THE BIOCHEMISTRY OF m-TYROSINE AND RELATED AROMATIC AMINO ACIDS

Thesis presented by

Jeffrey H.Y. Tong

to the

Division of Sciences

School of Graduate Studies

in partial fulfilment of the requirements

for the degree of Doctor of Philosophy.

Department of Biochemistry,

University of Ottawa.

June, 1972.

ACKNOWLEDGEMENTS

I wish to express my appreciation and gratitude to Professors N.L. BENOITON and A. D'IORIO for their guidance and advice throughout the course of this work.

I wish to extend my thanks to members of the laboratory for helpful discussions. I am also grateful to Mrs. R. Demayo for technical assistance, and Miss E. Chan for typing this manuscript.

ABSTRACT

A simple effective method for the resolution of ring-substituted phenylalanines was devised. The amino acids resolved include 3,4-dihydroxyphenylalanine (DOPA), tyrosine, m-hydroxyphenylalanine (m-tyrosine), o-hydroxyphenylalanine (o-tyrosine), p-chlorophenylalanine (p-CP), and p-fluorophenylalanine (p-FP). The resolution of the aromatic amino acids was effected by the asymmetric hydrolysis of their racemic ethyl esters with α-chymotrypsin at pH 5. The L-amino acids were obtained by crystallization from the concentrated reaction mixture, and the ethyl esters of the D-isomer were extracted with ethyl acetate followed by saponification or acid hydrolysis to give the D-amino acids. The optical purities of the isomers obtained by this method were greater than 99.5%, and the yields were generally above 60% for the L-isomers and about 60% for the D-isomers. Furthermore, the application of method to the large scale resolution of ring-substituted phenylalanines was tested with m-tyrosine. 70 g of L-m-tyrosine was prepared and no difficulties were encountered in the resolution method with respect to both yield and optical purity.

Studies on the hydroxylation of phenylalanine showed that m-tyrosine was formed along with tyrosine and DOPA when

L-phenylalanine-^{14}C was incubated with beef adrenal medulla homogenate in the presence of a pteridine co-factor and a DOPA decarboxylase inhibitor. The experimental data indicate that the conversion of phenylalanine to m-tyrosine was enzymic, and that tyrosine hydroxylase could be the enzyme responsible for the reaction. Incubation of L-m-tyrosine with rat liver or beef adrenal homogenates gave rise to a amino acid which was identified as DOPA on the basis of its fluorescence spectrum, chromatography, and the formation of 3-O-methyl-DOPA from L-m-tyrosine. Based on several properties of the reaction, amongst these the requirement for a pteridine co-factor, and the fact that m-tyrosine hydroxylating activity was present in partially purified preparations of tyrosine and phenylalanine hydroxylases, it is likely that the conversion of m-tyrosine to DOPA was effected by these two well known aromatic amino acid hydroxylases. These findings provide an explanation for the appearance of m-hydroxyphenyl compounds in human and animal urine and lead to the proposal of an alternative pathway for the biosynthesis of catecholamines from phenylalanine with m-tyrosine as an intermediate.

The effects in vivo of D- and L-p-CP on the hydroxylation of phenylalanine and m-tyrosine by rat liver were investigated. Injection of D- or L-p-CP into rats resulted

in identical decreases in the liver phenylalanine hydroxylase activity. The hepatic hydroxylating activity responsible for the conversion of L-m-tyrosine to DOPA was inhibited by p-CP in the same manner. No effects on the tyrosine hydroxylase activity of the brain and adrenals could be detected. The results further indicate that it is not necessary to invoke the presence of a new enzyme to account for the hydroxylation of m-tyrosine by rat liver. The significance of these findings in relation to the mechanism of action of p-CP is discussed.

The anti-reserpine action of DL-m-tyrosine was confirmed by using the optical isomers of m-tyrosine. L-m-tyrosine like L-DOPA can antagonize the sedative action of reserpine. D-m-tyrosine, and the D- or L-isomers of tyrosine or o-tyrosine had no awakening effect on reserpine-treated animals. The use of L-m-tyrosine as a therapeutic drug for the treatment of Parkinsonism is proposed.

As part of a study on the biochemistry of m-tyrosine, the transamination reaction of ring-substituted phenylalanines was investigated. m-Tyrosine, o-tyrosine, p-FP, and p-CP were tested as substrates for the cytosol and mitochondrial tyrosine aminotransferases with α-ketoglutarate, oxaloacetate and pyruvate as amino group acceptors. None of the above aromatic amino acids were found to be substrates for the cytosol enzyme.

On the other hand the mitochondrial enzyme was shown to be capable of transaminating m-tyrosine, p-CP, and p-FP with all three keto acids as amino group acceptors and at rates of 16-25% of that for tyrosine. A slow but definite transamination of L-o-tyrosine by the mitochondrial enzyme was demonstrated using labelled α-ketoglutarate as amino group acceptor.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS AND TRIVIAL NAMES

m-Tyrosine	m-Hydroxyphenylalanine
m-Tyramine	m-Hydroxyphenylethylamine
o-Tyrosine	o-Hydroxyphenylalanine
o-Tyramine	o-Hydroxyphenylethylamine
DOPA	3,4-Dihydroxyphenylalanine
DOPamine	3,4-Dihydroxyphenylethylamine
p-CP	p-Chlorophenylalanine
p-FP	p-Fluorophenylalanine
NADPH	Nicotinamide-adenine trinucleotide, reduced
$DMPH_4$	6,7-Methyl-5,6,7,8-tetrahydro-pterine·HCl·1½ H_2O
NSD-1055	3-Bromo-4-hydroxybenzyloxyamino phosphate
α-Ketoglutarate	2-Oxoglutarate
d.p.m.	Disintegrations per minute

INTRODUCTION

It is well established that the D- and L- forms of amino acids are not biologically equivalent, yet many investigators still utilize the DL forms because their optical isomers are not readily available. Although isolation from natural sources usually yields L-amino acid isomers, amino acids prepared by chemical methods are racemic. At the present time resolution of synthetic amino acids represents an important route for the preparation of L-isomers, and is particularly valuable for the preparation of D-isomers.

Ring-substituted phenylalanines have received increasing interest over the past ten years because of their biological, pharmacological and medical importance. For example 3,4-dihydroxyphenylalanine (DOPA), an intermediate in catecholamine biosynthesis, is now used for the treatment of a number of neurological diseases and in particular Parkinsonism (Cotzias et al., 1969) (Barbeau, 1969). L-m-Hydroxyphenylalanine (L-m-tyrosine) is gaining attention for the same reason (Sandler et al., 1971) (Benoiton et al., 1971). p-Fluorophenylalanine (p-FP), an anti-metabolite of phenylalanine and tyrosine, has been reported to inhibit tumor growth in mice when given with a phenylalanine deficient diet (Ryan and Elliot, 1968). p-Chlorophenylalanine (p-CP) is now well known as an inhibitor

of serotonin biosynthesis and as a possible drug for the treatment of malignant carcinoid syndrome (Sjoerdsma et al., 1970). However the optical isomers of these aromatic amino acids are not readily accessible, and relatively little is known about their biochemistry. This thesis describes the resolution of these aromatic amino acids and some of their biochemical properties.

The methods for the resolution of amino acids can be divided into two general categories, chemical and biological. An excellent review article on the resolution of amino acids was given by Greenstein (1954). The introduction here will be restricted to the discussion on the resolution of phenylalanine and its ring-substituted analogues.

CHEMICAL METHODS FOR RESOLUTION OF AMINO ACID RACEMATES

Selective crystallization: Selective crystallization of one optical isomer from a concentrated racemic solution was first observed by Pasteur (1852). Since then several observations of spontaneous crystallization of one isomer of a compound from a solution of the corresponding racemate have been recorded in the literature. L-tyrosine^{14}C-labelled was obtained by dilution of ^{14}C-labelled DL-tyrosine with an

excess of unlabelled L-tyrosine and by crystallization from water (Weinhouse and Millington, 1948). L-DOPA was separated from DL-DOPA in a similar manner (Brossi et al., 1970). An obvious drawback of this method is the danger of contamination of one isomer with its enantiomorph during crystallization.

Formation of diastereoisomeric salts: The resolution of racemic forms by conversion to diastereoisomers was first devised by Pasteur, and later Fischer applied the principle to the resolution of amino acids (Fischer, 1899). The separation is based on the differential solubilities of the diastereoisomeric salts of the racemate with optically-active bases or acids. The resolution method usually involves the use of amino acids acylated on the amino groups so as to cause them to be essentially acids and hence able to form salts with optically-active bases. After the separation of the diastereoisomeric salts, the base is regenerated and the acyl group is removed by hydrolysis to give the free amino acid.

D- and L-phenylalanine have been prepared from the resolution of N-carbobenzoxy-DL-phenylalanine with phenylethylamine (Overby and Ingersoll, 1960), N-formyl-DL-phenylalanine with brucine (du Vigneaud and Meyer, 1932), and N-acetyl-

DL-phenylalanine with α-fenchylamine (Overby and Ingersoll, 1951).

The preparation of D- and L-tyrosine by the resolution of the synthetic N-benzoyl and N-acetyl derivatives with brucine and cinchonine has been described (Fischer, 1900), (Sealock, 1946). Separation of D- and L-isomers of m-tyrosine has been achieved from the resolution of N-formyl-DL-m-tyrosine with brucine (Sealock et al., 1951).

D- and L-DOPA are available by the resolution of N-acetyl-(3,4-diacetoxyphenyl)-DL-alanine with cinchonin, followed by removal of the alkaloids and subsequent hydrolysis (Harington and Randall, 1931) (Vogler and Baumgartner, 1952). Recently the resolution of N-acetyl-(3,4-dimethoxyphenyl)-DL-alanine with optically-active organic bases has been reported for the preparation of L-DOPA (Berenyi et al., 1971) (Hever et al., 1970) (Nakamoto et al., 1971). D- and L-2,5-dihydroxyphenylalanine were obtained from the resolution of N-benzoyl-(2,5-dimethoxyphenyl)-DL-alanine, in a study to determine the origin of homogentisic acid (Neuberger, 1948).

There are a number of difficulties frequently encountered in the resolution of amino acids by the principle of diastereoisomerism. For successful resolution the procedure would require the resolving agent to form a relatively tightly bound salt with at least one of the two isomers of the racemate

and this salt must be quantitatively precipitable from the other diastereoisomer. However, this ideal condition was rarely met, and as in the case with selective crystallization there is always the danger of cross contamination of isomers. Thus after finding a suitable resolving agent, a tedious sequence of crystallizations and measurements on the degree of optical purity must be made to determine the best conditions for precipitation. Another disadvantage of this method is that it gives no clue to the optical configuration of the respective isomerides.

Chromatography on support with optical activity: The possibility that optical antipodes of a racemate might be separated on the surface of an optically-active solid material was first considered by Willstatter (1904). The separation of the D- and L-isomers of 2,3-, 2,4-, and 3,5-dihydroxyphenylalanine were noted on Whatman paper using a butanol-acetic acid-water mixture as solvent (Dalgliesh, 1952) (Lambooy,1954). However, no separation was observed for 3,4-dihydroxy-DL-phenylalanine. A column chromatography method for the resolution of DOPA has been described recently (Baczuk et al.,1971). The method was based on a "3 point attraction" theory between arginine and DOPA. The asymmetric support was prepared by bonding L-arginine through a cyanuric

chloride linkage to Sephadex G 25. DL-DOPA was resolved on the column by elution with water. Whether any of these methods might be of practical use remains to be seen.

BIOLOGICAL METHODS FOR RESOLUTION OF AMINO ACID RACEMATES

The essential problem of preparing one or the other optical isomer of the racemate by the use of biological method is that of specificity, both structural and optical.

Asymmetric oxidation or decarboxylation: This method stems from the classic observations by Pasteur on the preferential use by Penicillium of the dextro over the levo form of tartaric acid. A number of D-amino acids were prepared by the selective action of micro-organisms on the L-isomers of the corresponding racemates (Ehrlich, 1929) (Ehrlich and Wendel, 1908). It must be realized however that the D-isomers of certain amino acids could be metabolised just as well as their L-isomers (Bernheim et al., 1935) (Webster and Bernheim, 1936).

Stereospecific D- and L-amino acid oxidases from different sources have been used for the preparation of optically pure isomers by oxidation of the corresponding enantiomorph. D-Phenylalanine has been prepared by incubation of DL-phenylalanine with a L-amino acid oxidase of Proteus

vulgaris (Stumpf and Green, 1944). The D-amino acid oxidase of mammalian kidney and the L-amino acid oxidase of snake venom are powerful enzymes of precise optical specificity and have been employed for the preparation of the optical isomers of amino acids. Nevertheless it was observed that the pure L-isomers of tyrosine and tryptophan could not be obtained by the action of hog renal D-amino acid oxidase because of product inhibition (Parikh et al., 1958). Since asymmetric oxidation or decarboxylation leads to the preparation of only one of the two antipodes, it cannot be regarded as a resolution method in its accepted sense.

Asymmetric synthesis: Bergmann and his co-workers found that the proteolytic enzyme papain in the presence of N-carbobenzoxy-DL-amino acids and aniline catalyzed the synthesis of N-carbobenzoxy-L-amino acid anilides more rapidly than the corresponding D-anilides (Bergmann and Fraenkel-Conrat, 1937; 1938). The precipitation of the less soluble L-anilide from the incubation mixture leaving the N-acyl-D-amino acid in solution resulted in a method for the resolution of amino acid isomers. Isolation of the insoluble L-anilide followed by hydrolysis thus yielded the L-isomer of the free amino acid, and the D-isomer could be recovered from the filtrate. It was observed later that the acyl group has a marked influence on the optical course of the reaction. From a study

on the resolution of N-acyl-DL-phenylalanines by asymmetric synthesis of the hydrazides with papain, it was found that appreciable D-hydrazide was formed when the acyl group was carbobenzoxy but not when the acyl group was acetyl (Bennett and Niemann, 1950a).

Optical isomers of phenylalanine, tyrosine o-, m-, and p-fluorophenylalanine were obtained by asymmetric synthesis of L-anilides or L-hydrazides, from their racemic N-acetyl derivatives (Doherty and Popenoe, 1951) (Bennett and Niemann, 1950b). 3-Fluoro-DL-tyrosine was resolved by this method but in this case the acyl group used was the benzoyl group (Niemann and Rapport, 1946).

A condition for this method involves a compromise in solubilities, as the N-acyl-DL-amino acid must be soluble in the buffer mixture yet the N-acyl-L-amino acid anilide must be sparingly soluble if the reaction is to proceed to completion. The choice for the N-acyl group is also important as it could affect the optical course of the reaction. Another problem encountered in the resolution of amino acids by asymmetric synthesis with papain is that drastic conditions which may lead to racemization, are required for the hydrolysis of the isolated anilides.

Asymmetric hydrolysis: In this procedure a derivative

of the amino acid racemate is prepared and treated with an enzyme that selectively hydrolyzes the derivative of one enantiomorph.

Perhaps the most valuable and extensive studies on the resolution of amino acids have been carried out with the aid of amino acid acylases. When N-acylated amino acids are subjected to the hydrolytic action of renal acylase or pancreatic carboxypeptidase, only the L-isomer is attacked. The resulting free L-amino acid may be separated in a single step since the free L-amino acid is insoluble in nonaqueous solvents in which the acyl-D-amino acid is soluble. An alternative method for the separation of the free amino acid and its N-acyl derivative is the use of ion-exchange resins. Another advantage over the methods so far discussed except for asymmetric oxidation or decarboxylation is that in this method the course of the reaction can be followed by convenient analytical methods. The reaction is therefore complete when 50% of the racemic mixture has been hydrolyzed.

There are at least two acylases in the hog kidney referred to as acylase I, and acylase II. Acylase I refers to the fraction active toward the great majority of N-acylated aliphatic amino acids, and acylase II to the fraction active only toward N-acylated aspartic acid. Some 45 amino acids have been resolved into their isomerides through the action

of acylase I (or II) on their acyl derivatives (Greenstein, 1954). Recently L-m-tyrosine was obtained by resolution of N-phenylacetyl-(3-methoxyphenyl)-DL-alanine with an E. coli acylase, followed by hydrolysis with hydriodic acid in the presence of red phosphorus (Bamberg and Sjoberg, 1971).

In general asymmetric hydrolysis with acylase is not considered to be a suitable method for the resolution of aromatic amino acids. It was observed that hog kidney acylase was relatively unreactive toward the acetyl or chloroacetyl derivatives of aromatic amino acids (Fodor et al., 1949) (Birnbaum et al., 1952). A better method for the resolution of phenylalanine and tyrosine with a purified beef pancreas carboxypeptidase was described by Gilbert et al. (1949). They reported a 30% yield for L-phenylalanine and L-tyrosine and a 25% yield for their D-isomers. This resolution method of aromatic amino acids was employed in the preparation of L-p-fluorophenylalanine-1-^{14}C (Loftfield and Eigner, 1966).

Resolution by asymmetric hydrolysis of amino acid esters with pancreas esterase has been used by a number of workers. A number of racemic amino acid esters have been resolved using crude preparations of pancreas and also with chymotrypsin.

Warburg (1906) demonstrated that a lipase-free pancreatin preparation acted rapidly and asymmetrically on

DL-leucine propyl ester to yield L-leucine. The method was extended to the resolution of DL-tyrosine by the action of a crude pancreas secretion on DL-tyrosine ethyl ester (Abderhalden et al., 1923). Pancreas powder was used to resolve DL-phenylalanine by its hydrolytic action on the DL-phenylalanine isopropyl ester (Wretlind, 1950). DL-tryptophan has been resolved by the action of crystalline chymotrypsin on DL-tryptophan methyl ester (Brenner et al., 1948).

The resolution method by asymmetric hydrolysis with esterases was considered to have limited value for three reasons: (a) possible spontaneous hydrolysis of esters under the conditions employed (Wretlind, 1950), (b) because some of the amino acid esters tend to polymerize and form higher peptide esters in the presences of chymotrypsin (Brenner et. al., 1950) and (c) replacement of an α-acetamido group by an amino group would lead to partial loss in stereospecificity of chymotrypsin (Hein and Neimann, 1961).

Recently the metabolism of ^{14}C-labelled D- and L-o-tyrosine in rats has been studied in our laboratory. The resolution of DL-o-tyrosine was accomplished by the use of stereospecific hydrolytic action of α-chymotrypsin on the racemic amino acid ethyl ester at pH 5 (Petitclerc et al., 1969a; 1969b). Also a kinetic study of the interaction of

α-chymotrypsin with phenylalanine derivatives containing a free α-amino group showed no enzymatic hydrolysis of D-phenylalanine ethyl ester or D-phenylalanine p-nitrobenzyl ester at pH 6 or 6.5 (Purdie and Benoiton, 1970). The simplicity and other attractive features of this method have prompted us to apply it to the resolution of ring-substituted phenylalanines.

BIOCHEMISTRY OF SOME RING-SUBSTITUTED PHENYLALANINES

(o-tyrosine; m-tyrosine; p-fluorophenyalanine (p-FP); p-chlorophenylalanine (p-CP))

While a great deal has been learned about the biochemistry of phenylalanine, relatively little is known about its ring-substituted analogues except tyrosine and DOPA.

The enzymatic decarboxylation of a number of ring-substituted phenylalanines has been studied. It was found that 2,3-dihydroxyphenylalanine, 2,5-dihydroxyphenylalanine, m-tyrosine and o-tyrosine could be decarboxylated by extracts of mammalian kidney and liver (Blaschko et al., 1949) (Blaschko and Holton, 1950). That the decarboxylation of these aromatic amino acids was catalyzed by a single enzyme, DOPA decarboxylase, was later confirmed by Awapara et al. (1962). o-, m-, and p-Chlorophenylalanines however were not substrates for the mammalian or bacterial decarboxylases (Blaschko and Stiven, 1950). All these aromatic amino acids were found to be substrates of D-amino acid oxidase of hog kidney and L-amino acid oxidase of snake venom (Blaschko et. al., 1949) (Blaschko and Stiven, 1950).

The appearance of o- and m-hydroxyphenyl acids and amines in human urine have been constantly reported in the literature. m-Hydroxyhippuric acid, m-hydroxyphenylacetic

acid, m-hydroxybenzoic acid and m-hydroxyphenyl-propionic acid were detected in human urine (Armstrong et al., 1956a; 1956b) (Boyland et al., 1953). Abnormality in the excretion of m-hydroxyphenylacetic acid has been reported in patients of phenylketonuria (Boscott and Bickel, 1953) (Boscott and Kirman, 1955). o-Hydroxyphenylacetic acid in urines of normal and phenylketonuric patients was also observed (Armstrong et al., 1955) (Armstrong and Shaw, 1955). Normal human urine contains a very small amount of m-hydroxyphenylethylamine (m-tyramine) (Jepson et al., 1960) (Perry et al., 1962), and o-hydroxyphenylethylamine (o-tyramine) (Nishimura and Gjessing, 1966) (Jepson et al., 1960).

It has been suggested that m-tyramine may be of endogenous origin (Perry et al., 1966). The exact sequence of reactions leading to these o- and m-hydroxyphenyl compounds is not known. There are two theories concerning the origin of m-hydroxyphenylacetic acid : (a) a dehydroxylation reaction of 3,4-dihydroxyphenylacetic acid, a metabolite of DOPA, by the action of intestinal micro-organisms (Booth and Williams, 1963) (Sandler et al., 1969), and (b) a hydroxylation reaction of phenylalanine to form m-tyrosine by liver phenylalanine hydroxylase (Coulson et al., 1968). On the other hand, the appearance of o-hydroxyphenylacetic acid was attributed to the conversion of phenylalanine

to o-tyrosine (Fellman and Devlin, 1958), or the conversion of phenylpyruvate to o-hydroxyphenylacetate by the liver (Taniguchi and Armstrong, 1963).

The possibility that m-tyrosine may give rise to catecholamines was first put forth by Sourkes et al., (1961). On the other hand m-tyrosine has been rejected as an intermediate in the formation of DOPA (Ikeda et al., 1967) partly on the basis that m-tyrosine was reported not to be a substrate for the tyrosine hydroxylase (Nagatsu et al., 1964) or phenylalanine hydroxylase (Kaufman, 1962a). However, relating a few separate experiments m-tyrosine could indeed serve as a precursor for catecholamines via a rather obscure metabolic pathway. m-Tyrosine was decarboxylated to form m-tyramine (Blaschko et al., 1949) which could give rise to m-octopamine by the action of DOPamine β-hydroxylase (Creveling et al., 1962), m-octopamine could in turn give rise to norepinephrine by the action of a microsomal nonspecific hydroxylase (Axelrod, 1965). Also it has been shown that a cell-free preparation from Bacillus cereus and Bacillus thuringiensis could carry out the conversion of m-tyrosine to DOPA (Aronson and Vickers, 1965).

The hydroxylation of some halogenated phenylalanines has been described. p-FP can be converted to tyrosine

(Kaufman, 1961b), p-CP to m-chlorotyrosine, and p-bromophenylalanine to m-bromotyrosine (Guroff et al., 1966) upon incubation with phenylalanine hydroxylase.

There are a number of similarities between the pharmacological actions of L-DOPA and m-tyrosine which deserve mentioning. Like L-DOPA, m-tyrosine can overcome a neurological syndrome resembling Parkinsonism produced by treatment with reserpine in mice (Blaschko and Chrusciel. 1960) (Carlsson et al., 1957b). This pharmacological action of L-DOPA and m-tyrosine is thought to be mediated by 3,4-dihydroxyphenylethylamine (DOPamine) or m-tyramine (Blaschko and Chrusciel, 1960). However, the exact biochemical mechanism of action of L-DOPA and m-tyrosine is still unknown. That DOPamine might be capable of exerting a direct pharmacological action instead of merely playing the part of an intermediate in the synthetic pathway of norepinephrine has been suggested (Carlsson, 1959) (Everett and Wiegand, 1962) and may also be true for m-tyramine. A central hypotensive effect for both m-tyrosine and L-DOPA has been described. When rats were pretreated with a peripheral DOPA decarboxylase inhibitor MK 485 (DL-α-hydrazino-α-methyl-β-(3,4-dihydroxyphenyl)-propionic acid), m-tyrosine caused a lowering of blood pressure (Rubenson, 1971).

Halogenated phenylalanines have been studied with particular interest to find specific inhibitors for (a) tyrosine hydroxylase as one approach to anti-hypertensive drugs (Counsell et al., 1970) (McGeer and McGeer, 1967), (b) phenylalanine hydroxylase for the study of experimental phenylketonuria (DeGraw et al., 1967) and (c) tryptophan hydroxylase in order to elucidate the cerebral function of serotonin (Jequier et al., 1969) (McGeer and Peters, 1969). The most rewarding discovery so far came from Koe and Weissman (1966) when they described a strong, long-lasting depletion of serotonin from a variety of animal tissues with p-CP. Later it was found that this depleting action involves a decrease in the activity of tryptophan hydroxylase (Jequier et al., 1967), differing from the mechanism of action of reserpine (Pletscher et al., 1964a), and p-chloromethamphetamine (Pletscher et al., 1964b). The pharmacological and behavioral effects of p-CP on learning (Stevens et al., 1967), alcohol consumption (Myers and Veale, 1968), sleep pattern (Weitzman et al., 1968), and sexual behaviour (Ferguson et al., 1970) (Tagliamonte et al., 1969) were assumed to be mediated through its effect on brain serotonin. Moreover, p-CP decreases the hepatic phenylalanine hydroxylase activity (Koe, 1967) (Guroff, 1969). Gal and Millard (1971) demonstrated the incorporation of p-CP into proteins and they suggested that

the inactivation of phenylalanine and tryptophan hydroxylases may be due to the incorporation of p-CP at an enzymatic site essential for their activity. Recently it was reported that p-CP did not inactivate tryptophan hydroxylase in rat pineal, although it lowered the serotonin level in the pineal gland (Deguchi and Barchas, 1972). This raises the question whether the pineal tryptophan hydroxylase is different from the cerebral tryptophan hydroxylase. With the knowledge that p-CP is an effective inhibitor of 5-hydroxyindole synthesis, Engelman et al. (1967) studied its therapeutic value on the treatment of malignant carcinoid syndrome, a disease usually associated with a metastatic 5-hydroxyindole producing tumor, and reported an excellent effect on the diarrhea, a clinical characteristic of the disease.

The transamination of ring-substituted phenylalanines has been suggested to account for certain biological effects of their keto acid analogues. The pressor responses in the cat and rat caused by m-hydroxyphenylpyruvic acid was attributed to a transamination to m-tyrosine followed by decarboxylation to m-tyramine (Pogrund et al., 1961). A transamination reaction was also postulated as an essential step for the conversion of D-o-tyrosine to L-o-tyrosine (Petitclerc et al., 1969b). However neither m-tyrosine nor o-tyrosine were found

to be substrates for the purified hepatic tyrosine aminotransferase (Canellakis and Cohen, 1956a) (Jacoby and La Du, 1964). The inhibitory effect of p-chlorophenylpyruvic acid on tryptophan hydroxylase may also be due to a transamination to p-CP (Koe and Weissman, 1966) (Gal et al., 1970). The in vitro transamination of p-CP has been demonstrated with a partially purified rat brain aromatic amino acid transaminase (Gal et al., 1970) and with a purified rat liver mitochondrial tyrosine aminotransaminase (Miller and Litwack, 1971). The transamination reaction will be expected to become more important when a large concentration of an aromatic amino acid is present, for example when used as a chemotherapeutic drug, since this pathway competes with the amine synthesizing system or the incorporation of the amino acid into proteins.

The very interesting biological properties of the above aromatic amino acids, the lack of knowledge in their biochemistry and the availability of their optically pure isomers prompted us to do biochemical investigations along the following lines:

(1) The origin of m-hydroxyphenyl compounds

(2) The hydroxylation of m-tyrosine

(3) The transamination of ring-substituted phenylalanines.

The following is an account on the properties of phenylalanine hydroxylase, tyrosine hydroxylase, and tyrosine aminotransferase, the three best known enzymes responsible for the hydroxylation and transamination of aromatic amino acids.

Phenylalanine Hydroxylase (EC 1.14.3.1): The conversion of the aromatic amino acid phenylalanine to tyrosine is one of the best studied aerobic hydroxylation reactions. The reaction is believed to be first of all an obligatory step in the catabolism of phenylalanine to CO_2 and water, and secondly it provides an endogenous source for the aromatic amino acid tyrosine and indirectly for such tyrosine derived metabolites as melanin, norepinephrine and epinephrine.

The conversion of phenylalanine to tyrosine was postulated as early as 1901 by Neubauer (Neubauer, 1909). The first in vitro demonstration of the enzymatic conversion of phenylalanine to tyrosine was reported in 1913 (Embden and Baldes, 1913) in liver perfused with phenylalanine. Evidence for this conversion in vivo was first demonstrated by Moss and Schoenheimer in 1940 in rats by isolation of deuterium-labelled tyrosine after similarly labelled phenylalanine had been given.

Phenylalanine hydroxylase catalyzes the hydroxylation of a variety of aromatic amino acids (Kaufman, 1962a). The enzyme has been detected in the livers of all animals.

Extracts of mouse kidney and pancreas are reported to have comparable activity to that of mouse liver but no activity could be found in any other tissue or organ (Tourian et al., 1969).

The characterization of the phenylalanine hydroxylating system began when Udenfriend and Cooper (1952) reported that a soluble extract from rat liver could catalyze the hydroxylation of phenylalanine to tyrosine in the presence of NAD^+, oxygen and an aldehyde or alcohol. Further studies showed that the hydroxylating system was a complex one, involving two proteins, a pyridine-nucleotide and oxygen (Mitoma, 1956) (Kaufman, 1957). Sheep liver extract was found to be an excellent source for one of the proteins. Although lacking the overall activity, the sheep liver extract could stimulate the semi-purified preparation from rat liver. The two proteins were referred to as the "rat-liver" and "sheep-liver" enzymes (Kaufman, 1957).

Subsequent purification studies showed that a non-protein co-factor from a boiled extract of rat liver which was not NADPH was necessary for the hydroxylation reaction (Kaufman, 1958a). The co-factor was extensively purified from the boiled rat liver extracts and chemical evidence suggested the active compound might be a pteridine (Kaufman, 1958a; 1958b). Tetrahydrofolate was the first compound of this

class to show co-factor activity. However, several synthetic tetrahydropterins (Pterin is the trivial name for 2-amino-4-hydroxypteridine) e.g. 6-methyl- and 6,7-dimethyltetrahydropterin were found to be considerably more active than tetrahydrofolate (Kaufman and Levenberg, 1959). These results suggested that the natural co-factor might be tetrahydropterin without a p-aminobenzoic acid side-chain. Also, it was found that in the presence of the 6,7-dimethyltetrahydropterin, NADPH and the "sheep-liver" enzyme were no longer necessary for the hydroxylation of phenylalanine, although they did stimulate the reaction (Kaufman, 1962b). Thus it was clear that the "rat-liver" enzyme was the hydroxylase and that NADPH and the "sheep-liver" enzyme catalyzed the reduction of an unstable oxidized pterin intermediate back to the active tetrahydro level. The oxidized pterin intermediate was identified as the quinonoid dihydropterin, and the "sheep-liver" enzyme was referred to as the dihydropterin reductase (Kaufman, 1961a) (Kaufman, 1964a).

The natural co-factor isolated from rat liver turned out to be 7,8-dihydrobiopterin which must be reduced to the tetrahydro form for co-factor function by the dihydrofolate reductase (Kaufman, 1963; 1967), which was responsible for the characteristic lag period of the hydroxylation of phenylalanine

in the presence of the natural co-factor. Recently a new protein factor called phenylalanine hydroxylase stimulator (PHS) was isolated from rat-liver extracts (Kaufman, 1970). It stimulates the rate of the hydroxylation reaction in the presence of the natural co-factor. The mechanism for this stimulation is still not fully understood. Kaufman favored the idea that PHS prevents the concentration dependent association of the hydroxylase to form(s) having low catalytic activity. Kaufman and Fisher (1970) purified the rat liver phenylalanine hydroxylase 400-fold and suggested that the enzyme consists of two isozymes which differ in charge but not molecular weight, and that each of the isozymes is capable of existing as a monomer (mol. wt. = 51000-55000), a dimer, and a tetramer. A model has been proposed for the relationship between the different forms of phenylalanine hydroxylase (Kaufman, 1970). According to this hypothesis there is an equilibrium set up between the dimer and the tetramer with the dimeric form having a higher specific activity.

The mechanism of action of phenylalanine hydroxylase is still not fully understood. Whether the enzyme is a metalloprotein is still not clear at this stage. Available evidence indicates that the reaction proceeds through a quaternary complex involving enzyme, phenylalanine, oxygen, and

tetrahydropterin (Storm and Kaufman, 1968). A possible cationoid intermediate of phenylalanine has been postulated (Kaufman, 1961b) (Guroff et al., 1967).

Tyrosine Hydroxylase (EC 1.14.3.a): Tyrosine hydroxylase catalyzes the tetrahydropterin dependent conversion of tyrosine to DOPA, the first step in the sequence of reactions leading to norepinephrine synthesis in the adrenal medulla (Hagen, 1956) (Goodall and Kirshner, 1957). The reaction is believed to be the rate limiting step in the biosynthesis of catecholamines (Levitt et al., 1965) (Udenfriend, 1966). Thus this enzyme is probably the most important enzyme for the regulation of catecholamine biosynthesis, and yet relatively little is known about this enzyme.

A specific L-tyrosine hydroxylase from adrenal medulla and brain different from tyrosinase was first demonstrated with a cell-free system by Nagatsu et al., (1964). The enzyme showed the requirement for a tetrahydropteridine, Fe^{2+}, and oxygen. The pH optimum of bovine medulla tyrosine hydroxylase is 6.0 compared to 6.8 of the liver phenylalanine hydroxylase. The in vivo synthesis of DOPA from tyrosine in the adrenal gland was demonstrated by Hempel and Mannl (1966). The participation of tetrahydropterin as a co-factor in the tyrosine hydroxylating system is thought to be the same as in the phenyl-

alanine hydroxylating system (Brenneman and Kaufman, 1964) (Kaufman, 1964b). Recently the natural pterin co-factor from bovine adrenal medulla has been isolated and characterized. The results show that it is similar to dihydrobiopterin, the natural co-factor for phenylalanine hydroxylase in liver (Lloyd and Weiner, 1971).

Important as it is to the understanding of the regulation of catecholamine synthesis, reports on the subcellular distribution pattern of tyrosine hydroxylase have been rather conflicting. It was reported that the enzyme activity was found in the particulate fraction and that the enzymatic activity associated with the 100,000 x g supernatant fraction was considered to be due to solubilization by prolonged homogenization (Nagatsu et al., 1964) (Petrack et al., 1968). Contrary to this, there has been evidence indicating that the enzyme is localized in the soluble fraction (Laduron and Belpaire, 1968) (Musacchio, 1967; 1968). The matter was reinvestigated by Wurzburger and Musacchio (1971). They concluded that the enzyme is soluble and that "particle-bound" tyrosine hydroxylase could be due to adsorption of tyrosine hydroxylase to coarse particles during homogenization and that this tendency is exaggerated in sucrose solution.

The "particle bound" tyrosine hydroxylase has been partially purified using tryptic (Petrack et al., 1971) or chymotryptic (Shiman et al., 1971) digestion as a solubilization step. This solubilized enzyme was found to have similar properties to the supernatant enzyme (Petrack et al., 1968). However, whether the solubilized "particle bound" enzyme by method of chymotryptic or tryptic digestion truly represents the native tyrosine hydroxylase is still not clear. It was suggested that the trypsin-treated tyrosine hydroxylase could be only a fragment of the native enzyme (Musacchio et al., 1971).

The stimulation of tyrosine hydroxylase activity by iron salts has been reported constantly even though the mechanism of this stimulation is not known (Nagatsu et al., 1964) (Petrack et al., 1968). It has been suggested that this is due to the ability of ferrous ions to decompose a peroxide-mediated inactivation of the tetrahydropterin co-factor and tyrosine hydroxylase, and that this stimulation could be replaced by the enzyme catalase. The source of this peroxide formation could be generated from the non-enzymatic oxidation of tetrahydropterins by oxygen (Shiman et al., 1971).

An interesting property of tyrosine hydroxylase is its ability to catalyze the hydroxylation of phenylalanine to

tyrosine (Ikeda et al., 1965; 1967). The hydroxylation of 4-tritio-phenylalanine by phenylalanine or tyrosine hydroxylase showed the same characteristic migration of tritium to the 3 position (Daly et al., 1968). Moreover phenylalanine and tyrosine were hydroxylated at comparable rates in the presence of the natural co-factor (Shiman et al., 1971). The conversion of phenylalanine to tyrosine by tyrosine hydroxylase indicates that liver is not the sole source for tyrosine production.

Tyrosine Aminotransferase (EC 2.6.1.5): Tyrosine aminotransferase (L-tyrosine: 2-oxoglutarate aminotransferase, EC 2.6.1.5) catalyzes the reversible transamination of tyrosine with α-ketoglutarate, the first step in the major catabolic pathway for tyrosine. Tyrosine aminotransferase was first purified and characterized by Canellakis and Cohen (1956a; 1956b) from a soluble extract of dog liver. Apart from the liver, tyrosine aminotransferase activity was detected in the kidney, heart, (Wurtman and Larin, 1968) adrenals, brain (Lin and Knox, 1958), and thyroid (Rivlin et al., 1962).

It was found that hepatic tyrosine aminotransferase catalyzes the transamination of a number of aromatic amino acids including phenylalanine, tryptophan, DOPA and 3-iodo-

tyrosine (Jacoby and La Du, 1962; 1964). α-ketoglutarate appears to be the only keto acid which accepts the amino group of tyrosine. The enzyme has a pH optimum of 7.6. Hepatic tyrosine aminotransferase, an inducible enzyme, can be affected by a number of hormones : glucocorticoids (Kenney, 1962), glucagon and insulin (Holten and Kenney, 1967), cyclic 3',5'-AMP (Wicks et al., 1969), and indole amines (Deguchi and Barchas, 1971). The exact mechanism responsible for the stimulation of tyrosine aminotransferase activity is controversial and has been the subject of intensive study (Kenney, 1962), (Ray et al., 1964), (Tomkins et al., 1969). The enzyme exhibits a diurnal rhythm which could possibly result from hormonal, neural and metabolic influences (Wurtman and Axelrod, 1967).

Studies on a 2500-fold purified tyrosine aminotransferase from liver showed that the enzyme has a molecular weight of about 115,000 and binds four moles of pyridoxal-5'-phosphate per mole of protein (Valeriote et al., 1969). Kinetic studies indicate a Ping Pong mechanism for tyrosine aminotransferase similar to that found with other aminotransferases, but with the difference that the pyridoxal phosphate co-enzyme can dissociate freely from the enzyme (Litwack and Cleland, 1968).

Recently there have been indications that isoenzymic forms of hepatic tyrosine aminotransferase exist. By comparing the transamination of tyrosine and 3-iodo-tyrosine before and after induction with cortisol, Miller and Litwack (1969a) suggested that there could be different forms of the cytosol tyrosine aminotransferase. Disc gel electrophoresis of liver tyrosine aminotransferase from normal and flumethasone-(a synthetic glucocorticoid) treated rats showed very different electrophoretic patterns. Induction with flumethasone showed five bands of enzymic activities where only two were detected before treatment (Blake and Broner, 1970). Another report indicated that four forms of the hepatic tyrosine aminotransferase having different stimulation responses to hormones could be identified by means of hydroxylapatite chromatography (Iwasaki and Pitot, 1971). The possible regulatory role of these isoenzymic forms of tyrosine aminotransferase on the metabolism of tyrosine still needs clarification.

Apart from the tyrosine aminotransferase found in the soluble fraction there is yet another enzyme associated with tyrosine transamination activity found in the mitochondrial fraction. This enzyme is referred to as mitochondrial tyrosine aminotransferase. The liver mitochondrial tyrosine

aminotransferase has been extensively purified. (Miller and Litwack, 1971). It has a molecular weight of 90,000-114,000. Substrate specificity and inhibitor studies on the purified enzyme indicated that it is identical with the mitochondrial aspartate aminotransferase. Unlike the cytosol enzyme the mitochondrial enzyme can utilize oxaloacetate and pyruvate as amino group acceptors besides α-ketoglutarate (Miller and Litwack, 1969b). Although the liver mitochondrial enzyme can be induced by cortisol the stimulation however is small compared with the cytosol enzyme (Fellman et al., 1969). The tyrosine aminotransferase of the brain was reported to be different from that of the liver as it did not exhibit diurnal variations and was not affected by hormones or stress (Fuller, 1970). This could be explained on the basis that most of the tyrosine transamination activity in the brain is associated with the mitochondrial fraction (Miller and Litwack, 1969b) (Semba and Civen, 1970) (Mark et al., 1970).

Whether the transamination of aromatic amino acids is catalyzed by separate enzymes or by one enzyme exhibiting group specificity with a preference toward tyrosine is still not clear. It has been suggested that there might be three separate aromatic aminotransferases in the rat brain (Fonnum et al., 1964).

The significance of the mitochondrial tyrosine aminotransferase is still uncertain. It may make use of various amino acids including tyrosine to regulate keto acids such as pyruvate, oxaloacetate and α-ketoglutarate in the liver. On the other hand it could aid in the conservation of aromatic amino acids in tissues such as the brain and thus permitting aromatic keto-acids to act as precursors of the corresponding biological amines.

SECTION I

A: THE RESOLUTION OF RING-SUBSTITUTED PHENYLALANINE

INTRODUCTION

Parkinsonism, a neurological disease characterized by akinesia, rigidity, tremor and a low brain DOPamine level is thought to be caused by the degeneration of the Substantia nigra (Sourkes and Poirier, 1966). The discovery that L-DOPA is an effective chemotherapeutic drug for the disease stimulated a search for methods of obtaining this compound from natural sources (Enei et al., 1969) (Sih et al., 1969) (Wysong, 1966) (Daxenbichler, 1971), since no satisfactory resolution method was available for this amino acid. Existing methods for preparing optically active ring-substituted phenylalanines are often unsatisfactory especially when both forms are required in high purity and good yield. The demonstration in our laboratory that o-tyrosine could be resolved by the action of α-chymotrypsin led us to apply this method to the resolution of DOPA and other ring-substituted phenylalanines of biological interest which are not readily accessible, namely o-tyrosine, m-tyrosine, p-fluorophenylalanine, and p-chlorophenylalanine.

MATERIALS AND METHODS

Materials: DL-o-tyrosine and 3,4-dihydroxy-DL-phenylalanine (DL-DOPA) were purchased from Mann Research Laboratories, Orangeburg, N.Y.; DL-m-tyrosine, from Koch-Light Laboratories, Colnbrook, U.K.; DL-tyrosine, from General Biochemicals, Chagrin Falls, Ohio; and p-chloro-DL-phenylalanine (DL-p-CP) and p-fluoro-DL-phenylalanine (DL-p-FP) from Pierce Chemical Co., Rockford, Ill. α-Chymotrypsin was the Worthington CDI product obtained from Winley-Morris Co., Montreal, Que. It possessed an activity of 53 units/mg. Twice crystallized L-amino acid oxidase from Crotalus adamanteus venom was obtained from Sigma Biochemical Co. The aminex A-5 resin (control No. 5028) was obtained from Bio Rad Laboratories, Richmond, Calif. The automatic titrating system was a Radiometer pH-stat type ABUIa/TTTlla/PHM 28b. Amino acids were determined with a Beckman model 120B amino acid analyzer. Optical rotations were measured with a Perkin Elmer model 141 polarimeter using a 1-dm tube.

Resolution of p-substituted DL-phenylalanines (Tyrosine, p-FP, and p-CP): Hydrogen chloride (anhydrous) was bubbled through a suspension of 0.5 g of the DL-amino acid in 30-50 ml of absolute ethanol until a clear solution was obtained. The

solution was placed in an ice-bath and introduction of HCl continued for 10-15 min. The solution was then left at room temperature protected with a drying tube (Drierite). Next day, the solvent was evaporated off under vacuum, and the whole operation was repeated. Excess HCl was then removed by repeating the evaporation three times after the addition of ethanol (50 ml). The residue was dissolved in 15-20 ml of water, the pH of the solution was adjusted to 5.0 with 0.2 M LiOH. α-Chymotrypsin (Table 1) was added and the mixture was incubated at room temperature for 30-90 min (Table 1), the pH being kept constant by the automatic addition of 0.2 M LiOH from the titrator. After the digestion, the mixture was concentrated until crystals appeared, cooled for 1 h, filtered, and the precipitate was washed with ethanol. The L-isomer so obtained was recrystallized by dissolving in hot water containing 2 ml of N HCl, filtering the solution through Celite, and neutralizing to pH 5 with N LiOH.

The filtrate (containing the D-amino acid ethyl ester and a small amount of free L-amino acid) obtained after removal of the L-isomer was brought to pH 9.0 with 0.2 M LiOH, saturated with NaCl, and extracted with ethyl acetate (3 x 50 ml). The combined extracts were dried over $MgSO_4$ and filtered into ethyl acetate containing hydrogen chloride.

The solvent was removed by evaporation. The residue was dissolved in 0.2 M LiOH (20 ml) to pH 12 and the solution was kept at 45°C for 1 h. The solution was then adjusted to pH 5.0 with N HCl and evaporated to dryness. The residue was triturated in hot ethanol. After cooling at 4°C for several hours, the mixture was filtered to give the D-isomer.

Resolution of DL-m-tyrosine: This was carried out essentially as described above. The re-esterification step in this case was found not to be necessary. After the chymotryptic digestion, the reaction mixture was evaporated to dryness. To the residue was added 50 ml of ethanol and the mixture was kept at 4°C for 1 h. The L-m-tyrosine was filtered off, washed with ethanol, and recrystallized as described for the L-isomers of p-substituted phenylalanines. The ethanol filtrate, containing the D-m-tyrosine ethyl ester with a small amount of L-m-tyrosine, was evaporated to dryness and the residue was dissolved in 20 ml water. The D-m-tyrosine was obtained as described above; extraction of the D-ester at pH 9, saponification at pH 12, and isolation of the D-amino acid at pH 5.

Resolution of DL-o-tyrosine: This was carried out as for the m-tyrosine isomers except that the esterification step was repeated as for the p-substituted derivatives. In

addition, after saponification of the D-amino acid ester, the L-isomer present due to the incomplete enzymatic reaction was destroyed by digestion with L-amino acid oxidase. For this purpose, 5 ml of N HCl and 3 ml of 10% NaH_2PO_4 were added to bring the pH to 8.0 and the mixture was incubated at 37°C for 24 h, with oxygen being bubbled through, in the presence of 100 mg of Crotalus adamanteus L-amino acid oxidase. Dowex 50 (H^+) (≈120 ml) was added, the suspension was stirred for 20 min and filtered, the resin was washed with water, and the amino acid then eluted from the resin with 500 ml of 3 N NH_4OH. The eluate was evaporated to dryness several times after the addition of water, and the residue was collected with the help of ethanol.

Resolution of DL-DOPA: DL-DOPA (4 g) was suspended in absolute ethanol (240 ml) and hydrogen chloride was bubbled through for 20 min. Next day, the clear solution was evaporated to dryness, and the evaporation was repeated after the addition of ethanol (3 x 50 ml). The residue was dissolved in water (100 ml) and the pH was adjusted to 5.5 with sodium hydroxide. α-Chymotrypsin (140 mg) was added and the mixture was incubated at room temperature while the pH was being kept constant by the automatic addition of N NaOH from the titrator. The mixture was concentrated to

about 50 ml and the crystalline L-DOPA was filtered off after cooling the mixture for 1 h. The product was recrystallized by dissolving in a slight excess of boiling N HCl, filtering the solution (Celite), and neutralizing to pH 5.5 with sodium hydroxide.

The original mother liquor was brought to pH 9.0 and extracted with ethyl acetate (3 x 100 ml). The combined extracts were dried ($MgSO_4$) and filtered into 50 ml of ethyl acetate containing hydrogen chloride. The solvent was removed under vacuum and the residue was refluxed for 1 h in 120 ml of N HCl. The mixture was evaporated to dryness and the evaporation was repeated after the addition of water (3 x 30 ml). The residue was dissolved in water (20 ml), the solution adjusted to pH 5.5 with sodium hydroxide, and the crystallized D-DOPA was filtered with the aid of ethanol.

Determination of optical purity of isomers: The optical purity of the isomers was determined by chromatography of their L-alanine dipeptides formed by reaction with L-alanine N-carboxyanhydride according to the method of Manning and Moore (1968) as used in our laboratory (Coggins and Benoiton, 1970). A 20-μmol sample was dissolved in 2 ml of ice-cold 0.45 M sodium borate buffer, pH 10.2, in a 100 x 10 mm pyrex test-tube. L-Alanine N-carboxyanhydride in 2 x molar excess

was quickly added and the tube was vigorously agitated on a Vortex mixer at maximum speed for 2 min. In cases of phenolic amino acids, 0.05 ml 10 N NaOH was added to the reaction mixture which was left at 45°C for 30 min to hydrolyze any phenolic esters. The solution was brought to pH 2 with N HCl (1.5 ml) and filtered through Celite. For chromatographic analysis, an aliquot corresponding to 2 µmol of amino acid was taken from the above solution, diluted with half of its volume with 0.2 N sodium citrate buffer, pH 2.2, and analyzed on the analyzer.

RESULTS

The first step in the resolution procedures described above was the conversion of the DL-amino acids into their ethyl esters by the classical HCl/ethanol method. The esterification step was repeated to ensure completeness of reaction, except for m-tyrosine and DOPA for which it was found to be unnecessary. All the amino acid ethyl esters thus obtained were shown to contain no more than 0.1% free amino acid except for o-tyrosine ethyl ester which contained 0.4% of o-tyrosine.

After the removal of solvent and excess HCl by repeated evaporation, the ethyl esters obtained were not crystallized or isolated before subjection to chymotryptic digestion at pH 5.0. In most cases the enzymatic digestion was complete within 30 min as indicated by the alkali uptake from the pH-stat, but a longer time was required for o-tyrosine and p-CP.

The L-amino acids were obtained by crytallization from the concentrated aqueous digests or by the precipitation with ethanol when the amino acids were too soluble in salt solution as with L-o-tyrosine and L-m-tyrosine. The D-amino acids were obtained by saponification of their isolated

ethyl esters, except for D-DOPA because of its instability at alkali pH. D-DOPA was obtained by acid hydrolysis of its ethyl ester. The yields obtained were between 60-80% for the L-isomers and 50-70% for the D-isomers. The pertinent data for the resolution of ring-substututed phenylalanines effected by chymotryptic digestion on their racemic ethyl esters are shown on Tables 1 and 2.

The identification and chemical purity of the products was established by comparison with the racemic starting materials by chromatography with a Beckman Model 120B amino acid analyzer. All isomers gave essentially the same ninhydrin color constants as the starting materials (Table 2).

The optical purity of the isomers was established by the method of Manning and Moore (1968), involving chromatography on the amino acid analyzer of the dipeptides formed by the reaction with L-alanine N-carboxyanhydride. The elution conditions for the chromatographic separation of the diastereoisomeric dipeptides were tested with sodium citrate buffers, pH 4.25, 5.28 and 6.48, at flow rates of 34 ml/h and 68 ml/h. The optimum conditions for separation are recorded in Table 2. All isomers except D-o-tyrosine were shown to be at least 99.5% optically pure.

Two problems were encountered in the resolution of

o-tyrosine. Firstly, the enzymatic hydrolysis of the racemic o-tyrosine ethyl ester did not go to completion and hence the D-isomer had to be purified by the destruction of the remaining L-isomer with L-amino acid oxidase. The selective destruction of the L-isomer of o-tyrosine by L-amino acid oxidase from Crotalus adamanteus venom is shown on Table 3. Secondly, the optical purity of D-o-tyrosine could not be determined by the chromatographic method as used in the other cases because : (a) the diastereoisomeric dipeptides were not resolved when eluted with pH 4.25 buffer, (b) L-alanyl-L-o-tyrosine overlapped with the o-tyrosine peak when eluted with pH 5.28 buffer and (c) the L-alanyl-L-o-tyrosine peak overlapped with some unidentified by-product of the coupling reaction when eluted with pH 6.48 buffer.

Table 2

Resolution of ring-substituted phenylalanines
Chromatographic data from the Beckman amino acid analyzer

	0.9 x 15 cm Aminex A-5 resin, 57°, eluted with 0.20 N sodium citrate pH 4.25; 68 ml/h		0.9 x 50 cm AA-15 resin, 57°, eluted with 0.20 N sodium citrate pH 4.25; 68 ml/h	
	Elution time (min)	Constant[a]	Elution time (min)	Elution time of L-Ala·X dipeptide (min)
D-m-Tyrosine	23	19.9	71	141
L-m-Tyrosine	23	21.0	71	154
DL-m-Tyrosine	23	19.8	71	141,154
D-Tyrosine	25	23.0	85	193
L-Tyrosine	25	23.2	85	213
DL-Tyrosine	25	22.0	85	193,213
D-DOPA	22	18.8	65	130
L-DOPA	22	18.9	65	155
DL-DOPA	22	18.8	65	130,155
			0.35 N, pH 6.48; 34 ml/h	
D-o-Tyrosine	28	20.0	92	85
L-o-Tyrosine	28	18.5	92	68
DL-o-Tyrosine	28	18.8	92	85,68
D-p-CP	54	20.7	219	177
L-p-CP	54	21.0	219	126
DL-p-CP	54	21.0	219	177,126
D-p-FP	30	22.1	115	99
L-p-FP	30	22.3	115	76
DL-p-FP	30	22.8	115	99,76

[a]Peak height times width divided by concentration (Spackman, Stein and Moore, 1958)

Table 1

Data for the resolution of ring-substituted DL-phenylalanines

Amino acid	Incubation conditions[a]			L-isomer			D-isomer		
	Substrate (g)	Enzyme (mg)	Time (min)	Yield (%)	$[\alpha]_D^{25}$[b]	Optical purity (%)	Yield (%)	$[\alpha]_D^{25}$[b]	Optical purity (%)
o-Tyrosine	2.0	200	60	75	-26.8	100	50	+25.4	
m-Tyrosine	0.5	45	30	75	- 7.9	100	78	+ 7.9	>99.5
Tyrosine	0.5	20	30	80	-10.2	100	78	+10.0	99.8
DOPA	4.0	140	30	70	-11.7[c]	>99.8	60	+11.6[c]	99.5
					- 9.5[d]			+ 9.5[d]	
p-CP	0.5	50	90	64	- 3.5	100	60	+ 3.3	99.5
p-FP	0.5	60	30	60	- 5.6	100	60	+ 5.6	>99.5

[a] pH 5.0, room temperature
[b] c = 2, N HCl
[c] 20°C. Lit 11.6° (c = 3, 4% HCl) (Vogler and Baumgartner, 1952)
[d] 30°C

Table 3

Effect of L-amino acid oxidase (Crotalus adamanteus) on o-tyrosine[a]

L-o-tyrosine (μmol)	DL-o-tyrosine (μmol)	L-amino acid oxidase (mg)	% recovery after incubation[b]
20	0	0	100.0
20	0	6	0.6
15	10	6	20.7
0	40	6	51.0

[a]The reaction was carried out in 10 ml 0.1 M sodium phosphate buffer, pH 8.0, with oxygen being bubbled through, at 37°C for 24 h.

[b]Suitable aliquots were taken for amino acid analysis on the analyzer.

DISCUSSION

The results presented in the foregoing pages described a simple effective method for the preparation of both isomers of ring-substituted phenylalanines by asymmetric hydrolysis of their ethyl esters with α-chymotrypsin. It should be noted that the method also provides a convenient source for the D-esters starting from the racemic acid. Since all the ring-substituted phenylalanine ethyl esters tested were found to be substrates of α-chymotrypsin, the method should be of general applicability for the resolution of other ring-substituted phenylalanines as well as other aromatic amino acids.

The experiments described above arose from the successful resolution of o-tyrosine-2-^{14}C (Petitclerc et al.,1969a). During the study on the metabolism of o-tyrosine in our laboratory (Petitclerc et al., 1969b), a small-scale resolution of labelled DL-o-tyrosine was required. Since the resolution of o-tyrosine had not been reported in the literature at that time, all the resolution methods (Greenstein and Winitz, 1961) for obtaining aromatic amino acid isomers were considered. These included selective destruction of one of the isomers of a racemate using L- or D-amino acid oxidase, resolution of the racemate by the stereospecific hydrolytic

action of carboxypeptidase A on the O,N-diacylamino acid, the stereospecific synthetic action of papain on the N-substituted amino acid giving rise to a substituted amide, and fractional crystallization of the diastereoisomeric salts from the N-acylamino acid and an optically active organic base. However, each of these methods has its shortcomings and was judged not suitable for the resolution of o-tyrosine. Later it was found that the resolution of L-o-tyrosine-2-^{14}C could be achieved by the action of α-chymotrypsin on its ethyl ester (Petitclerc et al., 1969a). The resolution method stems from the work of Brenner et al. (1948) and Wretlind (1950). Brenner et al. have resolved DL-tryptophan by the action of chymotrypsin on the methyl ester, and Wretlind used an extract of pancreas powder to resolve the isopropyl ester of DL-phenylalanine. The general procedures of this method involve the action of an enzyme on an N-unprotected amino acid ester to give the L-amino acid, and extraction of the D-ester from the reaction mixture with an organic solvent, followed by saponification or acid hydrolysis to give the D-amino acid. Leucine (Warburg, 1906), tyrosine (Abderhalden et al., 1923), and methionine (Brenner and Kocher, 1949) have been partially resolved in a similar manner using a crude pancreatic preparation. However, the

application of this method as a general resolution procedure was considered to be of limited value (Greenstein and Winitz, 1961), because of the spontaneous hydrolysis of the esters (Wretlind, 1950) (Brenner et al., 1950) and the tendency of some amino acid esters in the presence of chymotrypsin to polymerize and form higher peptide esters under the conditions employed (Brenner et al., 1950). In our resolution method described above, the enzymatic digestion of the racemic ethyl esters was carried out at pH 5. By operating at this pH we have eliminated the danger of spontaneous hydrolysis, and moreover have taken advantage of the higher rate which chymotrypsin exhibits towards phenylalanine esters (optimum pH, 6.0-6.2) below pH 7 than it does above this pH (Purdie and Benoiton, 1970) (Goldenberg and Goldenberg, 1950).

The resolution of o-tyrosine-2-^{14}C which prompted the present investigation has been described by Petitclerc et al., (1969a). Of the aromatic amino acids included in this study, p-CP and DOPA have not previously been resolved. Thus our resolution method has provided a convenient way of obtaining the optical isomers of these two compounds of significant biological interest. The resolution of m-tyrosine*

* Recently a patent application on the treatment of Parkinsonism with L-m-tyrosine has appeared in the Chemical abstracts : L-m-tyrosine was obtained from the resolution of N-phenyl-acetyl-(3-methoxyphenyl)-DL-alanine by the action of an E. coli acylase (Bamberg and Sjoberg, 1971).

has been accomplished by fractional crystallization of the brucine salts of N-formyl-m-tyrosine (Sealock et al., 1951). Of the resulting amino acids, the levorotatory isomer was shown to possess the L-configuration by means of the D-amino acid oxidase and by the Lutz-Jirgenson's rule. Although a direct comparison of the rotation data is not possible, our assignment of configurations based on the susceptibility of the L-isomer to chymotrypsin is consistent with that reported (Sealock et al., 1951). p-FP has been resolved by the synthetic action of papain in the conversion of the acetyl-L-amino acid into the corresponding L-phenyl-hydrazide (Bennett and Niemann, 1950b). L-p-Fluorophenylalanine-1-^{14}C has been obtained by the hydrolytic action of carboxypeptidase A on the chloroacetyl derivative (Loftfield and Eigner, 1966). The optical isomers of DOPA** are available by the resolution of N-acetyl-(3,4-diacetoxyphenyl)-DL-alanine with brucine (Harrington and Randall, 1931) and N-benzoyl-(3-hydroxy-4-methoxyphenyl)-DL-alanine with cinchonin (Vogler and Baumgartner, 1952), followed by removal

**Stimulated by the successful treatment of Parkinsonism with L-DOPA, recently a number of patent applications on the preparation of L-DOPA have appeared in the Chemical Abstracts: the resolution of N-acetyl-(3,4-dimethoxyphenyl)-DL-alanine with optically active bases has been reported for the preparation of L-DOPA (Hever et al., 1970) (Berenyi et al., 1971).

of the alkaloids and subsequent hydrolysis. In both cases the resolution step was performed on the precursors for the chemical synthesis of DOPA. The present study provides the first resolution starting from DOPA itself.

It is generally accepted that the specific substrates of chymotrypsin are typically N-acylamino esters, amides, and peptides and that replacement of the acylamino group by acetoxy-, chloro-, or hydrogen leads to a decrease in the rate of hydrolysis as well as a decrease in stereospecificity (Bender and Kezdy, 1965) (Cunningham, 1965) (Holloway, 1968) (Ingles and Knowles, 1968). In a kinetic study of α-chymotrypsin, Hein and Niemann (1961) stated that the replacement of an acylamino group by an amino group "results in a decrease in the rate of hydrolysis as well as a partial loss of stereospecificity which normally favors the L-antipode". The results presented here are at variance with this statement, are consistent with the published kinetic data on the interaction of α-chymotrypsin with phenylalanine derivatives containing a free α-amino group from our laboratory (Purdie and Benoiton, 1970) and amply demonstrate that chymotrypsin is indeed stereospecific in its action on N-unsubstituted aromatic amino acid esters.

B: LARGE SCALE PREPARATION OF L-m-TYROSINE

The application of the above method to the large scale resolution of ring-substituted phenylalanines was tested in a large scale preparation of L-m-tyrosine. DL-m-tyrosine was prepared by the method of Sealock et al. (1951)

EXPERIMENTAL

Materials: The following chemicals were obtained as indicated.

Acetylglycine	(BDH)
m-Hydroxybenzaldehyde	(Aldrich)
Sodium acetate, anhydrous	(BDH)
Hydriodic acid (57%)	(BDH)
Acetic anhydride	(BDH)
Acetic acid, glacial	(BDH)
Phosphorous red, amorphous	(Fisher)
α-Chymotrypsin	(Worthington)

2-Methyl-4-(3'-acetoxybenzal)5-oxazolone: 122 g (1 mol) m-Hydroxybenzaldehyde, 117.2 g (1 mol) acetylglycine, 82 g (1 mol) anhydrous sodium acetate and 287 ml (3 mol) acetic anhydride were mixed in a 2-litre flask. The mixture was placed in a boiling water bath for 6 h with occasional swirling. The hot reaction mixture was transferred to a 2-litre beaker and was allowed to cool slowly to room temperature.

A solid dark brown mass was formed. 600 ml of water was gradually worked into the solid mixture which was then kept in the cold overnight. The product was filtered off, washed well with 200 ml portions of ice-cold water, and dried well over P_2O_5 under vacuum. The acetic odor which remained was removed by washing the product again in 600 ml of water. The yellow product obtained had a melting point of 115-117°, and weighed 175-180 g, corresponding to yields of 69-71%.

DL-m-tyrosine : A mixture of 98 g 2-methyl-4-(3'-acetoxybenzal)5-oxazolone, 500 ml acetic acid, 500 ml hydriodic acid, and 40 g red phosphorus was refluxed for 4 h. The hot mixture was filtered through a medium grade sintered glass funnel and the filtrate was evaporated to dryness under vacuum. A thick brown oil was obtained. This was again evaporated to dryness after the addition of water (2 x 200 ml). After addition of 75 ml water, the residue was brought to boiling and treated with charcoal and Celite. The hot solution was filtered through Celite on a sintered glass funnel. The filtrate was cooled in ice, neutralized to pH 5.5 with 2 N LiOH and one volume of ethanol was added one hour later. This was then stored in the cold for 3 days. The product was filtered off and washed with ethanol. Infrared spectroscopy and amino acid analysis showed that the DL-m-tyr-

osine obtained was pure . This was used as starting material for the enzymatic resolution. From different preparations, 35-40 g were obtained, corresponding to yields of 52-62%. In some cases of lower yields more product was obtained by adding ethanol to the mother liquor and cooling for another 7 days.

DL-m-Tyrosine ethyl ester·HCl: 30 g DL-m-tyrosine was suspended in 1500 ml absolute ethanol in a 2-litre flask. Hydrogen chloride (anhydrous) was bubbled through until the suspension was clear (20-30 min). The hot solution was placed in an ice-bath and the introduction of HCl was continued for another 20 min. The solution was allowed to stand overnight at room temperature. Next day, the solvent was removed by evaporation under vacuum at 40°C and the excess HCl was removed by repeating the evaporation three times after the addition of 250 ml ethanol. At this stage the DL-m-tyrosine ethyl ester·HCl was a brown oil. The percentage of non-esterified amino acid was usually less than 0.1%. The racemic ester·HCl was used without isolation.

Enzymatic hydrolysis of DL-m-tyrosine ethyl ester : 500 ml of water was added to the DL-m-tyrosine ethyl ester·HCl obtained as described above and the mixture was filtered through Celite. After adjusting the solution to pH 5 with

2 N LiOH, 800 mg α-chymotrypsin dissolved in 40 ml water was added. The reaction was kept at pH 5 for 60-90 min at room temperature. The reaction mixture was then evaporated to near dryness under vacuum at 50°C (0.2-0.3 ml octanoic acid was added to prevent foaming during evaporation) and 750 ml ethanol was added to the residue. This was kept in the cold for 3 h. The precipitate was filtered off, washed well with ethanol, boiled in 150 ml N HCl for 10 min and filtered hot through Celite. The filtrate was cooled and adjusted to pH 5 with 2 N LiOH. L-m-tyrosine started to come out of solution. After cooling the solution for 30-60 min, 600 ml ethanol was added. This was kept in the cold for 2 days. The product was filtered off and washed with ethanol. From different preparations 9-9.5 g were obtained corresponding to yields of 60-63% (based on DL-m-tyrosine). Infrared spectroscopy and amino acid analysis showed the product was chemically pure. The optical purity was determined according to the method of Manning and Moore (1968) and the product showed an optical purity of greater than 99.5%.

No difficulties were encountered in the above experiment with respect to both the yield and optical purity of the product. 70 g of L-m-tyrosine were prepared by the above procedure. Hence the resolution method is suitable for large scale preparation purposes.

SECTION II

A: SYNTHESIS OF O,N-DIMETHYL-m-TYROSINE AND O-METHYL-m-TYROSINE

These two compounds were synthesized to serve as standards in the studies on the metabolism of m-tyrosine. O,N-Dimethyl-m-tyrosine was prepared by the method developed in our laboratory for the synthesis of N-methylamino acids (Coggins and Benoiton, 1971). O-Methyl-m-tyrosine was prepared by the method for the synthesis of O-methyl-tyrosine (Izumiya and Nagamatsu, 1952).

EXPERIMENTAL

Materials: Carbobenzoxy chloride was purchased from Pierce Chemical Co. and dimethyl sulphate from Matheson Coleman and Bell Company Inc. Methyl iodide and formic acid were obtained from Fisher Scientific Co. Tetrahydrofuran was the J.T. Baker product containing butylated hydroxytoluene as stabilizer. Sodium hydride, 50% dispersion in oil was purchased from BDH Chemicals.

N-Carbobenzoxy-m-tyrosine*: 18.1 g (0.1 mol) DL-m-tyrosine was dissolved in 4 N NaOH (25 ml) at 40°C with stirring. The solution was chilled to 5°C and a total of 30 ml 4 N NaOH and 18.7 g (0.11 mol) carbobenzoxy chloride were added alternately over a period of 30 min with vigorous stirring and cooling in an ice-bath. The reaction mixture was kept alkaline at all times with 4 N NaOH. Upon completion of reaction, the reaction mixture was extracted with ether (2 x 20 ml) to remove the excess carbobenzoxy chloride. The aqueous phase was chilled and slowly acidified with 5 N HCl to congo red. A thick oil resulted and was extracted into ethyl acetate. The ethyl acetate extract was washed with N HCl and then with water, dried with anhydrous sodium sulfate, and evaporated under vacuum to give an oily residue. 24.6 g was obtained, corresponding to a yield of 78%.

N-Carbobenzoxy-O,N-dimethyl-m-tyrosine: To 10 g (0.032 mol) of N-carbobenzoxy-m-tyrosine was added 150 ml tetrahydrofuran, 15 ml (0.256 mol) MeI, and 5.2 g (0.128 mol) NaH. The mixture was refluxed at 80°C for 24 h. The solvent was removed under vacuum, the evaporation was repeated after the addition of ether to remove excess MeI. The residue was distributed between ether (100 ml) and water (25 ml). The

* The identification of the compounds was confirmed by n.m.r. spectroscopy.

ether layer was washed with water, dried ($MgSO_4$), and evaporated to give two immiscible oils (one is the oil from the sodium hydride dispersion).

O,N-Dimethyl-m-tyrosine methyl ester·HBr: To the suspension of oils obtained as described above was added 50 ml of 37% HBr in acetic acid and the mixture was left at room temperature for 2 h. The mixture was evaporated to give an oil which was dissolved in water (50 ml). The solution was washed with ether (2 x 20 ml), and then evaporated to dryness under vacuum.

O,N-Dimethyl-m-tyrosine*: To the residue obtained above was added 100 ml 2 N HCl, and the mixture was refluxed for 3 h. After cooling the solution was filtered with the aid of Celite. The filtrate was evaporated to dryness under vacuum and the evaporation was repeated after the addition of water (2 x 25 ml). The residue was dissolved in 50 ml water and neutralized to pH 6 with 2 N LiOH. The solution was evaporated to dryness and 200 ml ethanol was added to the residue. The product was filtered off and recrystallized from ethanol/water. 2.5 g was obtained corresponding to a yield of 37% based on N-carbobenzoxy-m-tyrosine. Anal. Calcd. for $C_{11}H_{15}NO_3$: C, 63.14; H,7.26; N, 6.70. Found: C, 62.84; H, 7.69; N, 6.63.

* The identification of the compounds was confirmed by n.m.r. spectroscopy.

N-Formyl-m-tyrosine*: To a solution of 6 g (0.033 mol) DL-m-tyrosine in 85% formic acid (75 ml) was added acetic anhydride (22 ml) over a period of 30 min with stirring. The reaction mixture was left at room temperature. After 2 h, the solution was evaporated to dryness and the evaporation was repeated after addition of water (3 x 20 ml). Ice-cold N HCl was added to the residue to dissolve any unreacted m-tyrosine. The product was filtered off and recrystallized from water; yield 4.5 g (65%); m.p. 136-138°.

O-Methyl-N-formyl-m-tyrosine*: 3 g (0.014 mol) N-formyl-m-tyrosine was dissolved in 4 N NaOH (7.5 ml). The solution was placed in an ice-bath and a total of 5.6 ml of 4 N NaOH and 2.8 ml dimethyl sulfate were added alternately with stirring. The reaction mixture was left at room temperature and stirring continued for 2 h. The solution was then acidified with 8 N HNO_3. The crystalline product was collected: yield 2.6 g (78% based on N-formyl-m-tyrosine); m.p. 146-148°.

O-Methyl-m-tyrosine*: To 2.4 g (0.011 mol) O-methyl-N-formyl-m-tyrosine was added 25 ml 3 N HCl and the mixture was refluxed for 2 h. The solution was evaporated to dryness

---

* The identification of the compounds was confirmed by n.m.r. spectroscopy.

under vacuum and the evaporation was repeated after the addition of water (3 x 20 ml). The residue was dissolved in a small amount of water, and the solution neutralized with NH_4OH. After cooling the crystals were collected and recrystallized from ethanol/water; yield 1.3 g (66% based on O-methyl-N-formyl-m-tyrosine).

B: THE METABOLISM OF m-TYROSINE IN VITRO

INTRODUCTION

(1) The formation of m-tyrosine from phenylalanine

The possibility that m-hydroxyphenyl compounds arise by a meta-hydroxylation of phenylalanine to m-tyrosine has never been investigated in detail. para-Dehydroxylation of 3,4-dihydroxyphenylacetic acid by intestinal micro-organisms may account for the appearance of some m-hydroxyphenyl acids in animals and human urine (DeEds et al., 1957) (Sandler et al., 1969). However, this does not explain the presence of m-tyramine which is believed to be of endogenous origin (Kakimoto and Armstrong, 1962). Also, a m-hydroxyphenylalkylamine, leptodactyline (m-hydroxyphenylethyltrimethylammonium), has been found in the skin of some South American amphibians (Erspamer, 1959). The possibility of para-dehydroxylation of DOPA or DOPamine to give m-tyrosine and m-tyramine has been suggested but has so far not been demonstrated (Sandler et al., 1969)

(2) The hydroxylation of m-tyrosine

The biosynthetic pathway beginning with tyrosine for the formation of the sympathetic neurotransmitter norepine-

phrine and sympathetic hormone epinephrine was first suggested by (Blaschko (1939), and finally established with the discovery of the enzyme tyrosine hydroxylase (Nagatsu, et al., 1964). The para-hydroxylation activity of phenylalanine hydroxylase and tyrosine hydroxylase was demonstrated by their ability to catalyze the conversion of phenylalanine to tyrosine (Udenfriend and Cooper, 1952) (Ikeda et al., 1967). On the other hand, this activity failed to effect the conversion of m-tyrosine to DOPA (Kaufamn, 1962a) (Udenfriend, 1966), and hence m-tyrosine was rejected as a precursor for catecholamines. However, the increase in DOPamine excretion in rats after administration of m-tyrosine was claimed to be due to a direct conversion (Sourkes et al., 1961).

MATERIALS AND METHODS

(1) Studies on the hydroxylation of phenylalanine

Compounds were purchased as follows: L-phenylalanine-^{14}C uniformly labelled, 384 mCi/mmol (New England Nuclear), DL-DOPA-2-^{14}C and L-tyrosine-^{14}C (U) hydrochloride (Nuclear Chicago), 6,7-dimethyl-5,6,7,8-tetrahydropterine·HCl·1½H_2O ($DMPH_4$) (Calbiochem), NADPH and α-methyl-DL-tyrosine (Sigma), and 3-iodo-L-tyrosine (Aldrich). DL-m-Tyrosine-2-^{14}C was a product synthesized in our laboratory by Dr. S. Sharma. 3-Bromo-4-hydroxybenzyloxyamino phosphate (NSD-1055) was a gift from Smith and Nephew Research, Gilson Park, Harlow, U.K. Female Sprague-Dawley rats (150-165 g) killed by decapitation and fresh beef adrenals obtained locally were used.

Fresh beef adrenal medulla was homogenized in two volumes of 0.32 M sucrose in a Servall Omni-mixer. The homogenate was centrifuged at 1,000 x g for 10 min and the sediment was discarded. Rat liver was homogenized in three volumes of 0.9% NaCl with a motor-driven teflon-pestle homogenizer and the homogenate was centrifuged for 60 min at 22,000 x g. Aliquots of the supernatant were used in the study on the

enzymatic hydroxylation of L-phenylalanine. Protein concentrations, determined by the method of Lowry et al. (1951), were 46-48mg/ml for the adrenal medulla homogenate and 30 mg/ml for the rat liver homogenate. Partially purified tyrosine hydroxylase of beef adrenal medulla was prepared from the 105,000 x g supernatant by ammonium sulfate (40%) precipitation (Nagatsu et al., 1964). The precipitate obtained by centrifugation at 10,000 x g was dissolved in 0.005 M sodium phosphate buffer, pH 7.0, before use. The protein concentration was 52 mg/ml. All steps were carried out at 0-4°C.

The assay system contained the following components: 1 µCi of L-phenylalanine-^{14}C (U), 2 µmol each of $DMPH_4$ and NSD-1055, 100 µmol of mercaptoethanol, 0.2 ml of buffer (N sodium citrate, pH 6.0, was used for the adrenal medulla homogenate and N sodium phosphate, pH 7.0, was used for the rat liver homogenate), and 0.5 ml of enzyme preparation. The reaction mixture (final volume, 2 ml) was incubated at 37°C in open flasks in a shaking water bath. The reaction was terminated by the addition of 0.5 ml of 35% sulfosalicylic acid containing cold carriers 0.5 µmol each of DOPA, m-tyrosine and tyrosine. 0.5 ml of 0.2 N sodium citrate, pH 2.2, was added and the denatured protein was removed by

centrifugation. 2 ml aliquots from the supernatant were analyzed for phenylalanine hydroxylation products on a 50 x 0.9 cm column of AA-15 resin of a Beckman amino acid analyzer which was eluted with 0.2 N sodium citrate, pH 4.25, at 57°C at a flow rate of 34 ml/h. The effluent from the outlet of the column was passed through a Nuclear Chicago model 4526 flow cell scintillation system. The amount of radioactivity corresponding to each product (Fig.2) was obtained by collecting the effluent from the column in 0.5 ml fractions, adding 10 ml Biosolv scintillation mixture*, and counting with a Beckman liquid scintillation spectrometer model LS-133. Counts were corrected for quenching and expressed as d.p.m. A >95% recovery of radioactivity was obtained with this procedure in a control experiment.The reaction blank consisted of the above reagents with boiled enzyme or 0.32 M sucrose substituted for the enzyme solution.

(2) Studies on the hydroxylation of m-tyrosine

L-Phenylalanine and L-tyrosine were purchased from General Biochemicals; DL-DOPA, D-phenylalanine, and D-tyrosine from Mann Research Laboratories; 3-O-methyl-DOPA and

*Composition of Biosolv scintillation mixture: the mixture contained 0.4% 2,5-diphenyloxazole and 10% (v/v) Biosolv BBS 3 (Beckman Instruments) in toluene.

p-methoxyphenylalanine from Sigma Chemical Co; and S-adenosyl-L-methionine-methyl-^{14}C (52.3 mCi/mmol) from New England Nuclear Co. L- and D-isomers of m-tyrosine and o-tyrosine were obtained by resolution of their racemic ethyl esters using α-chymotrypsin. O,N-Dimethyl-m-tyrosine and 3-O-methyl-m-tyrosine were synthesized as described in section IIA (page 54). The sources of other materials used have been described previously (page 61). Protein was determined by an automated biuret method (Lane and Mavrides, 1969).

Rat liver was homogenized in three volumes of 0.9% NaCl and centrifuged at 22,000 x g for 60 min. Beef adrenal medulla was homogenized in one volume of 0.25 M sucrose and centrifuged at 1,000 x g for 15 min. Aliquots of the supernatants* were used for the subsequent enzymatic assays. The rat liver 22,000 x g supernatant was fractionated with ethanol and ammonium sulfate to give a semi-purified preparation of phenylalanine hydroxylase by the method of Kaufman (1962a), purification was carried out up to the first ammonium sulfate fractionation step of his method. The precipitates* of the 10-21% ethanol fraction and the 20-25% ammonium sulfate fraction were dissolved in

* For the rat liver preparations, the protein concentrations were as follows: 22,000 x g supernatant, 29.2 mg/ml; 10-21%ethanol precipitate, 30 mg/ml; 20-25% ammonium sulfate precipitate, 5.4 mg/ml.

0.033 M Tris buffer, pH 6.8, before use. The 1,000 x g beef adrenal medulla supernatant was centrifuged at 105,000 x g for 45 min. The high speed supernatant was fractionated with ammonium sulfate (40%) to give a semi-purified tyrosine hydroxylase as previously described (page 62). The particles* precipitated by high speed centrifugation were resuspended in 0.005 M sodium phosphate buffer, pH 7.0, before use. Rat brain was homogenized in four volumes of 0.25 M sucrose.

The enzyme assay mixture for the rat liver preparations consisted of 2 μmol each of substrate, NADPH, $DMPH_4$, and NSD-1055, 100 μmol of mercaptoethanol, 0.5 ml enzyme preparation and 0.2 ml N sodium phosphate, pH 7.0, in a final volume of 2.0 ml. Assay conditions for the beef adrenal medulla preparations were the same as above except that the digests contained 0.5 μmol $FeSO_4$ and the buffer was N sodium citrate, pH 6.0. Incubations were carried out at 37°C. The reaction was terminated by the addition of 0.5 ml sulfosalicylic acid. 0.5 ml of 0.2 N sodium citrate, pH 2.2, was added. After the removal of the precipitated protein by centrifugation, 1 ml aliquots of the supernatant were analyzed with a Beckman amino acid analyzer.

---

* For the beef adrenal medulla preparations, the protein concentrations were as follows: 1,000 x g supernatant, 56 mg/ml; 105,000 x g particles, 73.6 mg/ml; 40% ammonium sulfate precipitate of the 105,000 x g supernatant, 55 mg/ml.

It has been shown that catechols are O-methylated by catechol-O-methyltransferase with S-adenosylmethionine serving as the methyl donor (Pellerin and D'Iorio, 1958)(Axelrod and Tomchick, 1958). Thus when monophenols are incubated with an enzyme preparation containing an aromatic hydroxylase and a catechol-O-methyltransferase together with S-adenosylmethionine-methyl-^{14}C, any catechol formed enzymatically will be methylated, thus giving radioactive products. A radioactive assay method for the studies on the hydroxylation of m-tyrosine was established by making use of the biochemical property of DOPA in the formation of 3-O-methyl-DOPA. The radioactive assay mixture contained the following: 0.5 µCi of S-adenosyl-L-methionine-methyl-^{14}C, 4 µmol substrate, 2 µmol NSD-1055, 20 µmol $MgCl_2$, 0.2 ml of 0.5 M sodium phosphate buffer pH, 8.0 and 0.5 ml rat liver homogenate, in a final volume of 1.7 ml. The assay mixture was modified for the studies with rat brain homogenate as follows: 0.2ml aliquot of rat brain homogenate was incubated with 0.2 ml of 0.28 M sodium phosphate buffer, pH 6.2, 7.0, or 8.0 (containing 0.6 µmol $DMPH_4$, 0.12 µmol mercaptoethanol, and 2.0 µmol substrate) and 0.2 ml radioactive S-adenosylmethionine solution (containing 0.6 µmol NSD-1055, 6 µmol $MgCl_2$, and 0.5 µCi of S-adenosyl-L-methionine-methyl-^{14}C).

Incubations were carried out at 37°C. The reaction was stopped by the addition of 0.4 ml sulfosalicylic acid and the final volume was adjusted to 2.5 ml by the addition of o.2 N sodium citrate, pH 2.2. After the removal of the precipitated protein, 0.5-1.0 ml aliquots of the supernatant were analyzed on a 15 cm Aminex A-5 resin column of a Beckman amino acid analyzer coupled to a Nuclear Chicago flow cell assembly model 4526. The amount of radioactivity corresponding to the 3-O-methyl-DOPA fractions (Fig. 3) was counted in a Beckman scintillation counter model LS-133 as previously described (page 63).

RESULTS

Hydroxylation of phenylalanine by beef adrenal medulla

Three radioactive products were formed on incubation of L-phenylalanine-^{14}C with a beef adrenal medulla homogenate (Fig. 1). The radioactive products were identified as DOPA, m-tyrosine, and tyrosine by co-chromatography with authentic labelled compounds using an amino acid analyzer coupled to a flow cell scintillation system (Table 4). However, this system does not separate o-tyrosine from phenylalanine therefore any o-tyrosine that might have been formed was not detected. The order of emergence of the pertinent amino acids was consistent with that observed with the ninhydrin system.

A time study on the hydroxylation of phenylalanine by beef adrenal medulla homogenate in the presence of a pteridine co-factor ($DMPH_4$) and the DOPA decarboxylase inhibitor NSD-1055 is shown in Fig. 2. The initial rate of formation of m-tyrosine was about 15% of that of tyrosine. The amount of m-tyrosine and tyrosine levelled off after 60 min and the amount of DOPA remained constant after 90 min at a level equal to twice that of tyrosine.

The formation of the three hydroxylation products of phenylalanine identified was dependent on the presence of pteridine co-factor. In Table 5 are shown the results for 30 min incubations of modifying the standard incubation mixture used. In the absence of $DMPH_4$, the formation of m-tyrosine, tyrosine, and DOPA was reduced by 96%, 92.3% and 99.8% respectively. That the reduction of DOPA was more prominent could be explained on the basis that its formation is dependent on the availability of tyrosine as well as $DMPH_4$. Addition of ferrous ions or NADPH did not stimulate the hydroxylation reactions. The reduction in the formation of DOPA and m-tyrosine but not tyrosine in the absence of the DOPA decarboxylase inhibitor NSD-1055 could be explained by the fact that these two compounds are susceptible to the action of DOPA decarboxylase. With the addition of the tyrosine hydroxylase inhibitors α-methyltyrosine or 3-iodotyrosine at a concentration of 10^{-3} M, the hydroxylation of phenylalanine was completely prevented.

The hydroxylation of phenylalanine by beef adrenal medulla was compared with the well known non-enzymatic hydroxylation of phenylalanine mediated by tetrahydropterin in the presence of ferrous ion. Incubations were as described under Materials and Methods with the addition of 0.5 μmol ferrous ion (as

ferrous sulfate). The results are presented in Table 6. It is seen that the non-enzymatic hydroxylation of phenylalanine could not account for more than 5% of the m-tyrosine nor for more than 1% of the other products formed during the first 90 min of incubation.

The possibility that the m-tyrosine present in the incubation mixture could have come from a para-dehydroxylation reaction of DOPA instead of a direct hydroxylation of phenylalanine was investigated. From an incubation identical to the standard assay system except that the substrate L-phenylalanine-^{14}C was substituted by DL-DOPA-2-^{14}C (0.1 μCi), neither the formation of m-tyrosine nor tyrosine was observed indicating that the labelled m-tyrosine appearing in the digests containing L-phenylalanine-^{14}C could not have come from DOPA.

Similar experiments were carried out using rat liver homogenate. Under conditions where phenylalanine hydroxylase was shown to be active, no conversion of L-phenylalanine-^{14}C to m-tyrosine nor to DOPA could be detected even though our method would have been able to detect the conversion of as little as 0.2% of phenylalanine.

Table 4

Elution times of ^{14}C amino acids from the Beckman amino acid analyzer

	0.9 x 50 cm AA-15 resin, 57°, eluted with 0.20 N sodium citrate (pH 4.25, 34 ml/h)
^{14}C-amino acid	Elution time (min)
DOPA	103.5
m-Tyrosine	115.5
Tyrosine	127.3
Phenylalanine	140.0

The ^{14}C amino acids were detected by the flow cell scintillation system as described under Materials and Methods.

Table 5

Effect of various substances on the hydroxylation of L-phenylalanine-^{14}C by beef adrenal medulla homogenate[a]

Modification to digest	Radioactivity (d.p.m./mg protein) accounted for as		
	m-Tyrosine	Tyrosine	DOPA
-	864	4370	5981
+NADPH[b]	823	4312	5925
+Fe^{2+}[c]	831	4409	6203
-NSD-1055	580	4080	3170
-$DMPH_4$	34	335	13
+α-Methyl-DL-tyrosine	12	29	6
+3-Iodo-L-tyrosine	14	61	11

[a]Incubations were as described in Materials and Methods (page 62), for 30 minutes

[b]2 μmol.

[c]0.5 μmol $FeSO_4$.

Table 6

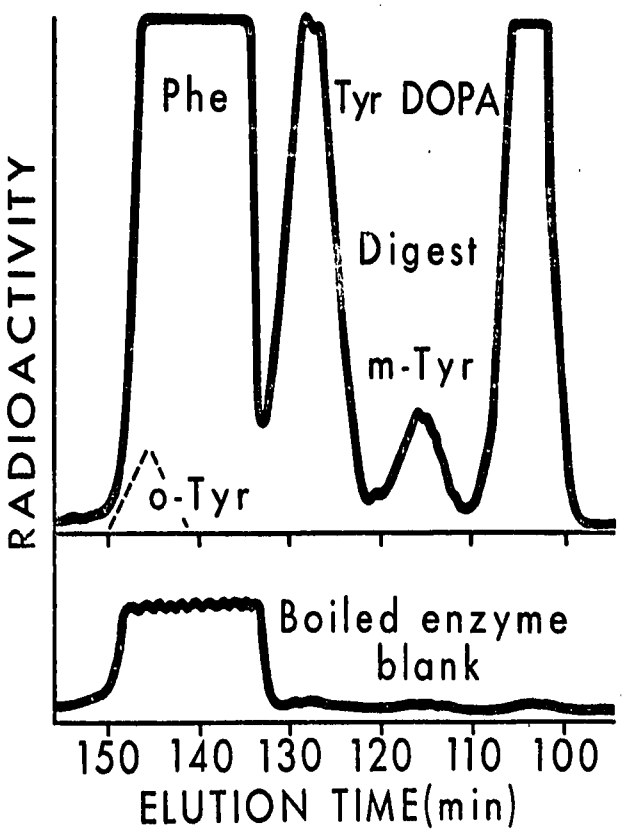
Comparison on the hydroxylation of phenylalanine with beef adrenal medulla homogenate and the non-enzymatic tetrahydropterin-Fe^{2+} system.

	Radioactivity (d.p.m. x 10^{-3}) accounted for as								
	m-tyrosine			tyrosine			DOPA		
Time (min)	60	90	180	60	90	180	60	90	180
Beef adrenal medulla homogenate	20.5	25.6	23.8	104.0	123.3	122.5	142.3	236.6	224.3
Boiled homogenate	0.8	1.0	2.5	0.2	0.3	0.8	0.9	1.6	4.8
No homogenate added	0.8	0.9	2.3	0.2	0.2	0.7	0.8	1.7	4.0

Incubations were as described under Materials and Methods with the addition of 0.5 µmol ferrous ion.

Figure 1

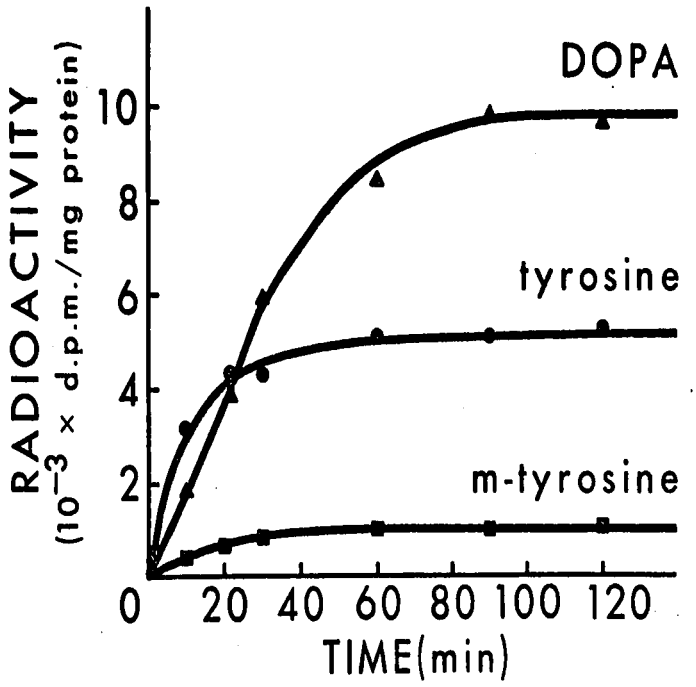
Chromatographic separation of the products from the incubation of L-phenylalanine-^{14}C (U) with beef adrenal medulla.



2-ml of a supernatant from a 30 min digest (Materials and Methods) (page 63) was placed on a 50 x 0.9 cm column of AA-15 resin of a Beckman amino acid analyzer which was eluted with 0.2 N sodium citrate, pH 4.25, at 57°C at a flow rate of 34 ml/h. The dotted line indicates the time of elution of o-tyrosine with this system.

Figure 2

Hydroxylation of L-phenylalanine-^{14}C by beef adrenal medulla.



Assay conditions were as described in Materials and Methods (page 62).

Hydroxylation of L-m-tyrosine by rat liver

Initial studies showed that when L-m-tyrosine was incubated with rat liver homogenate in the presence of the DOPA decarboxylase inhibitor NSD-1055 and a pteridine co-factor ($DMPH_4$), a new amino acid was formed which was indistinguishable from DOPA when chromatographed on the amino acid analyzer using the three systems described in Table 7. That the new amino acid formed from L-m-tyrosine was DOPA was confirmed by observation of its fluorescence spectrum and paper chromatography. A 2ml aliquot of a 3 h incubation was chromatographed on the analyzer using system B (Table 7) with the column effluent being directed into a fraction collector. Fractions were collected at 3-min intervals. m-Tyrosine was found in fractions 35-38 and the new amino acid in fractions 31-34. The fluorescence spectrum of fraction 37 (activation max at 280 nm; emission max at 305 nm) was identical with that of an authentic sample of m-tyrosine. The fluorescence spectrum of fraction 33 was identical with that of an authentic sample of DOPA (activation max at 285 nm; emission max at 320 nm) (Fig. 4; 5). Fraction 33 was extracted with N HCl-saturated n-butanol and was then chromatographed on Whatman No. 1 paper. Descending chromatography was carried

out at room temperature for 24 h using as solvent n-butanol-acetic acid- water (78:5:17) (Lissitzky and Roques, 1957). After drying, the chromatogram was developed with ninhydrin or diazotized sulfanilic acid. The new amino acid coincided with DOPA in this system which is reported to separate the 2,3-, 2,5-, and 3,4-isomers of dihydroxyphenylalanine (Lissitzky and Roques, 1957). No DOPA could be detected when L-m-tyrosine was incubated with rat brain homogenate.

The rate of hydroxylation of L-m-tyrosine in the formation of DOPA by rat liver homogenate was compared with the rate of formation of tyrosine from phenylalanine by the same preparation (Fig. 6). The initial rate of hydroxylation of L-m-tyrosine was approximately 25% of that of phenylalanine.

In Table 8 are shown the results on the hydroxylation of L-m-tyrosine by partially purified preparations of rat liver phenylalanine hydroxylase and some other experiments. The conversion of L-m-tyrosine to DOPA by rat liver homogenate could be carried out by the semi-purified preparations of phenylalanine hydroxylase. Upon purification of the enzyme, the reaction, like the hydroxylation of its normal substrate phenylalanine to tyrosine, became absolutely dependent on the presence of the reduced pteridine co-factor ($DMPH_4$). Also it

was observed that the DOPA decarboxylase inhibitor NSD-1055 was unnecessary in experiments with phenylalanine hydroxylase purified through the first ammonium sulfate step. No DOPA was detected in control experiments with boiled enzyme or with the D-isomer of m-tyrosine. The presence of D-m-tyrosine did not affect the formation of DOPA from L-m-tyrosine. No hydroxylation product was detected when L-o-tyrosine was tested as a substrate for the rat liver homogenate or with the partially purified phenylalanine hydroxylase.

The conversion of L-m-tyrosine to DOPA by rat liver was further confirmed by the radioactive assay method as described in Materials and Methods. When L-m-tyrosine was incubated with rat liver homogenate in the presence of S-adenosyl-L-methionine-methyl-^{14}C and NSD-1055, a new radioactive product was detected in the reaction mixture (Fig. 7). The radioactive product was identified as 3-O-methyl-DOPA from its elution time and by co-chromatography with an enzyme assay containing L-DOPA in place of L-m-tyrosine. A small amount of 3-O-methyl-DOPA was also detected from an incubation mixture with L-tyrosine. This is probably due to a small amount of tyrosine hydroxylase present in the rat liver homogenate. No methylation product was found with phenylalanine and o-tyrosine (Table 9). The presence of magnesium ion stimulated

the reaction as this metal ion is required for the activity of catechol-O-methyltransferase. It should be noted that no synthetic pteridine co-factor ($DMPH_4$) was added in the above radioactive assay method. The radioactive assay method was extended to the study of the hydroxylation of L-m-tyrosine by rat brain homogenate. Incubations were carried out in the presence of $DMPH_4$ and tested at three different pH's. However, no hydroxylation product from L-m-tyrosine or L-tyrosine could be detected by this assay method (Table 10).

Table 7

Elution times of aromatic amino acids and some of their Methyl-derivatives on the amino acid analyzer[a]

	0.2 N sodium citrate buffer, pH 4.25		
	50 cm AA-15 resin		15 cm Aminex A-5 resin[b]
	A: 68 ml/h	B: 34 ml/h	C: 34 ml/h
Phenylalanine	86	172	55
Tyrosine	79.5	158	51
o-Tyrosine	90.5	179	56
m-Tyrosine	72	145	46.5
DOPA	66	132	43.5
N-Methyl-m-tyrosine[c]			36
O,N-Dimethyl-m-tyrosine			49
O-Methyl-m-tyrosine			66
3-O-Methyl-DOPA			52
O-Methyl-tyrosine			75.5

a System A used for determining tyrosine, A or B for DOPA, and A, B and C when o-tyrosine was used as substrate.

b Bio. Rad Laboratories, Richmond, Calif.

c Determined from a hydrolysate of O,N-dimethyl-m-tyrosine with hydriodic acid.

Table 8

Formation of DOPA[a] from m-tyrosine

Substrate	Modification to digest	Rat liver[b]			Beef adrenal medulla[c]		
		Homogenate	Second ethanol fraction	First $(NH_4)_2SO_4$ fraction	Homogenate	Particles	40% $(NH_4)_2SO_4$ ppt. from sol. fraction
L-m-Tyrosine		42.1	86.6	193	2.8	3.3	17.4
L-m-Tyrosine	-NSD-1055	0	2.8	178	1.9	2.6	13.8
L-m-Tyrosine	$-DMPH_4$	21.7	8.9	0	0	0	0
L-m-Tyrosine	-NADPH	42.0	87.0	193	3.25	3.5	17.7
L-m-Tyrosine	boiled enzyme	0	0	0	0	0	0
-		0	0	0	0.5	0.7	0.6
L-m-Tyrosine	+D-m-Tyrosine[f]	41.0			3.0		
D-m-Tyrosine		0	0	0	0.55	0.8	0.5
L-o-Tyrosine		0			0.5		
L-Tyrosine		0	0	0	5.8	6.0	35.2
L-Tyrosine	$-DMPH_4$				0	0	0
L-Phenylalanine		49.7[g]	120[g]	293[g]			
L-Phenylalanine	$-DMPH_4$	28.7[g]	17.8[g]	0[g]			

a mμmol/3 h/mg protein; 37°C.
b Digests contained ingredients as described in Materials and Methods (page 65).
c Digests as for rat liver except that the buffer was sodium citrate, pH 6.0, and digest contained 2.5×10^{-4} M ferrous sulfate (page 65).
d Partially purified phenylalanine hydroxylase, as per Kaufman (1962a).
e Partially purified tyrosine hydroxylase, as per Nagatsu et al. (1964).
f 2 μmol.
g Tyrosine, mμmol/30 min.

Table 9

The formation of 3-O-methyl-DOPA from L-m-tyrosine by rat liver homogenate[a]

Substrate	3-O-methyl-DOPA Radioactivity (10^{-2} x d.p.m./mg protein)
-	4.43
L-m-tyrosine	57.10
L-m-tyrosine (-Mg^{2+})	7.43
D-m-tyrosine	4.56
L-tyrosine	9.21
D-tyrosine	4.27
L-o-tyrosine	4.50
D-o-tyrosine	4.23
L-phenylalanine	4.87
D-phenylalanine	4.77

[a] Assay conditions were as described in Materials and Methods (page 66), for 120 min.

Table 10

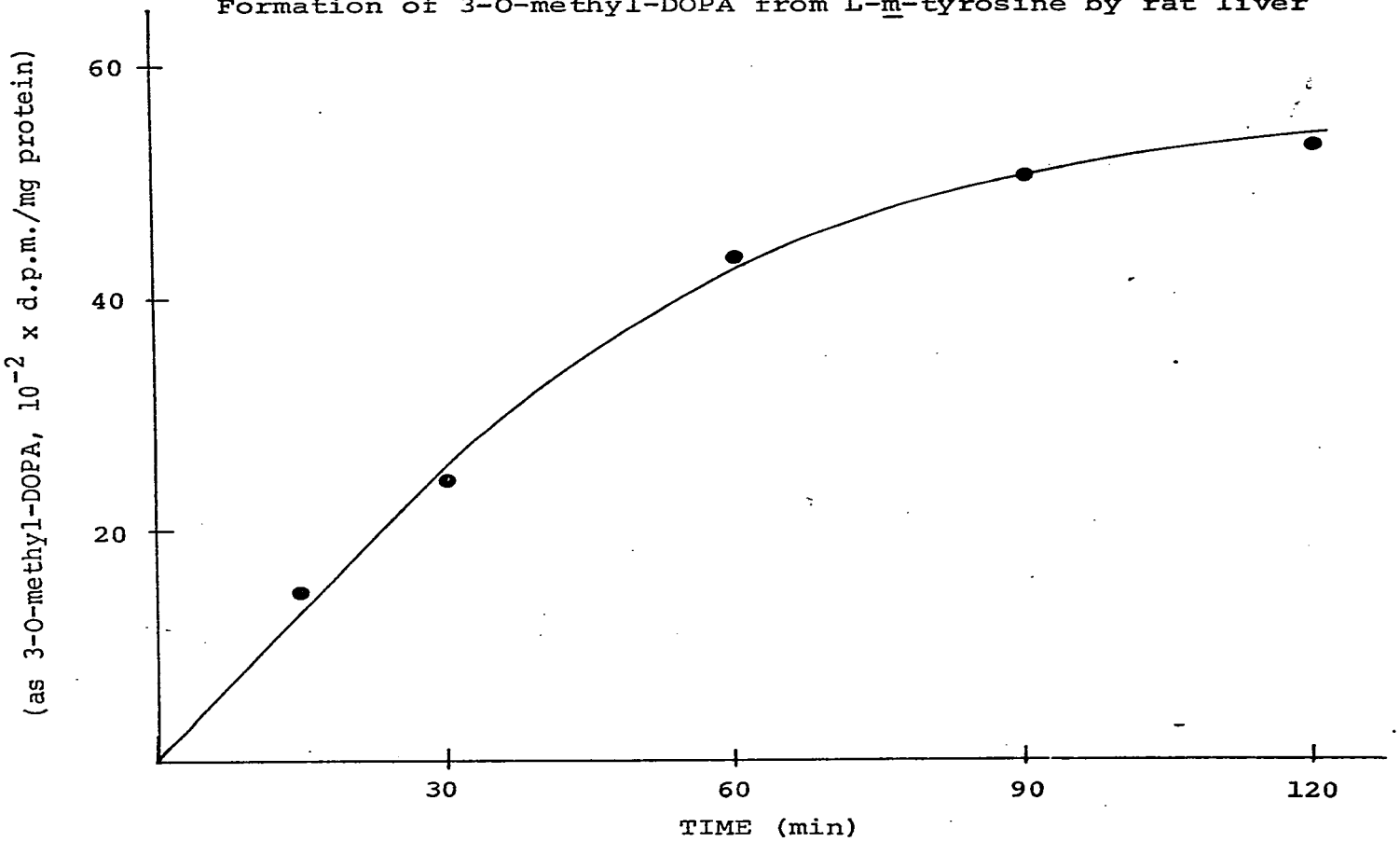
The formation of 3-O-methyl-DOPA by rat brain homogenate[a]

Substrate	3-O-methyl-DOPA Radioactivity d.p.m. x 10^{-2}		
	pH 6.2	pH 7.0	pH 8.0
-	11.21	11.18	11.65
L-DOPA	426.24	522.95	567.57
L-tyrosine	11.62	10.49	11.76
L-m-tyrosine	10.73	11.52	10.98

[a] Incubation of S-adenosyl-L-methionine-methyl-^{14}C with rat brain in the presence of aromatic amino acids. Assay conditions were as described in Materials and Methods (page 66), for 120 min.

Figure 3

Formation of 3-O-methyl-DOPA from L-m-tyrosine by rat liver[a]



[a]Incubation conditions and analytical methods were as described in Materials and Methods (Page 66).

Figure 4

Fluorometric identification of DOPA from the incubation of L-m-tyrosine with rat liver.

(A) Emission (activation at 285 nm; emission at 320 nm) profile of the effluent from a 50 x 0.9 cm AA-15 resin column eluted with 0.2 N sodium citrate, pH 4.25, at a flow rate of 34 ml/h of a 3-h incubation of m-tyrosine with rat liver. (——— L-m-tyrosine; - - - D-m-tyrosine) Incubation conditions were as described in Materials and Methods.

(B) Fluorescence spectra of fractions 33 and 37.

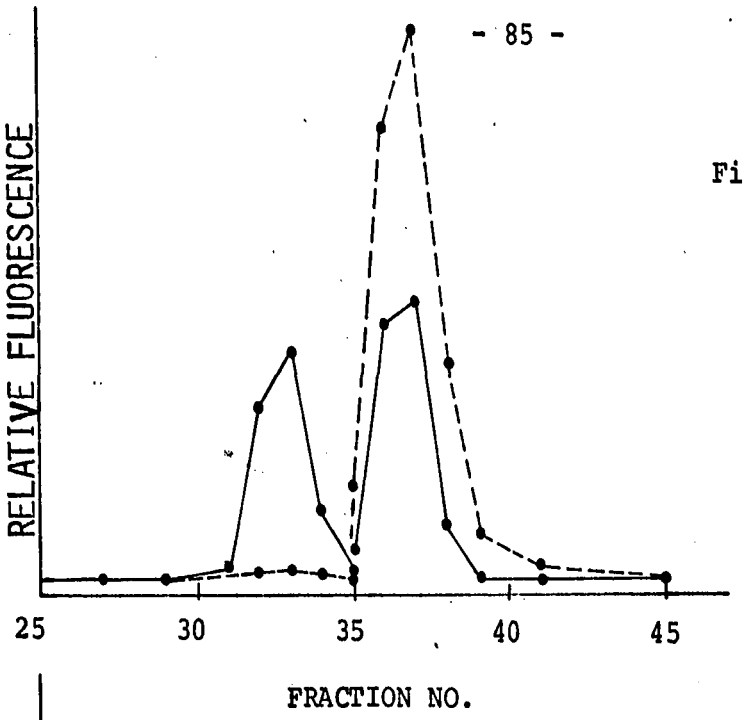


Figure 4A

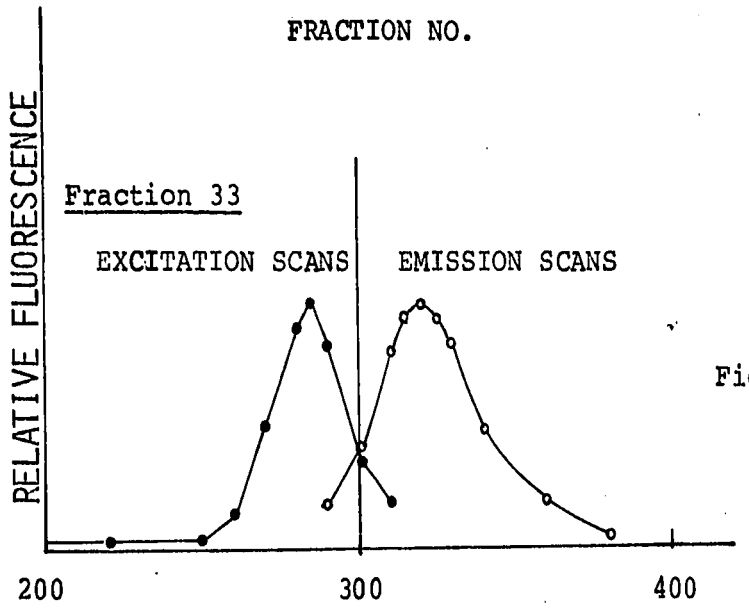


Figure 4B

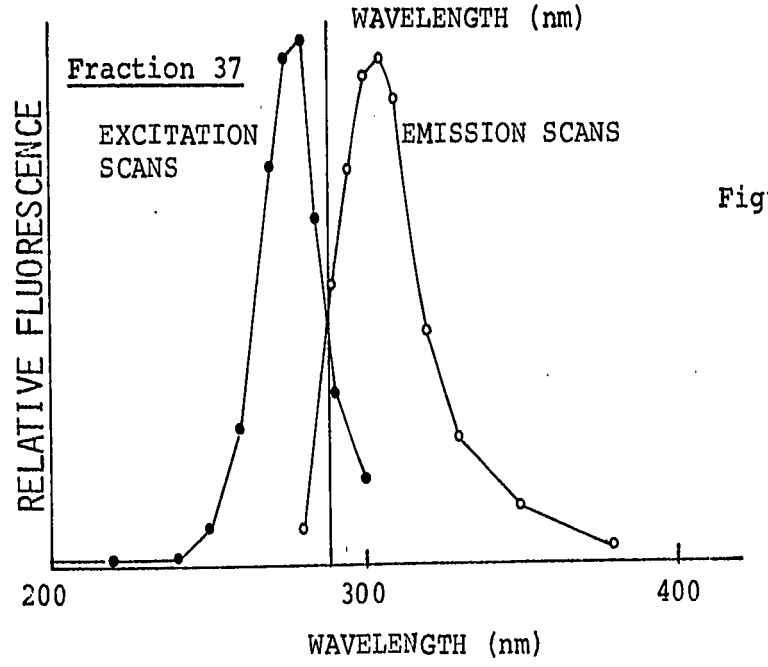


Figure 4B

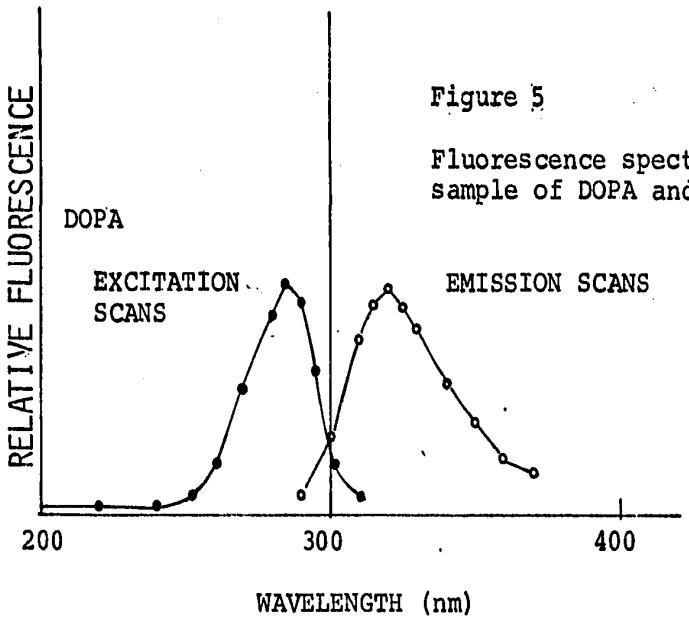


Figure 5

Fluorescence spectra of authentic sample of DOPA and m-tyrosine.

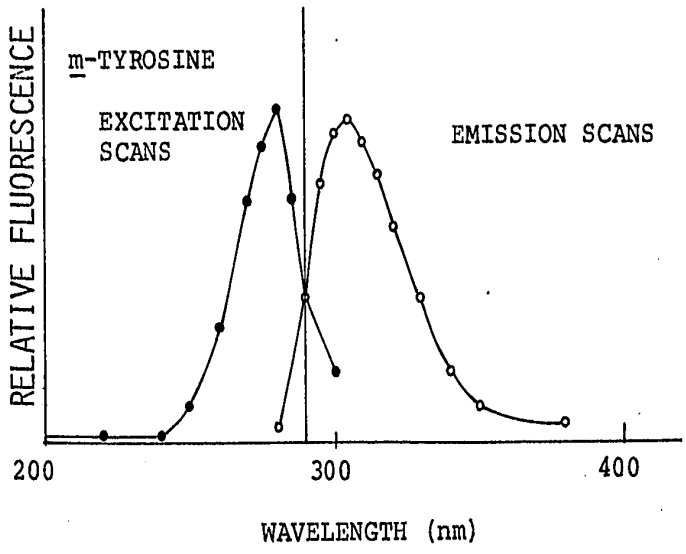
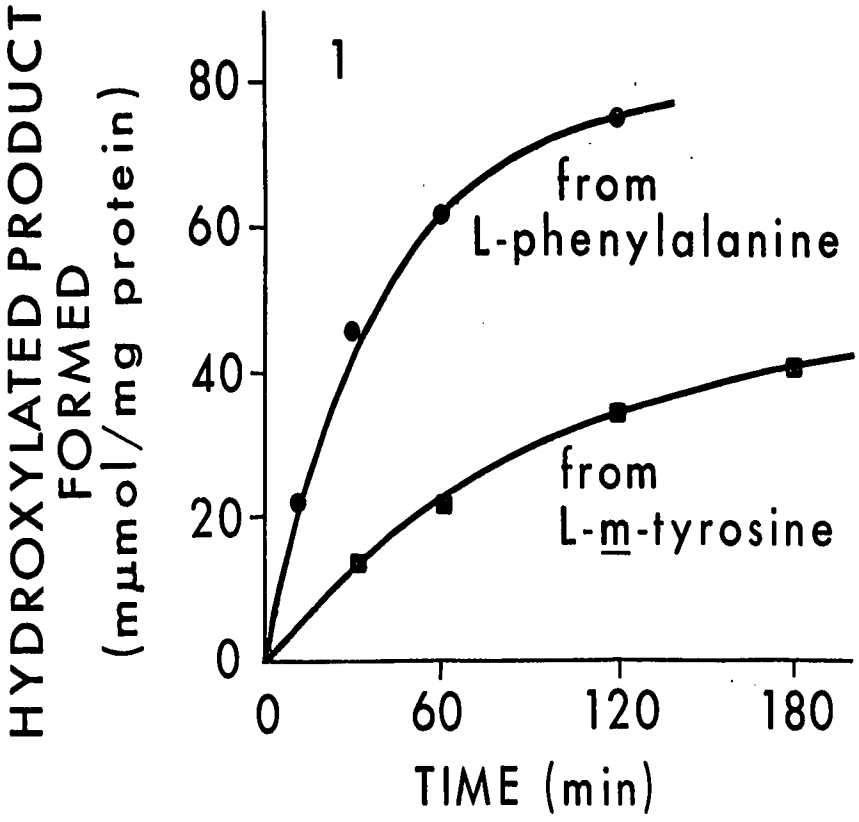


Figure 6

Formation of DOPA from L-m-tyrosine, and tyrosine from L-phenylalanine by rat liver homogenate.



Assay conditions were as described in Materials and Methods (page 65).

Figure 7

Chromatography of a 2 h incubation of m-tyrosine with rat liver in the presence of S-adenosyl-L-methionine-methyl-^{14}C

(A) L-isomer (B) D-isomer. Incubation conditions were as described in Materials and Methods.

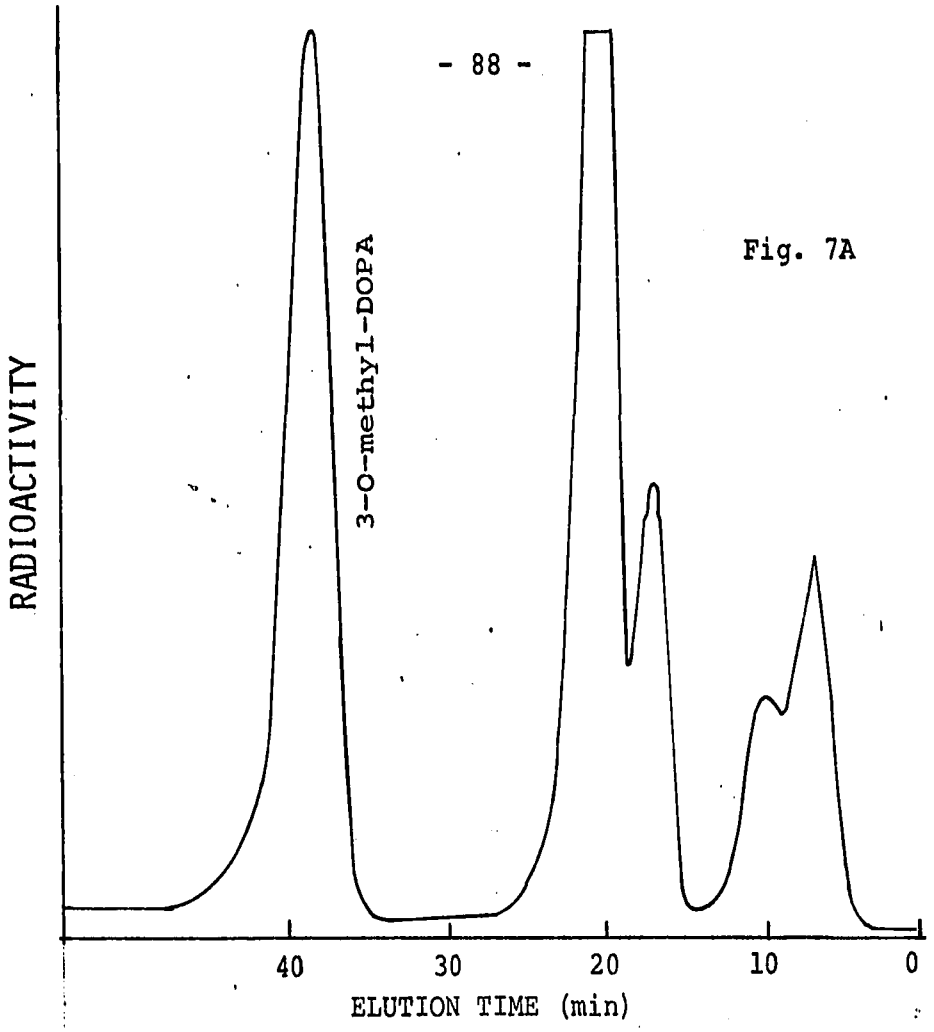

RADIOACTIVITY
3-O-methyl-DOPA
40
30
20
10
0
ELUTION TIME (min)

Fig. 7A

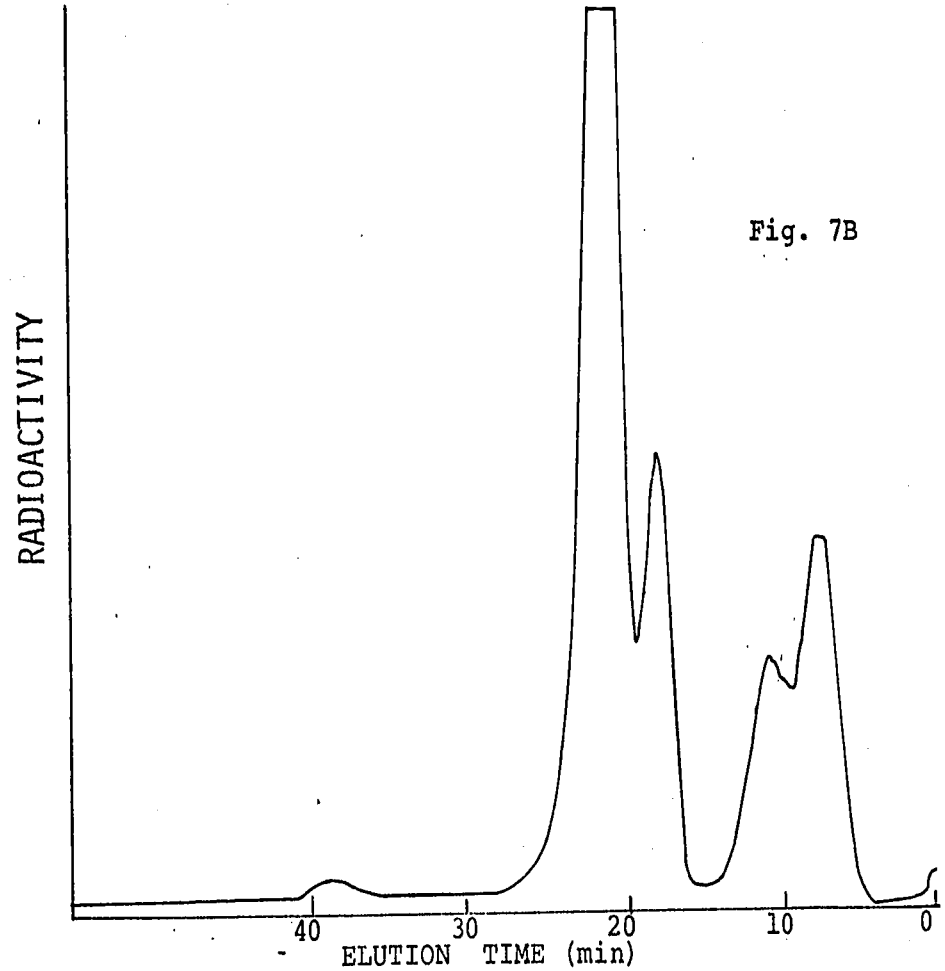

RADIOACTIVITY
40
30
20
10
0
ELUTION TIME (min)

Fig. 7B

Hydroxylation of L-m-tyrosine by beef adrenal medulla

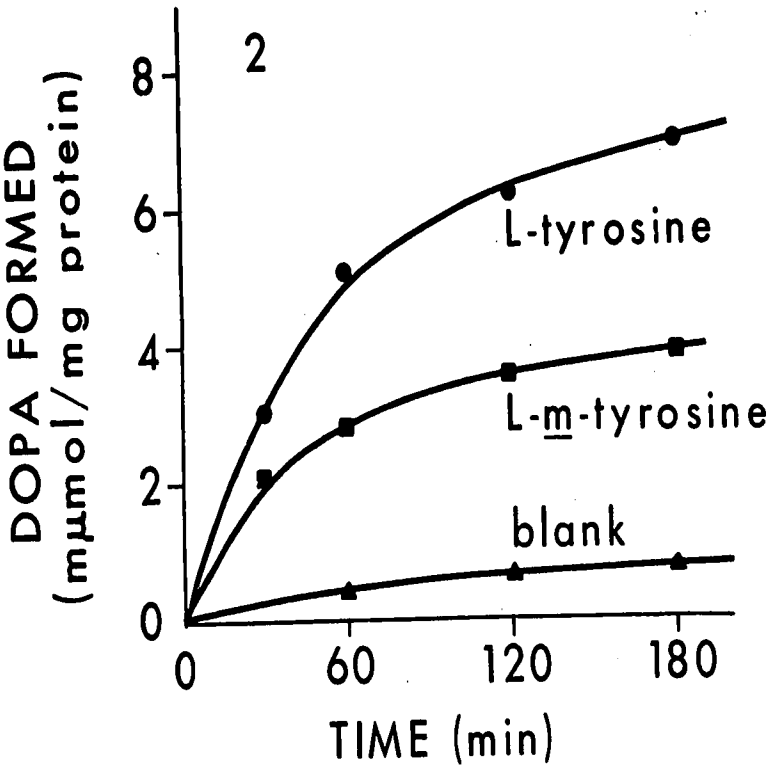
Similar experiments on the hydroxylation of m-tyrosine were carried out with beef adrenal medulla. When L-m-tyrosine was incubated with a beef adrenal medulla homogenate in the presence of $DMPH_4$ and NSD-1055, DOPA appeared in the medium in amounts significantly greater than that which originated from endogenous substrate (Fig. 8). The initial rate of hydroxylation of L-m-tyrosine to DOPA was approximately 60% of that of L-tyrosine.

In Table 8 are shown the results on the conversion of L-m-tyrosine to DOPA by different enzyme preparations of beef adrenal medulla. It is seen that the reaction was dependent on the presence of $DMPH_4$. In contrast to the rat liver homogenate, an appreciable amount of DOPA was formed when L-m-tyrosine was incubated with beef adrenal medulla homogenate in the absence of the DOPA decarboxylase inhibitor NSD-1055. The formation of DOPA from L-m-tyrosine and tyrosine by beef adrenal homogenate could be carried out by the semi-purified preparation of tyrosine hydroxylase and the particles precipitated by high speed centrifugation. The amount of DOPA detected when D-m-tyrosine was added as substrate was not greater than that detected in control

experiments in the absence of added substrate. As in the studies with rat liver the presence of D-m-tyrosine did not affect the formation of DOPA from L-m-tyrosine, nor was a new amino acid detected when L-o-tyrosine was the substrate.

Figure 8

Formation of DOPA from L-m-tyrosine and L-tyrosine by beef adrenal medulla homogenate.



Incubation conditions and analytical methods were as described under Materials and Methods (page 65).

DISCUSSION

It is well established that phenylalanine is converted into tyrosine by phenylalanine hydroxylase of liver (Udenfriend and Cooper, 1952) (Kaufman, 1962a), and into tyrosine and DOPA by tyrosine hydroxylase of brain and adrenals (Ikeda et al., 1965; 1967). Our results from the studies on the hydroxylation of phenylalanine showed that m-tyrosine was also present in a beef adrenal system carrying out the conversion of L-phenylalanine into tyrosine and DOPA. From carefully controlled experiments we demonstrated that neither the well known non-enzymatic hydroxylation of phenylalanine (Hamilton et al., 1964) (Bobst and Viscontini, 1966) (Viscontini and Mattern, 1970) (Coulson et al., 1970) nor the proposed para-dehydroxylation of DOPA (DeEds et al, 1957) (Sandler et al., 1969) could account for the appearence of m-tyrosine. Our conclusion is that the labelled m-tyrosine was formed directly from L-phenylalanine by beef adrenals.

In the presence of α-methyltyrosine and 3-iodotyrosine which are specfic inhibitors of tyrosine hydroxylase (Ikeda et al., 1967) at a concentration of 10^{-3} M, the hydroxylation reactions of L-phenylalanine was completely inhibited. Moreover, the same three hydroxylation products of phenyl-

alanine were observed using a semi-purified preparation of beef adrenal tyrosine hydroxylase. The logical inference is that the hydroxylating reaction of phenylalanine to m-tyrosine and tyrosine were catalyzed by the same enzyme. If this is accepted, it removes the apparent incongruity (Ikeda et al., 1967) that tyrosine hydroxylase oxidizes tyrosine at the meta-position and phenylalanine exclusively at the para-position.

The occurrence of m-hydroxyphenyl acids in animal and human urine has been known for some time (Lederer and Polonsky, 1948) (Bray et al., 1950) (Armstrong et al., 1956a; 1956b) Also, m-tyramine has been reported as a normal constituent of human urine (Jepson et al., 1960) (Perry et al., 1962) (Kakimoto and Armstrong, 1962) (Perry and Schroeder, 1963) (Coward et al., 1964). Our results provide an explanation for the appearance of these m-hydroxyphenyl compounds. The possibility that phenylalanine could give rise to m-tyrosine has been considered before (Jepson et al., 1960) (Ikeda et al., 1967) but was not explored in depth.

It has been shown that the hydroxylation of phenylalanine by rat liver phenylalanine hydroxylase occurs exclusively at the para-position. Our observation that no m-tyrosine was formed from phenylalanine in rat liver agrees with the

results of experiments using partially purified rat liver phenylalanine hydroxylase (Mitoma, 1956), though the formation of m-tyramine as a direct product of the hydroxylation of phenylalanine by liver preparations has been reported (Coulson et al., 1968).

The possibility of a para-hydroxylation reaction of m-tyrosine to give DOPA has been considered in order to explain the similarities in the pharmacological effects of DOPA and m-tyrosine (Pogrund et al., 1961) (Blaschko and Chrusciel, 1960) and to account for the conversion of m-tyrosine to DOPamine (Sourkes et al., 1961). However, it was reported that m-tyrosine was neither a substrate for the rat liver phenylalanine hydroxylase (Kaufman, 1962a) nor a substrate for the beef adrenal tyrosine hydroxylase (Nagatsu et al., 1964), and m-tyrosine has been rejected as an intermediate in the formation of DOPA from phenyalanine partly on this basis (Ikeda et al., 1967)

Based on the results from the studies on the hydroxylation of m-tyrosine, we concluded that L-m-tyrosine can be converted to DOPA by both rat liver and beef adrenals, and that the well known tyrosine hydroxylase and phenylalanine hydroxylase are likely to be responsible for the reaction. Such a transformation has been effected by a soluble, cell-

free preparation from Bacillus cereus and Bacillus thuringiensis (Aronson and Vickers, 1965) however, it has never been shown to take place in a mammalian system.

As in the case of phenylalanine hydroxylase and tyrosine hydroxylase, the conversion of L-m-tyrosine to DOPA requires the presence of a tetrahydropteridine co-factor. The formation of an appreciable amount of DOPA from m-tyrosine by the crude rat liver and beef adrenal homogenates in the absence of the synthetic pteridine co-factor ($DMPH_4$) indicates that the naturally occurring co-factor (tetrahydrobiopterin) (Kaufman, 1963) can act as the co-enzyme for this reaction.

That we could not detect the conversion of m-tyrosine to DOPA by rat brain could be accounted for on the basis that the assay methods used were not sensitive enough to measure the brain tyrosine hydroxylase activity. Thus the possibility that brain tyrosine hydroxylase could carry out such a reaction cannot be excluded.

It is generally accepted that catecholamines are formed in adrenals and brain as a result of hydroxylation of phenylalanine in the para-position to give tyrosine, followed by a meta-hydroxylation to DOPA and subsequent decarboxylation to DOPamine (Meister, 1965a). The conversion of phenylalanine

to DOPA by way of the same reactions in the alternative sequence, that is beginning with the formation of m-tyrosine, has never been shown. Our demonstration of the conversion of phenylalanine to m-tyrosine by beef adrenals and the conversion of m-tyrosine to DOPA by both beef adrenals and rat liver suggests that the alternative biosynthetic route to the catecholamines may indeed be operative in animals.

SECTION III

THE EFFECTS IN VIVO OF L- AND D-p-CHLOROPHENYLALANINE ON THE AROMATIC HYDROXYLATING ACTIVITY OF RAT LIVER.

The pharmacological and behavioral effects of p-chlorophenylalanine (p-CP) are believed to be mediated through its potent depleting action on brain serotonin. Administration of p-CP caused a long-lasting irreversible inhibition of the liver phenylalanine and the brain tryptophan hydroxylases (Guroff, 1969) (Jequier et al., 1967). The mechanism of inhibition of these two hydroxylases is still not clear. Moreover with one exception (Koe and Weissman, 1966), all the studies have been carried out using DL-p-CP; therefore the stereochemical requirements associated with its biochemical and behavioral effects remain unknown. With the availability of the D- and L-isomers of p-CP from our resolution method we decided to investigate the effects of D- and L-p-CP on the two hydroxylation reactions described in the previous section namely, the conversion of phenylalanine to tyrosine, and m-tyrosine to DOPA by rat liver.

MATERIALS AND METHODS

The optical isomers of p-CP were obtained by enzymatic resolution as previously described (page 33). Uniformly ^{14}C-labelled L-tyrosine (367 mCi/mmol) was purchased from New England Nuclear Co. Sources for other materials have already been given (page 61).

Female Sprague-Dawley rats (80 g) were injected intraperitoneally with a suspension of p-CP (180 mg/kg) in 0.9% saline (1 ml). At the times indicated, the animals were decapitated, livers were homogenized in three volumes of 0.9% saline. The homogenates were centrifuged at 22,000 x g for 60 min and the supernatant fluids were assayed for enzymatic activity on the hydroxylation of L-phenylalanine and L-m-tyrosine as previously described (page 65), and for protein content (Lowry et al., 1951). For the assay of the tyrosine hydroxylase activity of rat brain and adrenals, the animals received 369 mg/kg of p-CP, and the tissues were homogenized in 0.25 M sucrose. The enzymatic activity was determined by measuring the radioactivity of the DOPA formed from L-tyrosine-^{14}C. The reaction mixture contained the following components: 0.5 µCi of L-tyrosine-^{14}C, 1.0 µmol $DMPH_4$, 0.3 µmol NSD-1055, 12 µmol of mercaptoethanol, 0.14 ml of buffer

(0.5 N sodium phosphate pH 6.2 was used for the brain homogenate and 0.5 N sodium citrate pH 6.2 was used for the adrenal homogenate), and 0.2 ml of crude homogenate (brain at 100 mg tissue/ml; adrenal at 25 mg tissue/ml), in a final volume of 0.8 ml. The mixtures were incubated for 1 h with shaking at 37°C. The reaction was stopped by the addition of 0.2 ml 35% sulfosalicylic acid. 1 ml of 0.2 N sodium citrate, pH 2.2, was added and the precipitated protein was removed by centrifugation. 1 ml aliquots of the supernatant were analyzed for radioactive DOPA as previouly described (page 63). Boiled tissues served as controls.

RESULTS

The effects of a single dose of D- or L-p-CP on the rat liver phenylalanine hydroxylase activity are shown in Fig.9. It is seen that both isomers of p-CP lowered the enzyme activity to the same extent, down to about 15% of untreated animals after 24 h, and the activity rising back to about 60% after 48 h. Results on the hydroxylating activity of the same tissues towards L-m-tyrosine are shown in Fig. 10. It is seen that the rate of conversion of L-m-tyrosine to DOPA was inhibited by p-CP in the same manner as was observed on the hydroxylation of phenylalanine to tyrosine, that is to the same extent, as well as equally, by the two isomers. In both hydroxylation reactions, racemic p-CP produced the same inhibitory effects as those produced by either of the isomers. In these experiments, the concentration of p-CP ultimately appearing in the assay medium was about 3×10^{-5} M. In a control experiment, it was shown that under the conditions of the assay, p-CP at a concentration of 10^{-3} M inhibited the enzyme by less than 6%. The effect of p-CP on the tyrosine hydroxylase of rat brain and adrenals is shown in Table 11. Animals receiving twice the dose of p-CP showed no change in their brain and adrenal tyrosine hydroxylase activities. For

these experiments, three animals were sacrificed 12 h and 24 h after the injection, and one animals was killed after three consective injections at 24-h intervals. The phenylalanine levels in the tissues of these animals were much higher (100-300%) than in those of untreated animals (Table 11). This is a strong indication that the phenylalanine hydroxylase activity had indeed been inhibited.

Table 11

Effect of p-chlorophenylalanine on the tyrosine hydroxylase activity of rat brain and adrenal

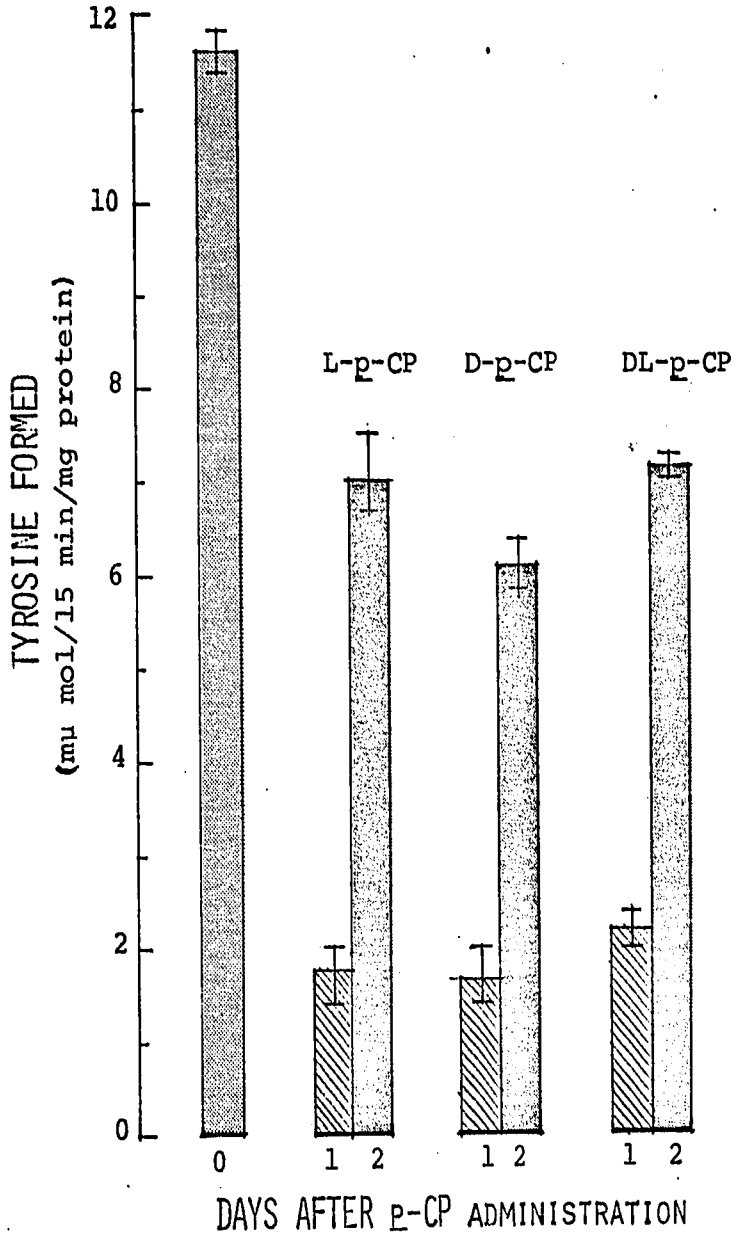
Tissue	Treatment	DOPA formed (d.p.m. x 10^{-2})	Tyrosine (mμmol/g tissue)	Phenylalanine (mμmol/g tissue)
Brain	saline	135.3	197	234
Brain (boiled)	saline	4.5		
Brain	p-CP[a]	130.6	210	453
Brain (boiled)	p-CP[a]	5.1		
Brain	p-CP[b]	137.3	195	654
Brain	p-CP[c]	134.6	177	288
Adrenal	saline	77.9	288	354
Adrenal (boiled)	saline	5.7		
Adrenal	p-CP[a]	76.8	328	803
Adrenal (boiled)	p-CP[a]	4.9		
Adrenal	p-CP[b]	78.1	291	1200
Adrenal	p-CP[c]	75.7	238	427

The values represent averages of three animals except in experiment p-CP where one animal was tested. Assay conditions for tyrosine hydroxylase activity were as described under Materials and Methods (page 98). Tyrosine and phenylalanine concentrations of the tissues were determined by the amino acid analyzer.

[a] Animals were killed 24 h after the injection of p-CP (316 mg/kg).
[b] Animals were killed 12 h after the injection of p-CP (316 mg/kg).
[c] One rat was killed 24 h after the last of three consecutive injections of p-CP (316 mg/kg) at 24 h intervals.

Figure 9

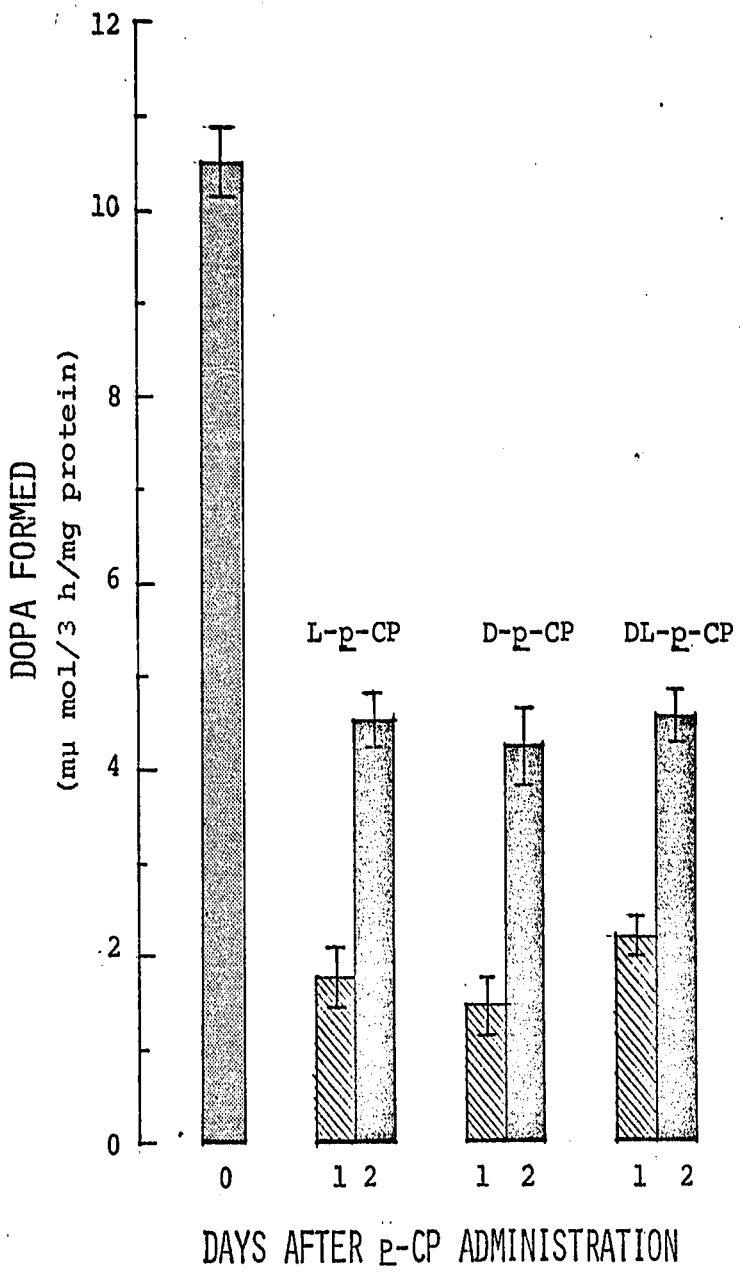
Effects of p-CP administration on the hydroxylation of L-phenylalanine by rat liver



Each bar represents duplicate determinations carried out on two animals.

Figure 10

Effects of p-CP administration on the hydroxylation of L-m-tyrosine by rat liver



Each bar represents duplicate determinations carried out on two animals.

DISCUSSION

The objectives of this study were to investigate the effects in vivo of the optical isomers of p-CP on the hydroxylation reactions of rat liver described in the previous section, namely the hydroxylations of phenylalanine and m-tyrosine.

It has been shown that the administration of p-CP markedly inhibits the hepatic phenylalanine hydroxylase (Koe and Weissman, 1966), and the cerebral tryptophan hydroxylase (Lovenberg et al., 1967), but has no inhibitory effect on the cerebral tyrosine hydroxylase (Koe and Weissman, 1966). Furthermore, this in vivo inhibition of phenylalanine hydroxylase and tryptophan hydroxylase was found to be irreversible and the enzyme activities remained low for a period of four to five days (Jequier et al., 1967) (Guroff, 1969). The parallelism between the serotonin content and tryptophan hydroxylase activity in the brain has been taken as evidence that the potent depleting action of p-CP on brain serotonin is caused by the inhibition of serotonin synthesis, at the level of tryptophan hydroxylase (Jequier et al., 1967).

The present study has shown that p-CP in vivo inhibits the conversion of L-m-tyrosine to DOPA as well as the con-

version of phenylalanine to tyrosine by rat liver. Our results have also shown that the inhibition of these two aromatic hydroxylation reactions is unaffected by steric differences in the p-CP. If p-CP inhibits the cerebral tryptophan hydroxylase in the same manner as we have shown for phenyalanine hydroxylase, (that is by both isomers) this phenomenon could account for the decrease in the serotonin levels of rat brain caused by D- and L-p-CP (Koe and Weissman, 1966). The behavioral effects of p-CP are generally assumed to be mediated through its effects on the brain serotonin. If this is true, they should be unaffected by steric differences in the p-CP. It should now be possible to establish if steric considerations are involved in the behavioral effects of p-CP.

The failure of p-CP to inhibit the tyrosine hydroxylase activity of brain and adrenals was consistent with the results reported previously for brain (Koe and Weissman, 1966) (Gal et al., 1970).

Very little is known about the exact mechanism of enzyme inactivation by p-CP *in vivo*. The *in vitro* inhibition of tryptophan hydroxylase by p-CP has been described as competitive with substrate (Jequier et al., 1967) and this inhibition can be reversed by the addition of a reduced pteridine

co-factor ($DMPH_4$) (Koe, 1967). On the other hand p-CP causes irreversible inactivation of the enzyme *in vivo* which cannot be reversed by the addition of $DMPH_4$. The inhibitory effect of p-CP on phenylalanine hydroxylase *in vivo* is not reversed by dialysis (Lipton et al., 1967) or by the addition of $DMPH_4$ (Koe, 1967) and could be prolonged by the administration of inhibitors of protein synthesis (Guroff, 1969). Whether p-CP or a metabolite thereof, is responsible for the observed *in vivo* effects of p-CP is not known. It has been observed that p-chlorophenylpyruvic acid *in vivo* is equally as active as p-CP (Koe and Weissman, 1966). Gal et al. (1970) have suggested that the inhibitory effect of p-chlorophenylpyruvic acid is solely due to its transamination to p-CP. Recently, it has been proposed that the mechanism of inhibition of aromatic hydroxylases *in vivo* by p-CP could be due to the incorporation of p-CP at an enzymatic site essential for their activity (Gal and Millar, 1971). However any mechanism proposed to explain the action of p-CP must reconcile these results with the apparently identical effects produced by the two optical isomers of p-CP. Oxidation of the D-isomer to the keto analoque, coupled with transamination of the product to the L-isomer, could account for the latter.

In the previous section we have demonstrated the conversion of L-m-tyrosine to DOPA by rat liver and beef adrenals.

The fact that the reaction requires a pteridine co-factor and that m-tyrosine hydroxylating activity is present in partially purified preparations of tyrosine hydroxylase and phenylalanine hydroxylase, has lead us to suggest that these two aromatic amino acid hydroxylases could be the enzymes responsible. The nonstereo-dependent inhibition of the hydroxylation of both phenylalanine and m-tyrosine by p-CP observed here, offers further evidence that it is not necessary to invoke the presence of a new enzyme to account for the conversion of m-tyrosine to DOPA by rat liver.

SECTION IV

THE AWAKENING EFFECT OF L-m-TYROSINE ON RESERPINE-TREATED MICE

INTRODUCTION

The depletion of catecholamines and serotonin from brain and from peripheral sites in animals by reserpine has been well established (Carlsson et al., 1957a) (Pletscher et al., 1955). The tranquilizing effect of reserpine is thought to be due to its depleting action on the catecholamine stores in the brain. Carlsson et al. (1957b) have shown that DOPA can antagonise the sedative action of reserpine while 5-hydroxytryptophan does not. The awakening effect of some ring-substituted phenylalanines on reserpine-treated mice has been investigated by Blaschko and Chrusciel (1960). They showed that 2,3- and 2,5-dihydroxyphenylalanine and D-DOPA did not have any awakening action on reserpine-treated animals. However, when m-tyrosine was administered to reserpine-treated animals, they observed an awakening effect similar to that of L-DOPA. The availability of the optical isomers of m-tyrosine prompted us to investigate the effects of D- and L-m-tyrosine on reserpine-treated mice.

MATERIALS AND METHODS

The plan of the experiments was similar to that of Carlsson et al. (1957b). Female Swiss mice (10-12 g) in groups of four were injected intraperitoneally with reserpine from Nutritional Biochemicals Corp (30 mg/kg). After 17 h, when the animals were markedly tranquilized and showed complete eyelid ptosis, 0.5 ml of amino acid (Table 12) suspended in 0.2% ascorbic acid was administered intraperitoneally. The amino acids used were obtained as previously described (page 35).

RESULTS AND DISCUSSION

The effects of the tyrosine analogues on the sedative action of reserpine are given in Table 12. The awakening effect of L-m-tyrosine in these experiments confirms the observations of Blaschko and Chrusciel (1960). The response of reserpine-treated mice to L-m-tyrosine was essentially the same as observed for L-DOPA, except that the beginning of the effect was a bit later and that the hyperactivity developed by the animals was less pronounced. Within 15-30 min after a dose of 300-1000 mg/kg of L-m-tyrosine the eyelids opened and the animals resumed normal activity. This was followed by a period of hyperactivity during which the animals were more active than normal. The animals gradually returned to the tranquilized state after approximately 3 h. D-m-tyrosine at a dose of 500 mg/kg did not have any awakening action on the reserpine-treated animals. Also the D- or L-isomers of tyrosine and o-tyrosine (500 mg/kg) had no awakening effect.

While some workers believe that the antagonistic action of L-DOPA on reserpine-treated animals is due to the increase in the 3,4-dihydroxyphenylethylamine (DOPamine) level in the brain (Everett and Wiegand, 1962) (Blaschko and Chrusciel, 1960), the mechanism responsible for the awakening effect of

m-tyrosine is not clear. Blaschko and Chrusciel (1960) suggested that the anti-reserpine action of m-tyrosine could be effected either by its conversion to DOPamine or to m-tyramine. The experiments here do not permit us to distinguish between the two alternatives. However, our results in section IIB (page 59) provides an explanation for the conversion of m-tyrosine to DOPamine. That D-m-tyrosine did not produce any awakening action is not at variance with the idea that the active agent could be an amine derived from m-tyrosine.

The earliest trials of DOPA for the treatment of Parkinsonism (Barbeau et al., 1962) (Birkmayer and Hornykiewicz, 1962) were motivated by the following reasons: (a) DOPamine may act as an independent neurotransmitter rather than as a precursor of norepinephrine (Carlsson, 1959); (b) DOPamine levels in patients with Parkinsonism were significantly lower than normal (Ehringer and Hornykiewicz 1960); (c) administration of DOPA would replenish the concentration of DOPamine and favour restoration of function. Whether DOPamine is indeed the anti-Parkinsonism agent was recently questioned by Sourkes (1970). The depleting action of reserpine on the catecholamine stores in brain induces a state of rigidity and akinesia in animals which is similar to that

observed in human Parkinsonism. Thus reserpine is a useful tool in the study of experimental Parkinsonism, and chemicals which can overcome the actions of reserpine may be of value in the treatment for Parkinsonism. Our demonstrations of the conversion of L-m-tyrosine to DOPA by rat liver and beef adrenals and that only the L-isomer of m-tyrosine is effective in reversing the tranquilized state in reserpine-treated mice have induced us to suggest consideration of L-m-tyrosine for the treatment of Parkinsonism. During the course of this work, Sandler et al. (1971) also suggested the possibility of using m-tyrosine for the treatment of Parkinsonism. Their proposal was based on the observation of an increased excretion level of m-tyramine in Parkinson patents treated with L-DOPA. Since then, there have appeared in Chemical Abstracts two patent applications on the treatment of Parkinsonism with m-tyrosine (Bamberg and Sjoberg, 1971) and m-tyrosine alkyl esters (Carlsson et al., 1971). Whether L-m-tyrosine will be proven to be an effective drug for Parkinsonism remains to be seen.

Table 12

Reversal of the tranquilizing effect of reserpine by L-m-tyrosine[a]

Amino acid administered	Dose (mg/kg)	Time (min) of maximum[b] effect	Time (min) when effect begins to wear off	Time (min) of return to tranquilized state
L-DOPA	500	30-60	120	180
L-DOPA	1000	30-60	150	210
L-Tyr	500		no awakening effect	
D-Tyr	500		no awakening effect	
L-m-Tyr	300	60-90	90	150
L-m-Tyr	500	60-90	120	180
L-m-Tyr	1000	60-90	150	210
D-m-Tyr	500		no awakening effect	
L-o-Tyr[c]	500		no awakening effect	
D-o-Tyr[d]	500		no awakening effect	

a Groups of 4 female Swiss mice (10-12 g) were injected intraperitoneally with reserpine (30 mg/kg; suspended in 0.2% ascorbic acid), and 17 h later, with 0.5 ml of amino acid (suspended in 0.2% ascorbic acid).

b Indicates complete awakening, with hyperactivity. The effect of L-m-tyrosine was identical with that of L-DOPA, but not as vigorous.

c Convulsions observed between 30-120 min; ptosis persisted.

d Three animals died after 90 min.

SECTION V

THE TRANSAMINATION OF RING-SUBSTITUTED PHENYLALANINES

INTRODUCTION

The reversible reaction of transamination is essential for the catabolism of amino acids as well as for their biosynthesis. It is generally accepted that the first step in the degradation of D-amino acids is their deamination by D-amino acid oxidase (Meister, 1965b). The asymmetric re-amination of the α-keto acid formed, to the L-amino acid can account for their biological actions and some of their metabolites which are otherwise limited to the L-isomer. Such a re-amination reaction could possibly involve transamination. Aminotransferases may therefore play a part in the conversion of D- to L-isomers. The susceptibility of ring-substituted phenylalanines to transamination is important because several of them are of biological interest. However, information of the transamination reaction of these amino acids is incomplete.

MATERIALS AND METHODS

2-Oxoglutaric acid (α-ketoglutaric acid), oxaloacetic acid, sodium pyruvate, and pyridoxal 5'-phosphate were purchased from Sigma Chemical Co. L-Tyrosine was obtained from Nutritional Biochemicals Corp. Sodium α-ketoglutarate-5-^{14}C (17.5 mCi/mmol), and glutamic-^{14}C (U) acid (10 mCi/mmol) were purchased from Nuclear Chicago. The optical isomers of p-chlorophenylalanine (p-CP), p-fluorophenylalanine (p-FP), o-tyrosine, and m-tyrosine were obtained by resolution of their racemic ethyl esters using chymotrypsin (page 32).

Sprague-Dawley female rats (150-165 g) were fasted for 16 h and killed by decapitation. The livers were removed and homogenized in three volumes of 0.25 M sucrose using a motor-driven teflon-pestle glass homogenizer. The homogenate was centrifuged at 600 x g for 10 min to remove cell debris and nuclei, and the mitochondria were then collected by centrifugation at 15,000 x g for 15 min. The post-mitochondrial fraction was centrifuged at 27,000 x g for one hour. The supernatant was fractionated with ammonium sulfate according to Jacoby and La Du (1964). The cytosol tyrosine aminotransferase refers to that fraction precipitating between 40% and 60% saturation. The mitochondrial tyrosine aminotransferase was

prepared essentially as described by Miller and Litwack (1971). The mitochondria were washed twice with 0.25 M sucrose, suspended in 0.1 M sodium phosphate buffer pH 7.6 containing 0.01 M pyridoxal 5'-phosphate and 0.1 mM α-ketoglutarate and the suspension was stored at -20°C for 48 h. After allowing the preparation to thaw, it was homogenized in a Waring Blendor for 2 min, centrifuged at 27,000 x g for 20 min and the supernatant fluid was fractionated with ammonium sulfate. The precipitate separating out at 30% saturation was discarded and the fraction precipitating at 70% saturation was collected. Both the cytosol and mitochondrial enzymes were suspended in 0.01 M sodium phosphate buffer, pH 7.6, and dialyzed overnight against 500 volumes of 0.01 M sodium phosphate buffer, pH 7.6, containing 0.01 mM pyridoxal 5'-phosphate and 0.1 mM α-ketoglutarate to remove most of the endogenous free amino acids. The dialyzed samples were lyophilized, stored frozen and redissolved in 0.1 M sodium phosphate buffer, pH 7.6, immediately before use. Protein concentration was determined by the method of Lowry et al. (1951). Protein concentrations of the enzyme preparations varied from 12-15 mg/ml for the cytosol enzyme and 3-6 mg/ml for the mitochondrial enzyme.

0.2 ml aliquots of the enzymes were assayed at 37°C,

with 0.5 ml 0.1 M sodium phosphate buffer, pH 7.6, 0.1 µmol pyridoxal 5'-phosphate, 30 µmol keto acids (α-ketoglutarate, oxaloacetate, pyruvate), and 6 µmol aromatic amino acid in a total volume of 2.1 ml. The reaction was stopped with 0.4 ml 35% sulfosalicylic acid. After the addition of 0.5 ml of 0.2 N sodium citrate buffer, pH 2.2, the precipitated protein was removed by centrifugation and 0.5 ml of the supernatant was analyzed for the product amino acid (glutamate, aspartate, and alanine) using the 15 cm Aminex A-5 resin column of a model 120 B Beckman amino acid analyzer eluted with 0.2 N sodium citrate, pH 3.28, at a flow rate of 34 ml/h (Lane and Mavrides, 1971) (Table 13).

For confirming the activities towards L-o-tyrosine and L-m-tyrosine, the following radiochemical assay was also used. This assay method is based on the conversion of α-ketoglutarate-^{14}C to glutamate-^{14}C. The reaction mixture containing 0.3 ml 0.1 M sodium phosphate buffer, pH 7.6, 12 µmol of α-ketoglutarate, 0.1 µmol pyridoxal 5'-phosphate and 0.2 ml of enzyme preparation was preincubated at 37°C for 15 min. The reaction was started with the addition of 6 µmol of aromatic amino acid and 1.0 µCi α-ketoglutarate-^{14}C. The final volume of the incubation mixture was 1.5 ml. After incubation at 37°C for various time intervals, the reactions

were terminated by the addition of 0.5 ml of 35% sulfosalicylic acid and 0.5 ml of 0.2 N sodium citrate, pH 2.2, was added. The mixture was centrifuged. 0.5 ml aliquots of the supernatant were analyzed for radioactive glutamate using the amino acid analyzer system described above, without ninhydrin, coupled to a Nuclear Chicago scintillation flow-cell assembly no. 4526. The identification of the radioactive product as glutamate was confirmed by co-chromatography with the labelled standard. The effluent from the column after passing through the scintillation flow-cell system was collected in 0.5 ml fractions. The fractions corresponding to glutamate-^{14}C were counted in 10% Biosolv scintillation mixture (page 63). Control experiments contained no added aromatic amino acid.

RESULTS

The transamination reactions of m-tyrosine, o-tyrosine, p-CP and p-FP with α-ketoglutarate, oxaloacetate, and pyruvate serving as amino group acceptors were investigated. In Table 14 are shown the results for the transamination of the various aromatic amino acids by both the cytosol and mitochondrial tyrosine aminotransferases of rat liver. With the cytosol enzyme, only L-tyrosine was transaminated in significant amounts utilizing α-ketoglutarate as the amino group acceptor. The results for all other substrate combinations were so low that they could be attributed to contamination by the mitochondrial enzyme (Miller and Litwack, 1969b). On the other hand, the mitochondrial enzyme was active on the five L-amino acid isomers utilizing each of the three keto acids as amino group acceptor. Except for o-tyrosine as substrate, the relative efficiency of the keto acid acceptors was about 0.17 : 0.60 : 1 in the order pyruvate : oxaloacetate : α-ketoglutarate. Progress curves for the reactions with α-ketoglutarate as amino group acceptor are shown in Fig. 11. Comparing the initial rates of the transamination reactions with α-ketoglutarate, m-tyrosine, p-CP, and p-FP were transaminated at rates of 26.4, 19.3, and 15.6% of that for tyrosine, respectively.

The very low but apparently significant results on the transamination of L-o-tyrosine by the mitochondrial enzyme was further investigated using labelled α-ketoglutarate as the keto acid. By measuring the formation of labelled glutamate, a very definite transamination of L-o-tyrosine by the mitochondrial enzyme was demonstrated (Fig. 12), the initial rate being 3% that for L-tyrosine. In Table 15 are shown the results on the transamination of o-tyrosine and m-tyrosine using the radioactive assay method. The results confirmed the resistance to transamination of the D-isomers of m-tyrosine and o-tyrosine by either enzyme, and the transamination of the L-isomers of m-tyrosine and o-tyrosine by the mitochondrial enzyme. The very small amount of activity associated with the cytosol enzyme in the transamination of L-m-tyrosine and L-o-tyrosine could be attributed to cross contamination by the mitochondrial enzyme.

Table 13

Elution times and constants of aspartate, glutamate and alanine from the Beckman amino acid analyzer

	0.9 x 15 cm Aminex A-5 resin, 57°, eluted with 0.20 N sodium citrate (pH 3.28, 34 ml/h)	
Amino acid	Elution time (min)	Constant[a]
Aspartate	35	36.6
Glutamate	41	40.0
Alanine	53	40.0

[a]Peak height times width divided by concentration

Table 14

Formation of glutamate, aspartate and alanine by transamination of aromatic amino acids with α-ketoglutarate, oxaloacetate and pyruvate.[a]

Substrate	Soluble enzyme			Mitochondrial enzyme		
	Glutamate	Aspartate	Alanine	Glutamate	Aspartate	Alanine
-	10.1	19.6	9.6	10.2	16.2	12.8
L-Tyrosine	214	36.5	13.1	610	368	107
L-m-Tyrosine	18.4	27.6	12.4	150	97.7	39.6
L-p-CP	17.6	28.0	12.3	123	86.2	35.5
L-p-FP	17.3	26.5	11.3	85.4	60.5	22.4
L-o-Tyrosine	12.0	20.0	11.0	16.5	19.5	18.7
D-m-Tyrosine				10.0		
D-p-CP				10.2		
D-p-FP				10.1		

[a] mμmol/2h/mg protein

Table 15

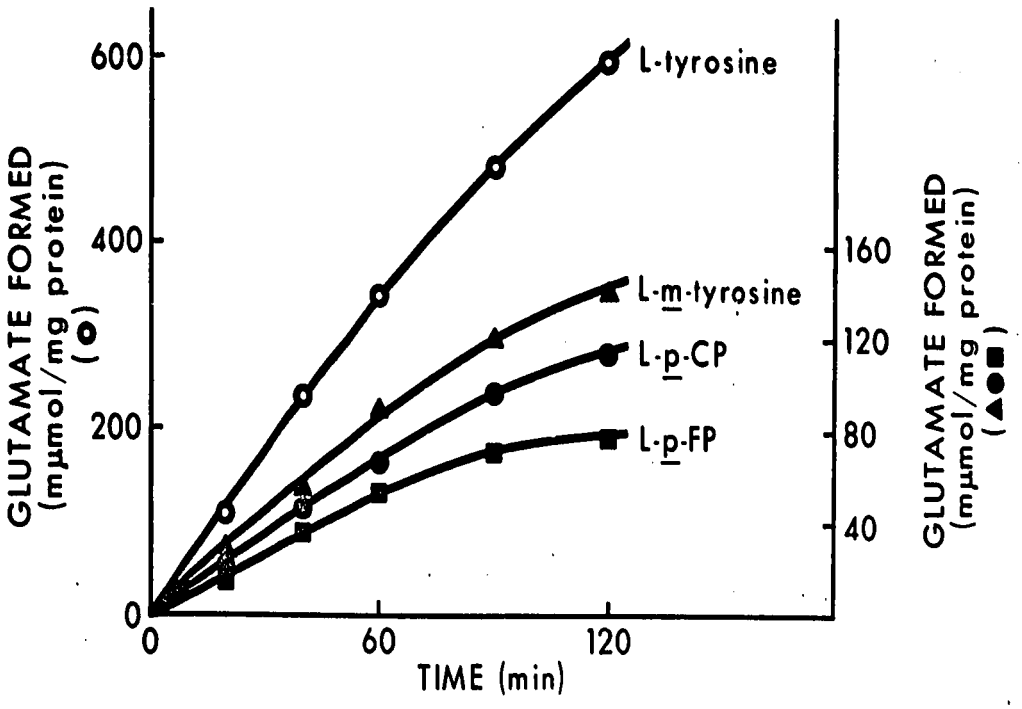
Formation of labelled glutamate from α-ketoglutarate-5-^{14}C and tyrosines.

Substrate	Labelled glutamate formed (d.p.m. x 10^{-2}/mg protein)[a]	
	Soluble enzyme	Mitochondrial enzyme
-	0.4	1.7
L-Tyrosine	88.9	280
L-m-Tyrosine	1.4	76.6
L-o-Tyrosine	0.5	9.4
D-Tyrosine	0.4	1.9
D-m-Tyrosine	0.4	1.8
D-o-Tyrosine	0.4	2.0

[a]Incubations were as described in Materials and Methods, for 2 h.

Figure 11

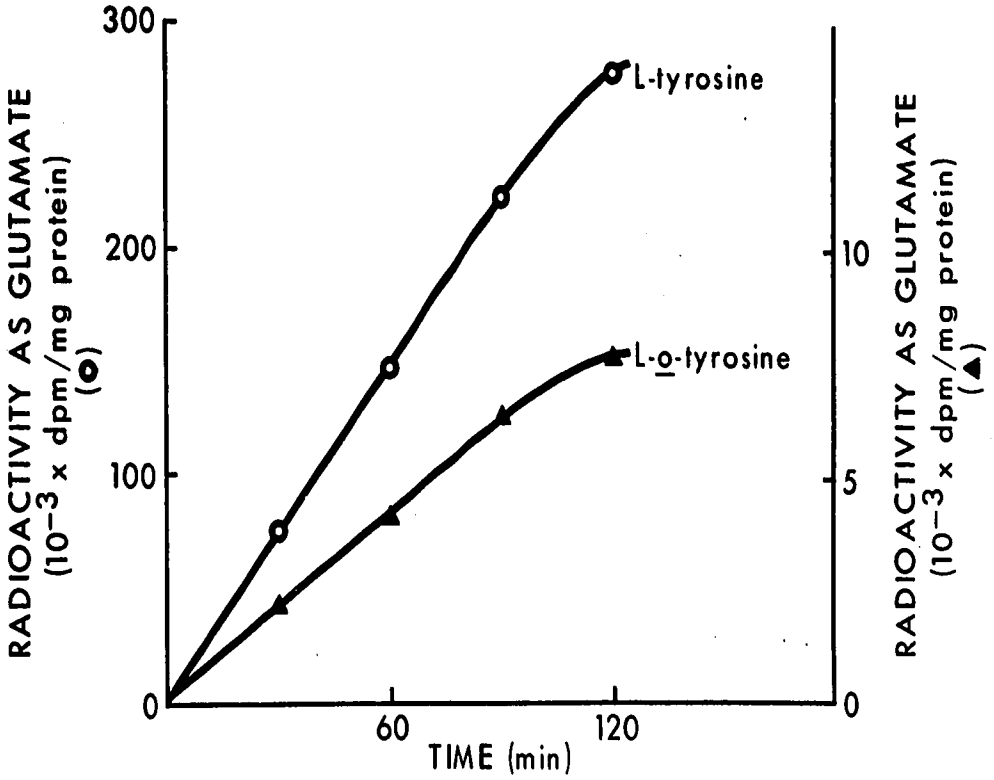
Transamination of ring-substituted phenylalanines with α-ketoglutarate by the partially purified mitochondrial tyrosine aminotransferase.



Assay conditions were as described in Materials and Methods, (page 117).

Figure 12

Transamination of L-o-tyrosine with α-ketoglutarate by the partially purified mitochondrial tyrosine aminotransferase.



Incubation conditions and analytical methods were as described in Materials and Methods (page 118).

DISCUSSION

The data obtained indicate that m-tyrosine, o-tyrosine p-FP, and p-CP are resistant to the action of the cytosol tyrosine aminotransferase of rat liver. However, the transamination of these aromatic amino acids can be effected by the mitochondrial tyrosine aminotransferase, adding a property to the metabolic role of this enzyme. Throughout this section, we have used the expediency of referring to the aromatic amino acid transaminating activity of the mitochondrial fraction used as deriving from mitochondrial tyrosine aminotransferase or the mitochondrial enzyme. The actual existence of an aminotransferase for aromatic amino acids distinct from aspartate aminotransferase (EC 2.6.1.1) and alanine aminotransferase (EC 2.6.1.2) in the mitochondria has been called into question. Miller and Litwack (1971) have extensively purified the tyrosine aminotransferase of rat liver mitochondria, and have found that aspartate is by far its best substrate, that its activity towards tyrosine is completely blocked by aspartate, and that it can be precipitated by antibodies specific to aspartate aminotransferases. Our results shed no light on this question, but are compatible with either view.

The experiments described in this section were initiated

as part of a study investigating the biochemistry of m-tyrosine. m-Hydroxyphenylpyruvic acid has been isolated from the urine of guinea pigs given m-tyrosine (Flatow, 1910). Also in a study on the metabolism of m-hydroxyphenylpyruvic and 3,4-dihydroxyphenylpyruvic acids, Pogrund et al. (1961) demonstrated the in vivo transamination of these two keto acids to m-tyrosine and DOPA. It has been reported that cytosol tyrosine aminotransferase can transaminate DOPA (Canellakis and Cohen, 1956a) (Jacoby and La Du, 1964) (Fellman and Roth, 1971). However, the demonstration of the transamination of m-tyrosine in vitro has been negative (Canellakis and Cohen, 1956a) (Jacoby and La Du, 1964). Our results on the transamination of m-tyrosine by the mitochondrial enzyme can now provide an explanation for the in vivo findings.

In the study on the metabolism of o-tyrosine from our laboratory, the oxidation of D-o-tyrosine by D-amino acid oxidase to o-hydroxyphenylpyruvic acid, followed by the asymmetric transamination of the keto acid to the L-isomer has been proposed to account for the appearance of o-tyramine in the urine of rats injected with D-o-tyrosine (Petitclerc et al., 1969b). The low but definite activity observed using the radiochemical assay for the transamination of L-o-tyrosine by the mitochondrial enzyme establishes

the feasibility of this proposal. The resistance of L-o-tyrosine to the action of the cytosol enzyme is consistent with the observation of Jacoby and La Du (1964) that DL-o-tyrosine was not a substrate for the 500-fold purified cytosol tyrosine aminotransferase.

p-FP has been reported to be transaminated at a rate of 7.9% of that for tyrosine by the cytosol tyrosine aminotransferase (Jacoby and La Du, 1964). Our experiments, however, showed that p-FP cannot be transaminated by the cytosol enzyme but that it can be transaminated, at a rate of 15.6% of that for tyrosine, by the mitochondrial enzyme. The reason for this discrepancy is not obvious. The possibility that their enzyme preparation could have been contaminated by the mitochondrial enzyme is overruled by the fact that their enzyme preparation was not active towards m-tyrosine which is a better substrate than p-FP for the mitochondrial enzyme according to our data.

The transamination of p-CP *in vitro* by rat brain (Gal et al., 1970) and by the purified rat liver mitochondrial tyrosine aminotransferase (Miller and Litwack, 1971) has been reported. Gal et al. (1970; 1971) have proposed that the inhibitory effects *in vivo* of p-CP on the phenylalanine and tryptophan hydroxylases involve the incorporation of

p-CP at or near the active sites of these enzymes, and that the same inhibitory effects produced by p-chlorophenylpyruvic acid are due to its transamination to p-CP. If the mechanism of action of p-CP is indeed as proposed above, our finding that D-p-CP has the same inhibitory effect on the phenylalanine hydroxylase as the L-isomer could be explained by the oxidation of D-p-CP by the D-amino acid oxidase followed by transamination of the keto acid to the L-isomer (page 100). Our finding that p-CP is a substrate for the mitochondrial but not the cytosol enzyme is in accord with the established specificities of the enzyme.

In view of the differences in substrate specificity (Miller and Litwack, 1971), binding between co-enzyme and apoenzyme (Miller and Litwack, 1969b) and sensitivity to inhibitors (Fellman and Roth, 1971) between the cytosol and the mitochondrial tyrosine aminotransferases, and together with the results presented in this section, it is conceivable that aromatic amino acids which are not substrates for the cytosol enzyme may be transaminated by the action of the mitochondrial enzyme.

BIBLIOGRAPHY

Abderhalden, E., Sickel, H., and Veda, H. (1923), Fermentforschung, 7, 91.

Armstrong, M.D., Shaw, K.N.F., and Robinson, K.S. (1955), J. Biol. Chem. 213, 797.

Armstrong, M.D., and Shaw, K.N.F. (1955), J. Biol. Chem. 213, 805.

Armstrong, M.D., Wall, P.E., and Parke, V.J. (1956a), J. Biol. Chem. 218, 921.

Armstrong, M.D., Shaw, K.N.F., and Wall, P.E. (1956b), J. Biol. Chem. 218, 293.

Aronson, J.N., and Vickers, R.S. (1965), Biochim. Biophys. Acta 110, 624.

Awapara, J., Sandman, R.P., and Hanly, C. (1962), Arch. Biochem. Biophys. 98, 520.

Axelrod, J., and Tomchick, R. (1958), J. Biol. Chem. 233, 702.

Axelrod, J. (1965), J. Pharm. Exp. Ther. 149, 16.

Baczuk, R.J., Landram, G.K., Dubois, R.J., and Delm, H.C. (1971), J. Chromatog. 60, 351.

Bamberg. P., and Sjoberg, B.O.H. (1971), Chem. Abst. 75, 129517b.(Ger. Offen. 2,100,445. July 20, 1971.)

Barbeau, A., Sourkes, T.L., and Murphy, G.F. (1962), in "Monoamine et systeme Nerveux Central" (J. de Ajuriaguerra Ed.) p. 247, Masson, Paris.

Barbeau, A. (1969), Can. Med. Ass. J. 101, 343.

Bender, M.L., and Kezdy, F.J. (1965), Ann. Rev. Biochem. 34, 49.

Bennett, E.L., and Niemann, C. (1950a), J. Am. Chem. Soc. 72, 1798.

Bennett, E.L., and Niemann, C. (1950b), J. Am. Chem. Soc. 72, 1800.

Benoiton, N.L., Tong, J.H., D'Iorio, A., and Petitclerc, J.C. (1971), Can. Patent application, no. 109471, April 2, 1971.

Berenyi, E., Budai, Z., Pallos, L., Benko, P., and Magdanyi, L. (1971), Chem. Abs. 75, 49579h. (Ger. Offen. 2,052,953. May 6, 1971)

Bergmann, M., and Fraenkel-Conrat, H. (1937), J. Biol. Chem. 119, 707.

Bergmann, M., and Fraenkel-Conrat, H. (1938), J. Biol. Chem. 124, 1.

Bernheim, F., Bernheim, M.L.C., and Webster, M.P. (1935), J. Biol. Chem. 110, 165.

Birkmayer, W., and Hornykiewicz, O. (1962), Arch. Psychiat. Nervenkr. 203, 560.

Birnbaum, S.M., Levintow, L., Kingsley, R.B., and Greenstein, J.P. (1952) J. Biol. Chem. 194, 455.

Blake, R.L., and Broner, J. (1970), Biochem. Biophys. Res. Commun. 41, 1443.

Blaschko, H. (1939), J. Physiol. London, 96, 50p.

Blaschko, H., Holton, P., and Sloane, S.G.H. (1949), J. Physiol. 108, 427.

Blaschko, H., and Holton, P. (1950), J. Physiol. 110, 482.

Blaschko, H., and Stiven, J. (1950), Biochem. J. 46, 88.

Blaschko, H., and Chrusciel, T.L. (1960), J. Physiol. 151, 272.

Bobst, A., and Viscontini, M. (1966), Helv. Chim. Acta 49, 884.

Booth, A.N., and Williams, R.T. (1963), Biochem. J. 88, 66p.

Boscott, R.J., and Bickel, H. (1953), Scand. J. Clin. Lab. Invest. 5, 380.

Boscott, R.J., and Kirman, B.H. (1955), Biochem. J. 60, iv.

Boyland, E., Manson, D., Solomon, J.B., and Wiltshire, G.H. (1953), Biochem. J. 53, 420.

Bray, H.G., Lake, H.J., Thorpe, W.V., and White, K. (1950), Biochem. J. 47, xiii.

Brenneman, A.R., and Kaufman, S. (1964), Biochem. Biophys. Res. Commun. 17, 177.

Brenner, M., Sailer, E., and Kocher, V. (1948), Helv. Chim. Acta 31, 1908.

Brenner, M., and Kocher, V. (1949), Helv. Chim. Acta 32, 333.

Brenner, M., Muller, H.R., and Pfister, R.W. (1950), Helv. Chim. Acta 33, 568.

Brossi, A., Heveran, J.E., McMullan, E.A., and Senkowshi, B.Z. (1970), Chem. Abs. 73, 80480b. (Ger. Offen. 2,003,101.)

Canellakis, Z.N., and Cohen, P.P. (1956a), J. Biol. Chem. 222, 63.

Canellakis, Z.N., and Cohen, P.P. (1956b), J. Biol. Chem. 222, 53.

Carlsson, A., Rosengren, E., Bertler, A., and Nilsson, J. (1957a), in Psychotropic Drugs (Garattini, S., and Ghetti, V., Eds) Elsevier, Amsterdam.

Carlsson, A., Lindqvist, M., and Magnusson, T. (1957b), Nature 180, 1200.

Carlsson, A., (1959), Pharmacol. Rev. 11, 490.

Carlsson, Per A.E., Corrodi, H.R., and Gaarder, J.O. (1971), Chem. Abs. 75, 151554h. (Ger. Offen. 2,109,657. Sept. 23, 1971.)

Coggins, J.R., and Benoiton, N.L. (1970), J. Chromatog. 52, 251.

Coggins, J.R., and Benoiton, N.L. (1971), Can. J. Chem. 49, 1968.

Cotzias, G.C., Papavasiliou, P.S., and Gellene, R. (1969), New Eng. J. Med. 280, 337.

Coulson, W.F., Henson, G., and Jepson, J.B. (1968), Biochim. Biophys. Acta 156, 135.

Coulson, W.F., Powers, M.J., and Jepson, J.B. (1970), Biochim. Biophys. Acta 222, 606.

Counsell, R.E., Desai, P., Smith, T.D., Chan, P.S., Weinhold, P.A., Rethy, V.B., and Burke, D. (1970), J. Med. Chem. 13, 1040.

Coward, R.F., Smith, P., and Wilson, O.S. (1964), Clin. Chim. Acta 9, 381.

Creveling, C.R., Daly, J.W., Witkop, B., and Udenfriend, S. (1962), Biochim. Biophys. Acta 64, 125.

Cunningham, L. (1965), in Comprehensive Biochem. Vol. 16. Edited by Florkin, M., and Stotz, E.H. : Elsevier, Amsterdam, p. 85, 1965.

Dalgliesh, C.E. (1952), J. Am. Chem. Soc. 137, 3940.

Daly, J., Levitt, M., Guroff, G., and Udenfriend, S. (1968), Arch. Biochem. Biophys. 126, 593.

Daxenbichler, M.A., Van Etten, C.H., Hallinan, E.A., and Earle, F.R. (1971), J. Med. Chem. 14, 463.

DeEds, F., Booth, A.N., and Jones, F.T. (1957), J. Biol. Chem. 225, 615.

DeGraw, J.T., Cory, M., Skinner, W.A., Theisen, M.C., and Mitoma, C. (1967), J. Med. Chem. 10, 64.

Deguchi, T., and Barchas, J. (1971), J. Biol. Chem. 246, 7217.

Deguchi, T., and Barchas, J. (1972), Nature New Biol. 235, 92.

Doherty, D.G., and Popenoe, E.A. Jr. (1951), J. Biol. Chem. 189, 447.

du Vigneaud, V., and Meyer, C.E. (1932), J. Biol. Chem. 98, 295.

Ehringer, H., and Hornykiewicz, O. (1960), Klin. Wschr. 38, 1236.

Ehrlich, F., and Wendel, A. (1908), Biochem. Z. 8, 438.

Ehrlich, F. (1929), Z. Physiol. chem. 181, 140.

Embden, G., and Baldes, K. (1913), Biochem. Z. 55, 157.

Enei, H., Matsui, H., Okumura, S., and Yamada, H. (1971), Biochem. Biophys. Res. Commun. 43, 1345.

Engelman, K., Lovenberg, W., and Sjoerdsma, A. (1967), New Eng. J. Med. 277, 1103.

Erspamer, V. (1959), Arch. Biochem. Biophys. 82, 431.

Everett, G.M., and Wiegand, R.G., (1962), in 1st. Intern. Pharmacol. Meeting, 8, 85. Pergamon Press, Oxford, London, N.Y., Paris.

Fellman, J.H., and Devlin, M. K. (1958), Biochim. Biophys. Acta 28, 328.

Fellman, J.H., Van bellinghem, P.J., Jones, R.T., and Koler, R.D. (1969), Biochemistry 8, 615.

Fellman, J.H., and Roth, E.S. (1971), Biochemistry 10, 408.

Ferguson, J., Henricksen, S., Cohen, H., Mitchell, G., Barchas, J., and Dement, W. (1970), Science 168, 499.

Fischer, E. (1899), Ber. 32, 2451.

Fischer, E. (1900), Ber. 33, 3638.

Flatow, L. (1910), Z. Physiol. Chem. 64, 367.

Fodor, P.J., Price, V.E., and Greenstein, J.P. (1949), J. Biol. Chem. 178, 503.

Fonnum, F., Haavaldsen, R., and Tangen, O. (1964), J. Neurochem. 11, 109.

Fuller, R.W. (1970), J. Neurochem. 17, 539.

Gal, E.M., Roggeveen, A.E., and Millard, S.A. (1970), J. Neurochem. 17, 1221.

Gal, E.M., and Millard, S.A. (1971), Biochim. Biophys. Acta 227, 32.

Gilbert, J.B., Price, V.E., and Greenstein, J.P. (1949), J. Biol. Chem. 180, 473.

Goldenberg, H., and Goldenberg, V. (1950), Arch. Biochem. 29, 154.

Goodall, Mc. C., and Kirschner, N. (1957), J. Biol. Chem. 226, 213.

Greenstein, J.P. (1954), Adv. Protein Chem. 9, 121.

Greenstein, J.P., and Winitz, M. (1961), in Chemistry of Amino Acids. John Wiley and Sons, N.Y., p. 715.

Guroff, G., Kondok, K., and Daly, J. (1966), Biochem. Biophys. Res. Commun. 25, 622.

Guroff, G., Daly, J., Jerina, D.M., Renson, J., Witkop B., and Udenfriend, S. (1967), Science 157, 1524.

Guroff, G. (1969), Arch. Biochem. Biophys. 134, 610.

Hagen, P.J. (1956), J. Pharmacol. Exp. Ther. 116, 26.

Hamilton, G.A., Woo, L., and Workman, R.J. (1964), J. Am. Chem. Soc. 86, 3390.

Harington, C.R., and Randall, S.S. (1931), Biochem. J. 25, 1028.

Hein, G.E., and Niemann, C. (1961), Proc. Nat. Acad. Sci. U.S. 47, 1341.

Hempel, K., and Mannl, H.F.K. (1966), Experientia 22, 689.

Hever, I., and Villanyi, E. (1970), Chem. Abs. 74, 64391p. (Hung. Teljes 383, June 6, 1970.)

Holloway, M.R. (1968), Ann. Rep. Chem. Soc. 65B, 601.

Holten, D., and Kenney, F.T. (1967), J. Biol. Chem. 242, 4372.

Ikeda, M., Levitt, M., and Udenfriend, S. (1965), Biochem. Biophys. Res. Commun. 18, 482.

Ikeda, M., Levitt, M., and Udenfriend, S. (1967) Arch. Biochem. Biophys. 120, 420.

Ingles, D.W., and Knowles, J.R. (1968), Biochem. J. 108, 561.

Iwasaki, Y., and Pitot, H.C. (1971), Life Sci. 10, 1071.

Izumiya, N., and Nagamatsu A. (1952), Bull. Chem. Soc. Japan 25, 265.

Jacoby, A., and La Du, B.N. (1962), Biochem. Biophys. Res. Commun. 8, 352.

Jacoby, A. and La Du, B.N. (1964), J. Biol. Chem. 239, 419.

Jepson, J.B., Lovenberg, W., Zaltzman, P., Oates, J.A., Sjoerdsma, A. and Udenfriend, S. (1960), Biochem. J. 74, 5p.

Jequier, E., Lovenberg, W., and Sjoerdsma, A. (1967), Mol. Pharmacol. 3, 274.

Jequier, E., Robinson, D.S., and Lovenberg, W. (1969), Biochem. Pharmacol. 18, 1071.

Kakimoto, Y., and Armstrong, M.D. (1962), J. Biol. Chem. 237, 208.

Kaufman, S. (1957), J. Biol. chem. 226, 511.

Kaufman, S. (1958a), J. Biol. Chem. 230, 931.

Kaufman, S. (1958b), Biochim. Biophys. Acta 27, 428.

Kaufman, S., and Levenberg, B. (1959), J. Biol. Chem. 234, 2683.

Kaufman, S. (1961a), J. Biol. Chem. 236, 804.

Kaufman, S. (1961b), Biochim. Biophys. Acta 5, 619.

Kaufman, S. (1962a), in Methods in Enzymology (C.H.W. Hirs, Ed.) Academic Press, N.Y., Vol. V, p. 809.

Kaufman, S. (1962b), J. Biol. Chem. 237, pc2712.

Kaufman, S. (1963), Proc. Nat. Acad. Sci. U.S. 50, 1085.

Kaufman, S. (1964a), J. Biol. Chem. 239, 332.

Kaufman, S. (1964b), Trans. N.Y. Acad. Sci. Ser. 2. 26, 977.

Kaufman, S. (1967), J. Biol. Chem. 242, 3934.

Kaufman, S. (1970), J. Biol. Chem. 245, 4751.

Kaufman, S., and Fisher, D.B. (1970), J. Biol. Chem. 245, 4745.

Kenney, F.T. (1962), J. Biol. Chem. 242, 1610.

Koe, B.K. and Weissman, K. (1966), J. Pharmacol. Exp. Ther. 154, 499.

Koe, B.K. (1967), Med. Pharmacol. Exp. 17, 129.

Laduron, P., and Belpaire, F. (1968), Biochem. Pharmacol. 17, 1127.

Lambooy, J.P. (1954), J. Am. Chem. Soc. 76, 133.

Lane, E.A., and Mavrides, C. (1969), Anal. Biochem. 27, 363.

Lane, E.A., and Mavrides, C. (1971), Anal. Biochem. 44, 106.

Lederer, E., and Polonsky, J. (1948), Biochim. Biophys. Acta 2, 431.

Levitt, M., Spector, S., Sjoerdsma, A., and Udenfriend, S. (1965), J. Pharmacol. Exp. Ther. 148, 1.

Lin, E.C.C., and Knox, W.E. (1958), J. Biol. Chem. 233, 1186.

Lipton, M.A., Gordon, R., Guroff, G., and Udenfriend, S. (1967), Science, 156, 248.

Lissitzky, S., and Roques, M. (1957), Bull. Soc. Chim. Biol. 39, 521.

Litwack, G., and Cleland, W.W. (1968), Biochemistry 7, 2072.

Lloyd, T., and Weiner, N. (1971), Mol. Pharmacol. 7, 569.

Loftfield, R.B., and Eigner, E.A. (1966), Biochim. Biophys. Acta 130, 449.

Lovenberg, W., Jequier, E., and Sjoerdsma, A. (1967), Science 156, 248.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951), J. Biol. Chem. 193, 265.

McGeer, E.G., and McGeer, P.L. (1967), Can. J. Biochem. 45, 115.

McGeer, E.G., and Peters, D.A. (1969), Can. J. Biochem. 47, 501.

Manning, J.M., and Moore, S. (1968), J. Biol. Chem. 243, 5591.

Mark, J., Pugge, H., and Mandel, P. (1970), J. Neurochem. 17, 1393.

Meister, A. (1965a), in Biochemistry of the Amino Acids, Academic Press N.Y. Vol. II, p. 909.

Meister, A. (1965b), in Biochemistry of the Amino Acids, Academic Press N.Y. Vol. I, p. 220.

Miller, J.E., and Litwack, G. (1969a), Biochem. Biophys. Res. Commun. 36, 35.

Miller, J.E., and Litwack, G. (1969b), Arch. Biochem. Biophys. 134, 149.

Miller, J.E., and Litwack, G. (1971), J. Biol. Chem. 246, 3234.

Mitoma, C. (1956), Arch. Biochem. Biophys. 60, 476.

Moss, A.R., and Schoenheimer, R. (1940), J. Biol. Chem. 135, 415.

Musacchio, J.M. (1967), Pharmacologist 9, 210.

Musacchio, J.M. (1968), Biochem. Pharmacol. 17, 1470.

Musacchio, J.M., Wurzburger, R.J., and D'Angelo, G.L. (1971), Mol. Pharmacol. 7, 136.

Myers, R.D., and Veale, W.L. (1968), Science, 160, 1469.

Nagatsu, T., Levitt, M., and Udenfriend, S. (1964), J. Biol. Chem. 239, 2910.

Nakamoto, H., Aburatani, M., and Inagaki, M. (1971), J. Med. Chem. 14, 1021.

Neubauer, O. (1909), Deut, Arch. Klin. Med. 95, 211.

Neuberger, A. (1948), Biochem. J. 43, 599.

Niemann, C., and Rapport, M.M. (1946), J. Am. Chem. Soc. 68, 1671.

Nishimura, T., and Gjessing, L.R. (1966), Scan. J. Clin. Lab. Invest. 18, 217.

Overby, L.R., and Ingersoll, A.W. (1951), J. Am. Chem. Soc. 73, 3363.

Overby, L.R., and Ingersoll, A.W. (1960), J. Am. Chem. Soc. 82, 2067.

Parikh, J.R., Greenstein, J.P., Winitz, M., and Birnbaun, S.M. (1958), J. Am. Chem. Soc. 80, 953.

Pasteur, L. (1852), Ann. Chim. Phys. 34, 30.

Pellerin, J., and D'Iorio, A. (1958), Can. J. Biochem. Physiol. 36, 491.

Perry, T.L., Shaw, K.N.F., Walker, D., and Redlich, D. (1962), Pediatrics 30, 576.

Perry, T.L., and Schroeder, W.A. (1963), J. Chromatog. 12, 358.

Perry, T.L., Hestrin, M., MacDougall, L., and Hansen, S. (1966), Clin. Chim. Acta 14, 116.

Petitclerc, C., D'Iorio, A., and Benoiton, N.L. (1969a), J. Labelled Comp. 5, 265.

Petitclerc, C., Benoiton, N.L., and D'Iorio, A. (1969b), Can. J. Biochem. 47, 1147.

Petrack, B., Sheppy, F., and Fetzer, V. (1968), J. Biol. Chem. 243, 743.

Pletscher, A., Shore, P.A., and Brodie, B.B. (1955), Science 122, 374.

Pletscher, A., Burkard, W.P., and Gey, K.F. (1964a), Biochem. Pharmacol. 13, 385.

Pletscher, A., Bartholini, G., Bruderer, H., Burkard, W.P., and Gey, K.F. (1964b), J. Pharmacol. Exp. Ther. 145, 344.

Pogrund, R.S., Drell, W., and Clark, W.G. (1961), J. Pharmacol. Exp. Ther. 131, 294.

Purdie, J.E., and Benoiton, N.L. (1970), Can. J. Biochem. 48, 1058.

Ray, P.D., Foster, D.O. and Lardy, H.A. (1964), J. Biol. Chem. 239, 3396.

Rivlin, R.S., Hollandar, C.S., and Asper, S.P. (1962), Endocrinology 71, 636.

Rubenson, A., (1971), J. Pharm. Pharmacol. 23, 228.

Ryan, W.L., and Elliott, J.A. (1968), Arch. Biochem. Biophys. 125, 797.

Sandler, M., Karoum, F., and Ruthven, C.R.J. (1969), Science 166, 1417.

Sandler, M., Goodwin, B.L., Ruthven, C.R.J., and Calne, B.L. (1971), Nature 229, 414.

Sealock, R.R. (1946), J. Biol. Chem. 166, 1.

Sealock, R.R., Spector, M.E., and Schweet, R.S. (1951), J. Am. Chem. Soc. 73, 5386.

Semba, T., and Civen, M. (1970), J. Neurochem. 17, 795.

Shiman, R., Akino, M., and Kaufman, S. (1971), J. Biol. Chem. 246, 1330.

Sih, C., Foss, P., Rosazza, J., and Lemberger, M. (1969), J. Am. Chem. Soc. 91, 6204.

Sjoerdsma, A., Lovenberg, W., Engelman, K., Carpenter, W.T. Jr., Wyatt, R., and Gessa, G. (1970), Ann. Intern. Med. 73, 607.

Sourkes, T.L., Murphy, G.F., and Rabinovitch, A. (1961), Nature 189, 577.

Sourkes, T.L., and Poirier, L.J. (1966), Can. Med. Ass. J. 94, 53.

Sourkes, T.L. (1970), Biochem. Med. 3, 321.

Stevens, D.A., Resnick, O., and Krus, D.M. (1967), Life Sci. 6, 2215.

Storm, C.B., and Kaufman, S. (1968), Biochem. Biophys. Res. Commun. 32, 788.

Stumpf, P.K., and Green, D.E. (1944), J. Biol. Chem. 153, 387.

Tagliamonte, A., Tagliamonte, P., Gessa, G.L., and Brodie, B.B. (1969) Science 166, 1433.

Taniguchi, K., and Armstrong, M.D. (1968), J. Biol. Chem. 238, 4091.

Tomkins, G.M., Gelehrter, T.D., Granner, D., Martin, D. Jr. Samuels, H.H., and Thompson, B.E. (1969), Science 166, 1474.

Tourian, A., Goddard, J., and Puck, T.T. (1969), J. Cellular Physiol. 73, 159.

Udenfriend, S., and Cooper, J.R. (1952), J. Biol. Chem. 194, 503.

Udenfriend, S. (1966), Pharmacol. Rev. 18, 43.

Valeriote, F.A., Auricchio, F., Tomkins, G.M., and Riley, D. (1969), J. Biol. Chem. 244, 3618.

Viscontini, M. and Mattern, G. (1970), Helv. Chim. Acta 53, 372.

Vogler, K., and Baumgartner, H. (1952), Helv. Chim. Acta 35, 1776.

Warburg, O. (1906), Z. Physiol. Chem. 48, 205.

Webster, M.D., and Bernheim, F. (1936), J. Biol. Chem. 114, 265.

Weinhouse, S., and Millington, R.H. (1948), J. Biol. Chem. 175, 995.

Weitzman, E.D., Rapport, M.M., Mcgregor, P., and Jacoby, J. (1968), Science 160, 1361.

Wicks, W.D., Kenney, F.T., and Leek, L. (1969), J. Biol. Chem. 244, 6008.

Willstatter, R. (1904), Ber. 37, 3758.

Wretlind, K.A.J. (1950), J. Biol. Chem. 186, 221.

Wurtman, R.J., and Axelrod, J. (1967), Proc. Nat. Acad. Sci. U.S. 57, 1594.

Wurtman, R.J., and Larin, F. (1968), Biochem. Pharmacol. 17, 817.

Wurzburger, R.J. and Musacchio, J.M. (1971), J. Pharmacol. Exp. Ther. 177, 155.

Wysong, D.V. (1966), Chem. Abs. 65, 5529a. (U.S. 3,253,023.)