The Metabolism and Binding of Catecholamines by the Hepatic Microsomal Mixed-Function Oxidase of the Rat

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(Received 29 December 1975)

Noradrenaline and adrenaline were metabolized by an NADPH- and oxygen-dependent process located within the hepatic microsomal fraction of the rat. Metabolism was inhibited by CO and compound SKF 525A, but not by pargyline, an inhibitor of monoamine oxidase, or by 3,4-dimethoxy-5-hydroxybenzoic acid, an inhibitor of catechol *O*-methyltransferase. It is concluded that the enzyme system responsible for the metabolism of the catecholamines was the microsomal mixed-function oxidase. The K_m for noradrenaline was 2.4 mM and for adrenaline 1.0 mM, and V 15.6 and 3.6 nmol/min per mg of microsomal protein respectively. Both catecholamines bound to the microsomal fraction, producing a type II spectral change, with a K_s for noradrenaline of 0.9 mM and for adrenaline of 1.0 mM, and showed other characteristics of type II compounds by inhibiting the reduction of cytochrome *P*-450 by NADPH and exhibiting an enhanced metabolism in the presence of acetone. The major product of catecholamine metabolism was an as yet unidentified alkali-labile compound, which did not correspond to any of the recognized catecholamine metabolites.

Catecholamines are usually considered to be metabolized almost exclusively by monoamine oxidase (EC 1.4.3.4) and catechol *O*-methyltransferase (EC 2.1.1.6) (Axelrod, 1965; Iversen, 1967; Nagatsu, 1973) or by glucuronide and sulphate conjugation (Nagatsu, 1973). Catecholamines circulating in the blood are thought to be metabolized largely by hepatic catechol *O*-methyltransferase (Axelrod *et al.*, 1958). The hepatic microsomal mixed-function oxidase, on the other hand, is known to metabolize a wide variety of substrates of both an endogenous and exogenous origin, most of which produce a type I spectral change (Mannering, 1971). The hepatic microsomal fraction will also bind a wide variety of amines of an exogenous nature, producing a type II spectral change (Mailman *et al.*, 1974), although relatively few of these amines are thought to be metabolized, unlike type I compounds, where the spectral change appears to be directly related to metabolism (Jansson *et al.*, 1972). The molecular specificity of type II binding might suggest, however, a role for the microsomal fraction in the metabolism of amines of an endogenous nature.

The present work is an investigation of the binding and the metabolism of noradrenaline and adrenaline by the hepatic microsomal subcellular fraction of the rat. A preliminary report of this work has appeared elsewhere (McKillop & Powis, 1976).

Experimental

Methods

Preparation of the microsomal fraction. A pooled microsomal subcellular fraction was prepared from the livers of four 200–250 g male albino rats of the Wistar strain by the method of Ernster *et al.* (1962) in 0.25 M-sucrose containing 0.05 M-Tris/HCl buffer, pH 7.4, and adjusted to give a protein concentration of 10 mg/ml. Protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as a standard.

Assays. The metabolism of noradrenaline and adrenaline by the freshly prepared microsomal fraction was measured over various times up to 30 min, at 37°C, with a reaction mixture containing 5 mg of microsomal protein, Tris/HCl buffer, pH 7.4 (112 μ mol), $MgCl_2$ (10 μ mol), glucose 6-phosphate (25 μ mol), glucose 6-phosphate dehydrogenase (EC 1.1.1.49) [2 units (μ mol/min)], $NADP^+$ (1 μ mol) and either (–)-noradrenaline or (–)-adrenaline (12 nmol), all in a final incubation volume of 2 ml. The unchanged noradrenaline remaining after the incubation was measured by a modification of the radiochemical assay procedure of Iversen & Jarrott (1970) as follows. Incubation medium (10 μ l) containing the noradrenaline was incubated for 60 min at 37°C with 10 μ l of a reaction mixture containing

sodium phosphate buffer, pH 7.6 (2 μ mol), phenylethanolamine *N*-methyltransferase (EC 2.1.1.28) (0.2 mg), 2-mercaptoethanol (36 pmol), EDTA (34 nmol), pargyline (20 nmol), pyrogallol (20 nmol), compound SKF 525A (20 nmol) and *S*-adenosyl-L-[methyl- 3 H]methionine (0.7 nmol, 2.5 μ Ci). [methyl- 3 H]Adrenaline was then separated by chromatography on Whatman 3MM paper with butanol/methanol/1 M-formic acid (3:1:1, by vol.) solvent and measured by liquid-scintillation counting (Patterson & Greene, 1965). Unchanged adrenaline in the incubation medium was measured by a similar method but by using catechol *O*-methyltransferase (0.2 mg) instead of phenylethanolamine *N*-methyltransferase and separating the [methyl- 3 H]metanephrine by chromatography on Whatman P81 cellulose phosphate paper with butanol/ethanol/water (1:1:1, by vol.) as solvent. The spectral changes produced by the interaction of catecholamines with the microsomal fraction were measured by the method of Schenkman *et al.* (1967). NADPH-cytochrome *P*-450 reductase was measured by a modification of the method of Gigon *et al.* (1969) as described by Powis (1975).

Identification of metabolites. Aldehyde-NAD(P)⁺ oxidoreductase (aldehyde dehydrogenase, EC 1.2.1.5) [6 units (μ mol/min)] was added to the incubation medium during studies to identify the products of catecholamine metabolism, to convert any aldehyde metabolites into the less-reactive acid form. This was in addition to the aldehyde dehydrogenase known to be present within the microsomal fraction (Tottmar *et al.*, 1973). Microsomal proteins were precipitated by adding an equal volume of 0.4 M-HClO₄ containing 0.1% EDTA and 0.12% Na₂SO₃ (w/v). The metabolites formed from 14 C-labelled catecholamines were then separated by chromatography on alumina and a cation-exchange resin as described by Graefe *et al.* (1973), followed by two-dimensional t.l.c. on cellulose (Fleming & Clark, 1970) and radioautography.

Materials

(-)-Noradrenaline bitartrate, (-)-adrenaline bitartrate, EDTA (disodium salt), pyrogallol, 2-mercaptoethanol, 3-amino-1,2,4-triazole, *S*-adenosyl-L-methionine, aldehyde dehydrogenase and crystalline bovine serum albumin were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Glucose 6-phosphate (disodium salt), glucose 6-phosphate dehydrogenase and NADP⁺ were from Boehringer Corp. (London) Ltd., Lewes, East Sussex, U.K. Bovine phenylethanolamine *N*-methyltransferase and bovine catechol *O*-methyltransferase were from Miles-Seravac (Pty.) Ltd., Maidenhead, Berks., U.K. Pargyline (*N*-benzyl-*N*-methylprop-2-ynylamine) hydrochloride was from Abbott Laboratories Ltd., Queenborough, Kent,

U.K., and 3,4-dimethoxy-5-hydroxybenzoic acid from Nipa Laboratories Ltd., Treforest, Mid Glamorgan, Wales, U.K. Compound SKF 525A was kindly given by Smith, Kline and French Laboratories, Welwyn Garden City, Herts., U.K. *S*-Adenosyl-L-[methyl- 3 H]methionine, (-)-[methylene- 14 C]noradrenaline bitartrate and (±)-[7- 14 C]adrenaline bitartrate were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. Solvents were obtained from BDH Chemicals, Poole, Dorset, U.K.

Results

Metabolism of catecholamines by the hepatic microsomal fraction

Both noradrenaline and adrenaline were metabolized by an NADPH-dependent process in the hepatic microsomal fraction (Fig. 1).

Effect of various factors on metabolism

The replacement of air by O₂-free N₂ during the incubation with the microsomal fraction resulted in a considerable decrease in the metabolism of noradrenaline and a smaller, although still marked, decrease in the metabolism of adrenaline (Table 1). O₂+CO (10:90), which is known to inhibit the hepatic microsomal mixed-function oxidase (Conney *et al.*, 1957), produced a considerable inhibition in the metabolism of noradrenaline, although a lesser

Fig. 1. *Metabolism of catecholamines by the hepatic microsomal fraction*

Metabolism was determined as described in the text with (○, ●) 6 μ M-noradrenaline and (□, ■) 6 μ M-adrenaline, in the presence (open symbols) and the absence (closed symbols) of an NADPH-generating system.

Table 1. *Factors affecting the metabolism of catecholamines by the hepatic microsomal fraction*

Metabolism was determined as described in the text over a period of 30 min. Values are expressed per mg of microsomal protein.

	Metabolism	
	Noradrenaline (pmol/min per mg)	Adrenaline (pmol/min per mg)
Control	93	86
N ₂	6	21
CO+O ₂ (90:10)	7	41
Compound SKF 525A (0.5 mM)	56	59
Pargyline (10 μ M)	89	80
3,4-Dimethoxy-5-hydroxy- benzoic acid (0.1 mM)	93	88
3-Amino-1,2,4-triazole*	90	79
Acetone (0.75 M)	122	92

* Rats were given 3-amino-1,2,4-triazole (1 mg/kg) by intraperitoneal injection 2.5 h before the preparation of the microsomal fraction.

inhibition in the metabolism of adrenaline. The dependence of metabolism on O₂ and NADPH and its inhibition by CO is usually taken as evidence for the involvement of the NADPH-dependent mixed-function oxidase (Omura & Sato, 1964). Compound SKF 525A is a known inhibitor of the microsomal mixed-function oxidase (Cooper *et al.*, 1954) and in the present study it inhibited the metabolism of both noradrenaline and adrenaline. Pargyline, an inhibitor of monoamine oxidase (Zeller, 1963), which in preliminary experiments produced a 99% inhibition of monoamine oxidase activity in whole hepatic homogenates, at 10 μ M, produced only a small inhibition of the metabolism of both noradrenaline and adrenaline. This may represent a small contribution by hepatic microsomal monoamine oxidase (Amar-Costesec *et al.*, 1974) towards metabolism or it could be a direct inhibitory effect of the pargyline on the microsomal mixed-function oxidase. Tranylcypromine [($\pm$)-*trans*-2-phenylcyclopropylamine], also an inhibitor of monoamine oxidase (Pletscher, 1966), similarly had very little effect on the metabolism of either catecholamine (results not shown). 3,4-Dimethoxy-5-hydroxybenzoic acid, an inhibitor of catechol *O*-methyltransferase (Nikodejevic *et al.*, 1970), had little effect on catecholamine metabolism. Catechol *O*-methyltransferase, although known to be present in the microsomal fraction (Inscoc *et al.*, 1965), would not be expected to be active in the absence of *S*-adenosyl-L-methionine. H₂O₂ produced by the mixed-function oxidase has been implicated in the microsomal metabolism of ethanol by a catalase-mediated reaction (Thurman & Scholz, 1973). Pretreating animals

with 3-amino-1,2,4-triazole, which is known to inhibit hepatic catalase (Lieber & DeCarli, 1970), failed to produce more than a small inhibition of the microsomal metabolism of either catecholamine. Acetone added directly to the incubation mixture is known to be able to enhance the metabolism of type II substrates, but to inhibit the metabolism of type I substrates (Anders, 1971). In the present study acetone enhanced the metabolism of both noradrenaline and adrenaline, suggesting that both are type II substrates.

Kinetics of microsomal catecholamine metabolism

Fig. 2 shows a Lineweaver-Burk plot of the initial rate of metabolism at various catecholamine concentrations. The *K_m* for noradrenaline was 2.4 mM and for adrenaline 1.0 mM, and *V* 15.6 and 3.6 nmol/min per mg of microsomal protein respectively.

Effect of catecholamines on NADPH-cytochrome P-450 reductase

Both noradrenaline and adrenaline inhibited the initial rate of reduction of hepatic microsomal cytochrome *P*-450 by NADPH (Fig. 3). Type I compounds are known to facilitate, and type II compounds to inhibit, the flow of electrons to cytochrome *P*-450 (Gigon *et al.*, 1969). This suggests therefore that catecholamines are acting as type II compounds.

Spectral changes produced by the binding of catecholamines

The spectral changes produced by the interaction of catecholamines with the hepatic microsomal

Fig. 2. *Kinetics of the hepatic microsomal metabolism of catecholamines*

Metabolism was determined as described in the text over a period of 10 min with (○) noradrenaline and (□) adrenaline.

Fig. 3. Effect of catecholamines on hepatic microsomal NADPH-cytochrome P-450 reductase

NADPH-cytochrome P-450 reductase was measured as described in the text. Each curve is the mean of four determinations on a pooled microsomal fraction from four rats at a microsomal protein concentration of 5 mg/ml. C, control, N, in the presence of 2 mM-noradrenaline and A, in the presence of 2 mM-adrenaline.

fraction have been described previously (McKillop & Powis, 1976). Noradrenaline produced a typical type II difference spectrum with a maximum absorption at 433 nm and a minimum at 390 nm. Adrenaline produced a similar, although smaller, spectral change with a maximum at 422 nm and a minimum at 382 nm. A noteworthy, but unexplained, observation is that below pH 4.0 both catecholamines produced a type I spectral change (results not shown). This cannot be explained by the ionization of the amine group, since catecholamines have a pK_a of about 9.0 (Nagatsu, 1973), and consequently are almost completely ionized at physiological pH. Ascorbate (0.1 mM), which itself produced no spectral change, had no effect on the spectral change produced by either catecholamine, indicating that autoxidation products, which might be formed at pH 7.4, were not involved in the spectral change produced by catecholamines. Neither catecholamine produced a spectral change with haemin or FAD, at concentrations of cytochrome or flavoprotein found in the microsomal fraction (Omura & Sato, 1963; Estabrook & Cohen, 1969).

An analysis of the peak-to-trough spectral changes at different catecholamine concentrations by means of a Lineweaver-Burk plot (Fig. 4) gave spectral dissociation constants, K_s , of 0.9 mM for noradrenaline and 1.0 mM for adrenaline.

The use of 30 mM-aniline as a spectral modifier, as described by Schenkman *et al.* (1973), failed to reveal any type I component in the binding of either noradrenaline or adrenaline to the microsomal fraction.

Products of microsomal catecholamine metabolism

The major metabolite formed by the microsomal metabolism of [14 C]noradrenaline was an unidentified alkali-labile compound (92%), with a much smaller proportion of 3,4-dihydroxyphenylglycol (3%) and 3,4-dihydroxymandelic acid (2%). The unidentified metabolite was separated along with normetanephrine by the conventional alumina and cation-exchange-resin fractionation procedure for catecholamine metabolites (Graefe *et al.*, 1973), but could be distinguished from normetanephrine by its extreme instability under alkaline conditions. It was unaffected by boiling with 6M-HCl for 1 h, but was spontaneously oxidized on coming into contact with the second solvent, chloroform/methanol/1M-NH₃ (12:7:1, by vol.), during the separation by t.l.c. [14 C]Adrenaline appeared to form a similar alkali-labile metabolite.

Discussion

Catecholamines are usually considered to be metabolized almost exclusively by monoamine oxidase and catechol O-methyltransferase or by the formation of glucuronide and sulphate conjugates (Iversen, 1967; Nagatsu, 1973). There is,

Fig. 4. Lineweaver-Burk plot of the peak-to-trough spectral changes produced by the interaction of catecholamines with the hepatic microsomal fraction

Difference spectra were recorded by using $\circ$, noradrenaline (peak 433 nm, trough 390 nm) and $\square$, adrenaline (peak 422 nm, trough 382 nm) with a pooled microsomal fraction from four rats at a microsomal protein concentration of 3 mg/ml.

however, one early report of the NADPH-dependent *N*-demethylation of metanephrine by the microsomal fraction of the rabbit (Axelrod, 1960). The present work shows that catecholamines can be metabolized by an oxygen- and NADPH-dependent process located within the hepatic microsomal fraction which is inhibited by CO and compound SKF 525A. Such evidence is usually taken as indicating the involvement of the NADPH-dependent microsomal mixed-function oxidase (Omura & Sato, 1964). Monoamine oxidase, which is known to be present in the microsomal fraction (Amar-Costesec *et al.*, 1974), was not involved to any appreciable extent in the metabolism of catecholamines. Microsomal catechol *O*-methyltransferase (Inscoc *et al.*, 1965) and other microsomal enzymes responsible for conjugation would not be expected to function in the absence of the specific activated form of the group to be transferred. An unexplained observation was that the microsomal metabolism of adrenaline was less affected than the metabolism of noradrenaline by the absence of cofactors or the presence of inhibitors. This suggests that there may be some other pathway involved in the removal of adrenaline. Axelrod (1964) has reported the enzymic oxidation of adrenaline to adrenochrome by the parotid gland of the cat, by a system that oxidizes adrenaline much more rapidly than noradrenaline. There was no similar activity in the liver of the cat. It is possible, however, that such an enzyme system might be found within the hepatic microsomal fraction of the rat.

The identity of the products of the microsomal metabolism of catecholamines remains to be established. The *N*-oxidation of primary and secondary arylalkylamines has, however, been shown to be a general metabolic pathway (Weisburger & Weisburger, 1973) and Beckett (1973) has suggested that hydroxylamines may be formed during the metabolism of both adrenaline and noradrenaline. There are considerable difficulties, however, in synthesizing suitable reference compounds (Beckett, 1973). It is well established that amphetamine will form the oxime and hydroxylamine (Beckett & Al-Sarraj, 1972; Lindeke *et al.*, 1973), although the hydroxylamine is unstable in air or under alkaline conditions (Beckett & Al-Sarraj, 1973) and undergoes spontaneous autoxidation (Lindeke *et al.*, 1975). The products of catecholamine metabolism found in the present study were also unstable, undergoing spontaneous oxidation under alkaline conditions which might suggest similarities to the metabolism of amphetamine.

Both noradrenaline and adrenaline were found to bind to the microsomal fraction, producing a type II spectral change. The type II spectral change is known to be characteristic of nitrogen-containing compounds with a sterically accessible pair of non-bonded electrons (Mailman *et al.*, 1974). It is not

unexpected therefore that adrenaline, with a methyl group restricting access to the nitrogen atom, should exhibit a lesser type II spectral change compared with noradrenaline. Both noradrenaline and adrenaline showed other characteristics of type II compounds, namely the ability to inhibit NADPH-cytochrome *P*-450 reductase (Gigon *et al.*, 1969), the enhancement of their metabolism by acetone (Anders, 1971) and the displacement of CO from dithionite-reduced cytochrome *P*-450 (D. McKillop & G. Powis, unpublished work), thus differentiating catecholamines from compounds producing a reverse type I spectral change (Schenkman *et al.*, 1972).

It is perhaps surprising that catecholamines should bind to and be metabolized by the microsomal mixed-function oxidase. Because of the relatively high phospholipid content of the endoplasmic reticulum (Stier & Sackmann, 1973), access to the enzymes of the system is thought to depend on a minimum degree of lipid solubility (Lu *et al.*, 1971). Catecholamines have a rather low lipid solubility (Dewhurst & Marley, 1965). Lipid solubility is not the only factor involved, however, in determining the affinity with which compounds bind, and the rate at which they are metabolized by the microsomal mixed-function oxidase (Jansson *et al.*, 1972). The maximal rate of catecholamine metabolism by the microsomal fraction is an order of magnitude greater than that reported for aniline, another type II substrate (Archakov *et al.*, 1974; Powis & Boobis, 1975). The affinity of the catecholamines for the mixed-function oxidase is, on the other hand, an order of magnitude lower than that reported for aniline (Archakov *et al.*, 1974; Powis & Boobis, 1975) or hexobarbitone (Sitar & Mannering, 1973). This would accord with the finding that catecholamines do not act as competitive inhibitors of the microsomal metabolism of either aniline or hexobarbitone (Boobis & Powis, 1974).

The K_m of both catecholamines for the mixed-function oxidase is also much higher than physiological concentrations of catecholamines in the blood, which are around 1.2 nM-noradrenaline and 0.3 nM-adrenaline (Engelman & Portnoy, 1970; O'Hanlon *et al.*, 1970). The concentrations of circulating catecholamines may rise under conditions of stress. The highest recorded values in the rat are 31.0 nM-noradrenaline and 68.7 nM-adrenaline (Anton & Sayre, 1962). Metabolism by the microsomal fraction may, notwithstanding this, be a factor in the removal of circulating catecholamines by the liver. Work in this laboratory has shown that at a catecholamine concentration of 6 μ M the maximum rate of catecholamine removal by the isolated perfused liver of the rat is 2 nmol/min per g of liver (D. McKillop & G. Powis, unpublished work). This compares with a maximal rate of catecholamine metabolism by the

microsomal mixed-function oxidase at a similar catecholamine concentration of 1 nmol/min per g of liver. Such comparisons are, of course, of only a limited value because of the problems of compartmentalization and competition between different pathways. More work is needed to determine the physiological significance, if any, of the metabolism of catecholamines by the mixed-function oxidase. Even if unimportant in absolute terms it is possible that the apparently unique metabolite produced by this pathway might mediate some of the effects of catecholamines found *in vivo*.

A grant from the Rankin Research Fund of the University of Glasgow is gratefully acknowledged.

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