions are designated as described in Fig. 3 with the abbreviations Sy. and My. representing the synaptosomal and myelin fractions respectively.

The components assayed were (a) DNA, (b) Acetylcholinesterase, (c) Succinate dehydrogenase, (d) Butyrylcholinesterase, (e) Lactate dehydrogenase, (f) Acid phosphatase (g) Occluded lactate dehydrogenase (fractions not frozen before assay) and (h) Catechol-*O*-methyltransferase.

Table 1. The regional distribution of catechol-*O*-methyltransferase in pig brain. The results represent the means from two separate brain dissections with duplicate or triplicate assays in each case

Brain region	Specific activity (mU/mg)	Percentage of total activity
Cerebellum	0.016	10.9
Thalamus	0.016	13.5
Medulla and Pons	Not detectable	
Frontal lobe	0.017	17.0
Parietal lobe	0.020	19.4
Temporal lobe	0.013	15.3
Occipital lobe	0.019	17.0
Corpus callosum	0.010	2.8
Hypothalamus	0.010	0.5
Pituitary gland	Not detectable	

purified to a specific activity of 2.5 mU/mg was used, a similar curved plot was obtained (Fig. 6). A number of treatments were used in order to investigate the possible cause of this curvature. Dialysis of a sample of the enzyme for 18 hr against 20 mM phosphate buffer, pH 7.2, at 4°, and the inclusion of 1 mM EDTA in the assay mixture had no effect on the curvature of these plots. Such curved velocity versus enzyme concentration plots could be due to the presence of a dissociable inhibitor in the enzyme preparation and the failure of dialysis to affect the curve would indicate such an inhibitor to be a relatively large molecule.

When a sample of the rat liver enzyme that had been purified by the method of Nikodejevic *et al.* [34] was centrifuged at 65,000 *g* for 13 hr a yellow coloured sediment was obtained which was usually devoid of enzyme activity although in some preparations a relatively small proportion of the enzyme activity sedimented with this material. The activity of the colourless supernatant showed a linear dependence on enzyme concentration as shown in Fig. 6, but when the sedimented material was resuspended in the supernatant the dependence showed a similar downward curvature to that shown by the starting material.

A qualitative investigation of the lipid composition of the yellow coloured sediment was carried out by thin layer chromatography of chloroform:methanol extracts prepared according to the method of Folch *et al.* [37, 38]. The material did not stain with either the specific phosphate stain used or the α-naphthol stain indicating the absence of phospholipids and glycolipids. It did however stain with iodine, rhodamine G and ninhydrin suggesting the presence of unsaturated lipids and either aminolipid or protein material. In solvent system I the iodine-staining material remained at the origin whereas triolein which was used as a neutral lipid marker had an R_f value of 0.39. This marker moved with the solvent front in solvent system II whereas the sample separated into three bands moving just behind the front. These results indicate that the sample contained charged lipid and protein or aminolipid but they do not allow a more positive identification to be made.

The molecular weight of the enzyme that had been purified by the method of Nikodejevic *et al.* [34] to a specific activity of 10 mU/mg was determined using a column of Sephadex G-100 which had been calibrated with Blue Dextran to allow determination of the void volume. The enzyme eluted from the column in a volume that corresponded to a molecular weight of about 25,000, determined by the method of Determann and Michel [36]. However when less highly purified preparations of the enzyme were used the apparent molecular weight of the enzyme was considerably greater. Thus a sample of the enzyme that had been purified to a specific activity of 0.5 mU/mg (from a crude pig liver homogenate with a specific activity of 7 μU/mg), by using the procedure of Nidodejevic *et al.* [34] up to and including the calcium phosphate treatment, was eluted in the void volume of the Sephadex G-100 column. The molecular weight of this enzyme preparation was found by gel-filtration on a column of Sephadex G-200 using the method of Andrews [35] with chymotrypsinogen, malate dehydrogenase, lactate dehydrogenase, pyruvate kinase and β-galactosidase as the molecular weight markers. The catechol-*O*-methyltransferase activity eluted from the column in a volume that corresponded to a molecular weight of about 325,000.

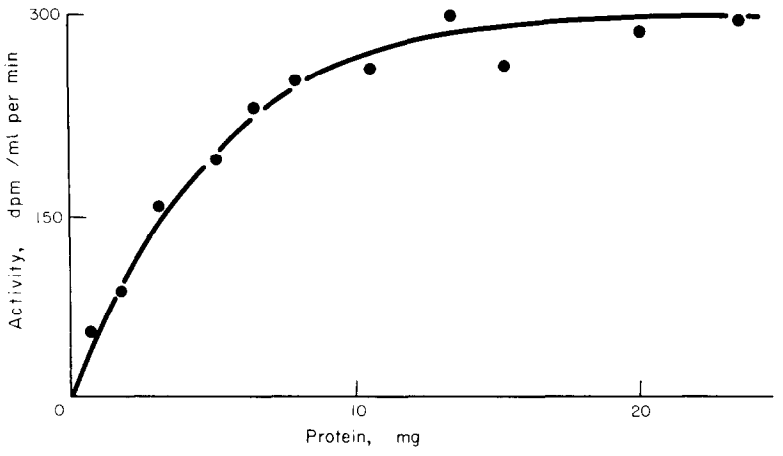

Fig. 5. The effect of protein concentration on the activity of pig liver catechol-*O*-methyltransferase. The radiochemical assay was used to determine the activity of the enzyme.

These results indicate that impure preparations of the enzyme exist in aggregated high molecular weight forms at the relatively high protein concentration (~11 mg/ml) used for the molecular weight determination. The existence of such a form was also demonstrated in an ultrafiltration experiment in which a preparation of specific activity 0.5 mU/mg remained above an Amicon XM 100 membrane (which allows materials with molecular weights below about 100,000 to pass through) with no detectable activity appearing in the filtrate. Attempts to cause disaggregation by varying the pH value (from pH 6.0–pH 8.0), the salt concentration (up to 0.5 M KCl or NaCl) or by the inclusion of either 0.1 per cent Triton X-100 or 1 mM dithiothreitol were unsuccessful in that no filtrate enzyme activity could be detected when the XM 100 membrane was used.

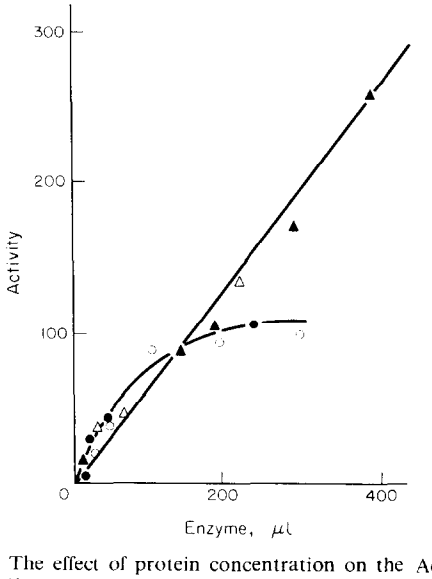

Fig. 6. The effect of protein concentration on the Activity of purified pig liver catechol-*O*-methyltransferase. The enzyme preparation purified by the method of Nikodejevic *et al.* (●, ○) and samples of this preparation that had been centrifuged at 65,000 *g* for a further 13 hr (▲, △) were used and activity was determined with either the radiochemical assay (●, ▲) or the coupled assay (○, △). The volumes of the centrifuged preparations were adjusted to be equivalent to that of the starting material (which had a protein concencentration of 4 mg/ml) and the activity is expressed as a percentage of that obtained with 200 μl of that preparation.

Since the enzyme is capable of aggregating with some high molecular weight material with inhibition of its activity, an attempt was made to see whether it would interact with any defined subcellular fractions obtained from rat liver homogenates. Samples of the purified enzyme (specific activity 8.5 mU/mg which gave linear velocity versus substrate concentration curves were assayed in the presence of the nuclear, heavy and light mitochondrial and microsomal fractions derived from pig liver. The subcellular fractions were dialysed against 20 mM phosphate buffer, pH 7.2, and were added to the enzyme sample to give a ratio of enzyme to particulate fraction that was close to that existing in the original homogenate as calculated from the data in Fig. 3. The microsomal and the two mitochondrial fractions were each able to induce curvature in the velocity versus enzyme concentration curves although the addition of these fractions resulted in an activation of the enzyme. The increase in the initial velocity at low enzyme concentrations was approximately two-fold in the presence of the light mitochondrial fraction, three-fold in the presence of the heavy mitochondrial fraction and nearly four-fold in the presence of the microsomal fraction. The presence of the nuclear fraction had no significant effect on the activity of the enzyme.

DISCUSSION

Catechol-*O*-methyltransferase appears to be largely confined to the soluble phase in subcellular fractionation studies of pig liver and brain. These results are in contrast to those of some other workers who have reported the presence of substantial amounts of membrane-bound enzyme in preparations from some other sources [6–12]. This may be due to differences

in the conditions employed for the fractionation studies since the amount of membrane-bound enzyme obtained from mouse liver appears to be very variable [compare 11, 44 with 45]. The results presented here indicate that the enzyme is capable of interacting with particulate material and that alterations in its properties result from this. The effects, however, appear to be quite complex. The studies on the effects of enzyme concentration upon the initial velocity indicate that the enzyme is capable of interacting with high molecular-weight material with inhibition of its activity whereas at lower concentrations a number of particulate fractionations from pig liver were shown to be capable of activating the enzyme. Thus the effects of membrane binding would appear to be concentration-dependent. Activation of the enzyme when it has bound to membrane material has been reported [9, 44] although Nikodejevic *et al.* [46] have observed the inhibition of the purified rat liver enzyme by a component of crude heart extract. In addition Tong and D'Iorio [45] have shown that membrane-bound mouse liver catechol-*O*-methyltransferase has different kinetic parameters from the soluble enzyme and the membrane-bound enzyme that has been subsequently solubilised, and effects such as these might explain the activation observed here.

Since the components separated in the subcellular fractionation studies are by no means homogeneous it is not certain whether the effects seen can be mediated by more than one component or whether they are due to a single component that is present as a contaminant in most fractions. In addition there is no evidence to suggest whether the activatory and inhibitory components are the same although the simplest model might involve binding of the enzyme resulting in activation with further polymerisation at higher concentrations causing inhibition. The nature of the inhibition has not been determined but the presence of a dissociable inhibitor in the enzyme preparation will result in hyperbolic plots of velocity versus enzyme concentration for all simple types of inhibition. Thus for example if this component (I) were a competitive inhibitor of the enzyme present in a fixed proportion to the enzyme, such that $I = xe$ (where e represents the enzyme concentration) the resultant kinetic equation would be

$$v = \frac{k}{\left(1 + \frac{K_m}{[S]}\right)\frac{1}{e} + \frac{K_m x}{K_i[S]}}$$

where $k = V/e$ and K_i represents the dissociation constant for the enzyme inhibitor complex. It is not however possible to determine a value for K_i without a knowledge of the type of inhibition given by the inhibitor or its molar proportion (x).

The relatively even distribution of catechol-*O*-methyltransferase in pig brain is consistent with the work of others [3, 47] and, in view of the wide differences in the type of innervation in different brain regions, this would argue against any specific association of the enzyme with areas associated with any particular type of innervation. The failure to detect significant amounts of this enzyme in the synaptosomal fraction of brain would also argue against a specific association with any given type of presynaptic transmitter system and are in agreement with the results of denervation experiments in brain [47–52] and metabolic studies that indicate that methylation of the catecholamines occurs at a different site from presynaptic oxidation [53, 54]. The subcellular fractionation studies reported here do not, however, indicate whether a proportion of the brain catechol-*O*-methyltransferase is extracellular, although the suggestion of Alberici *et al.* [2], that the enzyme that they found associated with the brain synaptosomal fraction might be bound to the outside of the synaptosomal membrane, would support such a localisation.

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