THE METABOLISM OF m-TYROSINE

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Randolph Gerald Smyth

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Department of Biochemistry,

University of Ottawa.

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ABSTRACT

The development of a method for the large scale synthesis and resolution of m-hydroxyphenylalanine (hereafter referred to as m-tyrosine for convenience) led to investigation of the biological differences between the optically pure isomers of this compound. Aspects of the biosynthesis, pharmacological actions, and metabolism of m-hydroxyphenyl compounds were studied using the rat as an experimental model.

The suggestion that m-hydroxyphenyl amines are responsible for the behavioral effects of m-tyrosine was tested. The activity of rats showed significant increases after L-m-tyrosine administration. The inhibition of dopa decarboxylase prevented this hyperactivity while the inhibition of monoamine oxidase potentiated it. m-Tyrosine could also reverse reserpine akinesia for a short period, lending support to speculations that m-hydroxyphenyl amines can mimic the actions of catecholamines.

A biochemical basis was then advanced for the effects of m-tyrosine. Its ability to deplete endogenous amine stores in the brain was confirmed and studied in detail. Levels of norepinephrine, dopamine, and serotonin fell 50% one hour after administration of 150 mg/kg L-m-tyrosine. D-m-tyrosine was less potent in effecting the depletion than the L-isomer under all

experimental conditions. The inhibition of dopa decarboxylase resulted in less m-hydroxyphenyl amine formation in the brain in vivo, and this was correlated with a reduction in the degree of depletion of brain monoamines.

The mechanism of depletion was further examined using brain homogenates. While m-tyrosine does not interfere with the exchange of biogenic amines in this tissue fraction, m-tyramine does displace the endogenous amines. Considerable evidence was thus obtained in support of the hypothesis that both the hyperactivity and depletion of endogenous amines observed after m-tyrosine administration is due to the action of m-hydroxyphenyl amines produced by the in vivo decarboxylation of the amino acid.

The metabolism of m-hydroxyphenyl compounds was examined using ^{14}C-labelled materials. The compounds synthesized were DL-m-tyrosine-2-^{14}C, m-tyramine-1-^{14}C, dl-m-octopamine-1-^{14}C, m-hydroxyphenylpyruvic acid-2-^{14}C, dl-m-hydroxymandelic acid-1-^{14}C, dl-m-hydroxyphenyllactic acid-2-^{14}C, and m-hydroxyphenylacetic acid-1-^{14}C. All were characterized to ensure their purity, and their identity with unlabelled commercial materials was confirmed whenever the latter were available. New syntheses in good yield for m-tyramine and m-octopamine were developed. DL-m-tyrosine-2-^{14}C was resolved to produce samples of the optically pure isomers.

Intraperitoneal administration of L-m-tyrosine-2-^{14}C resulted in its complete metabolism, with over 85% of the injected radioactivity excreted as acids in the urine. In contrast, 26% of the radioactivity from D-m-tyrosine-2-^{14}C was recovered unchanged, 30% was recovered as acidic products, while 40% was identified as m-tyramine. Several other radioactive m-hydroxyphenyl compounds were injected to examine the sequence of m-tyrosine metabolism.

The distribution of radioactive metabolites was determined in different tissues after m-tyrosine-2-^{14}C administration. This was correlated with the *in vitro* activity of various enzymes in the tissues which had accumulated the most radioactivity.

The ring hydroxylation of m-tyrosine-2-^{14}C to radioactive catechols was demonstrated *in vivo* and *in vitro*. After 24 hours, urinary catechols accounted for 0.17% of the radioactivity administered as m-tyrosine-2-^{14}C. Labelled dopa, dopamine, and dihydroxyphenylacetic acid were isolated from the kidney 15 minutes after administration of L-m-tyrosine-2-^{14}C. When the animals were pretreated with a dopa decarboxylase inhibitor, labelled dopa could also be detected in the liver and brain. D-m-tyrosine-2-^{14}C was not hydroxylated to the same extent as the L-isomer, and

pretreatment with p-chlorophenylalanine could also block the reaction. Phenylalanine hydroxylase may therefore be the enzyme involved in this conversion.

Ideas regarding the potential of m-tyrosine as a useful drug in the treatment of catecholamine-related disorders such as Parkinsonism have not been discarded. The results of these experiments have been related to several hypotheses in the literature concerning the role of m-hydroxyphenyl compounds in biochemical, diagnostic, and clinical fields.

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ABBREVIATIONS AND TRIVIAL NAMES

m-tyrosine	m-hydroxyphenylalanine
m-tyramine	m-hydroxyphenylethylamine
m-octopamine	m-hydroxyphenylethan-2-olamine
m-HPAA	m-hydroxyphenylacetic acid
m-HPPA	m-hydroxyphenylpyruvic acid
m-HMA	m-hydroxymandelic acid
o-tyrosine	o-hydroxyphenylalanine
o-tyramine	o-hydroxyphenylethylamine
dopa	3,4-dihydroxyphenylalanine
dopamine, DA	3,4-dihydroxyphenylethylamine
NE	norepinephrine
E	epinephrine
serotonin, 5HT	5-hydroxytryptamine
NSD-1034	N-methyl-N-(3-hydroxybenzyl)hydrazinium dihydrogen phosphate
NSD-1055	4-bromo-3-hydroxybenzyloxyamino phosphate
MK-486	L-α-hydrazino-α-methyl-β-(3,4-dihydroxyphenyl) propionic acid
FLA-63	bis-(4-methyl-1-homopiperazinylthiocarbonyl) disulphide
Catron	β-phenylisopropylhydrazine
H 44/68	DL-α-methyl-p-tyrosine ethyl ester

p-CP	p-chlorophenylalanine
ATP	adenosine triphosphate
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
FAD	flavin adenine dinucleotide
$DMPH_4$	dimethyl tetrahydropterin
MAO	monoamine oxidase
PPO	2,5-diphenyloxazole
POPOP	1,4-di-2-(5-phenyloxazolyl)benzene
EDTA	ethylene diamine tetraacetic acid
OPT	o-phthaldialdehyde
LAH	lithium aluminum hydride
d.p.m.	disintegrations per minute
c.p.m.	counts per minute
°C	Celsius degrees
g	gravities, grams
M	molar
Ci	curies
S.D.	standard deviation

REVIEW OF THE LITERATURE

IDENTIFICATION OF m-HYDROXYPHENYL COMPOUNDS IN NATURE

m-Hydroxyphenyl compounds have been identified from various sources and appear to be widespread in biological systems. However, as a class there is no easy method for their characterization, and they are normally present only in trace amounts. It was therefore not until the 1930s that the natural occurrence of m-hydroxyphenyl compounds was established. At that time, m-hydroxybenzoic acid was isolated from normal urine and other sources, and its metabolism examined (for a review see Boyland et al., 1953). The first reports of a compound containing a two-carbon side chain, m-hydroxyphenylacetic acid, appeared even more recently (Boscott and Bickel, 1953; Boscott and Kirman, 1955).

Since then, m-hydroxyphenyl compounds have been found in the urine and tissues of several species. Investigators have noted the presence of urinary m-tyramine (Jepson et al., 1960; Kakimoto and Armstrong, 1962a; Perry and Schroeder, 1963; Perry et al., 1962 & 1966). m-Tyramine has also been found in the brains of fowl (Juorio, 1976) and rat (Edwards and Blau, 1973). It is present in several tissues of the rat (Boulton and Dyck, 1974; Philips et al., 1975), although concentrations are low

except in the kidney. Leptodactyline (trimethylammonium m-hydroxyphenylethylamine) was discovered in the skins of South American amphibians (Erspamer and Glässer, 1960). Armstrong et al. (1956a & 1956b) reported several acidic compounds (m-hydroxy--benzoic, -hippuric, -phenylacetic, and -phenylpropionic acids) in human urine. The concept of a m-hydroxyphenyl amino acid (i.e. m-tyrosine) serving as a common precursor of both amine and acid products is an attractive theory. To date, however, m-tyrosine has never been detected in normal biological systems (eg. Hall et al., 1961).

ORIGIN OF m-HYDROXYPHENYL COMPOUNDS

m-Hydroxyphenyl compounds have been shown to arise in vivo by the hydroxylation of phenylethylamine to m-tyramine in the rat (Boulton et al., 1975) or the degradation of flavonoid compounds by microorganisms (Nakagawa et al., 1965). Early studies suggested that m-tyrosine was not formed by the action of phenylalanine hydroxylase on phenylalanine (Mitoma, 1956). Sensitive techniques showed that tyrosine hydroxylase could use phenylalanine as a substrate to effect the conversion (Bagchi and Zaricki, 1970), in contradiction to previous reports (Ikeda et al., 1965). The in vitro formation of m-tyrosine from phenylalanine (Tong et al., 1971b) and m-tyramine from phenylalanine (Coulson et al., 1968) has also been demonstrated. In vitro

systems are also capable of non-specific hydroxylation of various substrates (Lissitsky and Roques, 1957; Viscontini and Mattern, 1970).

The p-dehydroxylation of 3,4-catechols can also give rise to m-hydroxyphenyl compounds. m-Hydroxyphenylacetic acid is produced from catechols in the rabbit (Booth et al., 1955; DeEds et al., 1955). 3,4-Dihydroxycinnamic acid can be dehydroxylated anaerobically by intestinal bacteria (Booth and Williams, 1963a & 1963b). The ability of higher organisms to carry out the dehydroxylation is still open to question. m-Hydroxyphenyl products are produced by the normal rodent (DeEds et al., 1957), but the conversion from catechols has been attributed to the intestinal flora (Calne et al., 1969). Serious consideration of the contribution from gut microorganisms is necessary (Sandler et al., 1969), but studies with germ-free rats appear to confirm the ability of mammalian enzymes to carry out the p-dehydroxylation of catechols (Borud et al., 1973).

PHARMACOLOGICAL EFFECTS OF HYDROXYPHENYL COMPOUNDS

The administration of hydroxyphenyl compounds to experimental animals results in some striking physiological and behavioral responses. m-Tyrosine and 3,4-dihydroxyphenylalanine (usually abbreviated as dopa) exhibit parallel effects on blood pressure and activity (Minsker et al., 1971).

Intraperitoneal injection of m-tyrosine (Rubenson, 1971a & 1971b) or dopa (Henning and Rubenson, 1971b) produces hypertensive responses in rats. Although dopa is more potent than m-tyrosine in this case, blood pressure responses are difficult to interpret, since the magnitude and direction of the effects depends on the species and route of administration (for a review see Henning and Rubenson, 1970a).

However, after inhibition of peripheral dopa decarboxylase, the hypertensive action of both amino acids is abolished. At high doses of either m-tyrosine or dopa, a fall in blood pressure was demonstrated (Henning and Rubenson, 1970b; Minsker et al., 1971). Transections localized this hypotensive action to the lower brain stem (Henning et al., 1972). Minsker and Stokes (1974) found that m-tyrosine was considerably more potent as a hypotensive agent than was dopa, when administed intravenously to dogs after inhibition of peripheral dopa decarboxylase.

Both m-tyrosine and dopa have also been shown to reverse reserpine akinesia (Anden et al., 1973; Carlsson and Lindqvist, 1967; Blaschko and Chrusciel, 1960). The administration of m-tyrosine or dopa produces parallel behavioral modifications in 6-hydroxydopamine-treated Parkinsonian models (Ungerstedt et al., 1973). One of the consistent effects of m-tyrosine on

behavior in the normal rat is hyperactivity lasting several hours after administration (Mitoma et al., 1957).

THE BIOCHEMICAL BASIS OF THE EFFECTS OF HYDROXYPHENYL COMPOUNDS

Many of the actions of m-tyrosine have been known for some time (Barger and Dale, 1910). However, the most recent evidence all indicates that both m-tyrosine and dopa are pharmacologically inert. For instance, there is much support for a theory of mediation of both the hypertensive and hypotensive effects by the respective amine metabolites. Decarboxylation of the amino acids in the central nervous system results in a hypotensive action (Henning and Rubenson, 1970b). When the amines are also being formed in peripheral tissues, however, a rise in blood pressure will mask the central effect (Henning and Rubenson, 1970b). Several hydroxyphenyl compounds (3-hydroxyphenyl- and 3,4-dihydroxyphenyl- -pyruvates, -alanines, and -ethylamines) have hypertensive activity in cats (Pogrund et al., 1961). However, the keto acids and amino acids can be converted to the amines by transamination and/or decarboxylation. In experiments where the pure optical isomers of the amino acids were used, the L-isomers were twice as potent as the DL mixtures. Since dopa decarboxylase is specific for the L-configuration (Lovenberg et al., 1962), this lends further support to the theory that the

decarboxylated metabolites were responsible for the effects.

The catechol amines in the brain appear to be largely responsible for the level of activity in the rat (Maj et al., 1971). However, several other dopa metabolites including some catechol acids are also capable of improving motor performance in reserpinized animals (Ericsson et al., 1971). m-Tyramine has been effective in reversing reserpine akinesia (Engel, 1971; Anden et al., 1970b). A correlation between the stimulation of catecholamine receptors and the concentration of amine metabolites in the brain has been demonstrated (Anden et al., 1970a; Marsden et al., 1974).

The depletion of brain biogenic amines by administration of m-tyrosine is well known (Sourkes et al., 1961a; Carlsson and Lindqvist, 1967). m-Hydroxyphenylamine metabolite concentrations have been correlated with this depletion, as determined either by fluorometric assay of the amines or by fluorescence histochemistry (Barry, 1969; Jonsson and Sachs, 1971). The depletion may represent the biochemical basis for the physiological and behavioral effects of m-tyrosine. However, no detailed study relating the m-tyrosine metabolites to the depletion of cerebral catecholamines has been reported.

CLINICAL APPLICATIONS OF m-HYDROXYPHENYL COMPOUNDS

The role of m-hydroxyphenyl compounds in disorders of phenylamino acid metabolism has received some attention. The differences in the proportions of these compounds which are excreted by normal humans and by phenylketonuric patients have been known for some time (Boscott and Bickel, 1953; Jepson et al., 1960), although the causes and applications have not been apparent.

More recently, the usefulness of m-tyrosine as a drug for the treatment of Parkinson's disease has been examined (Barbeau et al., 1962). Results have been rather poor when compared to those obtained with dopa, which is now the drug of choice in the treatment of Parkinsonism (Cotzias et al., 1969; Yahr et al., 1969). Dopa replenishes the levels of dopamine in the corpus striatum (Ng et al., 1972a), which are depressed in Parkinsonian patients. These levels can be correlated with the appearance and severity of the symptoms associated with the disease (for reviews see Sourkes, 1971; Hornykiewicz, 1975). It has been suggested (Sandler et al., 1971) that m-tyrosine may be useful in combination with L-dopa for protection of the latter compound until it reaches the target tissues. Administration should not present a problem, since m-tyrosine may be absorbed against a concentration gradient from the gut by the

intestinal transport system (Lin and Wilson, 1960). Before this possibility can be further explored, a detailed study of the metabolism of m-tyrosine must be undertaken.

THE KNOWN METABOLISM OF m-TYROSINE

m-Tyrosine has undergone a fair amount of investigation in vitro, largely as a dopa analogue in studies on substrate specificity. The two amino acids have several reactions in common. The decarboxylation of m-tyrosine to m-tyramine by dopa decarboxylase (Lovenberg et al., 1962), β-hydroxylation of m-tyramine to m-octopamine (Creveling et al., 1962), transamination of m-tyrosine to m-hydroxyphenylpyruvic acid by tyrosine transaminase (Tong et al., 1973), and the reversal of the latter reaction (Pogrund et al., 1961) have all been demonstrated.

The decarboxylation of m-tyrosine has been known for some time, both in bacteria (Sloane-Stanley, 1949) and mammals (Blaschko et al., 1949). Several more recent experiments using preparations from various sources indicate that the enzyme shows comparable activity towards m-tyrosine and dopa (Davis and Awapara, 1960). Ox adrenal medulla, human phaeochromocytoma and argentaffinoma (Hagen, 1962), rat liver (Awapara, 1962), guinea pig kidney, mouse brain, and Streptococcus faecalis preparations (Ferrini and Glässer, 1964) all give similar

results.

A high pyridoxal phosphate concentration is necessary for m-tyrosine decarboxylation in vitro (Lancaster and Sourkes, 1972). Otherwise the pyridoxal phosphate is removed from the system by irreversible condensation with m-tyrosine, forming a tetrahydroisoquinoline which can no longer participate in decarboxylation or transamination reactions (Zenker, 1966). This type of condensation product may even act as an inhibitor of tyrosine transaminase, which is associated with the same cofactor (Fellman and Roth, 1971). Soluble tyrosine transaminase from rat liver is reported to have no effect on m-tyrosine in vitro (Jacoby and La Du, 1964). However, m-tyrosine is a substrate for the mitochondrial enzyme (Tong et al., 1973).

Tyrosine hydroxylase has no effect on m-tyrosine (Nagatsu et al., 1964). m-Tyrosine appears to be a substrate for phenylalanine hydroxylase (Kaufman, 1962). Several other results support the demonstration of ring hydroxylation. The formation of dopa from m-tyrosine has been shown in bacteria (Aronson and Vickers, 1965). Dopamine has been found in the urine of the rat after administration of m-tyrosine (Sourkes et al., 1961b). Dopa levels in rat brains and livers rise after administration of m-tyrosine and dopa decarboxylase inhibitor (Hollunger and Persson, 1974). Pretreatment with other specific inhibitors

indicates that phenylalanine hydroxylase may be the enzyme responsible (Persson, 1974). Non-specific microsomal hydroxylases may convert other m-hydroxyphenyl compounds to the corresponding catechols (Axelrod, 1963).

The degradation of m-hydroxyphenyl compounds may proceed still further in vivo. m-Octopamine has been detected after the administration of either m-tyrosine (Anden et al., 1970a) or m-tyramine (Engel, 1971). Bakke (1971) has demonstrated the decarboxylation of m-hydroxyphenylacetic acid to m-cresol. This reaction, and possible subsequent ones attacking the aromatic ring, account for only a small percentage of the total metabolism, and most compounds of this type retain their 8- or 9-carbon skeletons until they are excreted.

THE CHEMICAL SYNTHESIS OF m-HYDROXYPHENYL COMPOUNDS

m-Hydroxyphenyl compounds possessing a radioactive label are required for the exploration of their metabolism in vivo. m-Tyrosine and the m-hydroxyphenyl amines are commercially available, and the acidic compounds may be readily prepared by the methods of Shaw et al. (1956). However, there are no published reports on the total synthesis of m-hydroxyphenyl amines from one-carbon precursors. The synthesis of ^{14}C-labelled m-hydroxyphenyl compounds involves the selection of

several individual literature procedures.

(The applicability of material which is tritium-labelled is restricted by the possibility of loss or exchange of the label. These compounds also show kinetic isotope effects (Belleau et al., 1961) which may alter the normal metabolism of these compounds.)

Since many of the published syntheses of m-hydroxyphenyl compounds begin with the coupling of a one-carbon reagent to a m-hydroxybenzaldehyde derivative, ^{14}C label can be conveniently introduced at the position corresponding to C-2 of m-tyrosine. The final products of metabolism usually retain a skeleton of at least eight carbon atoms (Scheline, 1968), so that recovery of the label in the metabolites is virtually quantitative. m-Hydroxyhippuric and β-m-hydroxyphenylhydracrylic acid have been identified as the major m-hydroxyphenyl compounds in normal human urine (Armstrong and Shaw, 1957); but while benzoic acid derivatives can be found (Boyland, 1953), they are not considered to arise from the metabolism of m-tyrosine but from the breakdown of other dietary constituents. In vitro studies have shown several of the m-hydroxyphenyl compounds to be resistant to caecal bacteria (Scheline, 1968). Carbon-carbon cleavage, apart from amino acid decarboxylation, is a minor pathway at most (Bakke, 1971).

In contrast to other m-hydroxyphenyl compounds, m-tyrosine has been available from chemical synthesis for many years (Blum, 1908). There are now at least three routes for the synthesis, two of which have been attempted previously in this laboratory. Condensation of ethyl acetamidocyanoacetate-2-^{14}C with methoxybenzyl chloride and hydrolysis of the complex with hydrobromic acid has been established for tyrosine (Fields et al., 1951) and o-tyrosine (Petitclerc et al., 1969b), and reported for m-tyrosine (D'Iorio et al., 1974). Acetylation of glycine-2-^{14}C, formation of oxazolone from N-acetylglycine and m-hydroxybenzaldehyde, and hydrolysis with hydriodic acid (Sealock et al., 1951) is an attractive route for radioactive synthesis of m-tyrosine-2-^{14}C. Synthesis from a diketopiperazine dimer (Ueda, 1928) has proven successful for both o- and m-tyrosine, but the "glycine anhydride" used as a starting material, which must be labelled, is more difficult to obtain or synthesize than the other labelled precursors. Attempts to synthesize m-tyrosine-2-^{14}C were therefore restricted to the two former methods.

THE METABOLISM OF OTHER HYDROXYPHENYL AMINO ACIDS

This section is an account of the metabolism of dopa, tyrosine, and o-tyrosine (o-hydroxyphenylalanine).

Dopa: The first study of dopa metabolism using material ^{14}C-labelled at the 2-position was conducted by Pellerin and D'Iorio on the rat *in vivo* (1955, 1957b) and with bovine adrenal homogenate (1957a). Only 2% of the administered radioactivity was recovered as $^{14}CO_2$ in the *in vivo* study. 80% was regained in the urine after 24 hours and characterized chromatographically. In addition to unchanged dopa, significant quantities of dopamine and dihydroxyphenylpyruvic acid were identified, together with a product which appeared to be an indole. The *in vitro* experiment confirmed earlier demonstrations of the biosynthesis of norepinephrine from dopa (Demis et al., 1955; Hagen, 1956) and also noted that oxidation to dihydroxyphenylacetic acid is an important pathway in this system.

Fellman (1959) showed that dopamine could be converted to dihydroxyphenylacetaldehyde and oxidized to the acetate *in vitro*. The same mechanism was inferred *in vivo*, together with the methylation of dihydroxyphenylacetic acid to homovanillic acid in the brain (Carlsson and Hillarp, 1962). Reduction of the aldehyde to the alcohol was also demonstrated (Goldstein and Gerber, 1963).

Autoradiographic studies revealed that administered dopa-^{14}C concentrates initially in protein-synthesizing organs, but only the adrenal medulla retains the radioactivity for

several days, as norepinephrine and epinephrine (Rosell et al., 1963). The decarboxylation sites were identified as the adrenal medulla, pancreatic islets, liver, intestinal mucosa, and sympathetic ganglia (Tjalve and Ullberg, 1972).

Several authors (Weiss and Rossi, 1963; Gey and Pletscher, 1964; Wurtman et al., 1970) used a different methodology for identification of the individual metabolites in vivo. Catecholamines and other labelled metabolites were quantitated in several organs at various times after dopa-^{14}C administration. In general, dopa levels decline quickly after injection; only about 7% of the original dose is present as dopa in the animal four hours after injection. Unlike tyrosine, dopa is not concentrated in the brain, although brain levels of its decarboxylation product dopamine rise after dopa administration (Everett and Borcherding, 1970) and chronic administration of dopa results in increased turnover of cerebral catechols (Romero et al., 1972). Dopamine levels reach a maximum of 18% of the injected material after 90 minutes, but fall to 12% after 4 hours. Norepinephrine, epinephrine, and dihydroxyphenylacetic acid are never present in more than traces (2-3% of that injected) (Weiss and Rossi, 1963), explaining the lack of hypertension in humans on dopa therapy. The large percentage of dopa which is methylated (Wurtman et al., 1970) suggests that

such patients may require an unusually high methionine intake.

Several minor conjugates of dopa and its metabolites were also discovered upon closer study. N-acetyl derivatives of the amines were produced *in vivo* by rats (Goldstein and Musacchio, 1962). Perfused rat liver carries out the N-acetylation as well as the conjugation of free phenol to sulfate, glucuronide, and methyl groups (Tyce, 1971). Landsberg et al., (1975) demonstrated the glucuronide conjugate enzymatically in the course of *in vivo* studies on dopa-^{3}H intestinal uptake and metabolism. The labelled dopa was injected and metabolites separated chromatographically into catechols and noncatechols, acidic, basic, and neutral fractions. A similar methodology permitted the characterization of labelled monohydroxyphenyl compounds in the brain after intracerebral injection of dopa-^{14}C (Boulton and Quan, 1970). The latter study may be evidence of dopa dehydroxylation, but other more complex possibilities have not been ruled out.

Unlike the *m*-hydroxyphenyl compounds, dopa and other catechols may be methylated by catechol-O-methyl transferase (Pellerin and D'Iorio, 1958; Axelrod and Tomchick, 1958). Methylated metabolites of the catechols may inhibit other enzymes (Fellman et al., 1975), introducing secondary effects on the metabolism of dopa.

Tyrosine: The metabolism of tyrosine has been examined in some detail. It can be hydroxylated to catechols (Udenfriend et al., 1953) and is present in proteins (Bopp, 1849). Booth et al. (1960) developed a scheme to account for the degradation of tyrosine to acidic metabolites in vivo, beginning with a transamination reaction to p-hydroxyphenylpyruvic acid. This is the major quantitative pathway for the degradation of the amino acid in the brain (Fonnum et al., 1964; Guldberg and Guldberg, 1973).

The enzymes involved are quite sensitive to the general physiological state of the organism: for instance, scorbutic animals lose their capacity to degrade the pyruvate (Knox and Goswani, 1960). Genetic disorders of metabolism result in other abnormalities in the urinary constituents (Wadman et al., 1971). Phenylpyruvic, -lactic, -acetic, and -mandelic acids and o-hydroxyphenylacetic and -benzoic acids (free or conjugated) appear in the urine of phenylketonuric patients. Urinary homogentisic acid is characteristic of alcaptonuria; while disturbances of tyrosine metabolism may give rise to p-hydroxy-phenylpyruvic, -lactic, -acetic, or -mandelic acids in the urine.

The decarboxylation of tyrosine has not been detected in peripheral tissues; it is not normally considered a substrate

for dopa decarboxylase (Blaschko, 1950). However, p-tyramine has been detected and assayed after the introduction of radioactive dopa or tyrosine directly into the brain (Boulton and Wu, 1972; Philips et al., 1974). p-Tyramine has also been identified as the "pink spot" observed in the urine of schizophrenic patients (Boulton et al., 1967).

p-Tyramine is a substrate for the enzymes acting on other phenylethylamines. The report of p-octopamine in mammals (Kakimoto and Armstrong, 1962b) led to studies confirming its biosynthesis from p-tyramine (Masuoka et al., 1964; Carlsson and Waldeck, 1964). After intracerebral injection, the metabolites of p-tyramine include p-hydroxyphenylacetaldehyde and p-octopamine; the proportion of p-octopamine increases if monoamine oxidase is inhibited. Oxidation or reduction of the aldehyde occurs largely in peripheral tissues (Wu and Boulton, 1974).

The nature of the urinary p-tyramine metabolites varies with the species. Rats excrete 2-p-hydroxyphenylethanol (tyrosol), N-acetyl-p-tyramine, and probably p-hydroxyphenylaceturic acid (Nakajima and Sano, 1964). Rabbits degrade p-tyramine largely to p-hydroxyphenylacetic acid, with traces of tyrosol, p-hydroxymandelic acid, and the catechol derivatives vanillic acid and vanillylmandelic acid (Lemberger et

al., 1966). While p-tyramine is not a major product of tyrosine, these findings may provide a close parallel to the metabolism of m-tyramine.

o-Tyrosine: Apart from in vitro studies in parallel with m-tyrosine and dopa (for a review see Petitclerc et al., 1969a), the metabolism of o-tyrosine has received little attention. The urinary metabolites have been determined in the rat using D- and L-o-tyrosine-2-^{14}C. After 24 hours, 85% of the radioactivity injected intraperitoneally was excreted in the urine. There was no significant change in the proportions of the major metabolites with time. For the L-isomer, the metabolites included o-hydroxyphenylacetic acid and an unidentified conjugate thereof together with some o-tyramine and a small quantity of unchanged o-tyrosine. The D-isomer produced smaller proportions of the acetate and more unchanged amino acid in the urine (Petitclerc et al., 1969a). In man, o-tyrosine gives the same metabolites, as well as traces of o-hydroxyphenyllactic acid and o-hydroxyphenylethanol (Gjessing and Borud, 1966). In rat brain, it undergoes decarboxylation as a substrate of dopa decarboxylase (Mitoma et al., 1957).

INHIBITORS OF THE ENZYMES OF CATECHOLAMINE BIOSYNTHESIS

m-Hydroxyphenyl amines have been implicated as the active

agents in several of the effects of m-tyrosine. These amines are thought to be formed and destroyed by the same enzymes known to act on the corresponding catecholamines. Inhibitors of these enzymes have been developed, and have proven valuable in the study of catecholamine metabolism. The same inhibitors may help to clarify the metabolism of m-tyrosine. This section describes the inhibitors used in this study and the rationale for their use.

Dopa decarboxylase inhibition: NSD-1034 (N-methyl-N-3-hydroxybenzylhydrazinium dihydrogen phosphate) and NSD-1055 (4-bromo-3-hydroxybenzyloxyamino phosphate) both prevent the formation of amines in all tissues of the rat. At doses of 100 mg/kg, they have been shown to block decarboxylation of o-tyrosine *in vivo* (Petitclerc et al., 1969a), and both are also effective against the decarboxylation of several compounds *in vitro* (Levine and Sjoerdsma, 1964). An inhibitor which affects only the peripheral enzymes is valuable for determining the contribution of cerebral metabolism to the effects of the amines. MK-486 (L-α-hydrazino-α-methyl-β-(3,4-dihydroxyphenyl)-propionic acid) is a decarboxylase inhibitor *in vitro* (Porter et al., 1962; Porter, 1971) and in peripheral organs *in vivo*. However, it is unable to cross the blood-brain barrier, so central dopa decarboxylase is unaffected (Bartholini and

Pletscher, 1969). The use of these inhibitors may help to determine whether decarboxylation is a necessary step for the effect under investigation.

Dopamine-β-hydroxylase inhibition: Hydroxylation at the β-position of dopamine (Svensson and Waldeck, 1969) or m-tyramine (Engel, 1971) can be prevented by the administration of 40 mg/kg of FLA-63 (bis-(4-methyl-1-homopiperazinylthiocarbonyl)disulphide). This inhibitor can determine the effects of β-hydroxylated amines on blood pressure (Henning and Rubenson, 1970a) and motor activity (Anden et al., 1970a). It can thus be used to explore the contribution of m-octopamine to the effects of m-tyrosine.

Monoamine oxidase inhibition: The oxidation of amine to aldehyde by monoamine oxidase (MAO) is apparently not the major route for catecholamine metabolism (Kopin, 1964) but it does appear to regulate the levels of brain amines. A potent inhibitor of MAO in vivo is Catron (β-phenylisopropylhydrazine) which is effective at 10 mg/kg (Horita, 1959).

Aromatic amino acid hydroxylase inhibition: Two enzymes, phenylalanine hydroxylase and tyrosine hydroxylase, are responsible for the hydroxylation of phenylalanine and tyrosine respectively. p-Chlorophenylalanine inhibits phenylalanine

hydroxylase (Koe and Weissman, 1966). H-44/68 (DL-α-methyl-p-tyrosine methyl ester) is a specific inhibitor of tyrosine hydroxylase (Spector et al., 1965; Moore and Dominic, 1971). These enzymes will be implicated if the conversion of m-tyrosine to dopa is affected by their respective inhibitors.

PROPOSAL OF PROJECT

Most previous studies with m-tyrosine have described its effects rather than specifying the compounds responsible. While experiments with enzyme inhibitors have implicated the amine metabolites in many cases, little solid information is available on the biochemical actions of the products of m-tyrosine metabolism. The pharmacological activities of m-tyrosine and its metabolites, particularly those attributed to the m-hydroxyphenyl amines in the brain, need more detailed examination.

Tracing a potentially complex metabolic sequence in vivo involves the use of several labelled compounds. Suitable materials are not commercially available and must be chemically synthesized.

Apart from reports on the ability of certain enzymes to interconvert m-hydroxyphenyl compounds in vitro, almost nothing is known about the metabolism of these compounds. They have been regarded either as analogues of more prevalent enzyme substrates in studies of substrate specificity, or as drugs producing specific pharmacological responses usually mediated by the amines. Any clinical applications of m-hydroxyphenyl compounds will require some knowledge of the most important reactions which actually take place in vivo and the metabolites

produced under such conditions.

It is therefore proposed to: (1) examine the biochemical basis of the pharmacological actions of m-tyrosine; (2) synthesize and characterize several m-hydroxyphenyl compounds with a radioactive label; (3) trace the metabolic fate of administered m-tyrosine in the rat.

STUDIES ON THE DEPLETION OF BIOGENIC AMINES BY m-TYROSINE

INTRODUCTION

There has been considerable interest in m-tyrosine in the field of neuropharmacology, since its physiological and pharmacological actions show many similarities to those of dopa (pp. 3-5). The effects of these two amino acids are thought to be brought about by changes in the brain monoamine levels, mediated by their respective decarboxylation products (Rubenson, 1971b; Anden et al., 1970a). A correlation between m-tyramine levels and activity has been reported (Engel, 1971), but correlation with the depletion of endogenous amines is largely untested. Whether m-tyramine is the active agent in the observed decrease in brain catecholamine levels after administration of m-tyrosine (Carlsson and Lindqvist, 1967; Dahlstrom et al., 1965) has never been investigated. We have therefore studied the effect of optically pure isomers of m-tyrosine on the monoamines of the brain and other organs of the rat, using specific enzyme inhibitors to test this hypothesis.

MATERIALS

D- and L-m-tyrosine were kindly provided by Dr. N.L. Benoiton, University of Ottawa. m-Tyramine hydrochloride was purchased from Vega-Fox Biochemicals, Tucson, Arizona. dl-m-Octopamine hydrochloride was kindly donated by Sterling-Winthrop Research Institute, Renssaeler, New York. NSD-1055 and NSD-1034 were purchased from Sandev Ltd., Gilson Park, Harlow, U.K. MK-486 was a generous gift of Merck, Sharp, and Dohme, Rahway, New Jersey. FLA-63 was obtained from Labkemi AB, Stockholm, Sweden. Catron was donated by Lakeside Laboratories, Milwaukee, Wisconsin. l-^{3}H-Dopamine tartrate (8 Ci/mmole), PPO (2,5-diphenyloxazole), and Aquasol Liquid Scintillation Fluid were obtained from New England Nuclear Corp., Boston, Massachusetts. Uniformly labelled ^{3}H-5-hydroxytryptamine creatinine sulfate (serotonin) (16 Ci/mmole), 7-^{3}H-norepinephrine hydrochloride (15 Ci/mmole), and PCS Liquid Scintillation Fluid were purchased from Amersham-Searle Corp., Oakville, Ontario. Naphthalene, POPOP (1,4-di-2-(5-phenyloxazolyl)benzene), and alumina (Woelm) were purchased from ICN Pharmaceuticals, Cleveland, Ohio. Epinephrine, norepinephrine, dopamine, 5-hydroxytryptamine, L-dopa, D-dopa, L-tyrosine, DL-m-tyrosine, DL-o-tyrosine, dl-p-octopamine, DL-p-chlorophenylalanine, H 44/68, reserpine, and o-phthaldialdehyde were obtained from

Sigma Chemical Co., St. Louis, Missouri. Benzylamine and p-tyramine hydrochloride were purchased from Eastman Kodak Co., Rochester, New York. 1N Iodine solution, methanol (Spectranalysed), p-dioxane (Scintanalysed) and ethylene glycol were obtained from Fisher Chemical Co., Ottawa, Ontario. Amberlite CG-50, H^+ form, 100-200 mesh was from The Rohm & Haas Co., Philadelphia, Pennsylvania. Male Sprague-Dawley rats (85-100 g) were supplied by Bio Breeding Laboratories, Ottawa, Ontario. Activity measurements were made on 14-inch diameter activity wheels produced by Lafayette Instrument Co., Lafayette, Indiana.

METHODS

Isolation of monoamines from rat tissues

Rats were injected intraperitoneally, using either 0.9% NaCl or dilute HCl as a vehicle. The animals were sacrificed by decapitation. Organs were quickly removed and frozen in ethanol-dry ice to avoid losses of catecholamines (Grabarits et al., 1966; Sloviter and Connor, 1977). The frozen organs were weighed and homogenized with three volumes of 0.4 N perchloric acid containing 0.1% sodium metabisulfite and 0.05% disodium ethylenediaminetetraacetic acid (Shellenberger and Gordon, 1971). The homogenate was centrifuged at 20,000 x g for 10 minutes. The pellet was discarded and 200-250 mg of alumina, prepared according to the method of Anton and Sayre (1962) was added to the supernatant. The mixture was brought to pH 8.5-9.0 with NaOH. After further mixing for one minute, the alumina was allowed to settle and the supernatant applied to an Amberlite CG-50 (sodium form) (Bergstrom and Hansson, 1951; Kirshner and Goodall, 1957) column (4.0 x 0.5 cm) prepared as described by Pisano (1960) to separate the basic from the acidic and neutral compounds (Weil-Malherbe and Bone, 1959). The column was washed with 5 ml water to remove the acidic and neutral compounds, and the noncatechol amines were eluted with 3 ml 2N HCl (Davis et al., 1964). The alumina was washed with 5 ml water and the

adsorbed catecholamines were eluted with 2 ml 0.05 N perchloric acid.

Assay of specific fluorophores of the monoamines

The 5-hydroxytryptamine fluorophore was developed by condensation with o-phthaldialdehyde (OPT) (Maickel et al., 1968; Komesu and Thompson, 1971). To 1.0 ml of the eluate from the Amberlite columns was added 1.0 ml of OPT reagent (1% in 10N HCl). The mixture was heated in a boiling water bath for 10 minutes and cooled to room temperature. The fluorescence of the solutions was measured at an excitation wavelength of 370 nm and an emission wavelength of 500 nm.

o-Phthaldialdehyde was also used to determine the m-hydroxyphenyl amines in the same fraction by the method of Shore and Alpers (1964). To 1.0 ml of eluate is added 0.5 ml of sodium borate buffer (0.5 M, pH 9.0), followed by 0.1 ml OPT (1% in absolute methanol). After various times depending on the compound under investigation, the resulting mixture was acidified with 0.25 ml 2N HCl. Fluorescence was measured with an excitation wavelength of 370 nm for m-tyrosine or 360 nm for the amines (m-tyramine or m-octopamine) and an emission wavelength of 495 nm for m-tyrosine or 490 nm for the amines.

The oxidation of catecholamines to hydroxyindoles was

carried out by the procedures described by Laverty and Taylor (1968) and Shellenberger and Gordon (1971). To 1.0 ml of the sample eluted from alumina was added 3.0 ml sodium phosphate buffer (0.1 M, pH 7.0) followed by 0.4 ml 0.1 N iodine solution. After 2 minutes, 1.0 ml of alkaline sulfite solution (2.5 mg/ml $Na_2S_2O_5$ in 5N NaOH, fresh daily) was added; after a further 2 minutes, 0.8 ml glacial acetic acid was added. Samples were heated in a boiling water bath for 2 minutes, cooled to room temperature, and read at an excitation wavelength of 380 nm and an emission wavelength of 495 nm for norepinephrine fluorescence (Method A). The aliquots were replaced in the boiling water bath for 40 minutes, then chilled in ice. The dopamine fluorescence was read immediately at an excitation wavelength of 325 nm and an emission wavelength of 380 nm (Method B). Dopa was assayed as for dopamine except that the buffer was 3.0 ml sodium citrate (0.1 M, pH 3.5) and the fluorescence was read at an excitation wavelength of 330 nm (Method C). When epinephrine was assayed, 2.8 ml sodium citrate buffer (0.1 M, pH 3.5) was used; after addition of acetic acid, the samples were read immediately with no heating at an excitation wavelength of 410 nm and an emission wavelength of 500 nm (Method D).

An Aminco-Bowman spectrophotometer with 3 mm slits was

used for fluorometric assays; all wavelengths given are uncorrected. Glass-distilled water was used for all solutions involved in the fluorometric assays.

Liquid scintillation counting

Radioactive samples were quantitated by liquid scintillation counting, using a Nuclear-Chicago Mark I scintillation spectrometer. PCS, Aquasol, or Bray's solution (Bray, 1960) were employed as cocktails. The latter mixture was made up in 4-litre batches by mixing 240 g naphthalene, 16 g PPO, 0.8 g POPOP, 400 ml methanol, 80 ml ethylene glycol, and sufficient p-dioxane for a total volume of 4 litres.

Measurement of activity of experimental animals

The activity of rats after injection of m-tyrosine and/or enzyme inhibitors was measured every 15 minutes for two hours. One revolution of the activity wheel corresponds to a linear motion of approximately 44 inches. Further details of the method are presented in the footnote to Table 2.

In vitro experiments

A radiochemical method (Ng et al., 1972b) was employed to study the effect of m-tyrosine on biogenic amines in brain homogenates, using a buffer made up of 120 mM NaCl, 5 mM KCl,

3 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 20 mM Tris-HCl at pH 7.4 (White and Keen, 1971). Rat brains were homogenized in 9 volumes of 0.32 M sucrose and centrifuged at 1,000 x g for 10 minutes. For experiments on the uptake of labelled amines, 0.2 ml of the supernatant (S_1) was incubated for 20 minutes at 37°C with ^{3}H-norepinephrine, ^{3}H-dopamine, or ^{3}H-5-hydroxytryptamine (25 µCi, 1.0 µmole per 100 ml of suspension in each case), in a total volume of 5 ml buffer. Samples without the addition of other compounds (listed in Tables 6-8) served as control. After incubation, the samples were chilled in ice and centrifuged at 18,000 x g for 10 minutes. The precipitates were resuspended in 5 ml cold buffer and the centrifugation repeated. The resulting pellet (P_2) was suspended in 0.25 ml 0.4 N perchloric acid and counted in 10 ml Aquasol.

For the experiments on the release of labelled amines from brain homogenate, 4 ml of the S_1 supernatant was incubated with 96 ml buffer containing ^{3}H-labelled amines as above for 20 minutes at 37°C. The incubation mixture was centrifuged at 18,000 x g for 10 minutes. After washing, the accumulation of exogenous amines in the pellet (P_2) was approximately 1.0-1.6 nmoles. The P_2 fraction was resuspended in 100 ml buffer and the compounds listed in Tables 6-8 were added to 5 ml aliquots. Samples with none of these compounds added served as controls.

After incubation for 20 min at 37°C, the samples were treated as described above for the uptake experiment. All manipulations were carried out at 0-4°C except in the course of the incubation.

RESULTS

Fluorescence characteristics of amines

Since no separation of the catecholamines from each other was attempted prior to their development, the specificity of the procedures for the individual compounds was determined. Results are presented in Table 1. Only two cases of significant overlap (contribution of one compound to the fluorescence of another type of sample) were observed: (1) epinephrine and norepinephrine, and (2) dopamine and dopa. In most experiments, the amount of dopa and epinephrine in the samples was negligible. The contribution of epinephrine to the fluorescence of norepinephrine was corrected when determining the catecholamine levels in peripheral organs. The contribution of dopa to the fluorescence of dopamine was corrected when the use of dopa decarboxylase inhibitors resulted in the accumulation of the amino acid. The values of Table 1 were used to derive simultaneous equations of the type:

Fluorescence at pH 7.0 = 0.36 E + 0.68 NE

Fluorescence at pH 3.5 = 0.72 E + 0.24 NE

for epinephrine (E) and norepinephrine (NE), or

Fluorescence at pH 7.0 = 0.89 DOPA + 1.77 DA

Fluorescence at pH 5.4 = 1.12 DOPA + 0.91 DA

TABLE 1

Fluorescence of catechols after hydroxyindole development.

Compound	Fluorescence/ng (arbitrary units)			
	A	B	C	D
Norepinephrine	0.68	0.00	0.00	0.24
Dopamine	0.02	1.77	0.91	0.01
Dopa	0.00	0.89	1.12	0.01
Epinephrine	0.36	0.00	0.00	0.72

The methods (A-D) used for the development and detection of specific fluorophores are described in the text (p. 29).

for dopa (DOPA) and dopamine (DA). The sensitivity* of the fluorescence procedure was 6 ng (NE), 13 ng (DA), 33 ng (DOPA), and 5 ng (E).

Fluorescence due to 5-hydroxytryptamine after condensation with o-phthaldialdehyde was linear up to 5 µg/sample with a sensitivity of 9 ng. Neither m-hydroxyphenyl nor catechol compounds produced significant fluorescence after the 5-hydroxytryptamine development procedure. Similarly, 5-hydroxytryptamine made no contribution to the fluorescence after the procedures used for development of the other compounds.

The fluorescence characteristics of m-tyrosine, m-tyramine, and m-octopamine after development with o-phthaldialdehyde are virtually identical, and are shown in Figure 1. A linear relationship of fluorescence to concentration is observed up to 10 µg/sample with a sensitivity of approximately 5 ng. However, the time required to develop this fluorescence before acidification was measurably different for the three compounds, providing a convenient method for assaying m-tyramine and m-octopamine in the same sample. Figure 2 shows that while m-tyrosine reaches maximum fluorescence

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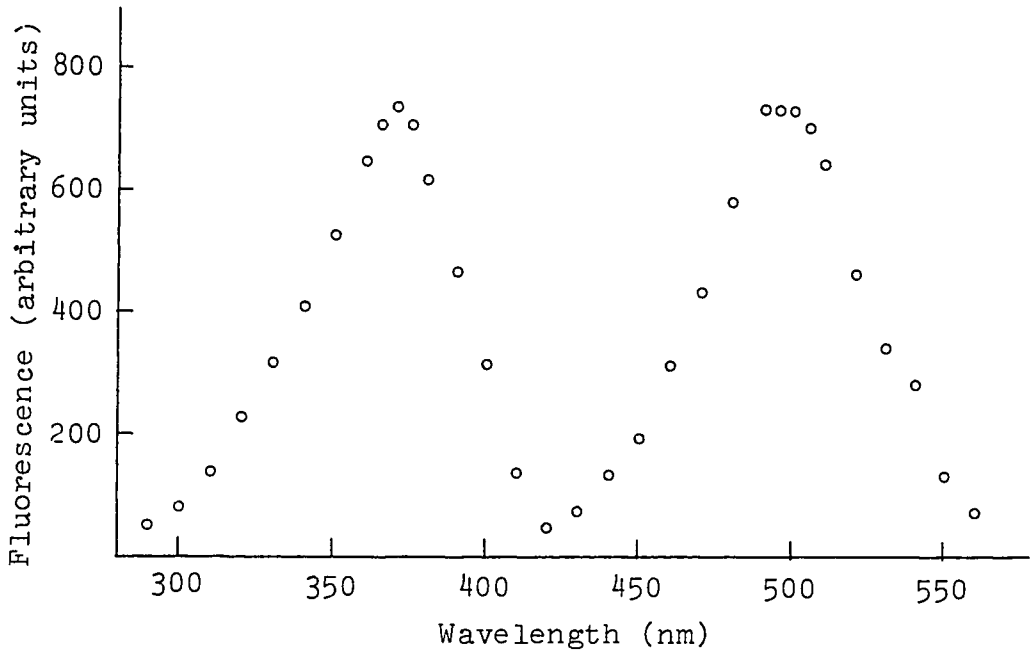
*: Sensitivity is defined as the quantity of a compound producing a fluorescence of double the blank value.

FIGURE 1

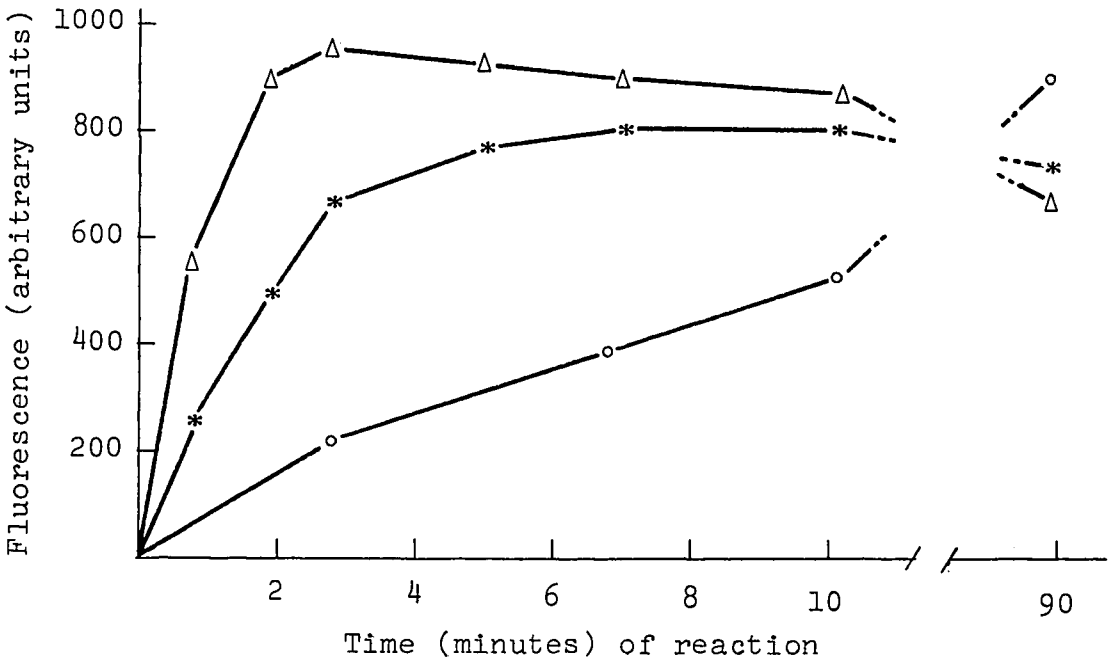
The fluorescence spectrum of m-tyrosine after the optimum time of condensation with o-phthaldialdehyde.



The maximum fluorescence of the complex of m-tyrosine with o-phthaldialdehyde is achieved 3 minutes after their mixing, with an excitation wavelength of 370 nm and an emission wavelength of 495 nm. The spectra of m-tyramine and m-octopamine are almost identical in position and shape, with peaks of fluorescence at an excitation wavelength of 360 nm and an emission wavelength of 490 nm.

FIGURE 2

Development of the fluorescence of the o-phthaldialdehyde condensation products of m-hydroxyphenylethylamine derivatives.



m-Tyrosine (Δ-Δ), m-tyramine (*-*), and m-octopamine (o-o) were mixed with o-phthaldialdehyde at pH 9.0 and left at room temperature for the indicated times before acidification with HCl.

after 3 minutes at pH 9.0, m-tyramine requires more time to reach a maximum (7 minutes), and the fluorescence due to m-octopamine requires 90 minutes for full development. If the m-tyramine : m-octopamine ratio is less than 10:1, the relative difference at 7 minutes and at 90 minutes permits the use of simultaneous equations of the type:

Fluorescence at 7 min = 0.76 m-tyramine + 0.36 m-octopamine

Fluorescence at 90 min = 0.71 m-tyramine + 0.81 m-octopamine

Liquid scintillation counting

The percentage of quenching was determined for both tritium and carbon-14 by the channels ratio method. For 1.0 ml of aqueous sample in 10 ml of Aquasol, counted in plastic vials at 3°C, approximately 81% of ^{14}C and 37% of ^{3}H disintegrations are registered.

Effects of m-hydroxyphenyl compounds on activity

Table 2 indicates the effect of enzyme inhibitors and reserpine on the L-m-tyrosine-induced hyperactivity. None of the inhibitors alone produced visible alterations in activity levels, but reserpine pretreatment leads to akinesia. D-m-tyrosine (300 mg/kg), m-hydroxyphenylacetic acid (180 mg/kg), m-hydroxyphenylpyruvic acid (180 mg/kg), m-tyramine

TABLE 2

The effect of enzyme inhibitors and reserpine on the activity of the rat after L-m-tyrosine administration.

Treatment of rats	Minutes after L-m-tyrosine injection				
	0-15	15-30	30-45	45-60	60-120
	(Number of revolutions of the wheel)				
Vehicle	33	2	1	1	1
L-m-tyrosine	226	354	303	77	4
L-m-tyrosine +					
MK-486	307	322	269	140	42
NSD-1034	66	35	21	10	5
FLA-63	207	368	323	64	7
Catron*	241	483	440	271	20
Reserpine	0	0	0	0	0
Reserpine +					
L-m-tyrosine	107	42	15	1	0
Reserpine +					
L-m-tyrosine +					
NSD-1034	3	1	0	0	0

Footnote to Table 2:

Reserpine (3 mg/kg) was injected intraperitoneally, 24 hours before m-tyrosine; Catron (10 mg/kg) 24 hours before m-tyrosine; FLA-63 (40 mg/kg) 2 hours before m-tyrosine; MK-486 (200 mg/kg) 90 minutes before m-tyrosine; NSD-1034 (100 mg/kg) 30 minutes before m-tyrosine. Rats were placed on activity wheels and their activity was monitored at 15 minute intervals for 2 hours after administration of L-m-tyrosine. Results are presented as single determinations, in number of revolutions of the wheel.

*: The dosage of L-m-tyrosine was 300 mg/kg in all cases except after Catron, when it was 100 mg/kg.

hydrochloride (300 mg/kg), and m-octopamine hydrochloride (300 mg/kg) were injected intraperitoneally without altering the normal level of activity. However, rats injected with L-m-tyrosine (300 mg/kg) were hyperactive for one hour after injection, this behavior reaching a peak between 15 and 45 minutes.

Together with L-m-tyrosine, the peripheral dopa decarboxylase inhibitor MK-486 gives little or no increase in activity over L-m-tyrosine alone, but it prolongs the duration of hyperactivity to 120 minutes after injection. The central dopa decarboxylase inhibitor NSD-1034 blocks the effect of L-m-tyrosine. FLA-63, a dopamine-β-hydroxylase inhibitor, has no apparent effect on the response to L-m-tyrosine. Catron, a monoamine oxidase inhibitor, potentiates the hyperactivity until the animal is exhausted; the usual dose of 300 mg/kg of L-m-tyrosine had to be reduced to 100 mg/kg in this case since the higher dose is lethal within 30 minutes of L-m-tyrosine administration.

Reserpine akinesia is reversed for approximately one hour after L-m-tyrosine injection, during which period the rat is even more active than the untreated control. Administration of D-m-tyrosine, m-tyramine hydrochloride, or L-m-tyrosine after NSD-1034, did not alleviate the reserpine akinesia.

DEPLETION OF ENDOGENOUS AMINES BY m-TYROSINE

The effect of a single injection of L-m-tyrosine (150 mg/kg) on rat brain amines as a function of time can be seen in Figure 3. Dopamine and serotonin levels of the L-m-tyrosine-treated animals were reduced to approximately 50% of control values at 60-90 minutes after the injection and returned to normal after 4 hours. Norepinephrine showed a similar type of response except that a much longer recovery period (roughly 24 hours) was required for its return to normal levels.

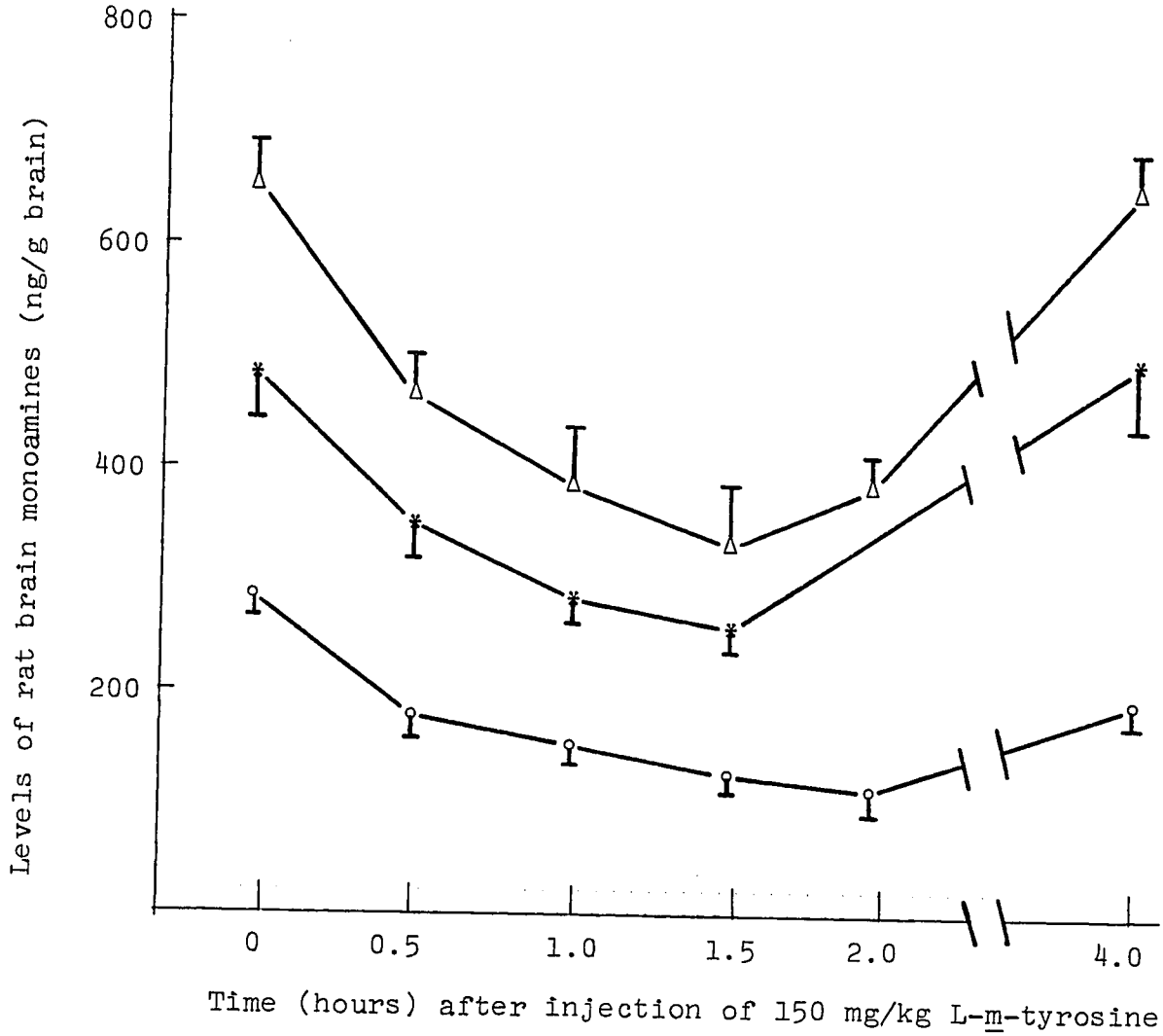
Reduction of brain amine levels was also observed with D-m-tyrosine at a dosage of 150 mg/kg (Figure 4). The relative potencies of the optical isomers of m-tyrosine on the depletion of brain amine stores are shown in Table 3. It was observed that the L-isomer was more potent than the D-isomer.

The response to dosage of m-tyrosine indicates that a maximum of 50-60% of the endogenous amines can be displaced, using 300 mg/kg of the L-isomer. 1000 mg/kg of either isomer results in death in approximately 50% of the cases. Among the survivors, there is a significant increase in brain dopamine at 1000 mg/kg when compared to 300 mg/kg.

Similar experiments on peripheral organs were conducted to determine whether depletion of endogenous stores with m-tyrosine was possible outside the brain. Levels of endogenous amines

FIGURE 3

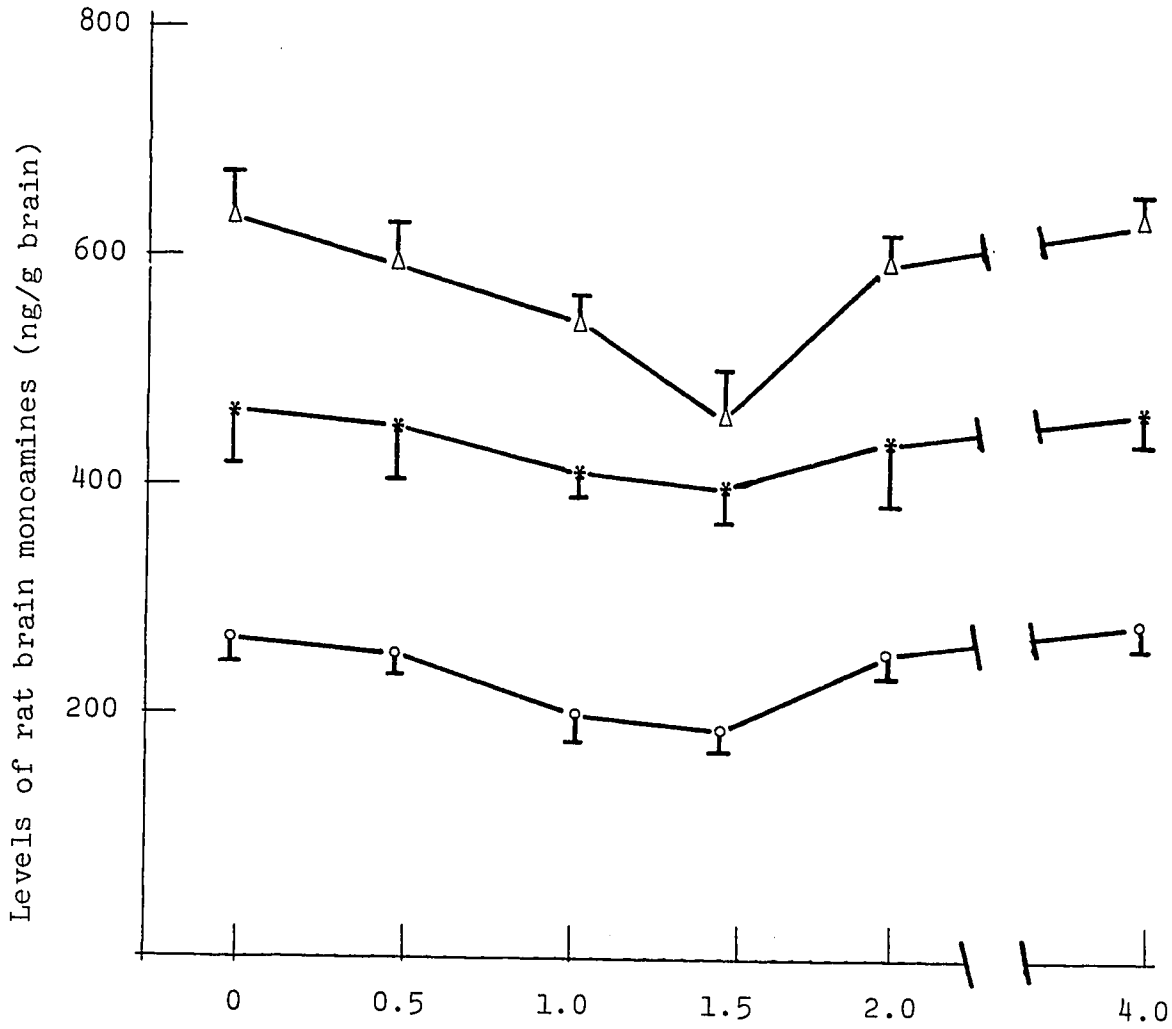
Effect of L-m-tyrosine administration on the levels of dopamine (Δ—Δ), norepinephrine (*—*) and serotonin (o—o) in rat brain.



Each value is given as the mean ± standard deviation of 6-8 individual determinations.

FIGURE 4

Effect of D-m-tyrosine administration on the levels of dopamine (Δ—Δ), norepinephrine (*—*) and serotonin (o—o) in rat brain.



Values are given as the means ± standard deviations of 5-6 individual determinations.

TABLE 3

Levels of monoamines in rat brain 60 minutes after administration of m-tyrosine.

Treatment of rats	Dose	Brain monoamines		
		DA	NE	5HT
	(mg/kg)		(% of control)	
L-m-tyrosine	30	84±4	72±5	88±1
L-m-tyrosine	100	70±6	59±2	69±5
L-m-tyrosine	300	49±3	41±6	44±4
L-m-tyrosine	1000	69±11	46±6	44±7
D-m-tyrosine	30	104±6	92±2	90±3
D-m-tyrosine	100	91±5	76±7	84±2
D-m-tyrosine	300	75±1	59±5	79±8
D-m-tyrosine	1000	67±3	48±4	66±5

Values are presented as percentages of controls without m-tyrosine, and are given as means ± S.D. of 5-7 animals. Control values are in the range indicated in Figures 3-4 (pp. 42-43).

were determined as seen in Table 4, but in several cases it was impossible to quantitate the effect of m-tyrosine. The depletion of amines resulted in their levels falling below the sensitivity of the method. Results for the catecholamines could be obtained in adrenals, and indicated that both depletion and repletion of the stores proceeds more rapidly than in the brain (Figure 5). In most cases the other peripheral organs showed similar results: fluorescence due to catechols disappeared quickly and reappeared at approximately control values within 1-4 hours.

It is known that L-m-tyrosine is a substrate for dopa decarboxylase (Blaschko, 1950). Experiments on animals pretreated with dopa decarboxylase inhibitor were carried out to determine whether a decarboxylation reaction is important in manifesting the depletion of brain amines. The results are given in Table 5. When both the central and peripheral dopa decarboxylases were inhibited with 100-200 mg/kg of NSD-1055 (Levine and Sjoerdsma, 1964), protection of brain amine stores from D- or L-m-tyrosine was observed. The degree of depletion of the endogenous brain amines appeared to be dependent on the presence of amine metabolites of m-tyrosine.

Other enzyme inhibitors were also tested to determine the reactions influencing the m-tyrosine-induced depletion of brain amines. Neither the peripheral dopa decarboxylase inhibitor

TABLE 4

Normal levels of catecholamines in rat organs.

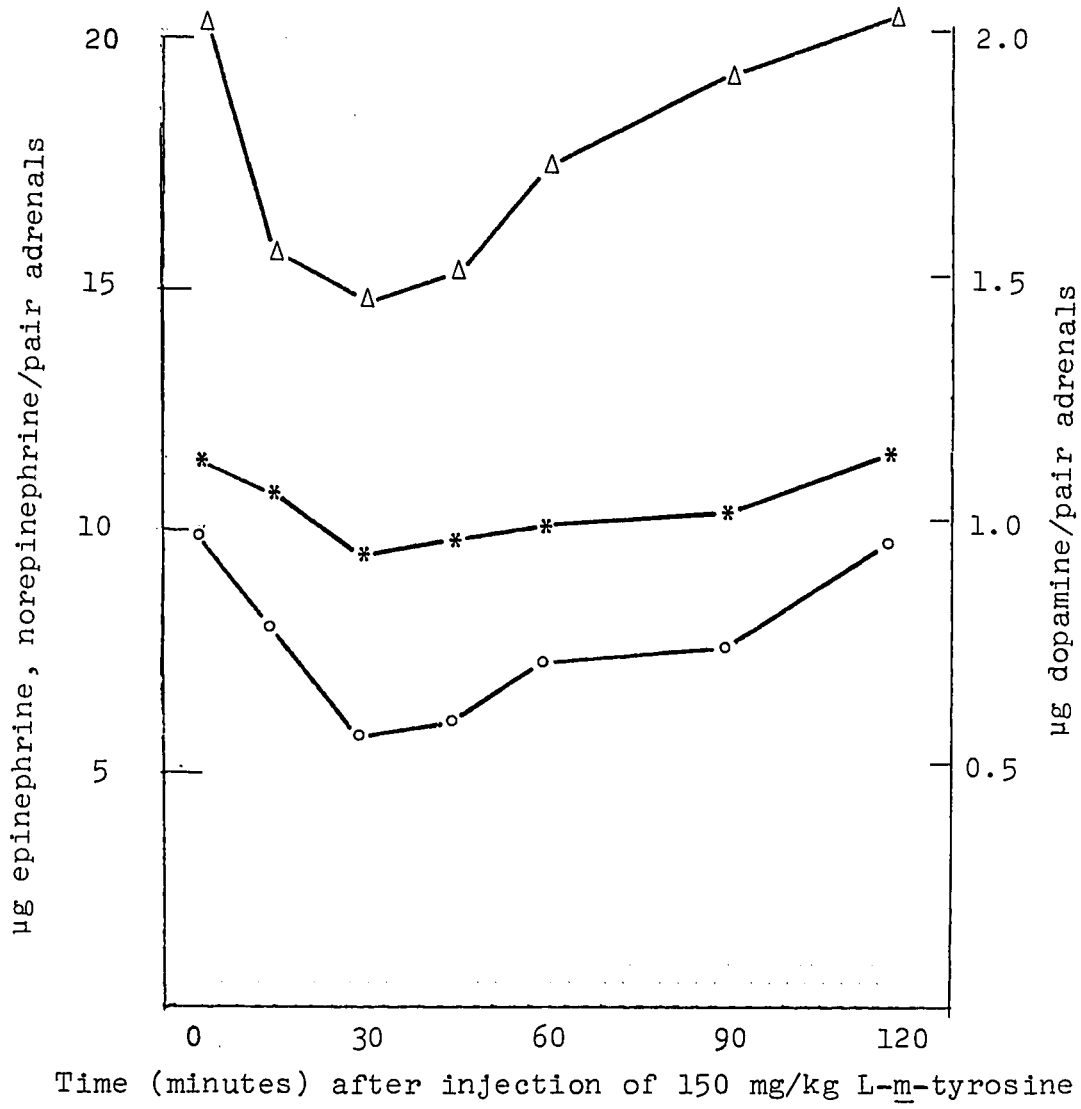
	Brain	Adrenals	Heart	Lungs	Liver
			(ng/g organ)		
Epinephrine	79	264,000	ND*	ND*	ND*
Norepinephrine	251	119,000	137	9	7
Dopamine	551	13,000	239	24	39

All organs were homogenized in 3 volumes 0.4N perchloric acid except the adrenals, weighing an average of 40 mg/pair, which were homogenized in 3 ml/pair. Recoveries from alumina were determined from internal radioactive standards and were identical for all organs. Results are the mean of four determinations.

*: The amine could not be detected with the assay procedures used.

FIGURE 5

Effect of L-m-tyrosine administration on the levels of dopamine (o—o), norepinephrine (*—*), and epinephrine (Δ—Δ) in rat adrenal.



Values are given as individual determinations.

TABLE 5

Protection of rat brain monoamines from L-m-tyrosine-induced depletion with the dopa decarboxylase inhibitor NSD-1055.

Treatment of rats	m-Tyrosine metabolites		Brain monoamines		
	m-Tyramine	m-Octopamine	DA	NE	5HT
(mg/kg NSD-1055)	(ng/g brain)		(% control)		
0	267±14	43±11	66±4	61±8	66±5
100	48±7	3±3	81±2	76±5	71±9
200	7±4	0±2	98±5	98±3	94±6

Rats pretreated with dopa decarboxylase inhibitor were given L-m-tyrosine (100 mg/kg) 30 minutes later. Animals without L-m-tyrosine served as controls. Rats were sacrificed by decapitation 60 minutes after the latter injection. Values are given as the mean ± S.D. of 4-6 animals. Control values of the monoamines are in the range indicated in Figures 3-4 (pp. 42-43).

MK-486 (200 mg/kg, 90 minutes before m-tyrosine) nor the dopamine-β-hydroxylase inhibitor FLA-63 (40 mg/kg, 2 hours before m-tyrosine) have any effect on the m-tyrosine action.

The effect of peripherally administered amines on the endogenous brain amines

Injection of various phenylethylamines intraperitoneally had little effect on the endogenous brain amines. Previous reports that phenylethylamine (Oldendorf, 1971) and p-chlorophenylethylamine (Koe and Weissman, 1966) can deplete the amines were confirmed, but neither m-tyramine nor m-octopamine (100 mg/kg, 60 minutes before sacrifice) entered the brain in measurable quantities or had any effect on the endogenous amines.

UPTAKE AND RELEASE OF LABELLED AMINES FROM BRAIN FRACTIONS

In order to explore further the mechanism of the depletion of monoamines from rat brain by m-tyrosine, experiments were carried out *in vitro*. The results are summarized in Tables 6-8. It was observed that L-m-tyrosine enhanced the efflux rates of exogenous labelled monoamines from rat brain particles. This effect could not be observed with D-m-tyrosine and could be blocked by the dopa decarboxylase inhibitor MK-486. m-Tyramine, the decarboxylation product of m-tyrosine, was more active than

TABLE 6

The effect of hydroxyaromatic amino acids on the efflux of exogenous labelled monoamines from rat brain homogenate.

Amino acid	Labelled amine in P_2 pellet*		
	DA	NE	5HT
	(% of control)		
L-dopa	52.8±6.3	71.5±1.9	77.8±4.9
L-dopa + MK-486	78.5±0.6	90.1±7.2	89.1±1.5
D-dopa	95.7±7.5	95.4±2.0	100.2±4.6
L-m-tyrosine	75.0±2.2	73.5±9.1	81.3±5.5
L-m-tyrosine + MK-486	87.7±2.4	91.0±9.7	90.2±2.8
D-m-tyrosine	92.7±8.1	99.5±8.5	99.4±6.2
L-p-tyrosine	103.6±4.4	98.4±1.5	99.1±5.7
DL-o-tyrosine	97.7±0.8	98.4±0.1	85.0±2.0

Efflux rates were measured in terms of the percentage of radioactivity (control = 100%, or 15,000 c.p.m.) remaining after incubation. Results are given as the means ± S.D. of four determinations. The final concentration in the incubation medium was 10^{-4} M for the L- and D-amino acids and the MK-486, and 2×10^{-4} M for DL-o-tyrosine.

*: The isolation of the P_2 fraction is described on p. 31.

TABLE 7

The effect of hydroxyphenylethylamines on the efflux of exogenous labelled monoamines from rat brain homogenate.

Amine	Labelled amine in P_2 pellet*		
	DA	NE	5HT
	(% of control)		
Dopamine	33.0±8.2	44.6±4.3	62.4±4.6
Norepinephrine	69.5±3.1	58.8±4.9	74.9±4.5
m-Tyramine	55.2±2.2	36.5±5.7	52.2±7.1
m-Octopamine	82.7±1.5	72.9±6.4	79.4±3.7
Tyramine	75.8±6.9	77.3±4.9	70.5±1.9
Octopamine	77.4±7.7	87.1±9.4	80.1±6.9

The amines were added to the incubation medium for a final concentration of 10^{-5} M. The data are presented as described in Table 6.

*: The isolation of the P_2 fraction is described on p. 31.

TABLE 8

The effect of amines on the efflux of exogenous labelled monoamines from rat brain homogenate.

Amine	Labelled amine in P_2 pellet*		
	DA	NE	5HT
	(% of control)		
5-Hydroxytryptamine	78.8±1.8	84.5±2.3	67.2±1.4
Benzylamine	92.3±2.1	96.6±2.4	90.7±8.1
Phenylethylamine	80.3±9.7	60.2±4.5	83.8±5.9
p-Chlorophenylethylamine	85.8±2.2	106.2±0.2	85.9±8.1

The amines were added to the incubation medium for a final concentration of 10^{-5} M. The data are presented as described in Table 6.

*: The isolation of the P_2 fraction is described on p. 31.

its precursor amino acid. These findings further support our in vivo observation that decarboxylation of m-tyrosine appears to be necessary for the depletion of amines in rat brain.

Dopa and m-tyrosine show many similar pharmacological effects (p. 3). We have compared their actions on the efflux rate of exogenous labelled monoamines from rat brain particles as seen in Table 6. Their actions on the three amines examined (dopamine, norepinephrine, and 5-hydroxytryptamine) are very similar. However, neither o-tyrosine nor p-tyrosine show a comparable effect on the efflux rate.

It is of interest to note that the β-hydroxylated analogues of dopamine and m-tyramine (norepinephrine and m-octopamine respectively) also enhanced the efflux rate of monoamines, though they are less active than the unsubstituted compounds (Table 7). Tyramine, octopamine, and other phenylethylamines produce similar effects.

The 3-hydroxy- and 3,4-dihydroxy- compounds listed in Tables 6-7 have been tested at several concentrations (10^{-4} M to 10^{-7} M). Within this range, higher concentrations in the incubation medium can be correlated with greater deviation of the efflux rate from control values.

DISCUSSION

EFFECTS OF m-HYDROXYPHENYL COMPOUNDS ON ACTIVITY

The activity of rats after L-m-tyrosine administration (Table 2) is consistent with previous reports and hypotheses concerning the behavioral effects of this compound (Blaschko and Chrusciel, 1960; Carlsson and Lindqvist, 1967). The antagonism by NSD-1034 indicates that a decarboxylated metabolite may be responsible for the hyperactivity observed. The differential effects of the central and peripheral dopa decarboxylase inhibitors support the idea that the cause of the hyperactivity resides in the brain (Anden et al., 1970a). Intraventricular injection of m-tyramine after depletion of endogenous catecholamines and pretreatment with a monoamine oxidase inhibitor is known to increase activity without affecting the levels of endogenous amines (Stoof et al., 1976). Since the peripheral injection of m-tyramine is ineffective in our studies, it appears that the blood-brain barrier is effective in excluding this compound from the brain.

The potentiation of the effect by MK-486 may indicate a protection of the amino acid from peripheral decarboxylation (Bartholini et al., 1968) and/or a temporary dysfunction of the enzymatic blood-brain barrier (Constantindis et al., 1969).

Either mechanism will result in higher brain concentrations of m-tyrosine and its metabolites. The ineffectiveness of m-hydroxyphenylacetic acid and the potentiation of the L-m-tyrosine response by the monoamine oxidase inhibitor Catron seem to rule out the production of an active metabolite by oxidation of an amine. Similar potentiation of m-tyrosine-induced activity by monoamine oxidase inhibition was also noted after treatment with chloral hydrate (Levy and Michel-Ber, 1962). FLA-63 fails to reverse the hyperactivity, implying that m-octopamine is not solely responsible for the effects of m-tyrosine. The observed effect therefore appears due to m-tyramine (and possibly m-octopamine) in the brain after the transport of the precursor m-tyrosine across the blood-brain barrier.

The results suggest that m-tyramine is also responsible for the temporary return of activity after reserpine pretreatment (Anden et al., 1973). The endogenous amines are metabolised after their release by reserpine to produce the depletion observed after reserpine administration (Colburn and Kopin, 1972). m-Tyramine is implicated by the ineffectiveness of the D-isomer (data not shown) and of the L-isomer after central dopa decarboxylase inhibition by NSD-1034 (Levine and Sjoerdsma, 1964). The inability of m-tyramine to reverse the akinesia after

intraperitoneal injection further supports the suggestion that the m-hydroxyphenyl amines mediate the effect only in the brain.

In addition to hyperactivity, m-tyrosine produces several effects noted by other investigators (Mitoma et al., 1957; Carlsson and Lindqvist, 1967): piloerection, shivering and tremors, salivation, hyperexcitability, and a characteristic posture with the head drawn back and the extremities stretched. When housed together (or during handling), aggressiveness was pronounced at 300 mg/kg, but higher doses led to physiological distress. At 1000 mg/kg the rats showed none of the above symptoms and became comatose after 15 minutes. Approximately half the rats so treated died within the first hour, but most of the survivors recovered somewhat after this period.

DEPLETION OF ENDOGENOUS AMINES BY m-TYROSINE

Administration of either the L- or the D-isomer of m-tyrosine was able to reduce brain monoamine levels. The determinations of amines in peripheral organs (Table 4, Figure 5) were consistent with the same effect of m-tyrosine. The fact that the depletion of brain monoamines was more pronounced with L-m-tyrosine (Table 1) is consistent with the idea that a decarboxylation product of m-tyrosine is involved in manifesting its action (Carlsson and Lindqvist, 1967), as the D-isomer is not a substrate for the stereospecific dopa

decarboxylase (Lovenberg et al., 1962).

This was further investigated by pretreating the animals with a dopa decarboxylase inhibitor, NSD-1055 (Hansson et al., 1964; Levine and Sjoerdsma, 1964). The results from these experiments showed that the depletion of brain monoamines appears to be related to the presence of m-tyramine and m-octopamine and that a decarboxylation reaction of m-tyrosine is indeed an essential step for its actions (Table 3). A possible explanation for the effect observed with D-m-tyrosine is its conversion to the L-isomer in vivo. Oxidation of the D-isomer to the keto analogue coupled with transamination of the product to the L-isomer is one established possibility (Pogrund et al., 1961; Tong et al., 1973; Yuwiler, 1973).

Apart from central dopa decarboxylase, enzyme action has little effect on the m-tyrosine-induced depletion of brain biogenic amines. The dopamine-β-hydroxylase inhibitor FLA-63 (Svensson and Waldeck, 1969; Corrodi et al., 1970) was studied to determine whether m-tyramine or m-octopamine was responsible for the depletion of endogenous amines. The use of the peripheral dopa decarboxylase inhibitor MK-486 (Porter, 1971; Hansson et al., 1964) elucidated the contribution of amines outside the central nervous system. Catron (Horita, 1959) was administered to indicate whether the levels of m-hydroxyphenyl

amines could be increased, and whether the endogenous amines could be depleted in the absence of a functioning degradative enzyme in the brain. While all these inhibitors have secondary actions arising from their inhibition of steps in amino acid and catecholamine metabolism, they give no new insight into the mechanism of depletion by m-tyrosine. The inability of these inhibitors to produce an alteration in the primary action of m-tyrosine suggests that only central dopa decarboxylase plays an important part in the depletion of brain amines by m-tyrosine.

It is possible that dopamine may be replaced by m-tyramine while norepinephrine is selectively displaced by m-octopamine; a similar theory was supported by experiments with the corresponding p-isomers in the heart (Poch and Kopin, 1966).

The parallels between these results and those on activity are apparent, and it is quite possible that the concentration of m-tyramine in the brain is responsible for both effects of m-tyrosine.

The effects of peripherally administered amines on the endogenous brain amines

Unlike epinephrine (Axelrod et al., 1959), dopamine (Weil-Malherbe et al., 1959), norepinephrine (Weil-Malherbe

et al., 1961), and 5-hydroxytryptamine (Udenfriend et al., 1957), the blood-brain barrier is permeable to β-phenylethylamine (Oldendorf, 1971; Jackson and Smythe, 1973; Wu and Boulton, 1975) and p-chlorophenylethylamine (Koe and Weissman, 1966). β-Phenylethylamine depletes the endogenous brain amines after its intraperitoneal injection (Jonsson et al., 1966; Fuxe et al., 1967; Jackson, 1971). Previous results (Figures 3-5, Tables 3 and 5) have shown a correlation between the levels of m-hydroxyphenyl amines and the depletion of endogenous brain amines after m-tyrosine administration. We therefore examined the endogenous brain amines for a possible effect of intraperitoneally injected m-tyramine. The results show that m-tyramine is ineffective in depleting the brain amines when administered intraperitoneally. m-Tyramine levels in the brain, determined after intraperitoneal injection of m-tyramine, are much lower than those found after injection of an equivalent dose of m-tyrosine (p. 49). The blood-brain barrier is therefore effective in preventing the entry of m-tyramine into the brain and only the decarboxylation of m-tyrosine within the brain will deplete the endogenous amines. This supports the deductions made from the results with dopa decarboxylase inhibitors (Tables 2 and 5).

IN VITRO EXPERIMENTS ON THE RELEASE OF BRAIN AMINES

Results from the in vitro experiments showed that the

action of L-m-tyrosine in enhancing the efflux of brain monoamines could be blocked by the dopa decarboxylase inhibitor MK-486 (Porter, 1971; Hansson et al., 1964). m-Tyramine is more active than L-m-tyrosine, and D-m-tyrosine is completely ineffective (Table 6). Thus it appears that the L-m-tyrosine-enhanced efflux rate of monoamines from brain particles is mediated by its amine metabolites m-tyramine and m-octopamine which may act by a simple displacement mechanism. Similar results have been reported for dopa and dopamine (Ng et al., 1972b). The similar effects of dopa and m-tyrosine or their amine metabolites on the efflux rate of brain monoamines (Tables 6-7) may account for some of the similar pharmacological actions of these two amino acids.

There are clear-cut quantitative differences in effects on the labelled amines in brain homogenates between the catechols and m-hydroxyphenyl amino acids on one hand, and the p- and o-substituted amino acids on the other (Table 6). This may be one explanation of the differences in physiological reactions to the two groups of compounds. Dopamine and m-tyramine are more effective at enhancing the efflux rate of other amines which are associated with brain homogenates than any other compounds tested. The nature of the amine being released appears to make little difference to the rate of exchange. Nor is the exchange

phenomenon limited to the brain: tyramine depletes other amines from bovine adrenal medullary granules (Schumann and Philippu, 1962) as well as from rat brain synaptosomes (Colburn and Kopin, 1972). Many binding sites appear to exhibit broad specificity for arylethylamines, but only those amino acids which readily undergo decarboxylation will produce significant physiological activity.

Results from the in vivo and in vitro experiments are consistent and suggest that a portion of the exogenous L-m-tyrosine may enter the central noradrenergic, dopaminergic, and serotonergic neurons and undergo decarboxylation to m-tyramine. m-Tyramine or its metabolites (e.g. m-octopamine) may displace the endogenous monoamines which are then quickly metabolised.

CONCLUSION

The results from all systems examined suggest the same conclusion: that m-tyrosine itself has little if any effect on endogenous brain amines or activity in the rat. Its amine metabolites, m-tyramine and perhaps m-octopamine, are the active compounds. These amines displace the endogenous amines from their storage sites; the latter compounds are then metabolised by monoamine oxidase and lost from the brain. The *in vitro* system provides another view of the depletion of biogenic amines. If the amines produced from m-tyrosine reach sufficient concentrations *in vivo*, they also produce hyperactivity and other behavioral changes.

The effects of m-tyrosine decarboxylation are produced largely in the central nervous system, and the blood-brain barrier is capable of excluding m-hydroxyphenyl amines. Peripheral administration of amines is therefore ineffective: only the amino acid can enter the brain, and its action there depends on its metabolism by a functioning dopa decarboxylase. Inhibition of peripheral decarboxylase potentiates the m-tyrosine action, since a major route of metabolism in peripheral organs is thus reduced. Similarly, inhibition of monoamine oxidase in the brain will restrict the metabolism of m-hydroxyphenyl amines after their formation there and enhance

the behavioral effects of m-tyrosine.

In conclusion, the data provide the first evidence supporting the commonly accepted hypothesis that the physiological and pharmacological actions of m-tyrosine are brought about by changes in the brain monoamine levels, mediated by its decarboxylation product m-tyramine. Whether the m-tyramine or m-octopamine formed could then be released and act as a "false" neurotransmitter is still uncertain, although the m-hydroxyphenyl amines have some independent actions in the modification of behavior.

THE CHEMICAL SYNTHESIS OF ^{14}C-LABELLED m-HYDROXYPHENYL COMPOUNDS

INTRODUCTION

m-Tyrosine-2-^{14}C was synthesized and resolved by a general method for ring-substituted phenylalanines (Tong et al., 1971c) (Figure 6) in order to trace its metabolism *in vivo* and examine its reactions *in vitro*. m-Hydroxyphenylpyruvic acid-2-^{14}C, m-hydroxyphenylacetic acid-1-^{14}C, dl-m-hydroxyphenyllactic acid-2-^{14}C, dl-m-hydroxymandelic acid-1-^{14}C, m-tyramine-1-^{14}C hydrochloride, and dl-m-octopamine-1-^{14}C hydrochloride were also synthesized (Figures 7-8) since none of the labelled compounds were commercially available.

FIGURE 6

The synthesis and resolution of ^{14}C-m-tyrosine.

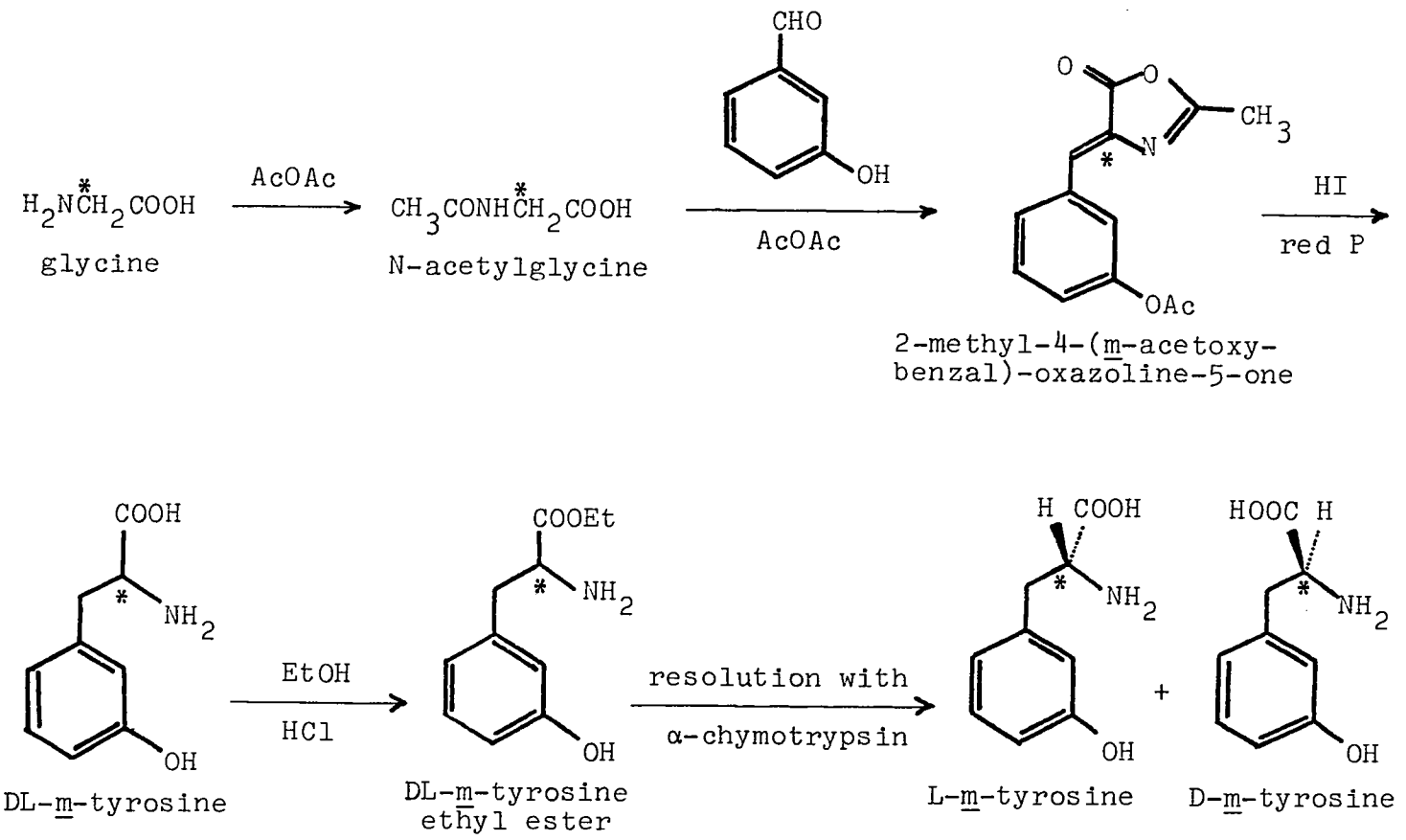


FIGURE 7

SYNTHESIS OF M-HYDROXYPHENYL ACIDS

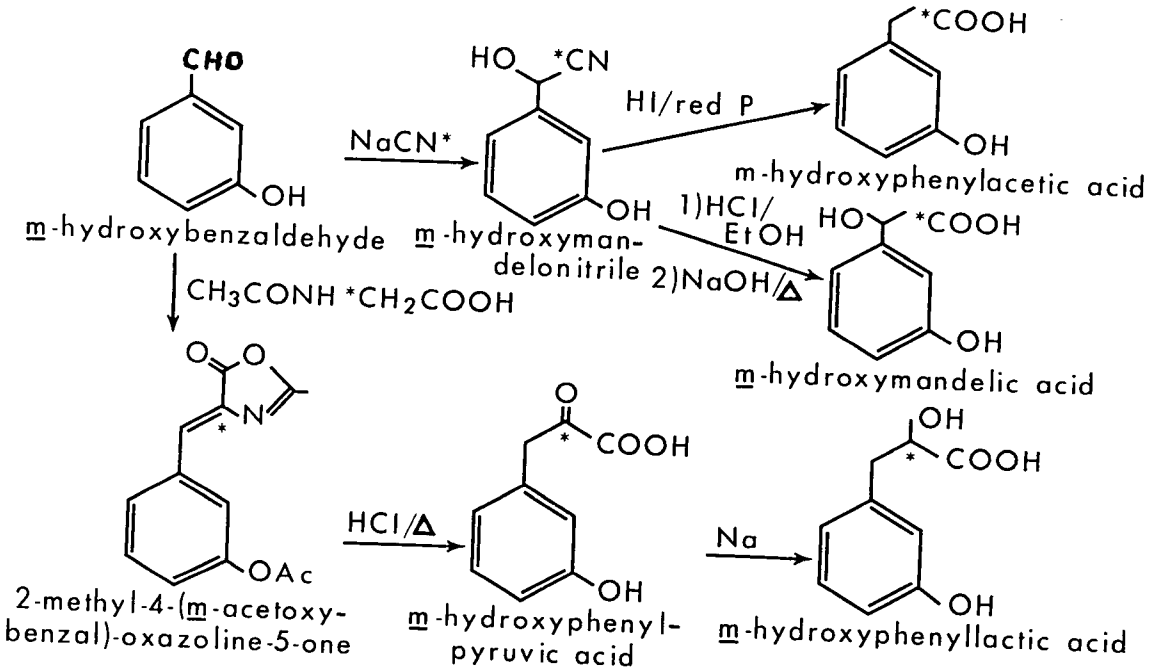
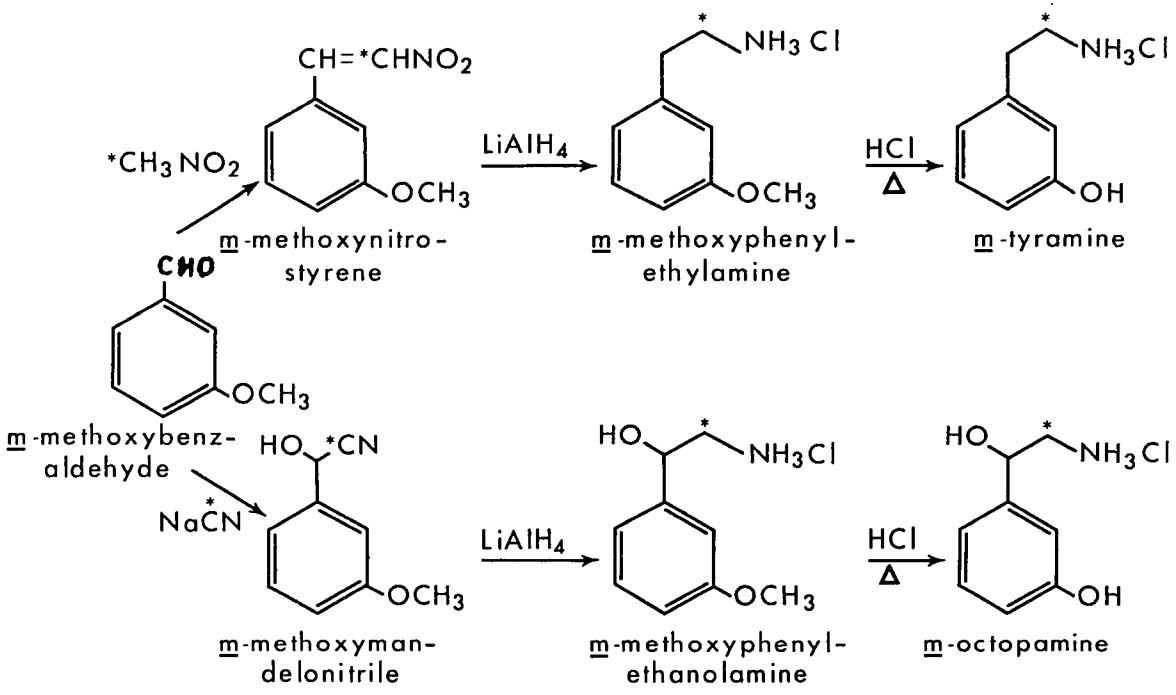


FIGURE 8

SYNTHESIS OF M-HYDROXYPHENYL AMINES



MATERIALS

m-Methoxybenzyl alcohol, glycine, α-chymotrypsin (one unit hydrolyses 1 μmole of N-benzoyl-L-tyrosine ethyl ester per minute at pH 7.8, 25°C), L-amino acid oxidase (one unit produces 1 μmole of hydrogen peroxide per minute at pH 7.0, 25°C), L-amino acid oxidase (one unit deaminates 1 μmole of L-phenylalanine per minute at pH 6.5, 37°C), D-amino acid oxidase (one unit deaminates 1 μmole of D-alanine per minute at pH 8.3, 25°C), and DL-m-tyrosine were purchased from Sigma Chemical Co., St. Louis, Missouri. Thionyl chloride, benzyl chloride, and red phosphorus were obtained from Fisher Chemical Co., Ottawa, Ontario. Ethyl acetamidocyanoacetate and m-hydroxybenzaldehyde were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin. Hydrobromic acid (48%) and Dowex 50W-X4 ion exchange resin were obtained from J.T. Baker Chemical Co., Phillipsburg, New Jersey. Anhydrous sodium acetate, acetic anhydride, sodium amalgam (5%), and hydriodic acid (7.6 N) were obtained from BDH Chemicals, Toronto, Ontario. Hydrogen chloride was purchased from Gas Dynamics, Scarborough, Ontario. Nitromethane was supplied by Matheson, Coleman, and Bell, Norwood, Ohio. m-Methoxybenzaldehyde was purchased from Koch-Light Laboratories, Colnbrook, Bucks, U.K. Glycine-2-^{14}C (anhydrous) was purchased from Amersham-Searle Corp., Oakville, Ontario. Sodium cyanide-^{14}C and

nitromethane-^{14}C were obtained from New England Nuclear Corp., Boston, Massachusetts. L-Alanine-N-carboxyanhydride was purchased from Miles Laboratories, Elkhart, Indiana.

METHODS AND RESULTS

THE SYNTHESIS OF m-TYROSINE-2-^{14}C

Initial attempts at the synthesis of unlabelled m-tyrosine were based on condensation of m-methoxybenzyl chloride with ethyl acetamidocyanoacetate, followed by cleavage with hydrobromic acid (D'Iorio et al., 1974).

m-Methoxybenzyl chloride: Using the procedure of Grice and Owen (1963), m-methoxybenzyl alcohol (50 g), thionyl chloride (45.3 ml) and pyridine (1 ml) were refluxed in benzene (400 ml) for 2 hours. The benzene was evaporated at reduced pressure at 40°C and the residue dissolved in cold diethyl ether (100 ml). This was washed quickly with cold water (3 x 50 ml), dried with magnesium sulfate, filtered, and evaporated. The residue was distilled into a liquid nitrogen trap and 37.0 g of liquid was recovered. The density was 1.54 g/ml, and the infrared spectrum of the product supported the assignment of a benzyl chloride structure. The product was stored at 4°C in a dessicator over Drierite to minimize its decomposition until used.

Ethyl-2-(3'-methoxybenzyl)acetamidocyanoacetate: Sodium (275 mg) was dissolved in absolute ethanol (20 ml). Ethyl acetamidocyanoacetate (2.0 g) was added and the mixture was stirred for 10 minutes. m-Methoxybenzyl chloride (as above)

(2.2 ml) was then added dropwise over 5 minutes and stirring was continued for 60 minutes. The solution was then refluxed for 3 hours and then chilled in ice. The ethanol was evaporated* at room temperature and the residue was dissolved in ethyl acetate (30 ml), washed with water (2 x 10 ml), dried with magnesium sulfate, and filtered. The solution was evaporated to leave a yellow oil; attempts at crystallization were unsuccessful. After driving off the last traces of solvent in a vacuum dessicator overnight, the next step was carried out without further characterization of the intermediate.

DL-m-tyrosine: The oil was refluxed for 18 hours with freshly distilled hydrobromic acid (48%) (40 ml). After cooling, the reaction mixture was evaporated to dryness at 50°C, and the excess acid was removed by repeated evaporation after the addition of water (5 x 20 ml). The residue was dissolved in water (20 ml), treated with charcoal (40 mg) and filtered through Celite. After evaporating the filtrate to dryness at 40°C, the residue was recrystallized from water. The product was identified as pure m-tyrosine by its melting point, infrared

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*: Immediate addition of cold water at this stage resulted in the precipitation of a brown oil which could not be crystallized. If this oil was refluxed in 48% HBr, a viscous gum precipitated and charred in the hot reaction vessel.

spectrum, fluorescence behavior, and its chromatographic behavior on thin layer cellulose plates using three different solvent systems and an amino acid analyser column. In all cases, the properties of the synthetic product were identical to those of standard commercial material. However, yield was poor (maximum 50 mg from the above quantities), so this procedure was not used for radioactive synthesis.

The coupling of m-hydroxybenzaldehyde to acetylglycine and cleavage of the product with hydriodic acid is also reported to yield m-tyrosine (Sealock et al., 1951) and has been carried out on a large scale in this laboratory in 60-63% overall yield (Tong, 1972). This method was therefore used for the radioactive synthesis (Figure 6).

N-Acetylglycine-2-^{14}C: Following the procedure of Herbst and Shemin (1943), glycine (2.0 g) containing 1 mCi glycine-2-^{14}C was dissolved in water (8 ml). Acetic anhydride (5.16 ml) was added to the solution with vigorous stirring. White crystals appeared over the next 20 minutes and the suspension was stored at 4°C overnight. The next day the N-acetylglycine-2-^{14}C was filtered off, washed with ice-cold water (7 ml), and dried over phosphorus pentoxide in vacuo. The filtrate was evaporated to dryness and the residue was recrystallized from water to give a second crop of the product. The combined products weighed

3.0 g (97% yield) and had a melting point of 208°C. The specific activity of the product was 3.18 μCi/10 mg.

2-Methyl-4-(m-acetoxybenzal)oxazoline-5-one-4-^{14}C: A mixture of N-acetylglycine-2-^{14}C (3.0 g), anhydrous sodium acetate (2.1 g), acetic anhydride (7.32 ml), and m-hydroxybenzaldehyde (3.13 g) was stirred in a water bath at 90°C for 6 hours. The reaction mixture was allowed to cool to room temperature, forming a solid which was triturated with water (15 ml). The mixture was stored at 4°C overnight. The bright yellow crystals of oxazolone were filtered, washed well with ice-cold water and dried over phosphorus pentoxide *in vacuo*. The oxazolone obtained had a m.p. of 117-118°C and weighed 3.7-4.5 g, corresponding to yields of 60-70% (Sealock et al. (1951) obtained 67-75% yield, m.p. 116-118°C). The specific activity of the product was 1.52 μCi/10 mg.

DL-m-Tyrosine-2-^{14}C: A mixture of freshly distilled hydriodic acid (20.4 ml), red phosphorus (1.65 g), glacial acetic acid (20.4 ml) and 2-methyl-4-(m-acetoxybenzal)oxazoline-5-one-4-^{14}C (4.0 g) was refluxed for 4 hours. The hot mixture was filtered through a sintered glass funnel and the filtrate evaporated to dryness at 40°C. The evaporation process was repeated after addition of water (5 x 50 ml) to the residue,

and the final oily product was dissolved in water (12 ml). The solution was brought to boiling, treated with charcoal, and filtered through Celite on a sintered glass funnel. After cooling, the filtrate was neutralized with 2N LiOH to pH 5.5 and an equal volume of ethanol was added. The mixture was left at room temperature for 1 hour and stored at 4°C for 3 days (crystals of the amino acid appeared after several hours in the cold). The product was filtered off, washed with ethanol, and recrystallized by dissolving in hot 1N HCl, treating the solution with charcoal (25 mg), filtering through Celite, and neutralizing to pH 5.0 with 2N LiOH. The product decomposed at 282°C, had a specific activity of 2.06 μCi/10 mg, and weighed 1.71-2.10 g corresponding to yields of 63-71%. Sealock et al. (1951) obtained 60-70% yield, m.p. 283°C. The product was identified by infrared spectroscopy and amino acid analysis. Ion exchange and thin layer chromatography showed that the DL-m-tyrosine-2-^{14}C was radiochemically pure. The chemical purity of the product was determined to be 96-113% by fluorescence assay using commercial DL-m-tyrosine as a standard.

Resolution of DL-m-tyrosine-2-^{14}C: Batches of DL-m-tyrosine-2-^{14}C (500 mg) were suspended in absolute ethanol (30 ml) and dry HCl gas was bubbled through the mixture for 2 minutes to dissolve the amino acid. The solution was cooled in ice and

the bubbling continued for 20 minutes. The mixture was left at room temperature overnight. The solvent was evaporated at 30°C to leave an oil, and the process repeated with addition of ethanol (3 x 20 ml) to remove excess HCl. Water (10 ml) was added to the residue and the solution was filtered through Celite and brought to pH 5.0 with 2N LiOH. α-Chymotrypsin (53 mg, capable of catalysing the hydrolysis of approximately 2 mmole of ester per minute) was dissolved in water (2 ml) and added to the ester solution. The pH of the stirred mixture was maintained at 5.0 ± 0.5 with 0.2 N LiOH for 25 minutes, at which time the pH stopped falling. One drop of octanoic acid was added and the mixture was evaporated to dryness at 35°C. The residue was suspended in ethanol (25 ml) and stored at 4°C overnight.

The next day the precipitate was filtered and washed with ethanol. Recrystallization of the precipitate from 1N HCl as described for the isolation of DL-m-tyrosine (p. 74) yields 150-182 mg of L-m-tyrosine-2-^{14}C, corresponding to yields of 60-73%. The ethanol filtrate and washings were re-filtered through Celite and evaporated to dryness at 30°C. 2N LiOH (25 ml) was added to the residue and the mixture was saponified at 50°C for 40 minutes. The solution was filtered through Celite, evaporated to dryness at 40°C, and triturated with hot ethanol (20 ml). The suspension was stored at 4°C overnight, filtered, washed with

ethanol, and recrystallized as described for the L-isomer. The crystals of D-m-tyrosine-2-^{14}C weighed 158-166 mg, corresponding to yields of 63-66%. Tests for chemical and radiochemical identity were repeated as described for the racemic product (p. 74) on the D- and L-isomers with similar results.

THE DETERMINATION OF THE OPTICAL PURITY OF m-TYROSINE

The oxidation of DL-m-tyrosine by stereospecific enzymes: Oxidation of a single amino acid isomer to keto acid with L- and D-amino acid oxidases was followed by separation of the components on a small ion exchange column. This depends on the ability of a given amino acid oxidase to selectively catalyse the oxidation of a specific isomer (Sealock, 1941). Details of the incubation mixtures are given in Table 9. 0.75 ml of 50% trichloroacetic acid was added to the control samples prior to addition of substrate. Other samples were incubated for 1 hour in a 37°C water bath before addition of the acid. All samples were then centrifuged at 25,000 x g for 10 minutes at 4°C. The supernatants were passed through columns of Dowex 50W-X4 (H^+ form) (5.0 x 0.5 cm) (Bertler et al., 1958) to adsorb the m-tyrosine. The resin was washed with 2 ml water and 1.0 ml of the combined effluent (5.0 ml total) containing the acidic product was counted. After washing the columns with a further

TABLE 9

The optical purity of m-tyrosine-2-^{14}C isomers.

Substrate	%L	%D
D-m-tyrosine-2-^{14}C	0.7	
DL-m-tyrosine-2-^{14}C	48.9	49.6
L-m-tyrosine-2-^{14}C		2.3

The quantity of each optical isomer was determined by its oxidation with the corresponding amino acid oxidase. 1.0 unit of L-amino acid oxidase and 3125 units of catalase were added to an incubation mixture containing 150 µmoles KCl, 500 µmoles Tris-HCl buffer at pH 7.6, and 25 µmoles m-tyrosine-2-^{14}C in a final volume of 3.0 ml. 2.0 units of D-amino acid oxidase and 3125 units of catalase were added to an incubation mixture containing 60 µmoles sodium pyrophosphate buffer (pH adjusted to 8.3 with HCl) and 25 µmoles m-tyrosine-2-^{14}C in a final volume of 3.0 ml. Further details of the methodology are given in the text (p. 76). Results are expressed as percentages of the total radioactivity (2 x 10^6 c.p.m.) in the two fractions, after single determinations of each sample.

5 ml water, the neutral material including m-tyrosine was eluted with 5 ml 2N ammonium hydroxide, and 1.0 ml was counted. The results are seen in Table 9, and show that both resolved materials are at least 97% pure by this determination. There is over 90% recovery of radioactivity from the columns. Using the experimental conditions described, approximately 0.01% of optical impurity can be detected.

The coupling of m-tyrosine and separation of the diastereoisomeric dipeptide products: L-Alanine-N-carboxyanhydride was coupled to m-tyrosine to produce two diastereoisomers with different physical properties (Manning and Moore, 1968). m-Tyrosine-2-^{14}C (3.6 mg) was dissolved in o.45 M sodium borate buffer, pH 10.2 (2.0 ml) at room temperature. The solution was chilled to 0°C and L-alanine-N-carboxyanhydride (3.1 mg) was added. The mixture was vortexed continuously for 2 minutes. 1N HCl (0.8 ml) was added to stop the reaction and the solution was stored at -20°C. On the day of analysis, the sample was thawed and 250 µl (containing approximately 50,000 c.p.m.) was made up to 1.0 ml with 0.2 M sodium citrate buffer pH 2.2. 0.8 ml of this solution was applied to a Beckman amino acid analyser. Elution conditions are given in Table 10. Roughly 60% of the radioactivity can be recovered as dipeptide, while 20% is eluted at 58 minutes (corresponding to m-tyrosine) and 20%

TABLE 10

The elution of radioactive dipeptides from the Beckman amino acid analyser.

Isomer	Elution time of L-Ala-X dipeptide (minutes)	%L	%D
D-m-tyrosine	128	0.4	
DL-m-tyrosine	128, 142	48.8	51.2
L-m-tyrosine	142		0.2

The radioactive materials were eluted from a 0.9 x 50 cm AA-15 resin column at 57°C. The buffer was 0.2 M sodium citrate, pH 4.25, with a flow rate of 68 ml/hour. Two-minute fractions were collected and aliquots were counted in Aquasol. Results are expressed as percentages of the total radioactivity (30,000 c.p.m.) in the dipeptide fractions, after single determinations of each sample.

appears in the sodium hydroxide fraction. This procedure detects 0.2% impurity in the resolved samples. The results shown in Table 10 indicate 98-99% optical purity for both L- and D-m-tyrosine-2-^{14}C.

SYNTHESIS OF ^{14}C-m-HYDROXYPHENYL ACIDS

The identity and purity of these compounds were determined by their chromatographic behavior in several solvent systems, the non-specific fluorescence of the phenolic products, and for radioactive compounds, the comparison of actual specific activities with those calculated. The syntheses described by Shaw et al. (1956) were modified using suitable ^{14}C-labelled starting materials (Figure 7).

m-Hydroxyphenylpyruvic acid-2-^{14}C: 2-Methyl-4-(m-acetoxybenzal)oxazoline-5-one-4-^{14}C (2.45 g), synthesized as described for m-tyrosine-2-^{14}C (p. 73) was suspended in 1N HCl (100 ml) and the system flushed with nitrogen. The oxazolone dissolved with heating and the solution was refluxed under nitrogen for 5 hours. Charcoal (25 mg) was then added to the hot liquid and the mixture was filtered through Celite. The product was extracted with ethyl acetate (6 x 25 ml) and the combined organic fractions were extracted with 1N sodium bicarbonate (3 x 20 ml). The bicarbonate solutions were pooled and acidified

with HCl to pH 1.5. The liquid was heated to boiling, cooled, and the product extracted with ethyl acetate (6 x 20 ml). The ethyl acetate was dried with magnesium sulfate, charcoal (25 mg) was added, and the mixture was filtered through Celite. The filtrate was evaporated at 30°C to an oily residue which was redissolved in ethyl acetate (20 ml). An equal volume of 1,2-dichloroethane was added dropwise, and the mixture was stored at 4°C for three days. The precipitated m-hydroxyphenylpyruvic acid-2-^{14}C was filtered and weighed at 950 mg, corresponding to a yield of 51%. The product decomposed at 164-166°C (Shaw et al. (1956) obtained a yield of 85% and a m.p. of 164-165°C). The specific activity was 2.08 μCi/10 mg. Storage at -20°C prevented measurable decomposition for three months.

m-Hydroxyphenyllactic acid-2-^{14}C: m-Hydroxyphenylpyruvic acid-2-^{14}C (630 mg) was dissolved in 1N NaOH (10 ml) with stirring, and 5% sodium amalgam (12 x 0.5 g) was added at intervals of 5 minutes for 1 hour. After an additional 1 hour of stirring, the supernatant was removed by aspiration and the amalgam washed with water (2 x 1 ml). The combined supernatants were brought to pH 7.0 with HCl and washed with ethyl acetate (3 x 10 ml). The aqueous solution was then acidified to pH 1.6 with HCl and extracted with ethyl acetate (4 x 10 ml). The pooled organic solutions were dried with magnesium sulfate,

charcoal (10 mg) was added, and the mixture was filtered through Celite. The filtrate was evaporated to dryness at 30°C, and addition of 1,2-dichloroethane (10 ml) to the residual oil produced a yellow solution and a white crystalline precipitate. This was evaporated to dryness once more, the flask was stoppered with a drying tube, and the mixture was chilled in ice. Cold diethyl ether (1 ml) was used to dissolve the crystalline residue. Addition of 1,2-dichloroethane (5 ml) produced a cloudy suspension which was stored at 4°C overnight. The crystals of dl-m-hydroxyphenyllactic acid-2-^{14}C were filtered; 293 mg was obtained, corresponding to a yield of 45%. The product had a melting point of 100-101°C and a specific activity of 2.01 μCi/10 mg. Shaw et al. (1956) obtained 55% yield with m.p. 100°C.

Synthesis of m-hydroxymandelic acid-1-^{14}C

m-Hydroxymandelonitrile-1-^{14}C: m-Hydroxybenzaldehyde (1.22 g) and sodium bisulfite (1.26 g) were dissolved in water (12 ml) at 50°C and chilled to -5°C in an ice-salt bath. Diethyl ether (7 ml) was added with stirring. Sodium cyanide (490 mg) containing 0.5 mCi ^{14}C-labelled compound was dissolved in cold water (2 ml) and added dropwise to the above mixture over 25 minutes. Stirring was continued for a further 45 minutes, the

phases were separated, and the aqueous solution was extracted with diethyl ether (4 x 7 ml). The combined ether fractions were washed with 1N $NaHSO_3$ (2 x 5 ml) and dried with magnesium sulfate. Charcoal (10 mg) was added and the mixture was filtered through Celite. The volume of the filtrate was reduced to approximately 8 ml at 30°C, benzene (36 ml) was added, and the total volume was reduced to roughly 20 ml. The cloudy suspension was left at 4°C overnight. The crystals of m-hydroxymandelonitrile-1-^{14}C were filtered, washed with cold benzene, and dried. The yield of this step was only 773 mg (52%), but further workup of the filtrate was unsuccessful. The melting point of the product was 108-109°C and the specific activity was 3.35 µCi/10 mg. Shaw et al. (1956) reported 84% yield with a melting point of 111-112°C.

m-Hydroxymandelic acid-1-^{14}C: m-Hydroxymandelonitrile-1-^{14}C (390 mg) was dissolved in a mixture of ethanol (165 µl) and diethyl ether (2 ml). Dry HCl gas was bubbled through the solution for 2 minutes while the mixture was cooled in ice. A yellow gum precipitated and the mixture was stored at 4°C for 3 hours. The solvent was decanted and discarded, and the precipitate was dried overnight in a vacuum dessicator over potassium hydroxide pellets. The next day the residue was dissolved in water (12 ml) and left at room temperature for

3 hours. 5N NaOH (4 ml) was added, the solution placed in a reflux flask, the system flushed with nitrogen, and the solution refluxed for 2 hours. The mixture was cooled and the pH brought to 7.5 with HCl. It was then washed with ethyl acetate (4 x 5 ml), the pH of the aqueous solution adjusted to 1.5 with HCl, and the product extracted with ethyl acetate (6 x 5 ml). The extracts were combined, dried over magnesium sulfate, charcoal (10 mg) added, and the mixture was filtered through Celite. The filtrate was evaporated to dryness at 30°C and the residual oil dissolved in ethyl acetate (1 ml). Cyclohexane (1.5 ml) was added and the cloudy suspension stored at 4°C overnight. dl-m-Hydroxymandelic acid-1-^{14}C was filtered and weighed at 286 mg, corresponding to a yield of 64%. The melting point of the product was 131°C and the specific activity was 2.97 μCi/10 mg. Shaw et al. (1956) reported a 50% yield with m.p. 130-131°C.

m-Hydroxyphenylacetic acid-1-^{14}C: m-Hydroxymandelonitrile-1-^{14}C (373 mg), redistilled 7.6 N hydriodic acid (15 ml), and red phosphorus (465 mg) were mixed and refluxed overnight. The reaction mixture was cooled, filtered through sintered glass, and evaporated to dryness at 40°C. Excess acid was driven off by repeated evaporation after addition of water (3 x 10 ml). The residue was dissolved in water (10 ml), charcoal (10 mg) was added and the mixture filtered through Celite. The filtrate was

adjusted to pH 7.5 with NaOH and washed with diethyl ether (3 x 10 ml). The aqueous phase was adjusted to pH 1.8 with HCl and extracted with diethyl ether (5 x 20 ml). The extracts were dried over magnesium sulfate, charcoal (10 mg) was added and the mixture was filtered through Celite. The filtrate was evaporated to dryness at 20°C and the residue dissolved in diethyl ether (5 ml). Cyclohexane (12 ml) was added and the mixture stored at 4°C overnight. m-Hydroxyphenylacetic acid-1-^{14}C was filtered and weighed at 270 mg, corresponding to a yield of 71%. The specific activity of the product was 3.16 μCi/10 mg, and the melting point was 128-129°C; Shaw et al. (1956) reported 93% yield, m.p. 130-131°C.

SYNTHESIS OF ^{14}C-m-HYDROXYPHENYL AMINES

The synthesized amines (Figure 8) are readily compared with the commercial samples by the specific fluorometric procedures used for the assay of biological samples (p. 28). Intermediates in the synthesis were tested for purity by their physical and chromatographic behavior.

Preliminary experiments using the alternative methods of Buck (1933a, 1933b) to produce mandelonitrile and Worrall (1941) to produce nitrostyrene indicated that the latter procedure was the only one which gave acceptable yields of the initial

product. Coupling to nitromethane was therefore chosen as the most practical initial step for the synthesis of m-tyramine-1-^{14}C. The reduction of the mandelonitrile, synthesized as previously described (p. 82) appeared suitable for the synthesis of dl-m-octopamine.

Synthesis of m-tyramine-1-^{14}C

m-Hydroxynitrostyrene: By the procedure of Worrall (1941), m-hydroxybenzaldehyde (1.76 g) was dissolved in methanol (4.0 ml), the solution chilled to -10°C in an ice-salt bath, and nitromethane (0.78 ml) added. 10 N NaOH (1.7 ml) was then added dropwise with swirling and the mixture was left standing for 15 minutes at -10°C. Ice-cold water (10 ml) was added, and the solution was transferred dropwise to a stirred solution of 4N HCl (8.3 ml). The crystals were filtered, washed with cold water, and dried overnight in a vacuum dessicator. The yield of m-hydroxynitrostyrene was 1.24 g (60%); the melting point was 133-135°C.

Reduction of m-hydroxynitrostyrene: m-Hydroxynitrostyrene (990 mg) was dissolved in anhydrous diethyl ether (100 ml) and added dropwise to a stirred suspension of lithium aluminum hydride (LAH) (594 mg) in anhydrous ether (50 ml). The mixture was refluxed for 18 hours, cooled to room temperature, and the

excess hydride was decomposed with water (0.2 ml), 5N NaOH (0.27 ml) and water (0.8 ml) added dropwise in that order. The suspension was filtered through Celite and aliquots were tested chromatographically on thin-layer cellulose plates. The reactant was absent from this solution, but a multitude of phenolic products were present and none could be identified as m-tyramine. Free phenols of this type are apparently labile in the presence of the LAH reagent, and later attempts involved the protection of the phenol during the reduction of the side chain. The basic reduction procedure (Ramirez and Burger, 1950; Finkelstein, 1951) was modified as necessary when reducing phenol-protected compounds with LAH.

Attempt to find a suitable protecting group: Benzylation: Benzyl chloride (2.7 ml), potassium iodide (130 mg), anhydrous potassium carbonate (3.0 g) and m-hydroxybenzaldehyde (2.44 g) were suspended in 95% ethanol (9.0 ml) and refluxed for 5 hours. Water (6 ml) was added to the cooled solution, and the resulting precipitate was filtered and washed well with cold water. 4.20 g of the product was obtained, corresponding to a 99% yield of m-benzyloxybenzaldehyde, with a melting point of 54-56°C. This melting point agrees with the report of Bristow (1957) after the same procedure, but is at variance with the 45°C reported by Cheng and Casida (1973) who employed a different route of

synthesis.

The benzyloxy-substituted compound was further manipulated as described below for m-methoxynitrostyrene, and produced m-benzyloxyphenylethylamine hydrochloride in good yield. However, several attempts at cleavage, including the hydrogenation of Philips et al. (1975) all failed to remove the benzyl group from this compound, and it was the methoxy ether which was used for the synthesis of the radioactive amines.

m-Methoxynitrostyrene-1-^{14}C: m-Methoxybenzaldehyde (1.36 g) was dissolved in 95% ethanol (80 ml), chilled in an ice-salt bath to 5°C, and nitromethane (610 mg) containing 1 mCi nitromethane-^{14}C was added with stirring. A 5% solution of NaOH in 95% ethanol (20 ml) was then added dropwise to the stirred solution over 20-30 minutes, followed by enough ice water to dissolve the precipitate which formed. This solution was then added dropwise to a stirred solution of 4N HCl (30 ml) at room temperature. The precipitate was filtered and washed with cold water (3 x 5 ml). The mother liquid was evaporated to dryness at 40°C and the residue washed well with water. The two crops were combined and dissolved in ethyl acetate (15 ml). Charcoal (15 mg) was added, the suspension was filtered through Celite, and the filtrate was dried with magnesium sulfate and re-filtered. This filtrate was evaporated at 30°C to leave a

residue of 1.30 g, m.p. 88-89°C, corresponding to yields of 75% of m-methoxynitrostyrene. (For the benzyloxy compound, the yield was 75% and the m.p. 108-110°C.) The specific activity of the m-methoxynitrostyrene-1-^{14}C was 5.75 μCi/10 mg.

m-Methoxyphenylethylamine-1-^{14}C hydrochloride: By the procedure of Lange and Hambourger (1931), m-methoxynitrostyrene-1-^{14}C (1.30 g) was dissolved in anhydrous diethyl ether (100 ml) and added to a stirred suspension of LAH (820 mg) in anhydrous ether (150 ml) over 30 minutes. The mixture was refluxed for 18 hours. After cooling to room temperature, the excess hydride was decomposed with water (0.3 ml), 5N NaOH (0.4 ml), and water (1.2 ml), added dropwise in that order. The suspension was stirred for 30 minutes and filtered. The filtrate was dried over magnesium sulfate, refiltered through Celite, and this filtrate evaporated to dryness. 2N HCl (5 ml) was added with considerable evolution of heat, and the mixture was re-evaporated at 40°C. The residue was dissolved in ethanol (12 ml) and added dropwise to diethyl ether (60 ml) with stirring. The white precipitate was filtered and washed with further ether. 910 mg was isolated, corresponding to a yield of 67% of m-methoxyphenylethylamine hydrochloride (95% was obtained with the benzyloxy compound). The melting point of the product was 130-131°C (146-148°C for the benzyloxy compound: Philips et al.

(1975) reported 149-150°C for deuterated m-benzyloxyphenylethylamine hydrochloride), and the specific activity was 5.54 μCi/10 mg.

m-Tyramine-1-^{14}C hydrochloride: Cleavage of the methoxy ether was attempted with several reagents including aluminum trichloride (Warden, 1970) and a hydrobromic acid/acetic acid mixture (Baker and Williams, 1959) but neither was successful. The method finally adopted was hydrochloric acid under pressure (Epstein et al., 1964).

m-Methoxyphenylethylamine hydrochloride-1-^{14}C (455 mg) was dissolved in 12 N HCl (1.5 ml) and the solution placed in a 200 ml pressure bottle. After flushing with nitrogen, the bottle was sealed and heated at 140-150°C for 4 hours in a glycerol bath. The solution was transferred to a flask by washing out the bottle contents with ethanol, and the mixture was evaporated at 30°C to leave an oil. Excess acid was driven off by the addition of ethanol (3 x 5 ml) and repeated evaporation. The final residue was in the form of crystalline flakes which were dissolved in minimal ethanol (roughly 1.5 ml). This solution was added dropwise to 10 volumes of diethyl ether. The precipitate was filtered and weighed at 282 mg, corresponding to a 70% yield of m-tyramine hydrochloride. The specific activity of the product was 6.20 μCi/10 mg, and the melting point was 134-136°C;

Epstein et al. (1964) gave m.p. 134-136°C.

Synthesis of m-octopamine-1-^{14}C

m-Methoxymandelonitrile-1-^{14}C: The method of Shaw et al. (1956) was used for the synthesis of mandelonitrile. m-Methoxybenzaldehyde (1.36 g) was treated as described for m-hydroxybenzaldehyde (p. 82) to the stage of filtering the ether extracts through Celite, except that for radioactive synthesis the sodium cyanide contained 0.5 mCi of the radioactive compound.

m-Methoxyphenylethanolamine-1-^{14}C hydrochloride: The organic filtrate containing m-methoxymandelonitrile-1-^{14}C was added directly to a stirred suspension of LAH (555 mg) in anhydrous diethyl ether (50 ml). The mixture was refluxed overnight and decomposed after cooling to room temperature, with water (1.0 ml), 5N NaOH (1.35 ml), and water (4.0 ml), added dropwise in that order. The suspension was filtered through Celite and evaporated to dryness, leaving a colourless oil. 2N HCl (3 ml) was added and the solution evaporated to dryness at 20°C once more. The residue was dissolved in ethanol (3.6 ml) and added dropwise with stirring to diethyl ether (20 ml). White crystals precipitated and were filtered for 1.02 g, corresponding to a yield of 50% for m-methoxyphenylethylamine hydrochloride. The melting point of this product was

108-110°C, and the specific activity was 4.89 μCi/10 mg.

m-Octopamine-1-^{14}C hydrochloride: m-Methoxyphenylethylamine-1-^{14}C hydrochloride (500 mg) was dissolved in 12 N HCl (5.0 ml) and heated at 140-150°C for 4 hours in a 200 ml pressure bottle under nitrogen. After cooling, ethanol (5 ml) was added and the mixture was evaporated to an oil; the evaporation procedure was repeated with more ethanol (3 x 5 ml). The residue was dissolved in minimal ethanol (roughly 2 ml) and reprecipitated by addition to 10 volumes of stirred diethyl ether. Filtering the product yielded 218 mg, corresponding to a yield of 57% of m-octopamine hydrochloride. The melting point of the product was 154-155°C (identical to the commercial product) and the specific activity was 4.46 μCi/10 mg.

THE METABOLISM OF m-TYROSINE IN THE RAT

INTRODUCTION

In spite of reports on the endogenous occurrence of m-hydroxyphenyl compounds of several types (Armstrong et al., 1956a & 1956b; Philips et al., 1975), little attention has been given to the identification of the products of m-tyrosine metabolism. After m-tyrosine administration, the m-hydroxyphenyl amine metabolite levels rise significantly in brain (Mitoma et al., 1957; Anden et al., 1970a; Engel, 1971) and in the urine (Pogrund et al., 1961). An increase in the levels of dopa (Hollunger and Persson, 1974) and the catecholamines (Sourkes et al., 1961b; D'Iorio et al., 1974) can also be correlated with the administration of m-tyrosine.

The possibility that m-tyrosine may give rise to catecholamines was first proposed by Sourkes et al. (1961b). Since then, the conversion of m-tyrosine to dopa has been demonstrated in Bacillus cereus (Aronson and Vickers, 1965) and in vitro in bovine adrenal medulla and rat liver (Tong et al., 1971a). There is evidence that phenylalanine hydroxylase could be the enzyme catalysing this conversion (Tong et al., 1971a; Fisher and Kaufman, 1973). Recently Hollunger and Persson (1974) reported

that when rats were pretreated with a dopa decarboxylase inhibitor, there was a further increase in the accumulation of dopa in brain and liver after the administration of m-tyrosine. They suggested that m-tyrosine could be converted to dopa in vivo, but as previously discussed (p. 62), it is conceivable that m-tyrosine also might affect catecholamine metabolism (Carlsson and Lindqvist, 1967). The question of whether the observed increases in dopamine (Sourkes et al., 1961b) and dopa (Hollunger and Persson, 1974) are indeed due to hydroxylation products of m-tyrosine is still open. It is therefore desirable to trace the reaction with labelled precursor.

Previous experiments (Table 2) identified the m-hydroxyphenylethylamines formed in the brain as the compounds responsible for some of the behavioral effects of m-tyrosine. Sandler et al. (1971) considered the possibility of using m-tyrosine as a supplement to L-dopa, to protect and potentiate the action of the catecholamines formed from dopa. The fate of administered m-tyrosine in the whole animal has been examined by quantitation of the urinary metabolites for the first time. The proportions of urinary products of labelled m-hydroxyphenyl compounds are also determined and some of the factors influencing the proportions of m-tyrosine-2-^{14}C metabolites are examined.

m-Tyrosine is known to be a substrate for several of the enzymes which act on similar compounds such as tyrosine, dopa, and their derivatives. Since metabolites may be distributed by the circulatory system after their formation, the appearance of a compound in a certain organ does not necessarily imply that this organ is the site of production. The enzyme activity of each organ should therefore be examined in isolation. Results from in vitro experiments reported in the literature are difficult to correlate, since only limited studies have been carried out on m-tyrosine and different preparative methods have been used by different authors. The in vitro enzyme activities of homogenates from various organs is examined with a standard methodology.

MATERIALS

3,4-Dihydroxyphenylacetic acid, m-hydroxyphenylacetic acid, nicotinamide, adenosine triphosphate (ATP), nicotinamide adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD), glucose-6-phosphate, pyridoxal phosphate, glutamine, and puromycin were obtained from Sigma Chemical Co., St. Louis, Missouri. Nicotinamide adenine dinucleotide phosphate (NADP) was obtained from Mann Research Laboratories, New York, New York. α-Keto-glutaric acid was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Labelled m-hydroxyphenyl compounds were synthesized as described previously (Figures 6-8). Ethylene-diamine was purchased from Fisher Chemical Co., Ottawa, Ontario. Protosol was a product of New England Nuclear Corp., Boston, Massachusetts. β-Mercaptoethanol, p-nitroaniline, and cellulose thin layer chromatography plates were purchased from Eastman Kodak, Rochester, New York. Silica Gel G plates were from Analtech, Newark, Delaware, or Brinkmann Canada, Rexdale, Ontario. Dimethyltetrahydrobiopterin hydrochloride ($DMPH_4$), 3,4-dihydroxymandelic acid, and Bio-Rad AG-50 resin (H^+ form, 200-400 mesh) were products of Calbiochem, San Diego, California. Other materials were obtained as previously described (pp. 25-26, 68-69).

METHODS

The collection of urine and preliminary separation of the metabolites with ion exchange resin

Rats were injected intraperitoneally with m-hydroxyphenyl compounds and placed in metabolic cages. Urine was collected over 1 ml 2N HCl and filtered through Celite. An aliquot of the filtrate was adjusted to pH 9.0 with NaOH and passed through an Amberlite column at the same pH to adsorb the amines as described previously (p. 27). The acidic and neutral compounds were washed from the resin with 5 ml water and discarded. The basic material was then eluted in 3 ml 2N HCl. The remainder of the urine filtrate was lyophilized and extracted into 1.2 ml sodium citrate buffer (0.2 M, pH 2.2).

Identification of hydroxyphenyl compounds on the Beckman amino acid analyser

The radioactive urinary metabolites of injected materials were identified by their elution times on a Beckman model 120B amino acid analyser column (0.9 x 50 cm of AA-15 resin at 57°C). The same conditions were used for all experiments. 1.0 ml of the buffered extract of the lyophilized urine samples described above was applied to the column, and the acidic and neutral

compounds were eluted at a flow rate of 68 ml/hour with sodium citrate buffer (0.2 M; first with pH 3.28 for 30 minutes, then with pH 4.25 for 150 minutes). Basic components were eluted by washing the column with 10 ml 0.2 M NaOH. The metabolites were identified by comparison of their elution times with those of authentic standard compounds. Fractions (2.27 ml) were collected at 2 minute intervals and 1 ml was taken from each fraction for counting in Bray's solution (Bray, 1960). The material eluted with 0.2 M NaOH, containing the urinary amines, was discarded after the initial quantitation since the amines are unstable in strong alkali. The remainder of the fractions which were found to contain significant radioactivity were lyophilized, together with the eluate from the Amberlite column (p. 97). The residues were extracted in a minimum volume of 0.01 N HCl and separated by thin layer chromatography to further characterize the radioactive metabolites.

Visualization of phenols on thin layer plates

Four solvent systems were used to characterize compounds on thin layer Silica Gel G plates: (A) n-butanol : acetic acid : water, 4:1:1 (B) i-propanol : ammonium hydroxide (37%) : water, 8:1:1 (C) t-amyl alcohol : ammonium hydroxide (37%), 4:1 (D) the organic (upper) phase of benzene : propionic acid : water,

2:2:1 (Acheson et al., 1958; Kakimoto and Armstrong, 1962a). After development, phenolic compounds on the plates were routinely visualized with diazotized p-nitroaniline (Acheson et al., 1958). This reagent was made up by adding 0.5 ml 0.2% p-nitroaniline in 1N HCl to 5 ml 0.1 N HCl, followed by the addition of 0.1 ml 5% $NaNO_2$ to the mixture. The resulting colourless solution was kept in ice until it was sprayed lightly on the dried chromatogram. After air drying the plate, it was sprayed with 20% K_2CO_3 to produce a red to violet colour characteristic of free phenols.

Determination of m-tyrosine metabolites in rat tissues

Rats were injected with 100 mg/kg of DL-m-tyrosine-2-^{14}C and sacrificed 15 minutes afterwards by decapitation. Adrenals, brain, heart, kidney, liver, and spleen were removed and homogenized in 0.4 N $HClO_4$ (15 ml for liver, 5 ml for all other organs). The homogenates were centrifuged at 18,000 x g for 10 minutes. The supernatants were adjusted to pH 9.0 with sodium hydroxide and passed through Amberlite columns at the same pH as previously described (p. 27). After washing with 5 ml water to remove the acidic and neutral compounds, the amines (largely m-tyramine, as determined by thin layer chromatography) were eluted from the column with 3 ml 2N HCl. The effluent and

washings from the column were combined, adjusted to pH 1.5 with HCl, and passed through columns of Dowex 50W-X4 (H^+ form) as previously described (p. 76). The acidic material was washed from the column with 5 ml water and the neutral material (m-tyrosine and the m-tyramine conjugate) was eluted with 3 ml 3N NH_4OH. The effluent and washings were combined for an acidic fraction containing m-hydroxyphenylpyruvic acid, m-hydroxyphenyllactic acid, m-hydroxymandelic acid, m-hydroxyphenylacetic acid, and the m-hydroxyphenylacetic acid conjugate.

STUDIES ON IN VITRO METABOLISM

Rats (100-200 g) were sacrificed by decapitation and livers, kidneys, and brains were homogenized. All later manipulations were carried out at 0-4°C except the incubations at 37°C. The enzyme-catalyzed conversions of m-hydroxyphenyl compounds were studied in several tissue fractions.

Decarboxylation of L-m-tyrosine to m-tyramine: The reaction mixture contained 0.5 ml of tissue homogenate (20% in 0.25 M sucrose), potassium phosphate buffer (100 µmoles, pH 7.2), Catron (10 nmoles), and L-m-tyrosine-2-^{14}C (3 µmoles containing 2.18×10^5 d.p.m.) in a total volume of 1.0 ml. Boiled tissue homogenates served as controls. Incubations were carried out for 15 or 30 minutes at 37°C, and the reaction was stopped by placing

all samples in boiling water for 2 minutes. The samples were then centrifuged at 20,000 x g for 10 minutes, and 0.5 ml of the supernatant was passed through an Amberlite column at pH 6.0 (see p. 27). The column was washed with 5 ml water to remove the remaining amino acid substrate and the amine product was eluted with 3 ml 2N HCl. A 1.0 ml aliquot of the acidic eluate was counted in 10 ml Aquasol scintillation fluid.

Oxidation of D-m-tyrosine to m-hydroxyphenylpyruvic acid: The resolution procedure (p. 74) leaves a small percentage (0.4-0.7%) of L-isomer in the D-m-tyrosine-2-^{14}C (Tables 9-10). To ensure the optical purity of the D-m-tyrosine-2-^{14}C, it was further purified by treatment with L-amino acid oxidase. D-m-tyrosine-2-^{14}C (50 mg) was incubated for 16 hours with L-amino acid oxidase at 37°C, in 30 ml of the medium described for the determination of optical purity (Table 9). The reaction was stopped with 10 ml 50% trichloroacetic acid. The mixture was passed through a Dowex AG-50 column (H^+ form) (10 ml wet resin volume) and the column washed with 500 ml water. The amino acid was then eluted with 30 ml 3N NH_4OH. Four 2-ml fractions were found to contain over 90% of the total eluted radioactivity; these were pooled and lyophilized to yield D-m-tyrosine-2-^{14}C free of the L-isomer.

D-amino acid oxidase was measured as described by Yagi (1971). The reaction mixture contained 0.25 ml of homogenate (20% in 0.25 M sucrose), sodium pyrophosphate buffer (100 µmoles, pH adjusted to 8.3 with HCl), FAD (0.1 µmoles), and D-m-tyrosine prepared as described (p. 101) (1.0 µmole containing 8.5×10^4 d.p.m.) in a total volume of 1.0 ml. Boiled homogenates served as controls. Incubation was carried out for 15 or 30 minutes at 37°C. The reaction was stopped by addition of 0.25 ml 50% trichloroacetic acid, and the mixture was centrifuged at 20,000 x g for 10 minutes. A 0.5 ml aliquot of the supernatant was passed through a Dowex AG-50 column (H^+ form) as described previously (p. 76). The amino acid substrate was adsorbed to the resin and the column was washed with 2 ml water to give a total of 2.5 ml effluent. The acidic product in 1.0 ml of this effluent was counted in 10 ml Aquasol liquid scintillation fluid.

Transamination of L-m-tyrosine to m-hydroxyphenylpyruvic acid: The reaction mixture was prepared following the directions of Granner and Tomkins (1970), and contained 0.1 ml of homogenate (20% in 0.5 M potassium phosphate buffer pH 7.7), potassium phosphate buffer (50 µmoles for a final concentration of 100 mM, pH 7.7), MK-486 (10 nmoles), pyridoxal phosphate (50 nmoles),

α-ketoglutaric acid (0.5 μmoles), and L-m-tyrosine-2-^{14}C (1.5 μmoles containing 8.0 x 10^4 d.p.m.) in a total volume of 1.0 ml. Samples without pyridoxal phosphate and α-ketoglutaric acid served as controls. Incubations were carried out for 20 or 40 minutes at 37°C, and the reaction was stopped by addition of 0.5 ml 2N HCl. The mixture was centrifuged at 20,000 x g for 10 minutes and 0.5 ml of the supernatant was passed through a Dowex column as previously described (p. 76). The resin with adsorbed m-tyrosine was washed with 2 ml water to elute the acidic products, and 1.0 ml of the combined effluent (total 2.5 ml) containing the m-hydroxyphenylpyruvic acid was counted in 10 ml of Aquasol liquid scintillation fluid.

Transamination of m-hydroxyphenylpyruvic acid to m-tyrosine: The incubation medium was similar to that used for the assay of tyrosine transaminase activity described above, except that glutamine (0.5 μmoles) replaced the α-ketoglutaric acid and m-hydroxyphenylpyruvic acid-2-^{14}C (5 μmoles containing 5.8 x 10^4 d.p.m.) replaced L-m-tyrosine-2-^{14}C. Samples without glutamine and pyridoxal phosphate served as controls. After obtaining the supernatant of the reaction mixture as described above, 0.5 ml was passed through a Dowex AG-50 column (H^+ form) (0.5 x 4.0 cm). The column was washed with 12 ml water to remove the remaining m-hydroxyphenylpyruvic acid-2-^{14}C. The

m-tyrosine product was then eluted with 2 ml 3N NH_4OH. A 1.0 ml aliquot of this eluate was counted in 10 ml Aquasol liquid scintillation fluid.

Oxidation of monoamines to acids: The conditions of Udenfriend et al. (1958) were used. The reaction mixture contained 0.5 ml of homogenate (20% in 0.25 M sucrose), potassium phosphate buffer (10 µmoles, pH 7.0), and m-tyramine-1-^{14}C or m-octopamine-1-^{14}C (1 µmole, containing 1.0 x 10^5 d.p.m.) in a total volume of 1.0 ml. Boiled homogenates served as controls. Incubation was carried out for 15 or 30 minutes at 37°C and the reaction was stopped by placing the samples in a boiling water bath for 2 minutes. The mixture was centrifuged at 20,000 x g for 10 minutes and 1.0 ml of the supernatant was passed through an Amberlite column at pH 6.0 (see p. 27). The column was washed with 2 ml of water to remove the acidic product, and a 1.0 ml aliquot of this eluate was counted in 10 ml Aquasol liquid scintillation fluid.

Hydroxylation of monoamines to the corresponding catechols: The conditions of Lemberger et al (1965) were followed. The reaction mixture contained 0.2 ml of supernatant (organs were homogenized in 2 ml/g of 1.15% KCl and centrifuged at 800 x g for 10 minutes), glucose-6-phosphate (3.2 x 10^{-7} moles), NADP (2.4 x 10^{-8} moles), NAD (3 x 10^{-8} moles), ATP (2 x 10^{-7} moles),

nicotinamide (12 µmoles), potassium chloride (20 µmoles), magnesium chloride (10^{-7} moles), Catron (2×10^{-7} moles), potassium phosphate buffer (100 µmoles, pH 7.4), and m-tyramine or m-octopamine (2 µmoles) in a final volume of 1.0 ml. Boiled homogenates served as controls. Incubation was carried out for 30 or 60 minutes at 37°C and reaction was stopped by addition of 0.25 ml 50% trichloroacetic acid. The acidified mixture was centrifuged at 20,000 x g for 10 minutes and 1.0 ml of the supernatant was made up to 2.5 ml for adsorption of catecholamine to alumina. The pH of these samples was adjusted to 8.0 as described previously (p. 27) and the catecholamine was eluted from the washed alumina with 2 ml of 0.05 M $HClO_4$. The fluorometric assay procedure for dopamine or norepinephrine (p. 29) was carried out on a 1 ml aliquot of this eluate.

The hydroxylation of m-tyrosine to dopa: The conditions used were those of Tong et al. (1971b). The reaction mixture contained 0.5 ml of homogenate (20% in 0.25 M sucrose), potassium phosphate buffer (100 µmoles, pH 7.0), β-mercaptoethanol (14 µmoles), $DMPH_4$ (6×10^{-7} moles), NADPH (2 µmoles), NSD-1055 (2 µmoles), and L-m-tyrosine-2-^{14}C (1 µmole, 4.3×10^4 d.p.m.) in a total volume of 1.0 ml. Boiled homogenates served as controls. Incubations were carried out for 30 or 60 minutes at 37°C, and the reaction was stopped by addition of 50%

trichloroacetic acid. The mixture was centrifuged at 20,000 x g for 10 minutes, and the catechols formed in 0.5 ml of supernatant were adsorbed on alumina (p. 27). The catechols were eluted from the washed alumina with 2 ml 0.05 M $HClO_4$, and 1.0 ml of this eluate was counted in 10 ml Aquasol liquid scintillation fluid.

In order to examine whether phenylalanine hydroxylase is responsible for the L-m-tyrosine-to-dopa conversion and to obtain a quantitative measurement of its activity, this enzyme was partially purified from rat liver. The livers of male and female Sprague-Dawley and Osborne-Mendel rats were treated by the method of Kaufman and Fisher (1970) to isolate the first ammonium sulfate fraction of the enzyme (40 mg/ml) in 33 mM Tris·HCl buffer pH 6.8. The incubation conditions used for experiments with m-tyrosine were those of Fisher and Kaufman (1973). The mixture contained potassium phosphate buffer (100 µmoles, pH 6.9), $DMPH_4$ (10^{-7} moles), NSD-1055 (5×10^{-8} moles) catalase (4×10^{-10} moles), L-phenylalanine or L-m-tyrosine (0.14 µmoles) and the phenylalanine hydroxylase solution (20 µl, or 8 nmoles) in a final volume of 1.0 ml. Boiled homogenates served as controls. The samples were incubated at 37°C for up to 60 minutes and the reaction was stopped by addition of 1 ml 0.8 N $HClO_4$. The tubes were chilled in ice and

centrifuged at 20,000 x g for 10 minutes. The supernatants were assayed for tyrosine by the nitrosonaphthol procedure (Waalkes and Udenfriend, 1957) or for dopa by adsorption to alumina and development of the hydroxyindole fluorescence (p. 29).

IDENTIFICATION OF CATECHOLS IN RAT URINE

Rats were injected intraperitoneally with 100 mg/kg of DL-m-tyrosine-2-^{14}C and urine was collected in individual metabolic cages for 6 hours. Radioactive DL-m-tyrosine (8 mg) was added to a control sample of urine to approximate the same level of radioactivity. Each sample was lyophilized, then made up to 5.0 ml with water, and the catechols were adsorbed to alumina as previously described (p. 27). The catechols were eluted from the washed alumina with 2 ml 50 mM $HClO_4$, and a 1 ml aliquot of this solution was counted in 10 ml Aquasol scintillation fluid.

The fluorometric determination of acidic catechols: After isolation from the urine, the acidic catechols 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxymandelic acid were assayed by the method of Drujan et al. (1966). A 1.0 ml aliquot of sample was mixed with 0.4 ml ethylenediamine reagent (ethylenediamine : 4M NH_4Cl, 2:3) and heated for 30 minutes at 60°C. The solutions were then diluted with 9 volumes of water and read on

an Aminco-Bowman spectrophotofluorometer. For 3,4-dihydroxyphenylacetic acid, the excitation wavelength was 420 nm and the emission wavelength was 540 nm; for 3,4-dihydroxymandelic acid, the excitation wavelength was 425 nm and the emission wavelength was 490 nm. The ethylenediamine complexes improved the sensitivities of the assays relative to the native fluorescence. In each case, approximately 5 µg of the acid per sample gave good results after condensation, with no significant overlap in the fluorescence contributions at the different wavelengths used.

Isolation of radioactive catechol metabolites: Compounds were administered to male Sprague-Dawley rats (85-100 g) by intraperitoneal injection, using either 0.9% NaCl or dilute HCl as a vehicle. The animals were sacrificed by decapitation. Brain, liver, and kidneys were removed, weighed, and homogenized in 15 ml 0.4 N $HClO_4$ containing 0.1% sodium metabisulfite and 0.05% disodium EDTA (Shellenberger and Gordon, 1971). The homogenate was centrifuged at 20,000 x g for 10 minutes. The pellet was discarded and a 1 ml aliquot of the supernatant solution was used to determine the quantity of labelled metabolites. Alumina (200-250 mg), prepared as described by Anton and Sayre (1961), was then added to the rest of the supernatant. The mixture was stirred for 4 minutes, the pH

being kept between 8.0 and 8.5 by the addition of NaOH. The alumina was allowed to settle (supernatant removed by aspiration), washed with water (3 x 15 ml) and transferred to a small column. The radioactivity adsorbed to alumina was eluted with 5 x 1 ml 0.05 N $HClO_4$ or 0.2 N HOAc and quantitated by liquid scintillation counting (1 ml in 10 ml Aquasol). In some experiments the alumina eluate was freeze-dried and the radioactive residue taken up in 1 ml of sodium phosphate buffer (0.1 M, pH 6.5), which was then applied to an Amberlite CG-50 column as previously described (p. 27). Two fractions were thus obtained, one containing the amines and one containing all non-basic compounds. Both fractions were lyophilized, the residues dissolved in a small volume of 10 mM HCl, and further separated on the amino acid analyser and thin layer plates.

ISOLATION AND WASHING OF PROTEIN FROM RAT ORGANS

Four rats received intraperitoneal injections: (A) 200 mg/kg L-m-tyrosine-2-^{14}C (B) 200 mg/kg D-m-tyrosine-2-^{14}C (C) 22.5 mg/kg puromycin, an inhibitor of protein synthesis (Godin, 1967) followed 60 minutes later by 200 mg/kg L-m-tyrosine-2-^{14}C (D) vehicles only. Rats were sacrificed by decapitation 15 minutes after the administration of m-tyrosine, and brains, livers, and kidneys were homogenized in 5 volumes

of water. The homogenates were centrifuged at 18,000 x g for 10 minutes, and 1 ml of the supernatant was counted in 10 ml PCS liquid scintillation fluid. DL-m-tyrosine-2-^{14}C was added to the control supernatants (from rats receiving vehicle only) to give the same initial counts per unit volume in all cases. Protein was precipitated by addition of one volume of 10% trichloroacetic acid, and the samples were centrifuged at 18,000 x g for 10 minutes. The precipitates were washed to extract all soluble radioactivity, first with 1.0 mg/ml DL-m-tyrosine in 5% trichloroacetic acid (3 x 40 ml), then following the procedure of Zamecnik et al. (1951) with ethanol (3 x 20 ml), ethanol : ether : chloroform 2:2:1 (3 x 20 ml), and finally with acetone (3 x 20 ml). After the last acetone wash, the remaining solid material was dried at room temperature overnight and weighed. A 5 mg sample from each source was solubilized with 1 ml Protosol in a 50°C water bath for 3 hours, then 10 ml Aquasol scintillation fluid was added and the samples were chilled and counted in a Nuclear-Chicago scintillation spectrophotometer.

RESULTS

Chromatography of phenolic compounds

R_f values for several phenolic compounds on thin layer Silica Gel plates are shown in Tables 11 and 12. Systems A and B (described on p. 98) were used for routine separation of all the compounds listed in the Tables, although several were oxidized under the alkaline conditions of system B. System C was particularly useful in the characterization of m-hydroxyphenyl amines; system D permitted the separation of several acidic compounds which were indistinguishable in the other systems. The use of cellulose supports made no difference to the observed R_f values in most systems. The running time for large (20 x 20 cm) plates was several hours, leading to the oxidation of catechols in the basic systems. On the large plates, a difference in R_f value of 0.10 was normally sufficient for a practical separation. Thin layer chromatography was the method of choice for the resolution of certain mixtures (e.g. m-tyramine and m-octopamine on system C; m-hydroxyphenylpyruvic, m-hydroxymandelic, and m-hydroxyphenyllactic acids in system D) as the amino acid analyser column would not separate these compounds satisfactorily under the conditions used. Most other compounds can be separated from each other on the column (Table

TABLE 11

Thin layer chromatography of phenolic amines and amino acids.

Compound	Solvent system		
	A	B	C
	(R_f values)		
m-Tyrosine	0.45	0.34	0.08
Dopa	0.32	-*	-*
m-Tyramine	0.61	0.65	0.58
Dopamine	0.50	-*	-*
m-Octopamine	0.59	0.64	0.42
Norepinephrine	0.40	-*	-*

Performed on precoated Silica Gel G plates with the solvent systems and development methods described on pp. 98-99.

*: All catechol compounds oxidized in the basic systems (B and C) as the chromatogram was running.

TABLE 12

Thin layer chromatography of phenolic acids and amino acids.

Compound	Solvent system		
	A	B	C
	(R_f values)		
m-Hydroxyphenylpyruvate	0.68	-*	0.36
m-Hydroxyphenyllactate	1.00	0.74	0.65
m-Hydroxyphenylacetate	1.00	0.42	0.52
m-Hydroxymandelate	0.66	0.37	0.18
m-Tyrosine	0.45	0.34	0.00
Dopa	0.32	-*	0.00

Performed on precoated Silica Gel G plates with the solvent systems and development methods described on pp. 98-99.

*: m-Hydroxyphenylpyruvate and dopa oxidized in the basic system (B) as the chromatogram was running.

13) with the exception of the amines: m-tyramine, m-octopamine, dopamine, and norepinephrine all appear in the sodium hydroxide fraction.

The native fluorescence of m-hydroxyphenyl compounds

The fluorescence spectra for m-hydroxyphenylacetic acid, m-hydroxyphenylpyruvic acid, m-hydroxymandelic acid, and m-tyramine in aqueous acidic solution are shown in Figure 9. The differences in the characteristic peaks of excitation and emission of these compounds was used to check the tentative assignments of identity to unknown metabolites. For characterization, the general shape of the fluorescence spectrum was as important as the position of the peaks.

RECOVERY OF RADIOACTIVITY IN THE URINE AFTER m-TYROSINE-2-^{14}C

m-Tyrosine-2-^{14}C metabolism *in vivo* was first studied by the determination of a suitable dosage and time for use in other experiments, and by the identification of the major urinary radioactive metabolites. Figure 10 shows the variation in recovery of administered radioactivity with time after the injection of 100 mg/kg DL-m-tyrosine-2-^{14}C. After 24 hours, 85% of the radioactivity had been excreted: similar values have been reported for recovery of dopa from the urine (Pellerin and D'Iorio, 1955; Shindo et al., 1973a).

TABLE 13

Ion exchange chromatography of hydroxyphenyl acids.

Compound	Elution time
	(minutes)
m-Hydroxyphenylpyruvate	14
m-Hydroxyphenyllactate	15
m-Hydroxymandelate	23
3,4-Dihydroxymandelate	26
m-Hydroxyphenylacetate	71
3,4-Dihydroxyphenylacetate	72
L-Dopa	101
L-m-Tyrosine	111

Elution was performed on a Beckman amino acid analyser under the conditions described in the Methods section (pp. 97-98).

FIGURE 9

The fluorescence spectra of native m-hydroxyphenyl compounds in aqueous acidic solution.

The wavelengths at which maximum fluorescence could be achieved were (A) m-hydroxyphenylpyruvic acid, excitation at 370 nm, emission at 470 nm (B) m-hydroxyphenylacetic acid, excitation at 305 nm, emission at 425 nm (C) m-hydroxymandelic acid, excitation at 310 nm, emission at 385 nm (D) m-tyramine, excitation at 290 nm, emission at 320 nm. All wavelengths are uncorrected. At the optimum conditions the sensitivity* of the assay for the acids is roughly 50 µg/ml; for m-tyramine (and m-octopamine which has an almost identical spectrum) it is roughly 500 ng/ml.

*: Sensitivity is defined as that quantity of compound required to produce a fluorescence reading equal to twice the blank value.

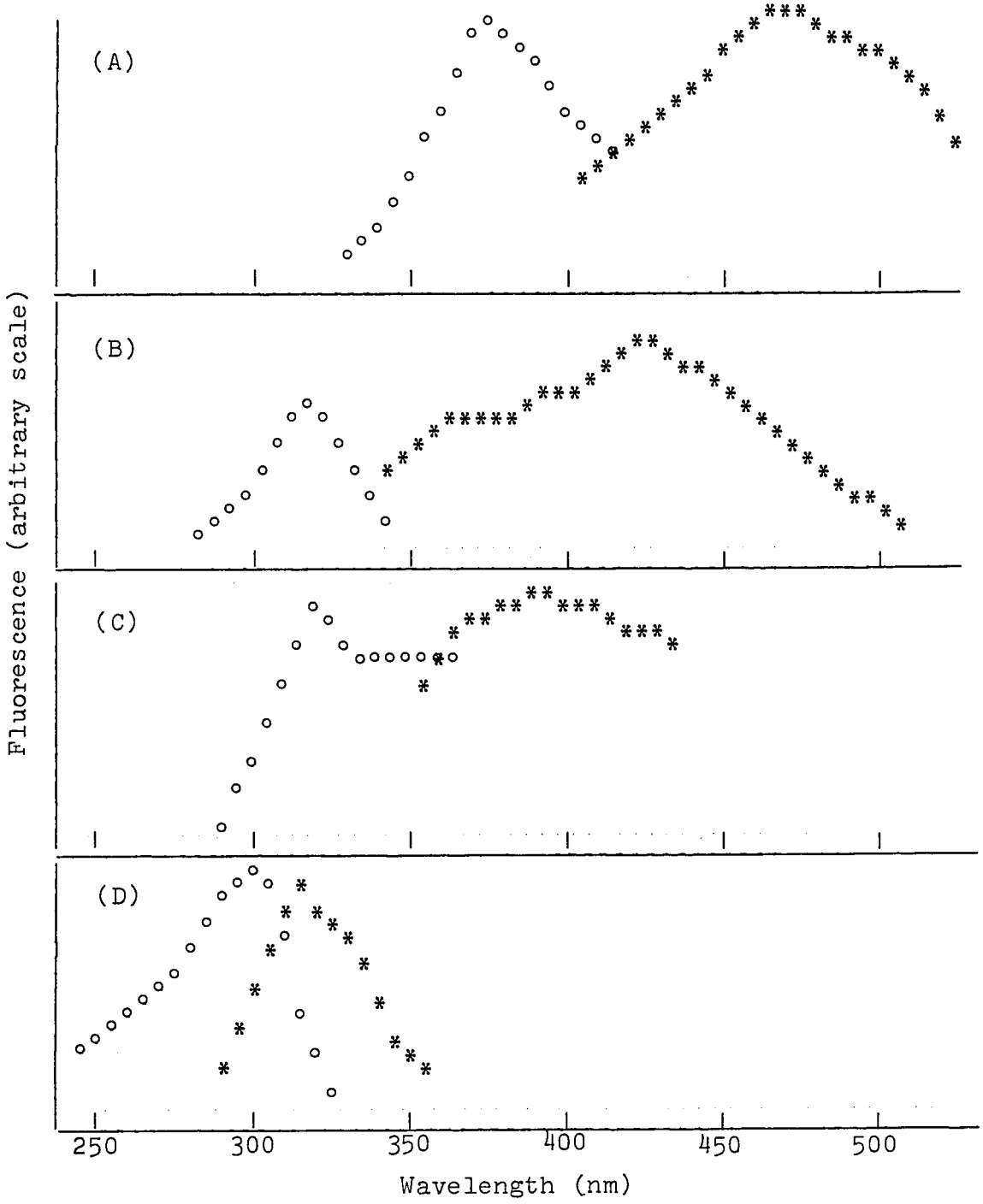
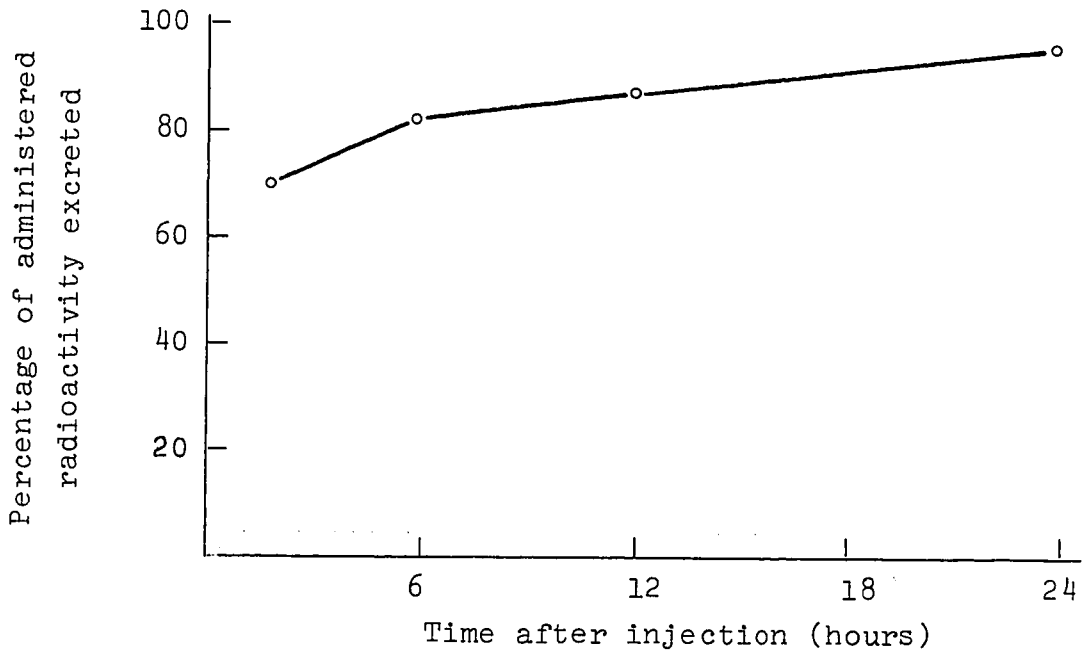

(A)
(B)
(C)
(D)
Fluorescence (arbitrary scale)
250
300
350
400
450
500
Wavelength (nm)

FIGURE 10

The excretion of radioactivity in rat urine as a function of time after the intraperitoneal administration of DL-m-tyrosine-2-^{14}C.



Urine was collected as described on p. 97 and the radioactivity quantitated without separation of the metabolites. Approximately 1.0×10^6 c.p.m. (100 mg/kg) was injected.

Isotopically labelled m-tyrosine-2-^{14}C was diluted with unlabelled compound to produce different dosages with a constant level of administered radioactivity (4.5×10^5 d.p.m.). In this experiment, changes in the dosage of DL-m-tyrosine-2-^{14}C made no significant difference in the recovery of urinary radioactivity. Administration of 100 mg/kg (4.5×10^6 d.p.m.) and a 24-hour collection period were therefore chosen for subsequent experiments. This permitted the recovery of 85% and the detection of 0.01% of the injected compounds.

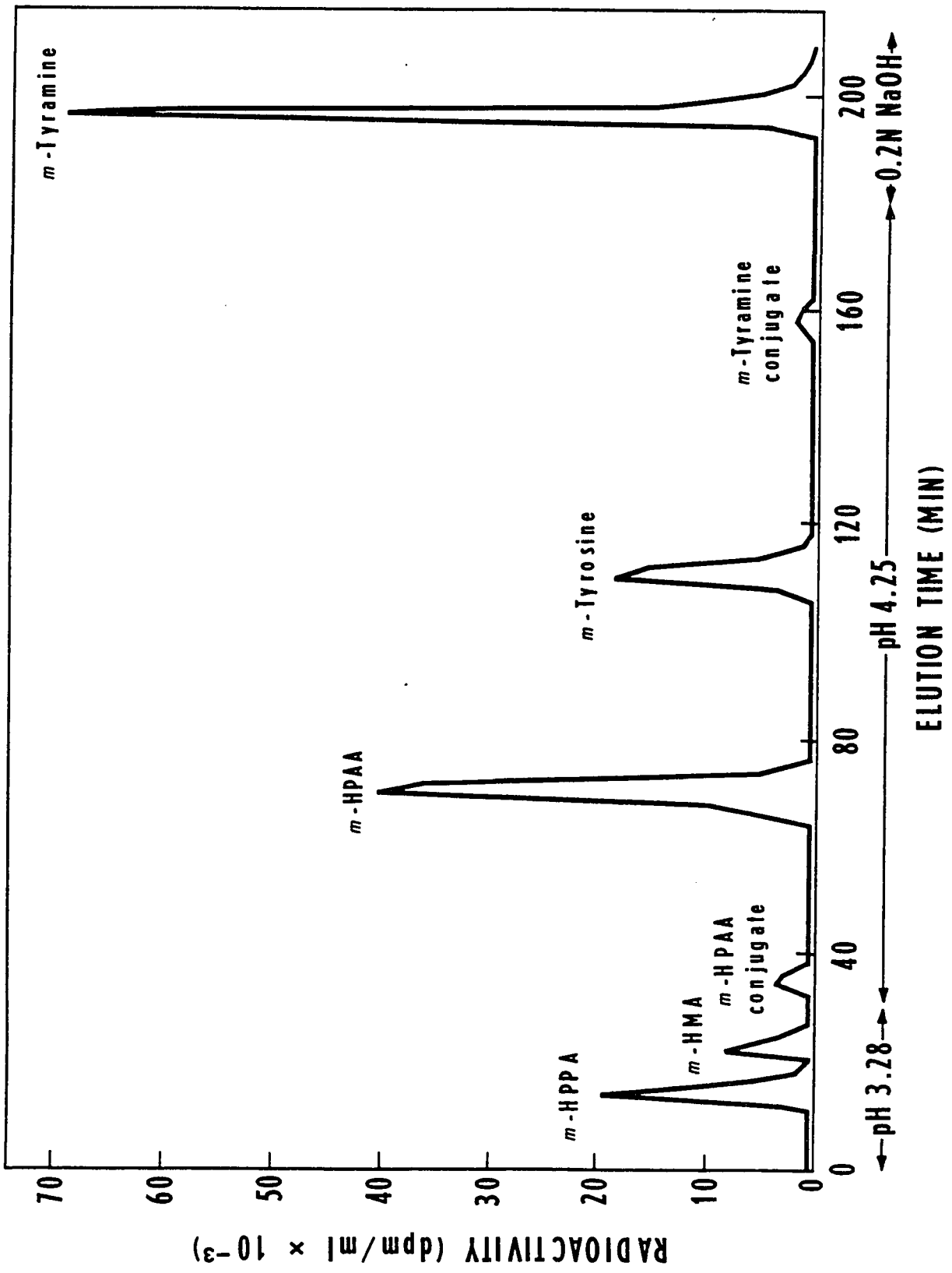
The urinary metabolites of m-tyrosine-2-^{14}C

Identification of the major urinary products of DL-m-tyrosine-2-^{14}C: A typical chromatographic elution pattern of the urinary metabolites of DL-m-tyrosine-2-^{14}C from the amino acid analyser is represented in Figure 11. Seven radioactive components are detected with this system (described on p. 97). m-Hydroxyphenylpyruvic acid, m-hydroxymandelic acid, m-hydroxyphenylacetic acid, and m-tyrosine can be identified by their elution times (Table 13). Two radioactive components which do not correspond to the elution times of any of the available compounds appeared after 35 minutes and 158 minutes. Upon hydrolysis in 6N HCl, the 35-minute peak yielded labelled m-hydroxyphenylacetic acid and the 158-minute peak yielded

FIGURE 11

Chromatography of the urinary metabolites of DL-m-tyrosine-2-^{14}C with the Beckman amino acid analyser.

The radioactive compounds were identified as: (1) m-hydroxyphenylpyruvic acid (2) m-hydroxymandelic acid (3) conjugate of m-hydroxyphenylacetic acid (4) m-hydroxyphenylacetic acid (5) m-tyrosine (6) conjugate of m-tyramine (7) m-tyramine. The elution conditions are described in the Methods section (p. 98).


m-Tyramine
m-HPAA
m-Tyrosine
m-Tyramine conjugate
m-HPPA
m-HMA
m-HPAA conjugate
pH 3.28
pH 4.25
0.2N NaOH
ELUTION TIME (MIN)
RADIOACTIVITY (dpm/ml × 10⁻³)
0
10
20
30
40
50
60
70
0
40
80
120
160
200

labelled m-tyramine as judged by co-chromatography on the analyser and thin-layer plates. Both components were therefore identified as acid-hydrolysable conjugates of the two compounds indicated. Tests for the phenolic functional group by ultraviolet spectroscopy and colour reaction with diazotized p-nitroaniline showed that the conjugate of m-hydroxyphenylacetic acid had a free phenolic group while that of the m-tyramine conjugate was blocked.

Effect of dosage of DL-m-tyrosine-2-^{14}C on the proportions of its urinary metabolites: At the four times indicated in Figure 10, the relative proportions of radioactive urinary metabolites, as determined by separation on the amino acid analyser, were virtually identical. However, changes in the dosage resulted in alterations in these proportions. Table 14 shows the variation in the four major radioactive urinary metabolites with increasing dosage. A slight but consistent decrease in the percentage of m-hydroxyphenylpyruvic acid excreted in the urine can be seen, together with a marked increase in the excretion of m-tyramine.

Contribution of D- and L-m-tyrosine-2-^{14}C to the proportions of urinary metabolites observed with DL-m-tyrosine-2-^{14}C: It is well known that the optical isomers of dopa are metabolized differently due to variations in transport or enzyme specificity.

TABLE 14

Major urinary metabolites of DL-m-tyrosine-2-^{14}C.

Metabolite	DL-m-tyrosine-2-^{14}C (mg/kg)			
	10	30	100	300
	(% of excreted radioactivity)			
m-Hydroxyphenylpyruvate	13.5	11.1	8.9	8.1
m-Hydroxyphenylacetate	30.4	26.0	29.0	25.0
m-Tyrosine	14.0	17.3	17.5	17.6
m-Tyramine	26.8	28.4	31.2	36.0
Total	84.7	82.8	86.6	86.7

Urine was collected for 24 hours after intraperitoneal administration of DL-m-tyrosine-2-^{14}C containing a constant quantity of radioactivity (4.5×10^6 d.p.m.). All values are given in percentages of the radioactivity placed on the column as described in the Methods section (p. 97). Results are expressed as individual determinations except at 100 mg/kg, where they are the mean of five determinations.

The D antipode may act as an enzyme inhibitor in some cases. Table 15 shows the differences in the proportions of metabolic products arising from 100 mg/kg of the optical isomers of m-tyrosine. L-m-tyrosine was metabolised mainly to acids, either through decarboxylation or transamination followed by oxidation; about 88% of urinary metabolites are acidic. In contrast, only about 30% of the urinary metabolites of D-m-tyrosine are acidic; a considerable proportion (26%) was excreted unchanged, while 40% was isolated as m-tyramine. The administration of the D-isomer produces more amine in the urine than the same dose of the L-isomer; Sourkes et al. (1960) reported a similar finding with D- and L-dopa. It is noteworthy, however, that although free m-tyramine was a major metabolite after D-m-tyrosine administration, none of the conjugated amine could be detected.

Effect of the dopa decarboxylase inhibitor NSD-1034 on the proportions of the urinary metabolites of DL-m-tyrosine-2-^{14}C:

The use of enzyme inhibitors may also alter the proportions of urinary metabolites. Decarboxylation appears to be the major route of metabolism of m-tyrosine in the normal rat, and its prevention may permit the selective observation of other pathways. Table 16 shows the effect of pretreatment with the general dopa decarboxylase inhibitor NSD-1034 (Levine and

TABLE 15

Metabolites of D-, L-, and DL-m-tyrosine-2-^{14}C (100 mg/kg) in 24-hour rat urine after intraperitoneal administration.

Metabolite	Isomer		
	D-m-tyrosine	DL-m-tyrosine	L-m-tyrosine
	(% of urinary radioactivity)		
m-Hydroxyphenylpyruvate	6.2	8.9	11.3
m-Hydroxymandelate	5.2	3.4	7.3
Conjugate of m-hydroxyphenylacetate	2.3	3.4	12.2
m-Hydroxyphenylacetate	16.7	29.0	57.3
m-Tyrosine	26.1	17.5	0.0
Conjugate of m-tyramine	0.0	6.8	8.4
m-Tyramine	40.5	31.2	3.3

Results are expressed as percentages of the radioactivity placed on the column from the lyophilized urine samples (3×10^6 c.p.m.), as individual determinations except for DL-m-tyrosine, where the results are expressed as the mean of five determinations.

TABLE 16

Urinary metabolites of DL-m-tyrosine-2-^{14}C after inhibition of dopa decarboxylase with NSD-1034.

Metabolite	Compounds injected	
	DL-m-tyrosine	NSD-1034 + DL-m-tyrosine
	(% of urinary radioactivity)	
m-Hydroxyphenylpyruvate	8.9	8.1
m-Hydroxymandelate	3.4	0.0
Conjugate of m-hydroxyphenylacetate	3.4	0.3
m-Hydroxyphenylacetate	29.0	3.2
m-Tyrosine	17.5	81.7
Conjugate of m-tyramine	6.8	0.0
m-Tyramine	31.2	6.4

NSD-1034 (100 mg/kg) was injected 30 minutes prior to administration of DL-m-tyrosine-2-^{14}C (100 mg/kg, 2.8 x 10^6 d.p.m.). Control animals received saline. Urine was collected for 24 hours. All values are given as percentages of the radioactivity placed on the column from the lyophilized urine samples. For DL-m-tyrosine alone, results are expressed as the mean of five determinations; those results with NSD-1034 are presented as a single determination.

Sjoerdsma, 1964). The urinary levels of amines and their metabolites dropped after the administration of inhibitor, and over 80% of the radioactivity was excreted as unchanged m-tyrosine. No new metabolites were discovered.

Administration of ^{14}C-labelled m-hydroxyphenyl compounds.

Examination of the products after the administration of each intermediate in m-tyrosine metabolism in vivo should permit the elucidation of the reaction sequence. The products of radiochemical synthesis (pp. 80-92) (m-hydroxyphenylpyruvic acid, m-hydroxyphenylacetic acid, m-hydroxymandelic acid, m-hydroxyphenyllactic acid, m-tyramine, and m-octopamine) were all injected and their metabolites examined.

The administration of m-hydroxyphenylpyruvic acid-2-^{14}C (100 mg/kg, 4.6×10^6 d.p.m.) resulted in the excretion of the compounds shown in Table 17. The appearance of m-tyrosine and m-tyramine strongly implied that a portion of the keto acid was transaminated, giving rise to the amino acid and its decarboxylation product. The reactions involved in the conversion of the pyruvate to m-tyramine were examined further by pretreatment with NSD-1055 to block dopa decarboxylase (Levine and Sjoerdsma, 1964). All major metabolites, particularly m-tyrosine, rose at the expense of m-tyramine.

TABLE 17

Major urinary metabolites of m-hydroxyphenylpyruvic acid-2-^{14}C.

Metabolites excreted	Compounds injected	
	m-HPPA*	NSD-1055 + m-HPPA*
	(% urinary radioactivity)	
m-Hydroxyphenylpyruvic acid**	32	39
m-Hydroxyphenylacetic acid	26	29
Conjugate of m-hydroxyphenylacetic acid	6	5
m-Tyrosine	1	18
m-Tyramine	34	7

The urine was collected for 24 hours after injection of m-hydroxyphenylpyruvic acid-2-^{14}C (100 mg/kg, 4.5×10^6 d.p.m.). NSD-1055 (100 mg/kg) was injected 30 minutes before the m-hydroxyphenylpyruvic acid. Control animals received saline. Results are given as single determinations.

*: m-Hydroxyphenylpyruvic acid-2-^{14}C was abbreviated as m-HPPA.

**: The m-hydroxyphenylpyruvic acid fraction contained roughly 20% m-hydroxyphenyllactic acid-2-^{14}C as determined by thin layer chromatographic separation (Systems A and D, Table 12). The remaining 80% had an R_f value corresponding to m-hydroxyphenylpyruvic acid.

After administration of m-hydroxyphenylacetic acid-1-^{14}C (100 mg/kg, 7.0 x 10^6 d.p.m.), 81% was excreted unchanged, 12% appeared as the acidic conjugate, while the remainder was eluted very quickly (less than 20 minutes) from the column as unidentified acidic products. Both m-hydroxyphenyllactic acid-2-^{14}C (100 mg/kg, 4.5 x 10^6 d.p.m.) and m-hydroxymandelic acid-2-^{14}C (100 mg/kg, 6.6 x 10^6 d.p.m.) were excreted unchanged.

m-Tyramine-1-^{14}C (40 mg/kg, 5.5 x 10^6 d.p.m.) and m-octopamine-1-^{14}C (40 mg/kg, 4.0 x 10^6 d.p.m.) were also injected and the urinary products examined. The majority of these radioactive compounds were oxidized to m-hydroxymandelic acid and/or m-hydroxyphenylacetic acid (Table 18). Although m-hydroxymandelic acid is a product of m-tyramine, no m-octopamine could be demonstrated in the amine fraction after m-tyramine administration. Similarly, after administration of m-octopamine, no m-tyramine or m-hydroxyphenylacetic acid could be recovered.

Distribution and metabolism of DL-m-tyrosine-2-^{14}C in tissues

Fifteen minutes after administration of DL-m-tyrosine-2-^{14}C (100 mg/kg) 5-7% of the radioactivity could be recovered from the six organs examined. Thereafter the radioactivity declined, and at 60 minutes, only 1-2% was present in these organs. The amount of radioactivity accumulated per organ at 15 minutes was

TABLE 18

Major urinary metabolites of ^{14}C-m-hydroxyphenyl amines.

Metabolite excreted	Compound injected	
	m-Tyramine	m-Octopamine
	(% urinary radioactivity)	
m-Hydroxymandelic acid	16	91
m-Hydroxyphenylacetic acid	68	0
Conjugate of m-tyramine	11	0
m-Tyramine	5	0
m-Octopamine	0	9

Urine was collected for 24 hours after injection of 40 mg/kg (4.0×10^6 d.p.m.) of m-tyramine-1-^{14}C or m-octopamine-1-^{14}C. Results are expressed as individual determinations. When the quantity of metabolite was less than 0.2% of the total, results were not included in the table.

found to be in the order: kidneys (3%), liver (3%), brain (0.4%), spleen (0.2%), heart (0.2%), adrenals (0.07%). These values were based on the radioactivity administered to the rat (4.5×10^6 d.p.m.=100%).

Table 19 shows that over 85% of the total radioactivity recovered was accumulated in the kidney and liver. Essentially all radioactivity was found in the neutral fraction (containing m-tyrosine) except in the kidney, where 27% of the total had been converted to other radioactive compounds.

After pretreatment with 100 mg/kg of the general dopa decarboxylase inhibitor NSD-1034 (Levine and Sjoerdsma, 1964), little change could be found in any of the proportions described above or in Table 19, except that the recovery of radioactivity in the acidic and amine fractions of most organs was negligible. Only in the kidney was as much as 2.1% of the radioactivity found in the acidic fraction in this case.

Similar experiments were carried out with liver and kidney using m-tyramine-1-^{14}C, since this is apparently the major initial metabolite of m-tyrosine-2-^{14}C. Fifteen minutes after injection of 100 mg/kg, the liver accumulates 2.0% of the total radioactivity injected, including 0.5% in the acidic fraction and 1.5% in the amine fraction. In the kidney, 2.9% was recovered, including 0.8% as amine, 2.0% as acid, and 0.14% in

TABLE 19

Classes of DL-m-tyrosine-2-^{14}C metabolites in rat tissues.

Organ	Metabolite fraction			Proportion of total
	Amine	Acid	Neutral	
	(% in organ)			(%)
Adrenal	5.1	0.8	94.1	5.1
Brain	1.0	2.5	96.5	5.1
Heart	3.4	4.8	91.7	2.9
Kidney	14.2	12.8	72.9	53.3
Liver	0.3	6.3	93.4	33.7
Spleen	1.5	0.7	97.7	3.9

All values are calculated from the radioactivity in the fractions of each organ, 15 minutes after injection of 100 mg/kg DL-m-tyrosine-2-^{14}C (4.5×10^6 d.p.m.). The metabolites are separated on Amberlite and Dowex columns as described in the text (p. 27, 77). The "Acid" fraction is not retained on either Amberlite or Dowex, and includes m-hydroxyphenylpyruvic acid, m-hydroxyphenyllactic acid, m-hydroxymandelic acid, m-hydroxyphenylacetic acid, the corresponding catechol acids, and the conjugate of m-hydroxyphenylacetic acid. The "Amine" fraction is adsorbed on Amberlite and includes m-tyramine, m-octopamine, and the catecholamines. The "Neutral" fraction is adsorbed to Dowex and includes m-tyrosine, dopa, and the conjugate of m-tyramine.

the neutral fraction.

THE *IN VITRO* METABOLISM OF *m*-HYDROXYPHENYL COMPOUNDS

In vitro studies were carried out to supplement the *in vivo* results described above. Brain, kidney, and liver were chosen for these studies since over 90% of the radioactivity recovered from tissues was accumulated in these three organs. Tables 20 and 21 indicate that under the conditions appropriate for the *in vitro* metabolism of similar compounds, *m*-tyrosine can be decarboxylated to *m*-tyramine, oxidized or transaminated (depending on the isomer used as the substrate) to *m*-hydroxyphenylpyruvic acid, or hydroxylated to dopa. The enzyme activities in the brain were considerably lower than those of kidney or liver when assayed under identical conditions, with the exception of the deamination of *m*-hydroxyphenyl amines. The highest activity for the oxidation of D-*m*-tyrosine was found in the kidney, where D-amino acid oxidase is mainly localized (Shindo and Maeda, 1974). The activity of dopamine-β-hydroxylase and sulfotransferase towards *m*-tyramine, producing *m*-octopamine and *m*-tyramine-3-O-sulfate respectively, could not be determined reproducibly using procedures established for similar compounds (Creveling et al., 1962; Meek and Neff, 1973).

The compounds found in the urine after *m*-tyramine injection

TABLE 20

The metabolism of m-hydroxyphenyl compounds in vitro.

Substrate	Product	Quantity of product formed		
		Brain	Kidney	Liver
		(nmoles/g tissue/minute)		
L-m-tyrosine	m-Tyramine	7.3	395.8	441.7
D-m-tyrosine	m-HPPA*	2.6	124.5	18.0
L-m-tyrosine	m-HPPA*	8.8	39.7	61.0
m-HPPA*	m-Tyrosine	20.2	127.8	144.1
m-Tyramine	m-HPAA**	54.3	29.1	148.2
m-Octopamine	m-HMA***	16.1	8.5	45.0

The values are derived from the reaction rates (linear over the time periods studied) based on the methods described in the text (pp. 100-104).

*: m-Hydroxyphenylpyruvic acid is abbreviated as m-HPPA.
**: m-Hydroxyphenylacetic acid is abbreviated as m-HPAA.
***: m-Hydroxymandelic acid is abbreviated as m-HMA.

TABLE 21

The conversion of hydroxyphenyl compounds to the corresponding catechols *in vitro*.

Substrate	Product	Quantity of product formed	
		Kidney	Liver
		(nmoles/g tissue/minute)	
L-*m*-tyrosine	Dopa	3.6	16.6
L-*p*-tyrosine	Dopa	0.1	1.2
m-Tyramine	Dopamine	0.0	0.3
m-Octopamine	Norepinephrine	0.0	0.2
p-Tyramine	Dopamine	0.0	0.6

The values are derived from the reaction rates (linear over the time periods studied) based on the methods described in the text (pp. 104-106).

(Table 18) indicate that the oxidation of m-tyramine is also important in in vivo metabolism. The conversion of D-m-tyrosine to L-m-tyrosine through m-hydroxyphenylpyruvic acid has been hypothesized as an explanation for some of the actions of the D-isomer in vivo. These two reactions have been examined in vitro, and the results (Table 20) show that these conversions are indeed possible.

Table 21 shows the conversion of m-hydroxyphenyl compounds to the corresponding catechols. The reactions are readily demonstrable in liver, particularly when radioactive substrates are used for greater sensitivity.

The hydroxylation of phenylalanine to tyrosine and of m-tyrosine to dopa by a partially purified phenylalanine hydroxylase preparation showed no significant differences among male and female Sprague-Dawley or Osborne-Mendel rats. Conversion of phenylalanine to tyrosine proceeds at roughly 3.0 nmole/minute/mg protein; m-tyrosine is converted to dopa at 0.9 nmole/minute/mg protein.

THE ISOLATION OF RADIOACTIVITY FROM RAT LIVER PROTEIN

The only protein fraction showing significant radioactivity after the washing procedure described previously (p. 110) was the sample from the liver of the rat injected with L-m-tyrosine-2-^{14}C alone. Approximately 100 c.p.m. per 5 mg of dry residue

was recovered, corresponding to 100 mg wet weight of liver tissue. The lack of radioactivity of the sample from the puromycin-treated rat is evidence that protein synthesis is necessary for the incorporation; the similar negative results after D-m-tyrosine injection indicates that some aspect of this incorporation is stereospecific.

The remaining solid from the L-m-tyrosine sample was hydrolysed in HCl, the solution lyophilized, and the residue chromatographed on the amino acid analyser and on thin layer plates. The radioactivity appeared in the alkali fraction on the amino acid analyser, and was immobile (R_f=0.00) in system A (Table 11, p. 98). Subjecting DL-m-tyrosine-2-^{14}C to the hydrolysis and lyophilization had no effect on its usual chromatographic behavior.

CONVERSION OF m-TYROSINE-2-^{14}C TO RADIOACTIVE CATECHOLS

The possibility that m-tyrosine and/or its metabolites may be hydroxylated to dopa and other catechols *in vivo* has received considerable attention. Most recent studies have supported this theory (Sourkes et al., 1961b; Hollunger and Persson, 1974) but none have made use of a radioactive label.

It is possible to recover 70-80% of the radioactivity from injected DL-m-tyrosine-2-^{14}C after 6 hours (Figure 10).

Of this radioactivity, 0.23% was recovered after adsorption of catechols to alumina at pH 8.0 and elution with 0.05 M $HClO_4$ (p. 107). The extent of conversion to dopa-^{14}C rose to 0.65% when dopa decarboxylase was inhibited by pretreatment with the inhibitor NSD-1034 (100 mg/kg) (Levine and Sjoerdsma, 1964).

When m-tyrosine-2-^{14}C was added directly to the urine, no radioactivity was found in the eluate from alumina. The radioactive eluate from the sample of the injected rat was lyophilized and the residue applied to the amino acid analyser (p. 97). Radioactivity was eluted at 100 minutes, the same time determined for authentic dopa (Table 13). To confirm this identification, the fluorescence of the fractions was examined relative to their radioactivity. As seen in Table 22, the correlation gives further support to the assignment. The ratio also indicates that the specific radioactivity of eluted dopa is identical to that of the injected m-tyrosine-2-^{14}C.

Effect of enzyme inhibitors on the conversion of m-tyrosine-2-^{14}C to ^{14}C-catechols: Fifteen minutes after administration of DL-m-tyrosine-2-^{14}C (100 mg/kg), approximately 0.5% of the radioactivity was recovered in the brain, 3.3% in liver and 2.8% in kidneys. These values were based on the radioactivity initially administered (4.5×10^6 d.p.m.) and did not change

TABLE 22

Comparison of radioactivity and fluorescence due to catechols in the amino acid analyser fractions from rat urine.

Fraction number	Radioactivity	Fluorescence	$\frac{\text{Radioactivity}}{\text{Fluorescence}}$
	(d.p.m./ml)	(arbitrary)	
48	470	2960	0.159
49	1069	6522	0.163
50	599	3709	0.161

Urine was collected for 24 hours after administration of DL-m-tyrosine-2-^{14}C as described in the text (p. 97). The radioactive material eluted from the Beckman amino acid analyser using the conditions described on p. 97 was adjusted to pH 8.5, treated with alumina (p. 27) and the catechols eluted with 2.5 ml 50 mM $HClO_4$. 1.0 ml of this eluate was counted in 10 ml Aquasol, and 1.0 ml was treated by procedure C (Table 2) to develop the fluorescence due to dopa. Values are given as d.p.m./ml of the alumina eluate, and in arbitrary fluorescence units, as single determinations.

significantly when the animals had been pretreated with the dopa decarboxylase inhibitors NSD-1034 (Levine and Sjoerdsma, 1964) or MK-486 (Porter, 1962) or the monoamine oxidase inhibitor Catron (Horita, 1959). The ^{14}C-catechols were determined by specific adsorption on alumina (p. 27) and the recovery of dopa (10-300 nmoles) is 22% for liver and 28% for kidneys and brain. The results are summarized in Table 23. It was found that in control and Catron-treated animals, the kidney was the only organ which accumulated significant amounts of radioactive catechol metabolites. However, when the animals were pretreated with dopa decarboxylase inhibitors, the conversion of m-tyrosine-2-^{14}C to radioactive catechols also became apparent in both brain and liver.

Identification of radioactive catechol metabolites

The radioactive catechols were separated using Amberlite CG-50 into two fractions: (a) basic and (b) acidic and neutral. They were then identified by co-chromatography with authentic compounds. In the kidneys of control animals, approximately 56% of the radioactive catechols was in the form of dopamine, 22% as dihydroxyphenylacetic acid, and 14% as dopa. In animals pretreated with NSD-1034, over 95% of the radioactive catechols was dopa in the three organs examined.

TABLE 23

Effect of dopa decarboxylase and monoamine oxidase inhibitors on the *in vivo* conversion of m-tyrosine-2-^{14}C to catechols.

Treatment	Radioactivity adsorbed to alumina		
	Brain	Liver	Kidneys
	(d.p.m./organ)		
-	12 ± 2	64 ± 11	529 ± 58
NSD-1034	228 ± 24	2482 ± 235	526 ± 90
MK-486	118 ± 17	1726 ± 102	700 ± 86
Catron	10 ± 3	101 ± 13	745 ± 71

Dopa decarboxylase inhibitor NSD-1034 (100 mg/kg) or MK-486 (100 mg/kg) was injected intraperitoneally into rats. Control animals received saline. DL-m-tyrosine-2-^{14}C (100 mg/kg) was similarly administered 60 minutes later. At 15 minutes after DL-m-tyrosine-2-^{14}C injection, the animals were sacrificed. Radioactive catechols in brain, liver, and kidneys were extracted and quantitated as described in the text (p. 108). Similar experiments were carried out on animals pretreated with monoamine oxidase inhibitor (Catron, 10 mg/kg) 24 hours prior to the DL-m-tyrosine-2-^{14}C. All values are expressed as d.p.m./organ and given as means ± standard deviation for four animals.

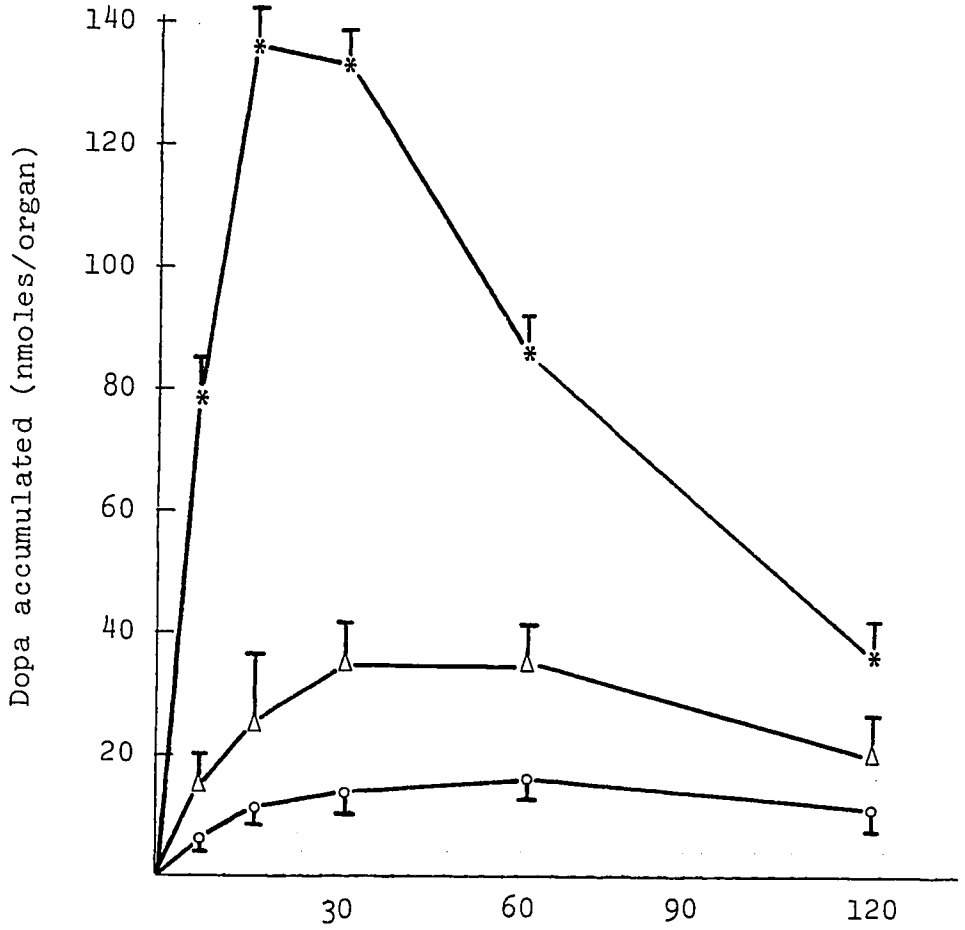
The *in vivo* conversion of *m*-tyrosine-2-^{14}C to ^{14}C-dopa

Experiments were performed on animals pretreated with the dopa decarboxylase inhibitor NSD-1034 (100 mg/kg) and radioactive dopa was quantitated. The accumulation of dopa after a single injection of DL-*m*-tyrosine-2-^{14}C (100 mg/kg) as a function of time can be seen in Figure 12. It was observed that the amount of dopa accumulated in the liver reached a maximum at 15-30 minutes with a steady decline over the next 90 minutes. In the brain and kidneys the maximum was at 30-60 minutes. Figure 13 shows the accumulation of labelled dopa from DL-*m*-tyrosine-2-^{14}C as a function of dosage, which was roughly linear up to 200 mg/kg. Of the three organs examined, the amount of dopa accumulated per gram organ was highest in the liver.

There is evidence that phenylalanine hydroxylase is able to catalyse the hydroxylation of *m*-tyrosine to dopa *in vitro* (Tong et al., 1971b; Fisher and Kaufman, 1973). It was therefore of interest to examine whether an aromatic amino acid hydroxylase was important in the *in vivo* conversion of *m*-tyrosine to dopa. The results are shown in Table 24. Animals receiving the tryptophan and phenylalanine hydroxylase inhibitor *p*-chlorophenylalanine (Koe and Weissman, 1966; Gal et al., 1970) showed a marked decrease in the formation of radioactive dopa. By contrast, the tyrosine hydroxylase inhibitor α-methyltyrosine

FIGURE 12

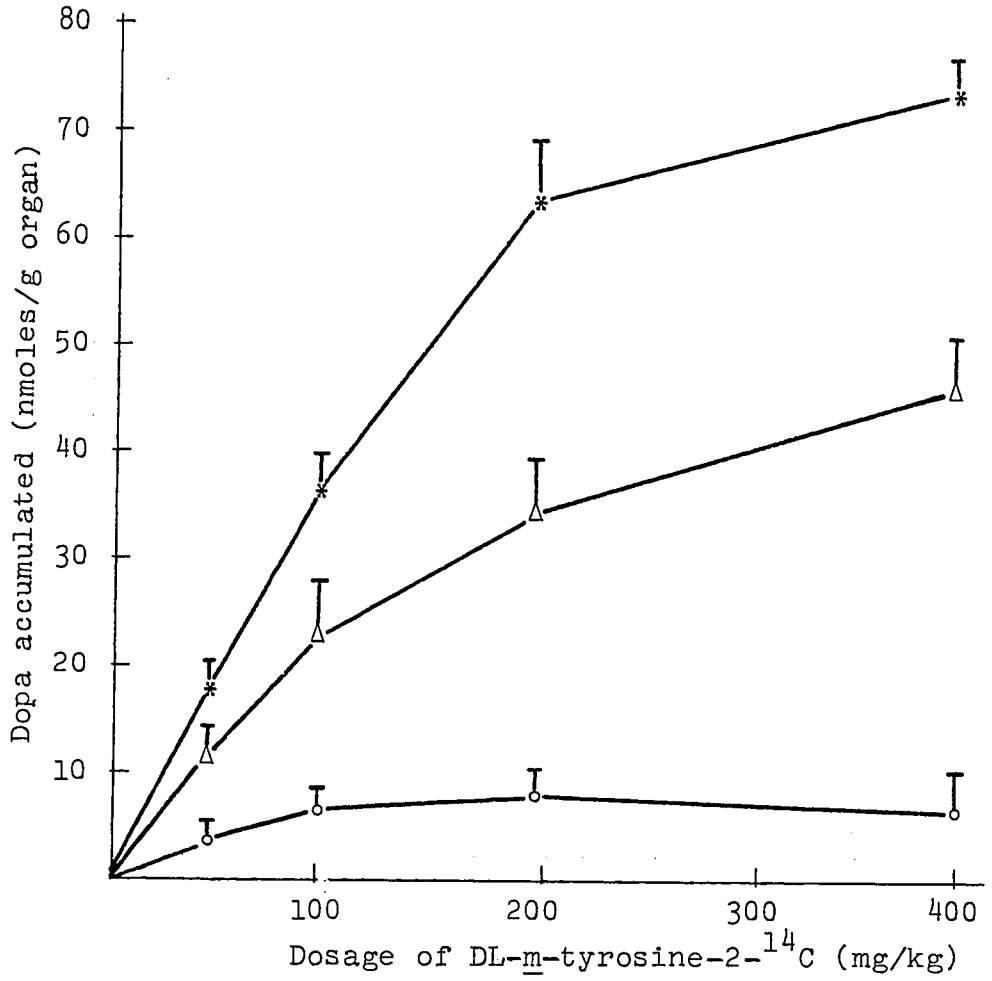
The accumulation of radioactive dopa in rat organs after administration of DL-m-tyrosine-2-^{14}C as a function of time.



Experiments were performed in rats pretreated with NSD-1034 (100 mg/kg) as described in Table 23. Values are expressed as nmoles of dopa per organ in liver (*-*), kidneys (Δ-Δ), and brain (o-o) and given as means ± standard deviation for four animals.

FIGURE 13

The accumulation of radioactive dopa in rat organs after administration of DL-m-tyrosine-2-^{14}C as a function of dosage.



Experiments were performed in rats pretreated with NSD-1034 (100 mg/kg) as described in Table 23. Values are expressed as nmoles of dopa per organ in liver (*-*), kidneys (Δ-Δ), and brain (∘-∘) and given as means ± standard deviation for four animals.

TABLE 24

Effect of aromatic amino acid hydroxylase inhibitors on the *in vivo* conversion of DL-*m*-tyrosine-2-^{14}C to dopa.

Inhibitor	Radioactive dopa		
	Brain	Liver	Kidneys
	(% of control)		
p-Chlorophenylalanine	28 ± 5	47 ± 5	24 ± 4
α-Methyltyrosine	90 ± 6	84 ± 10	70 ± 10

DL-*p*-chlorophenylalanine (360 mg/kg) was administered intraperitoneally to one group of rats, and 24 hours later, experiments on the conversion of DL-*m*-tyrosine-2-^{14}C were carried out on NSD-1034-treated animals as described in Table 23. DL-α-methyltyrosine methyl ester hydrochloride (200 mg/kg) was given to a second group of animals 60 minutes prior to the experiment. Values are presented as percentages of controls without the hydroxylase inhibitors, and are given as means ± standard deviations for four animals. Control values are in the range indicated in Table 23 (p. 139).

(Spector et al., 1965) had very little effect.

In agreement with previous in vitro results (Tong et al., 1971a), the in vivo conversion of m-tyrosine to dopa appears to be stereospecific. It was observed that the amount of dopa formed in the liver from D-m-tyrosine-2-^{14}C was only 25 ± 10% of that formed from the L-isomer.

DISCUSSION

IDENTIFICATION OF THE URINARY METABOLITES OF m-TYROSINE-2-^{14}C

Of the total radioactivity injected intraperitoneally as m-tyrosine-2-^{14}C, over 85% is normally excreted in the urine within 24 hours by the rat (Figure 10). Rat organs examined 24 hours after DL-m-tyrosine-2-^{14}C administration revealed no residual radioactivity. It may be concluded that the recovery of 85% represents the total metabolite excreted by the urinary route. This recovery is independent of dosage within the range of 30-1000 mg/kg.

Most of the radioactive material separated into distinct fractions by the amino acid analyser could be positively identified by co-chromatography with standard compounds: m-tyrosine, m-hydroxyphenylpyruvic acid, m-hydroxyphenylacetic acid, and m-tyramine were the major metabolites. However, two others were not completely identified (Figure 11). These metabolites were conjugates labile to hot acid, but the nonradioactive portions of the molecules were not characterized. The radioactive compound eluted from the amino acid analyser after 35 minutes was phenolic, suggesting that it was the carboxylic acid group of the m-hydroxyphenylacetic acid which was conjugated. Chandler and Lewis (1932) identified phenylaceturic acid (N-phenylacetylglycine) as a urinary product in rabbits, but Woolf (1951) and

Wadman et al. (1971) found that phenylacetic acid conjugated primarily with glutamine in phenylketonuric humans. m-Hydroxy-hippuric acid has been discovered in rabbit urine as a m-hydroxybenzoic acid metabolite (Bray et al., 1950) and in human urine as an endogenous product (Armstrong et al., 1956a, 1956b). Nakajima and Sano (1964) found that administration of p-tyramine resulted in increased excretion of p-hydroxyphenyl-aceturic acid in rats; Pogrund et al. (1961) demonstrated the formation of m-hydroxyphenylaceturic acid after administration of m-tyrosine to cats. The nature of the conjugate may be species-specific as well as a characteristic of the parent compound.

The material eluted from the amino acid analyser at 158 minutes gave none of the colour tests for phenols, implying that the phenol group of m-tyramine was masked by the conjugate. One of the possibilities considered was sulfoconjugation. It is known that the sulfoconjugates of dopamine are produced at both the 3- and the 4-positions in humans, and that the 3-O-sulfate is metabolically inert in rats (Jenner and Rose, 1974). However, no sulfotransferase activity towards m-tyramine was detected in preliminary experiments using the *in vitro* system recommended by these authors, despite the use of radioactive substrate which would permit the detection of 0.1% of this product.

Several minor acidic components were noted in the fractions eluted at the 8-29 minute interval on the amino acid analyser. The yield of these materials was too low for characterization.

They may represent oxidation or breakdown products, or the experimental conditions may be responsible for the destruction of unstable minor urinary components, but their total contribution to the radioactivity is negligible.

Alterations in the proportions of m-tyrosine metabolites

The proportions of the major metabolites of m-tyrosine excreted in the urine can be altered by changing the dosage or the optical isomer used, but no changes in these proportions could be observed at different times after the injection. Alterations in the dose of DL-m-tyrosine-2-^{14}C administered (Table 14) result in the highest proportion of acidic metabolites at low dosage (10 mg/kg), where they accounted for over half of the total radioactivity excreted. With increasing dosage (to 300 mg/kg), the percentage of unchanged m-tyrosine rose somewhat, but the greatest increase was for m-tyramine, which rose from 27% (at 10 mg/kg) to 36% (at 300 mg/kg) of the total excreted. Kochar et al. (1974) noted a similar correlation between the amine-to-acid ratio in the urine and the dosage of L-dopa administered to Parkinsonian patients. One explanation of this phenomenon may be that the capacity of the enzymes responsible for oxidation to acidic products is exceeded at high doses of the amino acid.

Significant differences in the fates of the optical isomers of m-tyrosine were observed (Table 15). L-m-tyrosine-2-^{14}C is completely metabolized, as shown by its absence from the urine when the pure isomer is injected. m-Tyramine is not the major urinary product; most appears to be oxidized to m-hydroxyphenylacetic acid. This suggests that the L-isomer is freely metabolized in various organs by several enzymes.

The m-tyramine conjugate does not form after administration of the D-isomer, although m-tyramine itself is the major urinary product. While studying the effect of vitamin deficiencies on metabolism, Sourkes et al. (1960) found no dopamine conjugate from D-dopa; this was corroborated by Shindo et al. (1973a, 1973b). D-m-tyrosine may inhibit the enzyme responsible for the conjugation reaction. Alternatively, differences between the fates of the isomers may be due to different sites of metabolism. m-Tyramine can only be produced from D-m-tyrosine by oxidation, transamination, and decarboxylation, and one or more of these steps may only occur in the kidney just prior to excretion. For instance, the in vitro studies indicated that the D-amino acid oxidase is localized almost entirely to the kidney; the enzyme responsible for conjugate formation may not be present there.

Pretreatment with NSD-1034 (Table 16) and other dopa decarboxylase inhibitors restricts the formation of most m-tyrosine metabolites, leaving over 80% of the injected compound to be excreted unchanged. The differences from the

untreated case can be attributed to the inhibition of dopa decarboxylase, and imply that the functioning of this enzyme is essential for the significant metabolism of the amino acid. The major urinary metabolite of L-m-tyrosine, m-hydroxyphenylacetic acid, seems to arise almost entirely from an m-tyramine intermediate rather than through an oxidative cleavage of m-hydroxyphenylpyruvic acid after transamination. Previous reports of the effectiveness of NSD-1034, NSD-1055, and MK-486 as dopa decarboxylase inhibitors (Porter et al., 1962; Levine and Sjoerdsma, 1964; Petitclerc et al., 1969a) have been supported with m-tyrosine as substrate.

The metabolism of m-hydroxyphenyl acids and amines

The metabolism of m-hydroxyphenylpyruvic acid-2-^{14}C (Table 17) confirms that the oxidation of m-tyramine is not the only source of urinary m-hydroxyphenylacetic acid. The pyruvate may also be converted directly into the acetate by oxidative decarboxylation. The change in the ratios of metabolites after NSD-1055 pretreatment may be explained by inhibition of either the transaminase or the decarboxylase or both, since a pyridoxal phosphate cofactor is required for both enzymes. The unusually high yield of m-tyramine compared to the amount of its supposed intermediate m-tyrosine implies that the transamination forming the amino acid is followed by almost quantitative decarboxylation to the amine.

The conversion of m-hydroxyphenylacetic acid to its conjugate is a likely explanation for the formation of the same conjugate from m-tyrosine. The finding that neither m-hydroxyphenyllactic acid-2-^{14}C nor m-hydroxymandelic acid-2-^{14}C undergo further reaction in vivo (p. 127) suggests that their proportions in the urine after administration of m-tyrosine represent the total formed from the amino acid.

Results of the studies on the metabolism of the amines can be seen in Table 18. The m-tyramine conjugate found after m-tyrosine injection is also present after administration of m-tyramine. Of the administered m-tyramine, 16% is converted to m-hydroxymandelic acid, but the logical intermediate m-octopamine was not detected. Although 9% of injected m-octopamine can be recovered unchanged from the urine, 91% is oxidized to m-hydroxymandelic acid. A small amount of m-octopamine which was produced from m-tyrosine may not be detectable due to its further metabolism.

ANALYSIS OF TISSUE METABOLITES OF DL-m-TYROSINE-2-^{14}C

Results from the analysis of the organs generally reflected the findings in the urine. After administration of 100 mg/kg of DL-m-tyrosine-2-^{14}C, 73% of the radioactivity in the kidney is neutral (presumably unchanged m-tyrosine) after 15 minutes. In

the urine collected over 24 hours, m-tyrosine accounts for no more than 20% of the total. The absence of unchanged amino acid in the urine after L-m-tyrosine administration suggests that much of the amino acid is metabolized in the kidney immediately before its excretion. This appears to be true particularly after the inhibition of dopa decarboxylase, when the kidney is the only organ to accumulate a non-neutral metabolite of DL-m-tyrosine-2-^{14}C. The appearance of the m-tyramine conjugate only in the kidney implies that the amine is conjugated there and likewise excreted immediately. However, the values obtained in Table 19 do not necessarily reflect the metabolic activity of the organs, but simply the nature and location of the radioactive metabolites.

THE IN VITRO METABOLISM OF m-HYDROXYPHENYL COMPOUNDS

In vitro studies were undertaken to confirm the existence of the reactions hypothesized in vivo from the urinary metabolites. These studies were also intended to identify the tissues which carried out the transformations, to complement the results obtained for the accumulation of radioactive metabolites in the tissues.

The values obtained in vitro (Table 20) suggest that a major route of metabolism of D-m-tyrosine is its oxidation to

m-hydroxyphenylpyruvic acid in the kidney. As seen in vivo, however, the keto acid is not the only product of D-m-tyrosine metabolism. Transamination of m-hydroxyphenylpyruvic acid to m-tyrosine (presumably the L-isomer) has also been demonstrated in vitro in all organs examined, accounting for the formation of the other products of D-m-tyrosine metabolism. Similar results were reported by Shindo and Maeda (1974) using D-dopa as the substrate. At a low concentration of D-dopa in vitro, dopamine was found as the major metabolite, whereas at higher concentrations dihydroxyphenylpyruvic acid was dominant, probably due to inhibition of dopa decarboxylase by the keto acid.

The transamination of L-m-tyrosine to m-hydroxyphenylpyruvic acid can also be demonstrated in all three organs, supporting the deduction from in vivo results that the transamination reaction is readily reversible.

Results from the experiments with liver, kidney, and brain homogenates showed that all three organs have the enzymes necessary to catalyse the transamination (Tong et al., 1973) and decarboxylation (Lovenberg et al., 1962) of L-m-tyrosine, the oxidation of D-m-tyrosine (Shindo and Maeda, 1974), the transamination of m-hydroxyphenylpyruvic acid (Pogrund et al., 1961), and the oxidative deamination of m-tyramine and m-octopamine

(Alles and Heegaard, 1943). While the conversion of m-tyramine to m-octopamine could not be detected in our crude homogenates in vitro, a possible pathway for the appearance of m-hydroxymandelic acid in rat urine is through the β-hydroxylation of m-tyramine to m-octopamine (Creveling et al., 1962) which is then acted upon by monoamine oxidase.

INCORPORATION OF RADIOACTIVITY FROM m-TYROSINE-^{14}C INTO PROTEIN

While m-tyrosine is not a substrate for tyrosyl ribonucleic acid synthetases in Escherichia coli and Bacillus subtilis (Calendar and Berg, 1966), the labelled amino acid is incorporated into bacterial peptides. The cyclization to an indole and condensation with serine are steps in the biosynthesis of gliotoxin in Trichoderma (Winstead and Suhadolnik, 1960). m-Tyrosine can be substituted for phenylalanine in certain Bacillus proteins. The substitution results in a reduced growth rate and other morphological changes (Aronson and Wermus, 1965). No data on higher organisms is available, although m-tyrosine is not considered as a normal constituent of proteins.

Radioactivity was recovered from a rat liver protein fraction after injection of L-m-tyrosine-2-^{14}C. Since incorporation of the radioactivity into protein may be preceded by metabolic transformation of the injected m-tyrosine, the

chemical nature of this radioactive material was also tested by its chromatographic behavior after liberation from the complex by hydrolysis.

Approximately 2% of the radioactivity from the initial liver supernatant was retained in the protein fraction throughout the washing procedure. Protein synthesis is necessary for this incorporation to occur. However, two types of chromatographic analysis after hydrolysis indicated that the radioactive material was no longer m-tyrosine-2-^{14}C; the quantity isolated was too small to permit its identification. The basicity of the compound and its stereospecific production from the L-isomer suggest a non-hydrolysable derivative of m-tyramine. It must be concluded that m-tyrosine is not chemically associated with protein as such, although a basic metabolite appears to be incorporated in newly synthesized protein.

CONVERSION OF m-TYROSINE-2-^{14}C INTO CATECHOLS

Although the percentage conversion of m-tyrosine to catechols is small, it has been possible to demonstrate and quantitate the formation of radioactive catechols after administration of m-tyrosine-2-^{14}C in tissues and urine, particularly when dopa decarboxylase is inhibited.

Recovery of dopa from urine: The results on the recovery of

urinary catechols are consistent with those of Sourkes et al. (1961b), supporting the theory of m-tyrosine to dopamine conversion and indicating that release of endogenous amines or effects on catechol metabolism is not the only source of excreted dopamine after m-tyrosine administration. In our experiments, even after inhibition of dopa decarboxylase, the contribution from endogenous dopa to that excreted in the urine was negligible since the specific radioactivities of injected m-tyrosine and the dopa isolated from urine were identical.

Accumulation of ^{14}C-catechols in tissues: The tissues in which ^{14}C-dopa accumulates have been determined. The appearance of radioactive catechols after the administration of DL-m-tyrosine-2-^{14}C is further evidence that m-tyrosine gives rise to catechols in vivo (Table 23). While p-hydroxylation of m-tyrosine will result in the formation of dopa and subsequent catechol metabolites, the possibility remains that hydroxylation of its metabolites such as m-tyramine and m-hydroxyphenylacetic acid may also contribute to the appearance of dopamine and dihydroxyphenylacetic acid (Axelrod, 1963).

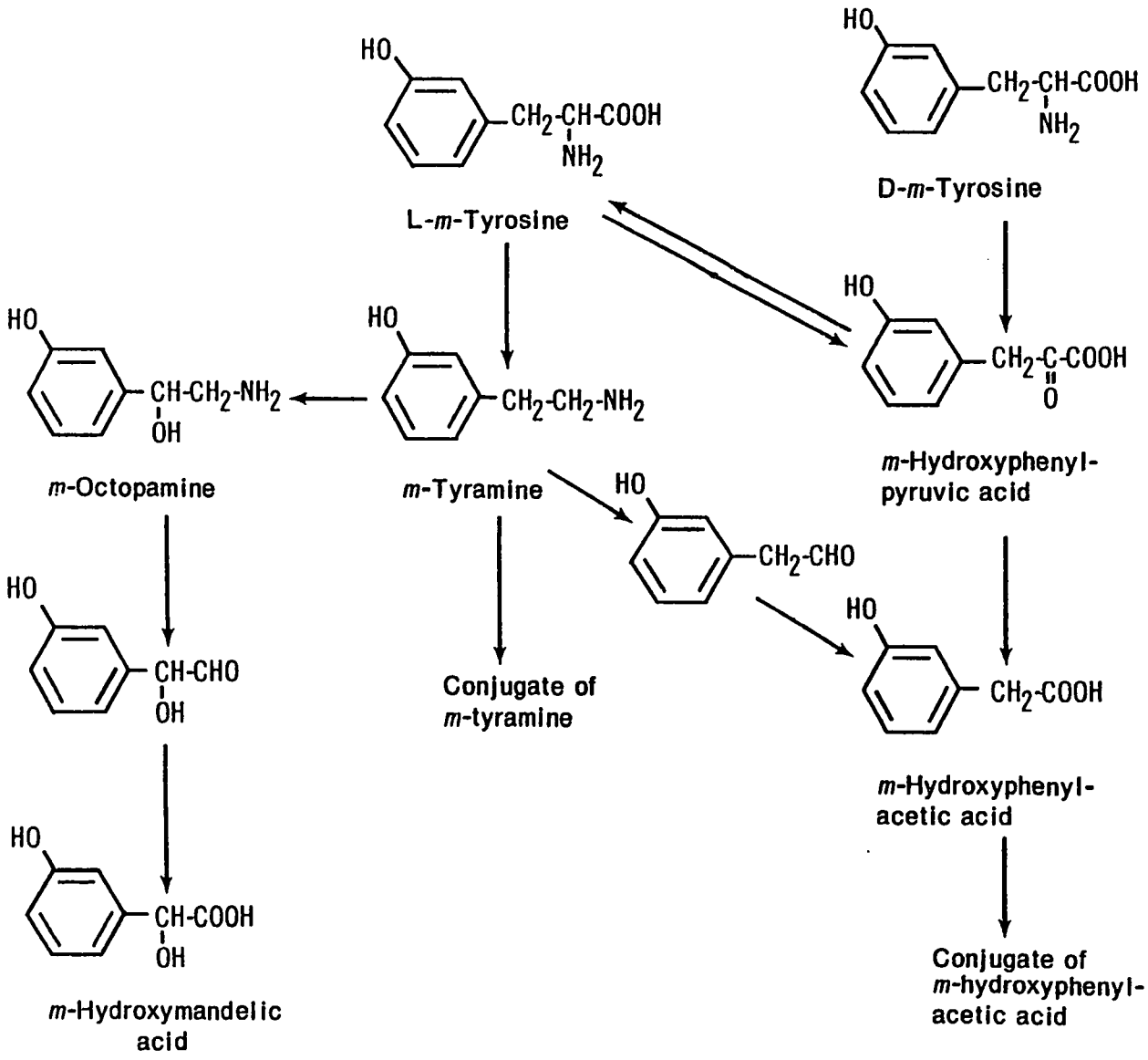
The fact that dopa accounted for most of the labelled catechols in animals pretreated with dopa decarboxylase inhibitor is not unexpected since decarboxylation is a major metabolic pathway for both dopa and m-tyrosine (Holtz et al., 1942; Pogrund

et al., 1961). The mechanism of the *in vivo* conversion of *m*-tyrosine to dopa was therefore investigated with animals pretreated with a dopa decarboxylase inhibitor, NSD-1034 (Levine and Sjoerdsma, 1964). The observed decline in the amount of labelled dopa 60 minutes after a single injection of DL-*m*-tyrosine-2-^{14}C (Figure 13) is probably due to excretion and the availability of substrate. Approximately 70% of the radioactivity administered initially was excreted within two hours. Results from the experiment using the enantiomers of *m*-tyrosine-2-^{14}C suggest that a stereospecific enzyme may be involved in the reaction. Studies on animals pretreated with *p*-chlorophenylalanine or α-methyltyrosine (Table 24) showed that phenylalanine hydroxylase could be the enzyme catalysing the hydroxylation of *m*-tyrosine *in vivo*, and this is further supported by the *in vitro* results (Tong et al., 1971a; Fisher and Kaufman, 1973; Table 21).

Our results provide the first unequivocal demonstration of the conversion of *m*-tyrosine to catechols *in vivo* and further data on various *in vitro* hydroxylations. Therefore, the physiological importance of these reactions and their contributions to the pharmacological actions of *m*-tyrosine can now be examined with confidence.

CONCLUSION

The above studies give some idea of the metabolism of m-tyrosine and its excretion in the urine, as seen in Figure 14. The pathways described are a satisfactory explanation for most of the observations, and the occurrence of these compounds in trace amounts in the normal animal can now be explained. It is now feasible to investigate the pharmacological importance of several of these metabolites after the in vivo synthesis and metabolism of m-hydroxyphenyl compounds.



MAJOR METABOLIC PATHWAYS OF *m*-TYROSINE IN THE RAT

SUMMARY

1. Hyperactivity can be induced in rats after the administration of L-m-tyrosine. Experiments with several other m-hydroxyphenyl compounds and after pretreatment with enzyme inhibitors suggest that the level of m-hydroxyphenyl amines in the brain is responsible.
2. Endogenous biogenic amine levels in the brain and peripheral tissues are lower after administration of m-tyrosine, the L-isomer being more potent than the D-isomer in effecting this depletion. Inhibition of the enzymes involved in the formation and metabolism of the m-hydroxyphenyl amines in the brain will change the pharmacological actions of m-tyrosine, in ways which suggest that these amines are the active agents.
3. Peripheral administration of hydroxyphenylethylamines has no significant effect on endogenous brain amine levels or behavior. However, these compounds have been shown to compete with each other in their association with brain homogenates in vitro. The amino acids do not compete in this fashion if decarboxylation is prevented with inhibitors. This further supports the hypothesis that only the amines can produce the observed effects directly, although they may be unable to

cross the blood-brain barrier.

4. Several radioactive (^{14}C) m-hydroxyphenyl compounds have been chemically synthesized, including m-tyramine and m-octopamine by novel routes. Some of the chromatographic and other properties of these compounds have been described.
5. The metabolism of several labelled m-hydroxyphenyl compounds in vivo was examined, and the identities and amounts of the major urinary products have been determined. The ratios of the m-tyrosine metabolites can be altered with changes in dosage, with the optical isomer used, or after pretreatment with enzyme inhibitors. The metabolism of the amino acid proceeds mainly through the decarboxylation or transamination of L-m-tyrosine-2-^{14}C and the oxidation of D-m-tyrosine-2-^{14}C.
6. The activities of several enzymes using m-tyrosine or its metabolites as substrates were determined in vitro. The demonstrated conversions could account for the urinary products observed. The distribution of m-tyrosine-2-^{14}C in rat organs, determined 15-60 minutes after injection, confirms that the substrate is available throughout this period.
7. A basic metabolite of m-tyrosine appears to become associated with the protein of rat liver. However, m-tyrosine itself is not incorporated into mammalian proteins.

8. Conversion of m-tyrosine to catechols *in vivo* has been demonstrated unequivocally with labelled precursor. The inhibition of selected enzymes indicates that phenylalanine hydroxylase may be responsible for this conversion.

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