# BRAIN MONOAMINES
# IN PYRIDOXINE DEFICIENCY

A Thesis Presented To
The Faculty of Graduate Studies
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In Partial Fulfillment of the
Requirements For The Degree Of
Master of Science

by
Willa Doreen Le Blancq
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for my mother

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## Abstract

Vitamin $B_6$ or pyridoxine is of particular importance in the nervous system, since pyridoxal phosphate (PALP) acts as a coenzyme for various amino acid decarboxylases. One of these, namely, aromatic L-amino acid decarboxylase, (AADC) is thought to catalyze the decarboxylation of the amino acid precursors of both the catecholamines as well as serotonin. One would expect, then, that one of the effects of dietary pyridoxine deprivation would be a parallel reduction in norepinephrine (NE), dopamine (DA), and serotonin (5-HT) levels in the brain. However, it has been found that there is a very significant reduction in brain 5-HT without alterations in either NE or DA levels of the pyridoxine-deficient growing rat. Although the deficiency was instituted at birth, rats of chronological age of 3 to 8 weeks were used for this study, since the critical period in the development of the central nervous system (CNS) extends from just before birth until the 20th day of life.

The purpose of this thesis is to investigate these nonparallel changes in brain monoamines. It was found that pyridoxine deficiency produced significant reductions in body and brain weights, deep body temperature, and motility, in addition to its effect on brain 5-HT. The specificity and reversibility of the deficiency was established by measurement of body and brain weights and brain 5-HT after dietary restriction and pyridoxine supplementation respectively. Further evidence was provided by experiments in which the deficiency was produced by deoxypyridoxine, a structural analogue and antagonist of pyridoxine. It was found that the lower 5-HT levels did not result from a reduction either in the plasma or the brain

levels of tryptophan (TRY), its precursor amino acid. Neither did they result from a lowered activity of tryptophan-5-hydroxylase (TPOH), the enzyme which is thought to be rate-limiting in 5-HT synthesis. Finally, the possibilities of increased degradation or elimination of 5-HT were also excluded by measurement of 5-HT as well as its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), after administration of pargyline or probenecid respectively. From the results obtained, it seems that the decarboxylation of 5-hydroxy-tryptophan (5-HTP) is decreased in pyridoxine deficiency.

Hence, these observations of a reduction in brain 5-HT without concomitant changes in the catecholamines, offer an experimental set up for the study of factors which regulate brain 5-HT exclusively.

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## Abbreviations

| | |
|---|---|
| AADC | Aromatic L-amino acid decarboxylase |
| $BH_4$ | Tetrahydrobiopterin |
| CNS | Central nervous system |
| COMT | Catechol-O-methyltransferase |
| CSF | Cerebrospinal fluid |
| DA | Dopamine |
| DBH | Dopamine $\beta$-hydroxylase |
| $DMPH_4$ | 6,7-dimethyltetrahydropterin |
| DOPA | 3,4-dihydroxyphenylalanine |
| DOPADC | 3,4-dihydroxyphenylalanine decarboxylase |
| EDTA | Ethylenediamine tetraacetic acid |
| 5-HIAA | 5-hydroxy-indoleacetic acid |
| 5-HT | 5-hydroxytryptamine (serotonin) |
| 5-HTP | 5-hydroxytryptophan |
| 5-HTPDC | 5-hydroxytryptophan decarboxylase |
| LSD | Lysergic acid diethylamide |
| MAO | Monoamine oxidase |
| $6\text{-}MPH_4$ | 6-methyltetrahydropterin |
| NAD | Nicotinamide adenine dinucleotide |
| NADH | Nicotinamide adenine dinucleotide, reduced |
| NE | Norepinephrine |
| OPT | o-pthalaldehyde |
| PALP | Pyridoxal-5'-phosphate |
| TCA | Trichloroacetic acid |
| TOH | Tyrosine hydroxylase |
| TPOH | Tryptophan-5-hydroxylase |
| TRY | Tryptophan |
| TYR | Tyrosine |

# I. Introduction

The thesis has been divided into four main sections - Introduction, Review of the Literature, Experimental, and Discussion. This Introduction explains the general organization of the thesis.

The Review of the Literature is sub-divided into five major sections. The first section deals with the discovery of each of the monoamines. It includes a brief outline of the evidence for their role as neurotransmitters, their topographical localization and possible functions in the CNS. The second and third sections discuss the regulation of monoamine metabolism - serotonin versus the catecholamines. The next section outlines some of the difficulties and discrepancies encountered in studies of the ''aromatic L-amino acid decarboxylase'', including evidence for and against the existence of one enzyme. Finally, a brief conclusion summarizes the Review of the Literature and states the objective of this investigation.

The section entitled Experimental outlines both the methods and the results of this work. The initial methods include the production of pyridoxine deficiency, the criteria used to evaluate the extent of this deficiency, and the basic method for measuring brain monoamine levels. This is followed by several experiments aimed at establishing the specificity and reversibility of pyridoxine deficiency. Measurements of 5-HIAA and TRY were then done to investigate the possibilities of increased 5-HT catabolism and decreased substrate availability respectively. Finally, the method for measuring the activity of TPOH is described. The results of the above experiments are then presented.

Lastly, the Discussion attempts to show that the results of this investigation provide additional evidence for the possible existence of two distinct decarboxylating enzymes involved in monoamine synthesis in the rat brain.

# II. Review Of The Literature

# Serotonin, Norepinephrine And Dopamine As Putative Neurotransmitters

## A. Discovery and Criteria for a Neurotransmitter Role

The biogenic amines are a group of ''putative'' neurotransmitters located within the central nervous system (CNS). This thesis deals with 3 of these amines, namely, the catecholamines, dopamine (DA) and norepinephrine (NE), and the indolealkylamine, 5-hydroxytryptamine (5-HT) or serotonin.

The initial indication that the amines might be important in nervous system function was Lewandowsky's report in 1899 of the similar effects of administering adrenal extract and stimulating sympathetic nerves (Lewandowsky, 1899). The substance secreted by the sympathetic nerves was eventually identified as NE (Van Euler, 1948).

Blaschlo was the first to suggest the biological importance of DA as a precursor of NE (Blaschlo, 1939). However, the lack of a more sensitive method of detection for DA prevented investigation of its possible role as a CNS neurotransmitter. It was the development of the spectrophotofluorometer which permitted its determination in brain, specifically, the basal ganglia (Bertler, 1961). This suggested a possible involvement of DA in the control of movement.

A role of 5-HT in the nervous system was suggested by the observation that the hallucinogen, lysergic acid diethylamide (LSD) inhibits its action on smooth muscle (Gaddum, 1953). Evidence suggesting a role in the CNS was found in the demonstration of a rather specific distribution of 5-HT in the brain (Amin et al, 1954).

More recently, histofluorescent techniques have revealed NE, DA and 5-HT neuronal tracts in the CNS (Hillarp et al, 1966), while specific enzyme assays in addition to various fluorometric analyses have demonstrated the presence

in the CNS of the enzymatic machinery, precursors, and intermediates involved in the synthesis and degradation of these monoamines. A great deal of evidence has accumulated which implicates these monoamines in the etiology of various disorders such as schizophrenia, manic depression, and Parkinson's disease, as well as in maintenance and control of bodily functions such as emotional state, hunger, body temperature, sleep and wakefulness. Such evidence, although impressive, awaits confirmation by electrophysiological, biochemical and pharmacological investigations of the specific cells and/or neuronal tracts involved.

## B. Topographical Localization Studies

The regional mapping out of monoamine neurons in the CNS was made possible due to the development of histochemical fluorescence by Fuxe (1965) and Hillarp (1966). This method involves the reaction of NA, DA, and 5-HT with formaldehyde to form strongly fluorescent isoquinolines (NE and DA) and carbolines (5-HT).

Because the axons contain very low amounts of amines, special procedures have been developed for exact mapping. In order to elevate monoamine levels, monamine oxidase (MAO) inhibitors such as nialamide or pargyline have been injected intracisternally. Also, local injections of amines into the brain have been used. Of particular interest in mapping 5-HT neurons is a 5-HT analogue, 6-hydroxytryptamine. This drug is taken up into 5-HT neurons, and the fluorescent product has, in contrast to that of 5-HT, a very high fluorescence yield (Hokfelt and Ljungdohl, 1972). 6-Hydroxydopamine, a DA analogue, has also become an important

tool in such studies, due to its selective destruction of catecholamine neurons. Another interesting approach involves studies on very young animals. Their monoamine axons contain such extremely high amounts of amines that they can be seen directly in the microscope. For example, Nobin and Bjorklund (1973) have recently completed such a study in human fetuses in which they noted that the organization of the monoamine neuronal tracts are quite similar to those of the rat brain.

Schematic illustrations of the central monoaminergic tracts are seen in Figures 1-4 (Hokfelt and Ljungdohl, 1972). Central NE neurons (Fig. 1) originate from cell bodies in the pons and medulla oblongata, giving rise to descending and ascending tracts. The A6 cell group (locus coeruleus) seems to innervate both the cerebral and cerebellar cortex (- - - - ). There is evidence that one single cell body in the locus coeruleus may innervate both cortices (loc. cit.). The hypothalamus is mainly innervated via the ascending ventral tract originating from the A1 (-.-.-), A5 and A7 (....) cell groups. The spinal cord is innervated by descending tracts from the A1 and A2 cell groups. It is an interesting feature that NE neurons thus give rise to large collateral networks offering a morphological basis for control of activity in widespread brain areas.

Although the 5-HT neural tracts are less well traced (Fig. 4), it seems as if they have the same principal construction as the NA neurons, that is, large collateral systems with widespread innervation areas. The 5-HT cell bodies are localized to the raphe nuclei in the mesencephalon and the medulla oblongata. These cell bodies give rise to descending ( -.-.-) and ascending (- - - -) tracts. There probably also exist short 5-HT neuronal tracts localized mainly in the pons (....).

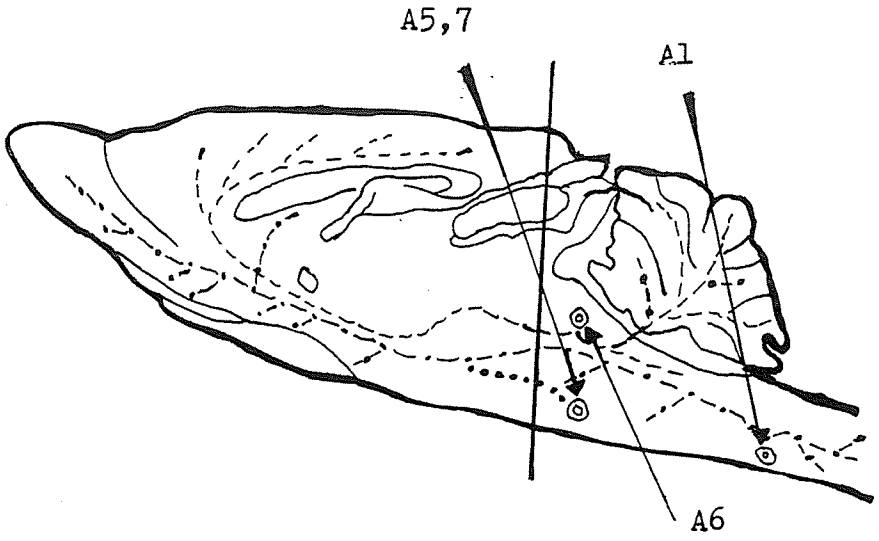



Fig.1 . Schematic illustration of the central NE tracts (Hokfelt and Ljungdohl, 1972).

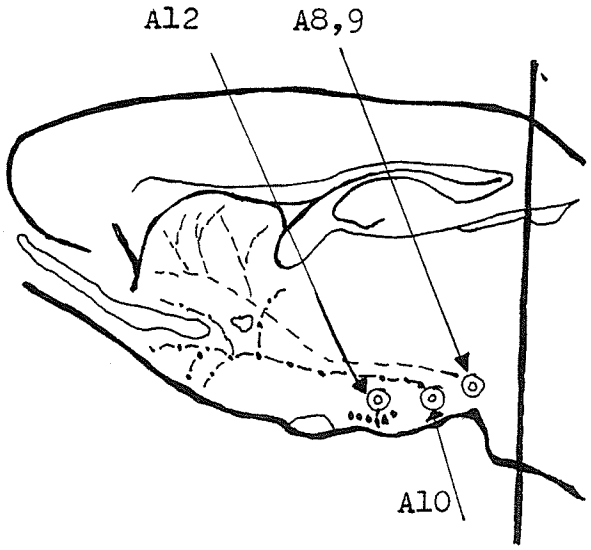



Fig.2 . Schematic illustration of the central DA tracts (Hokfelt and Ljungdohl, 1972).

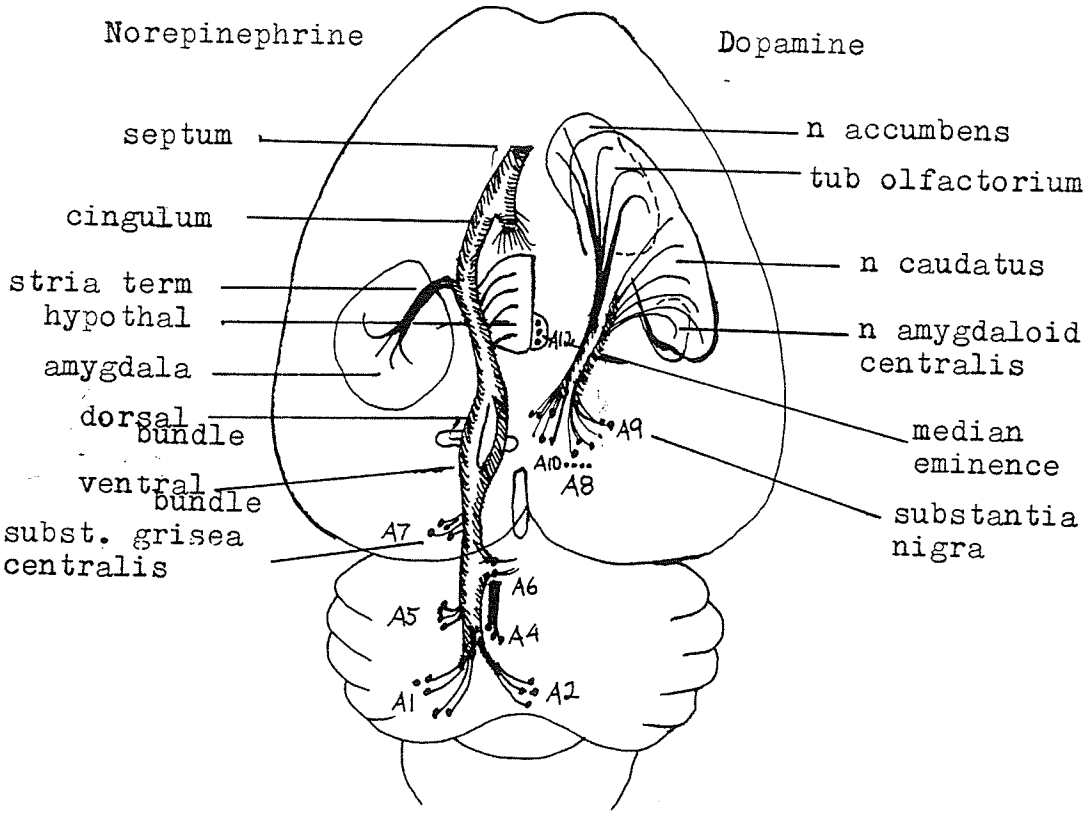



Fig. 3. Horizontal projection of the ascending NE and DA tracts (Hokfelt and Ljungdohl, 1972).

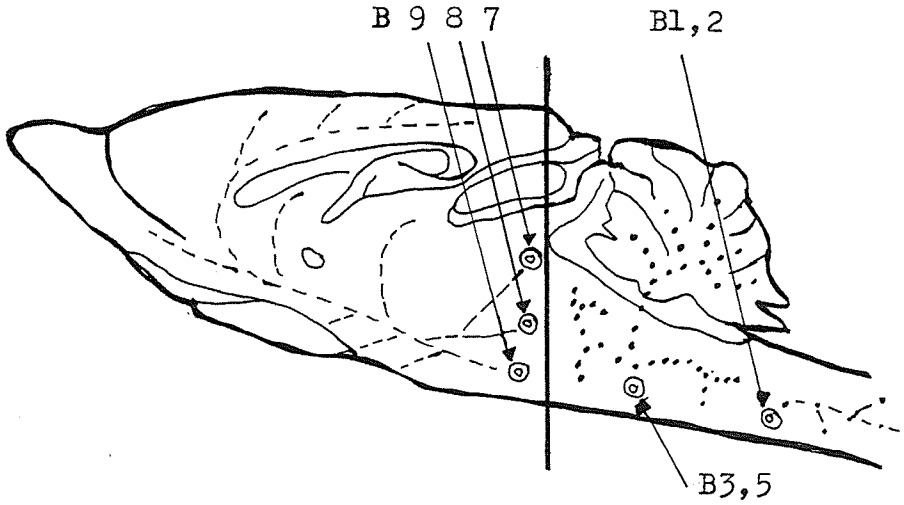



Fig. 4. Schematic illustration of the central 5-HT tracts (Hokfelt and Ljungdohl, 1972).

In contrast, the DA systems have much more restricted innervation areas (Fig. 2). The nigro-neostriatal DA neurons from the substantia nigra (A9, probably also A8) to the neostriatum (- - - -), and the mesolimbic DA neurons from cell group A10 (. - . - .) form two large ascending systems. The best known hypothalamic DA systems are the tuberoinfundibular DA neurons (A12) terminating in the external layer of the median eminence (.... . .). Now the basis of the distinction between the catecholamines and 5-HT in this method is a difference in color, the 5-HT-containing neurons appearing yellow and the catecholamines giving a green fluorescence (Aghajanian, 1972). However, the chemical identity of the ''yellow'' fluorophore in raphe neurons has not been established. One difficulty is the fact that a number of indole compounds, for example, tryptamine and tryptophan, can form yellow fluorophores in the formaldehyde condensation reaction. Their presence in raphe neurons has not been ruled out. Furthermore, Barrett (1972) has reported that p-chlorophenylalanine, a selective depletor of 5-HT, does not seem to alter the fluorescence in the raphe neurons. Such discrepancies must be considered in the interpretation of histofluorescent data.

Nevertheless, more recent histochemical techniques have served to confirm the monoamine localization data obtained with the Falck-Hillarp technique. For example, immunohistochemical techniques have shown that the distribution of dopamine-β-hydroxylase(DBH) correlates exactly with that of NE (Fuxe et al, 1971). In fact, the use of fluorescent labelled antibodies has resulted in the localization of all the major enzymes in monoamine biosynthesis (Goldstein et al, 1972; Hokfelt et al, 1973). Autoradiographic techniques have similarly confirmed that the distribution of

catecholamine and 5-HT uptake sites is in close agreement with that of both endogenous monoamines and of the biosynthetic enzymes (Fuxe et al, 1968; Bloom, 1973; Lapierre et al, 1973).

## C. Functions in the CNS

In terms of distribution of the cell bodies and axons, NE and 5-HT are topographically close, whereas the DA distribution pattern is different. Thus, while central functions such as sleep and wakefulness, emotion, neuroendocrine control, and temperature regulation are ascribed to NE and 5-HT, the DA neurons are mainly involved in motor functions and hypothalamic pituitary control (Hillarp et al, 1966). Turning first to studies concerning functions of NE and 5-HT, it is seen that most work has centered on their role in sleep and temperature regulation.

Jouvet (1972) has proposed a biochemical model of sleep control. He suggests that the occurrence of slow-wave or non-REM sleep depends upon brain levels of 5-HT, while the induction of fast wave or deep REM sleep depends on the combined action of a 5-HT metabolite, NE, and acetylcholine. Jouvet's theory is supported by brain lesion studies (selective destruction of the raphe nuclei), as well as pharmacological studies (administration of monoamine precursors such as tryptophan (TRY), 5-hydroxytryptophan (5-HTP), and dihydroxyphenylalanine (DOPA) and/or enzyme inhibitors such as parachlorophenylalanine, alpha-methylparatyrosine, and pargyline) (Jouvet, 1967, 1969, 1972).

However, several groups of researchers have used approaches similar to those of Jouvet and have come up with opposite effects (Wyatt et al, 1970,1972; Griffiths et al, 1972; Hartmann, 1976). A number of possible reasons have been cited to account for these discrepancies. Two of the major explanations are species differences (Wyatt, 1972) and possible multiple metabolic actions of parachlorophenylalanine (Miller et al, 1970; Mendelson et al, 1975).

Similarly, studies concerning the role of NE and 5-HT in thermoregulation have exhibited various discrepancies. Histochemical studies have shown that NE and 5-HT are present in the nerve terminals in the preoptic area and hypothalamus (Hillarp et al, 1966). Investigators have thus assumed that their injection in these areas will simulate the effects of the endogenous monoamines. This assumption, along with further validation by studies involving the effects of drugs such as reserpine, parachlorophenylalanine, levomethylmetatyrosine, 6-hydroxydopamine, and 5,6-dihydroxytryptamine, led Feldberg and Myers to postulate the monoamine theory of thermoregulation (Feldberg and Myers, 1964; Myers and Yaksh, 1969; Myers,1975). This theory states simply that the control of body temperature depends on the balance between the release of catecholamines and 5-HT in the anterior hypothalamus. The release of 5-HT is thought to activate a heat production pathway which is probably cholinergic in nature, while NE has an opposite effect (Myers and Waller, 1973).

On the other hand, Bligh et al (1971) have found that in sheep, goats, and rabbits intraventricular administration of 5-HT has an effect opposite to that proposed by Feldberg and Myers in the cat, dog, and monkey. They have proposed another model which is based essentially on the effects of ambient temperature (loc. cit.). Other variables

such as species, route and locus of administration, dosage, and even gender and hormonal state have been proposed to account for this lack of agreement in the literature (Bruck, 1976; Lacoste et al, 1976; Burks and Rosenfeld, 1979). In a study by Cronin (1976) on the reversal of parachlorophenylalanine -induced hyperthermia by 5-HTP, it is suggested that there may be both a peripheral and central level of defense acting via the 5-HT brain system against hyperthermia. Perhaps the cause (s) of some of the present discrepancies will be found when more information is available on the actual cells which are affected by NE and/or 5-HT, as well as the inhibitory or excitatory action of each of the monoamines on these cells.

In addition to the extensive research on sleep and thermoregulation, mention must be made of the numerous studies concerning behavior correlates of 5-HT depletion. Reports of 5-HT involvement in general neuroendocrine control include behavior correlates such as sexual and other biological rhythms (Axelrod and Wurtman, 1968; Popova et al, 1972; Benkert, 1976; Hyyppa et al, 1976; Carruba et al, 1977; Hamburger - Bar et al, 1978), and appetite (Blundell and Leshem, 1975; Weinberger et al, 1978; Wurtman and Wurtman, 1979). The role of 5-HT in pain sensitivity has been extensively studied in rats by measurement of their response to painful electric shock (Harvey et al, 1975; Fernstrom, 1976; Hole et al, 1976; Messing et al, 1976). Possible involvement of 5-HT (both peripherally and centrally ) in migraine headaches, migraine therapy, and locomotor activity have been areas of intensive research (Anselmi et al, 1975, 1976; Jacobs and Wise, 1975; Kangasmiemi, 1976). Studies in humans have also revealed abnormalities in platelet MAO activity in a variety of conditions, including arterial hypertension (Sicuteri et al, 1961),

iron deficiency anaemia (Collander et al, 1975), and intestinal carcinoid (Dini and Bartolini, 1971).

Such studies, however, have at times failed to produce convincing results, not only in experimental animals, but also in clinical studies. The problem in studies on humans could very well be a methodological one. Nevertheless, the presence of over 90% of the body's 5-HT in the intestine plagues many investigators and suggests that its role as a putative neurotransmitter may be that of a modulator on various other neurohumoral systems.

In contrast to much of the work on 5-HT, both animal and human studies of the DA system have met with relative success. Interest in the role of DA in brain function was aroused by the finding that a selective loss of this monoamine occurs in Parkinson's disease (Hornykiewicz, 1973). It was found that this loss occurs in the nigrostriatal DA tract which ends in various regions of the corpus striatum, a brain region known to be involved in the central control of movements (Iverson, 1976). The loss can be partly restored by administration of the DA precursor, DOPA (Birkmayer and Hornykiewicz, 1961). More recent treatment involves the simultaneous administration of peripheral DOPA decarboxylase inhibitors such as benserazide and carbidopa (Pinder et al, 1976), and drugs such as amantadine and methylphenidate (Rinne et al, 1973; Hornykiewicz, 1975).

Another possible role of DA (as well as NE and 5-HT) is in the etiology of affective disorders such as depression and mania. Evidence has been derived from four major sources - cerebrospinal fluid, blood, urine, and postmortem studies of depressive suicides. Despite the fact that such investigations present a host of practical as well as theoretical problems (Jimerson et al, 1976), many studies point to a decrease in brain 5-HT and catecholamine con-

centrations in depressive illness (Schildkraut, 1965; Coppen, 1972; Asberg et al, 1975; Coppen et al, 1976), and an increase in both 5-HT and the catecholamines in mania (Murphy et al, 1972; Prange et al, 1974). These studies have led Prange et al (1974) to postulate their ''permissive biogenic amine hypothesis of affective disorders''. The authors maintain that a deficit in central indoleaminergic transmission ''permits'' such disorders, but is insufficient to cause them. However, such a deficit, combined with changes in catecholaminergic transmission, will cause affective disorders - catecholaminergic transmission being elevated in mania and diminished in depression. Unfortunately, there still exist many discrepancies in the literature (Darver and Davis, 1979). Although it is plausible that disorders of monoamine metabolism play a role in the pathogenesis of depression, the exact nature of this role remains obscure.

# Regulation of
# Brain Serotonin Metabolism

## A. Pathways of Synthesis and Degradation

The pathway of major importance in this thesis for the biosynthesis and catabolism of 5-HT in rat brain is illustrated in Figure 5 (Fuller and Steinberg, 1976). Tryptophan (TRY), the amino acid precursor, is first hydroxylated in the 5 position by the enzyme, tryptophan-5-hydroxylase (TPOH), to form 5-hydroxytryptophan (5-HTP). 5-HTP is then almost immediately decarboxylated by an aromatic L-amino acid decarboxylase (AADC or 5-HTPDC) to yield 5-hydroxytryptamine (5-HT). 5-HT is deaminated by the enzyme monoamine oxidase (MAO) to an aldehyde product which is further oxidized to 5-hydroxyindoleacetic acid (5-HIAA) or reduced to 5-hydroxytryptophol depending on the $NAD^+/NADH$ ratio in the tissue.

## B. Tryptophan - 5 - Hydroxylase

Tryptophan-5-hydroxylase (TPOH) (EC 1.99.14) occurs in very low concentrations in brain and is thought to be the rate-limiting step in 5-HT biosynthesis (Jéquier et al, 1967). It appears to have an absolute requirement for molecular oxygen ($O_2$) and reduced pteridine as cosubstrates (loc. cit.). Although the exact chemical structure of the natural pteridine cofactor in brain is not known, studies indicate that it is probably tetrahydrobiopterin ($BH_4$)(Kaufman, 1971). However, most studies have used synthetic cofactors, either 6-methyltetrahydropterin ($6MPH_4$) or 6,7-dimethyltetrahydropterin ($DMPH_4$)(Friedman et al, 1972; Gál and Patterson, 1973). Thus, problems in interpretation arise when kinetic constants using these synthetic cofactors are applied to the in vivo situation. Nevertheless, it seems that the $K_m$ values obtained with $BH_4$ for TRY and $O_2$ are similar to the

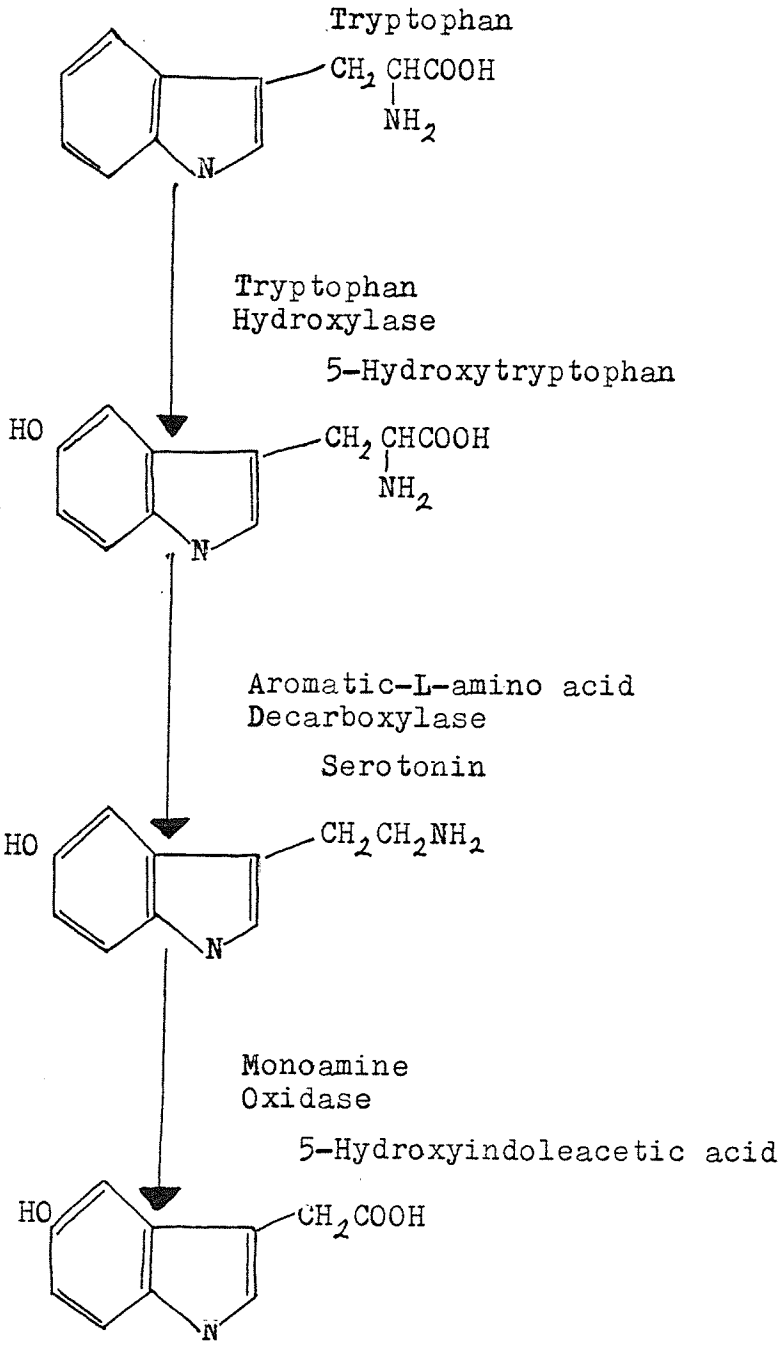



Fig.5. The pathway of 5-HT biosynthesis and catabolism (Fuller and Steinberg, 1976).

concentrations of these substrates in vivo (Costa and Meek, 1974; Gál, 1974). Levine et al (1979) have also reported that the regional distribution of $BH_4$ in rat brain correlates very well with that of TPOH.

Although the level of pteridine cofactor is not limiting for 5-HT synthesis, there is still the possibility that its reduction by dihydropteridine reductase could be a rate-determining factor (Musacchio et al, 1971; Craine et al, 1972). This seems unlikely, however, in view of the fact that it is orders of magnitude more active than the known hydroxylases in brain. Nevertheless, there are no doubt other pterin-dependent reactions in the brain still to be discovered, which could be important in the regulation of the reductase. Also, several studies indicate that $pO_2$ may be involved in the regulation of TPOH (Diaz et al, 1968; Davis and Carlsson, 1973). Inferences seem premature until more is known about the changes which can occur in the regional and cellular distribution of both cofactors.

Another question, the regulatory significance of which must be investigated, is the possible existence of two forms of TPOH in brain. Knapp and Mandell (1972) reported that the TPOH in brain stem is soluble, as opposed to other brain regions where it appears to be particulate, since it is associated with synaptosomes. A more recent study by Meek and Lofstrandh (1976) showed that the in vivo activity of TPOH is 30 times greater in the raphe nuclei ( 5-HT cell bodies) than the hippocampus or caudate (5-HT terminals). The synaptosomal TPOH activity was found to be distributed almost equally between the crude mitochondrial fraction and the supernatant, suggesting the presence of both soluble and particulate enzyme in the synaptosomes (Grahame-Smith, 1967). Furthermore, the lack of inhibition of septal TPOH by either parachlorophenylalanine administra-

tion or by transection of the medial forebrain bundle, suggests that the enzyme which is actively synthesized by the septum may be an isoenzyme of TPOH (Knapp and Mandell, 1972).

Finally, the possibility of TPOH regulation through ''feedback'' inhibition of 5-HT, 5-HTP or 5-HIAA is not likely in view of existing evidence. 5-HT has not been found to inhibit TPOH in vitro (even at levels of $10^{-4}$M to $10^{-3}$M) or in vivo (even after increasing its concentration threefold by MAO inhibition) (Jéquier et al, 1969; Lin et al, 1969; Millard and Gál, 1971). More recently, however, Macon et al (1971) and Hamon et al (1972) demonstrated a 37% reduction in 5-HT synthesis after the concentration of the amine was increased 2.5-fold. Their results are difficult to reconcile with previous work on partially purified TPOH, since their experiments were done both on whole animals and brain (striatum) slices. Their results suggest that perhaps the intracellular environment of TPOH somehow makes it more sensitive to inhibition by 5-HT. Numerous studies have not been able to confirm any negative feedback effect on TPOH by increasing the concentration of 5-HTP up to 0.1mM (Jéquier et al, 1969). Similarly, brain levels of 5-HIAA which have been shown to increase linearly after probenecid administration do not affect 5-HT synthesis (Diaz et al, 1968). Although the enzyme is inhibited by catecholamines, such inhibition is thought to be significant only under artificial conditions such as after administration of large doses of DOPA (Costa and Meek, 1974).

A few words must be said about developmental changes in TPOH activity. Its activity in the neonate rat is approximately 80% of adult activity (Karki et al, 1960). However, this level has been observed to drop to 20% of

adult levels during the first few postnatal days (Baker and Quay, 1969), with a rise to the earlier level only after the fifth week. Since the postnatal development of brain TPOH lags behind that of both AADC and MAO, it is considered to be rate-limiting in the synthesis of 5-HT and probably accounts for the low 5-HT concentrations in the neonate rat. The low TPOH activities during the first few weeks of life may be due to low pteridine availability, but this possibility awaits further investigation (Lanier et al, 1976). There appear to be two forms of the enzyme in the neonate - particulate and soluble - with the soluble form (located in the perikaryal cytoplasm of the brainstem) reaching adult levels of activity before the particulate form (located in the synaptosomes of the cerebellum, cortex, and midbrain) (Hoff et al, 1974). Furthermore, Bourgoin et al (1974) postulate that differences in the relative proportions of these possible free and bound forms of TPOH in newborn rats may account for their finding of an apparent Km for TRY that was twice that of adults. Characterization of these different forms should yield information as to whether they are indeed different isoenzymes of TPOH, or whether their differing rates of maturation are merely a consequence of the time required for axonal growth and synaptogenesis.

C. Availability of Tryptophan

The results of several investigations have opened up the possibility that the rate of tryptophan (TRY) hydroxylation in vivo depends on the availability of the amino acid itself. First of all, the Km for TRY of TPOH (using $DMPH_4$ as cofactor)(Jéquier et al, 1969) was found to be much greater than the concentration of TRY in brain (Fern-

strom and Wurtman, 1971a). Then wnen $BH_4$, the naturally occurring cofactor, was used, the Km, although much lower, still suggested that TPOH is not saturated with its substrate *in vivo* (Friedman et al, 1972). Finally, single injections of L-TRY have been shown to elevate brain 5-HT and 5-HIAA (Ashcroft et al, 1965; Moir and Eccleston, 1968). Such elevations were not only dose-related, but there was also a high degree of correlation between the increases in levels of the two 5-hydroxyindoles (Moir and Eccleston, 1968).

Fernstrom and Wurtman (1971b) have shown that the small daily fluctuations in brain TRY levels are sufficient to influence the rate of synthesis of 5-HT. Furthermore, they have found that a diet low in TRY leads to a decrease in brain TRY and 5-HT (Fernstrom and Wurtman, 1971c; Fernstrom and Lytle, 1976). In their studies of the factors that influence brain TRY concentration, they first of all reported that a single injection of insulin rapidly elevates serum TRY and then brain TRY and 5-HT levels (Fernstrom and Wurtman, 1971c). This led them to hypothesize that the actual uptake of TRY into brain is influenced by competing neutral amino acids in plasma, the serum concentrations of these neutral amino acids showing a decrease after an insulin injection (Fernstrom et al, 1975).

Several studies have shown that another important factor which influences this carrier-mediated transport of TRY into brain is the degree of TRY binding to serum albumin (Knott and Curzon, 1972; Tagliamonte et al, 1973). Although it was predicted that changes in brain TRY levels were proportional to the size of the free or ''available'' TRY pool in serum, it has now been demonstrated (Fernstrom and Wurtman, 1972) that these changes reflect the *ratio* of total plasma TRY to the sum of the competing neutral amino acids

rather than the total TRY concentration of the serum or the smaller free pool (Madras et al, 1974).

Hence, a major controversy exists as to whether or not physiological variables that affect 5-HT synthesis in brain do so by means of a direct effect on brain TRY concentrations. Tagliamonte et al (1971, 1972) have demonstrated that many drugs such as amphetamine, lithium salts, reserpine, and parachlorophenylalanine produce parallel changes in brain TRY and brain 5-HT turnover rate. In addition, a recent study by Fratta et al (1977) has shown that homosexual mounting behavior may be induced in male rats and rabbits by a TRY-free diet. This study is significant in that it suggests an association between reduced brain TRY levels and the behavior itself.

Further complicating factors include evidence (Shields and Eccleston, 1973; Lane and Aprison, 1978) for two pools for the synthesis of 5-HT (which implies two pools of TRY for such synthesis), and then the demonstration by Hery et al (1972) in brain slices that daily changes in 5-HT synthesis are related to changes in TRY transport. Thus, it must be kept in mind when interpreting such studies, that factors other than total brain TRY levels are involved.

It should be added that availability of TRY is not rate-limiting for brain 5-HT synthesis in the neonate rat. TRY levels fall from 300% of the adult value at birth down to 150-200% within the first week, but are still at more than 150% on the sixth week (Gornicki et al, 1963; Baker and Quay, 1969). Bourgoin et al (1974) have shown not only that most of the TRY is in the free form in the plasma of newborn rats, but also that the affinity of the uptake process for TRY is higher in newborns than in adults. Thus, it would be very difficult to explain either reduced levels of 5-HT or its developmental pattern in terms of TRY availability.

D. 5-Hydroxytryptophan Decarboxylase

5-Hydroxytryptophan decarboxylase (5-HTPDC) (EC4.1.1.28) appears to be present in excess so that levels of 5-HTP in brain are very low (Fuller and Steinberg, 1976). Even though earlier attempts to measure 5-HTP fluorometrically yielded low values that were due to interfering compounds (Lindquist, 1971; Carlsson et al, 1972), more recent studies have confirmed that the amounts of endogenous 5-HTP in rat brain are very small (0.23 ± 0.018nmole/g) compared to the levels of 5-HT and 5-HIAA (Aprison et al, 1974). Therefore, it is assumed that 5-HTPDC is present in large excess in both neural and non-neural tissue and is never rate-limiting in the synthesis of 5-HT. *In vivo* evidence (Brodie et al, 1962) supports this in that 5-HTPDC can be inhibited by 90% or more for several hours without affecting the level of brain 5-HT.

However, few studies have dealt specifically with this brain enzyme due to the difficulties encountered when attempts are made at its purification (Lovenberg et al, 1962; Sims, 1974). A relatively high concentration (1 mM) of its coenzyme, pyridoxal phosphate (PALP), is necessary for its stability *in vitro*. Sims et al (1973) have found that the experimental conditions have a great effect on 5-HTPDC activity in brain. Although they did not report any substrate or product inhibition with concentrations as high as 10 mM 5-HTP or 2.0 mM 5-HT respectively, they did find a 40% inhibition of activity when 5 mM DOPA was present (loc. cit.). They concluded that this inhibition of 5-HTPDC by DOPA could be due to the direct interaction of DOPA and PALP as reported by Schott and Clark (1952).

It would seem that 5-HTPDC capacity exceeds the 5-HT concentration during the critical stages of CNS develop-

ment (Baker and Quay, 1969). However, even though its activity is 80-85% of adult levels at birth, there is a sharp drop on the second day of life and adult values are not reached until after the fifth week (Smith et al, 1962; Bennett and Giarman, 1965). Furthermore, Hakanson et al (1967) have reported an early high TPOH level in pineal, indicating that 5-HTPDC is the rate-limiting enzyme during development of this region.

E. Monoamine Oxidase

Another area of controversy, the regulatory significance of which remains to be elucidated, is the possible existence of multiple forms of monoamine oxidase (MAO) (EC1.4.3.4). Since the enzyme is tightly bound to the mitochondrial outer membrane, it is very difficult to solubilize (Achee et al, 1977; Jain, 1977; Lyles, 1978; Murphy, 1978). The soluble enzyme also has different properties from the membrane-bound enzyme. These properties include its kinetic mechanism and its sensitivity towards inhibitors (Houslay and Tipton, 1976). Thus, most present evidence suggests that the multiple forms of MAO which can be separated electrophoretically are mainly a result of the rather vigorous preparative procedures and do not necessarily represent the *in vivo* situation (Neff and Yang, 1974). Furthermore, attempts to clarify the function (neuronal vs non-neuronal) of the two major MAO activities via denervation experiments have met with conflicting results (Goridis and Neff, 1971; Jarrott, 1971). The rise in amine concentrations after selective inhibition of the neuronal MAO species (type A) results in inhibition of the reuptake process (Trendelenburg et al, 1972). However, the presence of the non-neuronal MAO species (type B) in brain as well

as other tissues, and the existence of the neuronal MAO species in poorly innervated tissue suggest an additional function of MAO which is as yet undefined. For example, even though type A is totally absent in pig brain and blood platelets (Long et al, 1976), the latter exhibit active metabolism of 5-HT to 5-HIAA and 5-hydroxy tryptophol.

Discrepancies also arise in the study of the multiple forms of MAO during development. The total MAO activity of the rat brain at birth is less than one-third of the adult value (Kurzepa and Bojanek, 1965). Substrate specificity studies have shown that most of this activity is type A (Mantle et al, 1976), while electrophoresis has revealed that adult brain possesses all the MAO bands seen in neonatal samples and some additional ones (Shih and Eiduson, 1971). In addition, these MAO activities which differ with the age of the animal do not have the same substrate specificity (Gripois, 1975). Although adult levels of MAO are not achieved until 2-3 weeks postpartum, most studies have shown that MAO has a higher relative activity at birth and reaches adult levels sooner than TPOH (Lanier et al, 1976).

## F. Feedback Control Via a Neuronal Loop

Electrophysiological measurement of drug-induced changes in the activity of 5-HT-containing neurons has led to a neuronal loop model for the short-term feedback control of the firing rate of these neurons (Costa and Meek, 1974). Figure 6 illustrates some of the drugs that have been used, as well as their possible sites of action, namely, TPOH, uptake, release, receptors, re-uptake, and MAO (Cooper et al, 1974). For example, extracellular recordings of the raphe nuclei have shown that both MAO inhibitors and LSD decrease the firing rate of serotonergic neurons. However, LSD also decreases the rate of 5-HT turnover via a

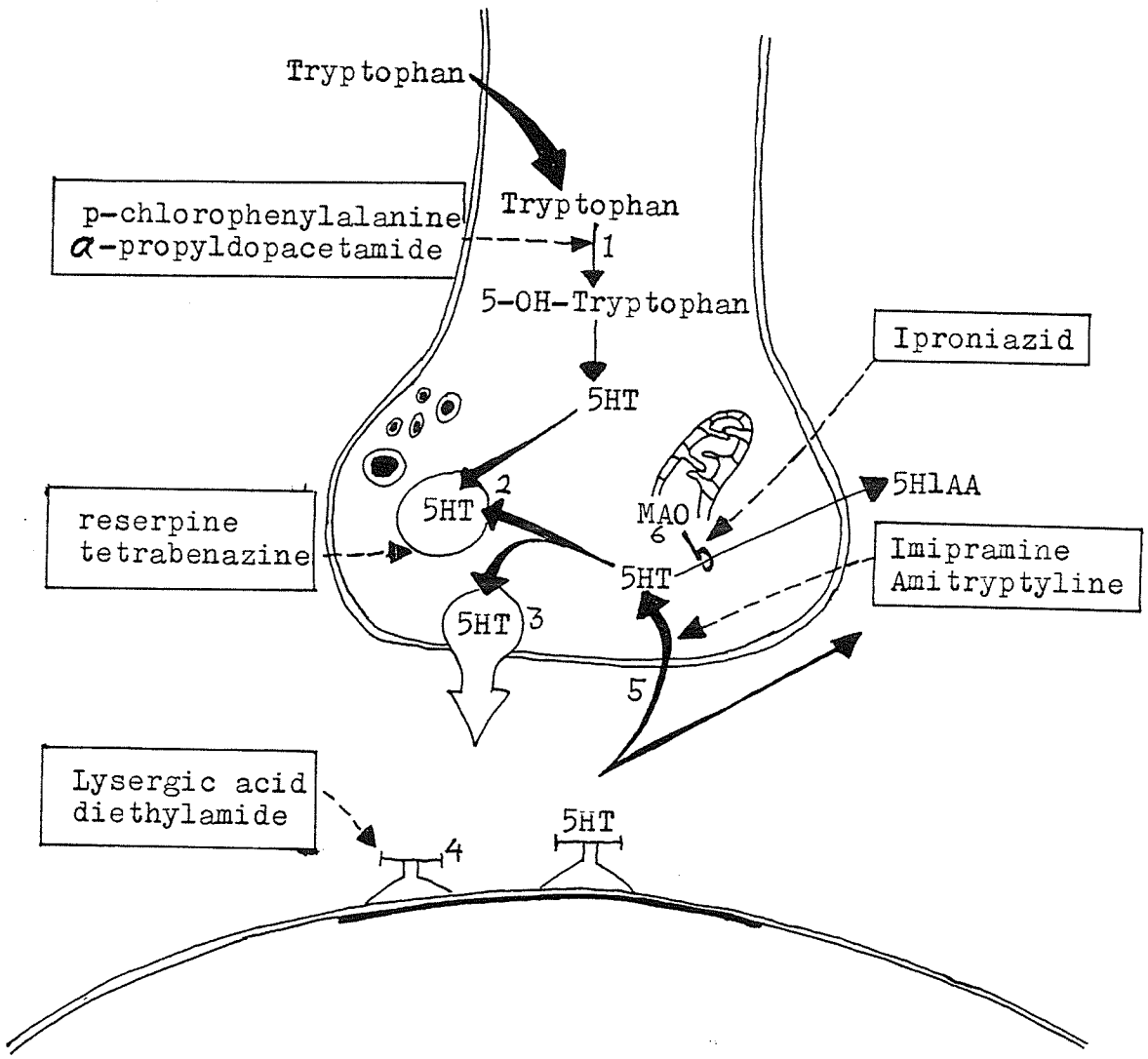



Fig.6. Schematic model of a central serotonergic neuron indicating possible sites of drug action (Cooper et al, 1974).

direct effect on the TPOH (Anden et al, 1968; Zivkovic et al, 1974). It is thus postulated that serotonergic neurons might be controlled presynaptically through either an interneuron or a collateral of the target cell. These are shown in Figure 7 (Costa and Meek, 1974). Although they can be activated by other synaptic inputs, they are thought to be responsible for the presynaptic inhibition of 5-HT-containing neurons (loc. cit.). This inhibition continues or even increases after axotomy, in spite of the fact that results from different areas of the CNS are conflicting (loc. cit.). Since a reduction in TPOH activity has been observed as early as one hour after transection, this effect is attributed to the cessation of impulse traffic, rather than to the loss of enzyme molecules (Sheard and Aghajanian, 1968).

G. Competing Metabolic Pathways

There are alternative routes of metabolism for 5-HT as well as its precursors TRY and 5-HTP. Some of these are shown in Figure 8. The extraneuronal AADC can decarboxylate TRY to tryptamine which is then methylated to N, $N^1$-dimethyltryptamine, a potent hallucinogenic agent. (David, 1975). This pathway is thought to compete with the conversion of TRY to 5-HT (Hayaishi, 1976). In addition, TRY may be metabolized via a transaminase (Minatogawa et al, 1973) or an indoleamine 2,3-dioxygenase, the latter cleaving the indole ring of 5-HTP, 5-HT, tryptamine, melatonin, and other indoleamine derivatives as well (Tsuda et al, 1972; Hayaishi, 1976). Another important route of TRY metabolism occurs in both brain and liver via tryptophan 2,3-dioxygenase (pyrrolase) (Badawy, 1977; Gál and Sherman, 1978). Green and Curzon (1975) have observed that the induction of

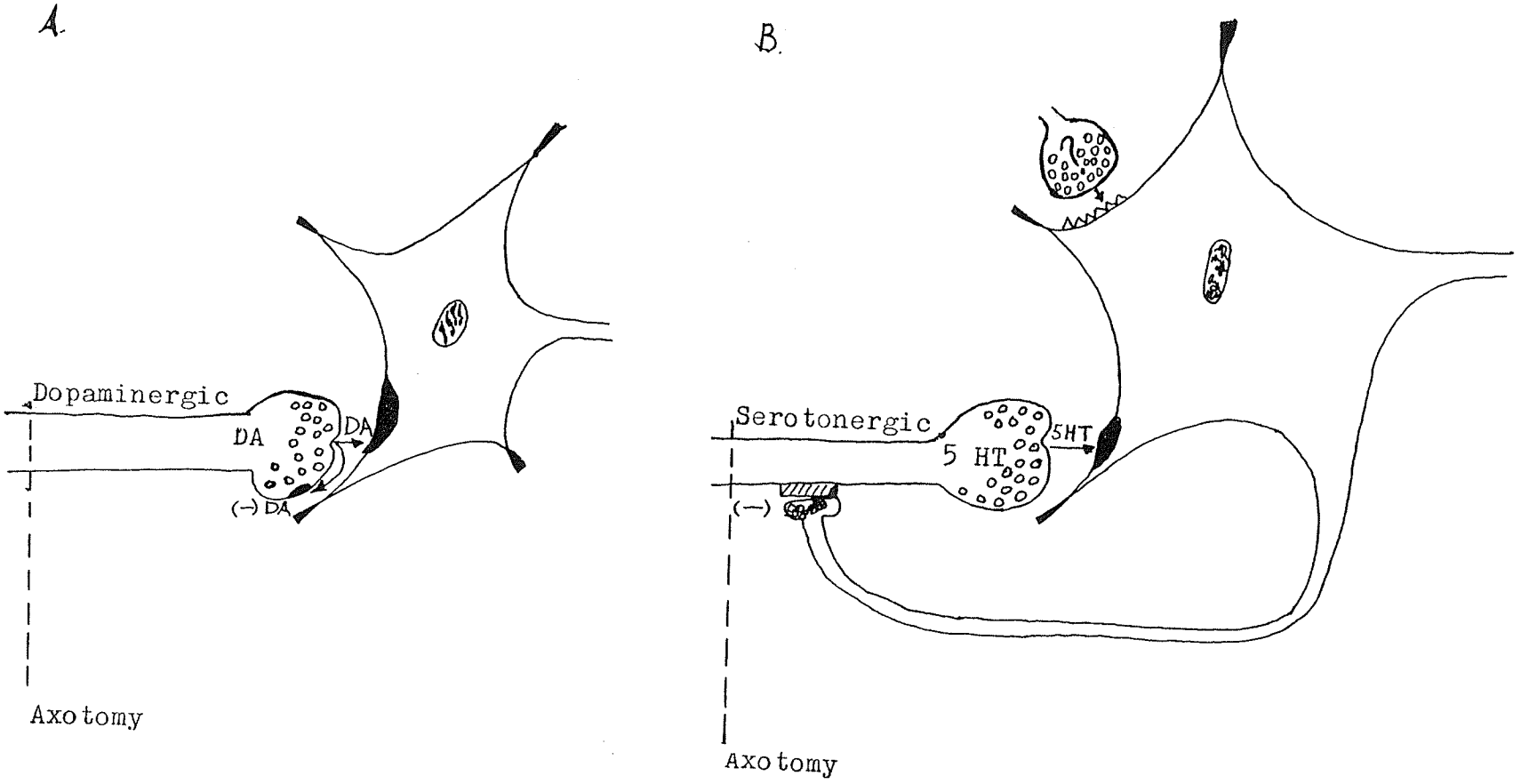



Fig. 7. Model illustrating possible feedback loops in (A) dopaminergic terminals of the striatum, and (B) serotonergic terminals in the spinal cord (Costa and Meek, 1974).

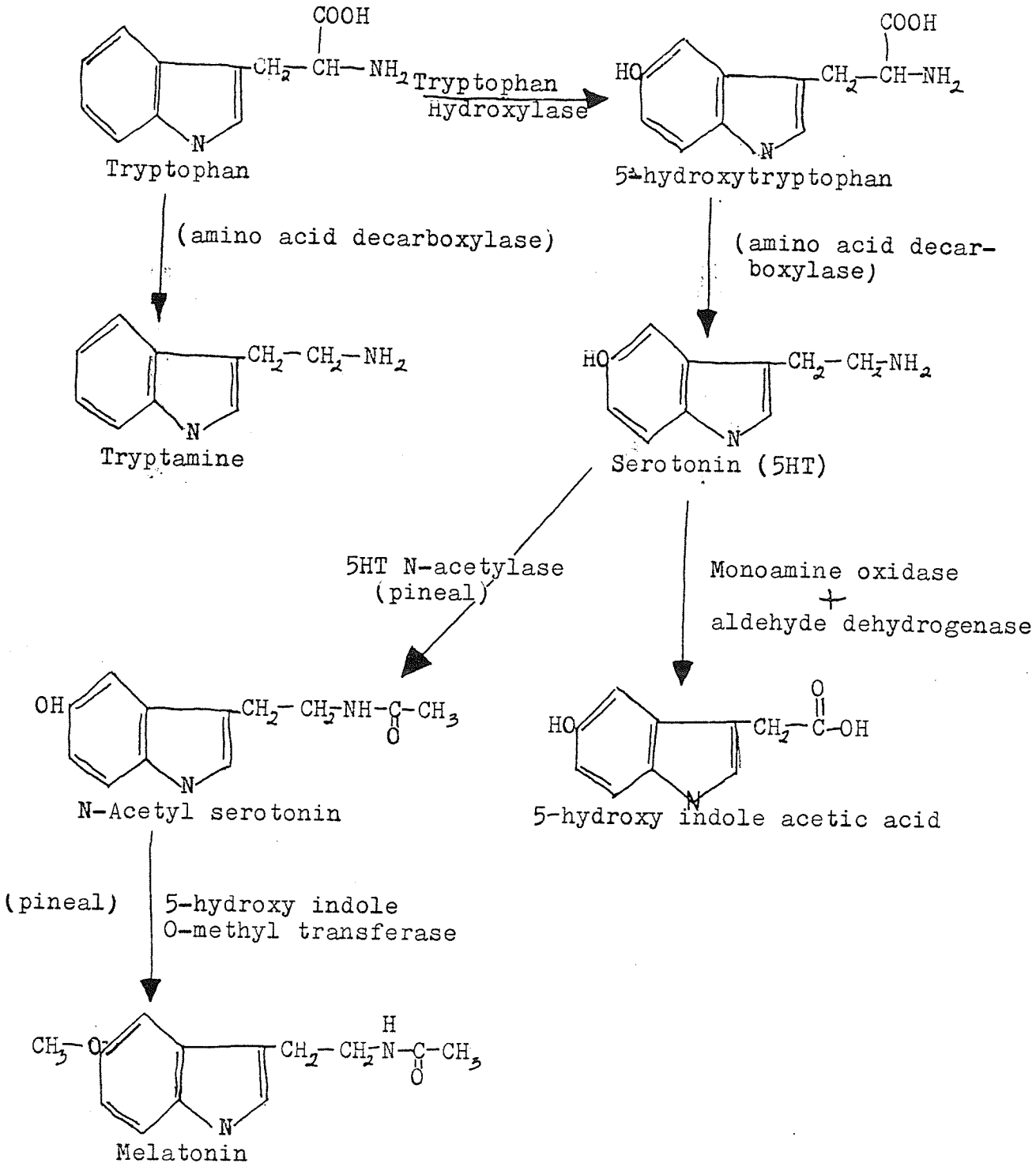



Fig. 8. The metabolic pathways available for the synthesis and catabolism of serotonin (Cooper et al, 1974).

tryptophan 2,3-dioxygenase by such factors as immobilization and/or glucocorticoid administration decreases both plasma TRY levels and brain 5-HT levels.

The three alternate routes of catabolism of 5-HT (in order of importance) are O-methylation, N-methylation and 6-hydroxylation. Although the function of the metabolites formed after 6-hydroxylation and O-methylation is still unknown (Lemberger et al, 1971; Snyder et al, 1974), N-methylation of 5-HT produces bufotenine which is thought to be a factor in generating schizophrenia-like symptoms (Matthysse et al, 1972). Finally, regulation by NE of the N-acetyltranferase which converts 5-HT to N-acetylserotonin (the direct precursor of melatonin) provides a unique example of interregulation in the catecholamine and indoleamine pathways. Although this route of catabolism for 5-HT is found only in the pineal gland, melatonin does have effects on the entire 5-HT system and on sleep mechanisms (Marczynski et al, 1964).

## H. Long-Term Regulatory Mechanisms

Chronic morphine administration is thought to produce an immediate decrease in TPOH activity, followed by its long-term increase in 5-HT nerve endings (Knapp and Mandell, 1972). However, there was no change in the activity of TPOH in 5-HT nerve cell bodies. Other studies have attempted to correlate TPOH activity and TRY uptake after chronic lithium chloride treatment. However, the delay in effect from the 5-HT cell bodies to the nerve terminals does correspond to the time required for axoplasmic transport of TPOH (Meek and Neff, 1972).

It has been found (Azmitia and McEwen, 1969) that bilateral adrenalectomy reduces both TPOH activity as well as the turnover rate of 5-HT in the midbrain. Although the

effects of many drugs such as reserpine and pargyline appear to be mediated by adrenocortical secretion (Zivkovic et al, 1974), the functional significance of these long-term changes in TPOH activity remains to be established (Costa and Meek, 1974).

# Regulation Of Brain Norepinephrine And Dopamine Metabolism

## A. Pathways of Synthesis and Degradation

The major pathways of catecholamine biosynthesis and catabolism in rat brain are illustrated in Figures 9 and 10 (Fuller and Steinberg, 1976; Curzon, 1972). The first step in biosynthesis, namely, the hydroxylation of tyrosine (TYR) to 3,4-dihydroxyphenylalanine (DOPA), is catalyzed by tyrosine hydroxylase (TOH). This enzyme is similar to TPOH in that it is dependent on $O_2$ and a reduced pteridine for activity. Decarboxylation of DOPA by an aromatic amino acid decarboxylase (AADC or DOPADC) leads to formation of DA which is then transformed to NE by dopamine β-hydroxylase (DBH).

The enzymatic inactivation of both DA and NE involves two major enzymes, monoamine oxidase (MAO) and catechol-oxygen-methyltransferase (COMT). MAO converts DA and NE to their appropriate aldehydes. These in turn are converted to acids, homovanillic acid (HVA) and 3-methoxy-4-hydroxy-mandelic acid (VMA), respectively. COMT (in the presence of magnesium ions) introduces a methyl group, substituting with it the hydrogen in the hydroxyl group at the carbon atom in postion 3 of the catechol ring. This leads to the formation of 3-methoxy-derivatives of the catecholamines. As shown in Figure 10, either MAO or COMT may act first. Thus, a part of deaminated metabolites is later methylated and a part of methoxy-derivatives is deaminated later. Carlsson and Hillarp (1962) have shown that MAO primarily acts first on DA. However, the sequence of action of MAO and COMT on NE differs in various centers of the brain (Matsuoka et al, 1964).

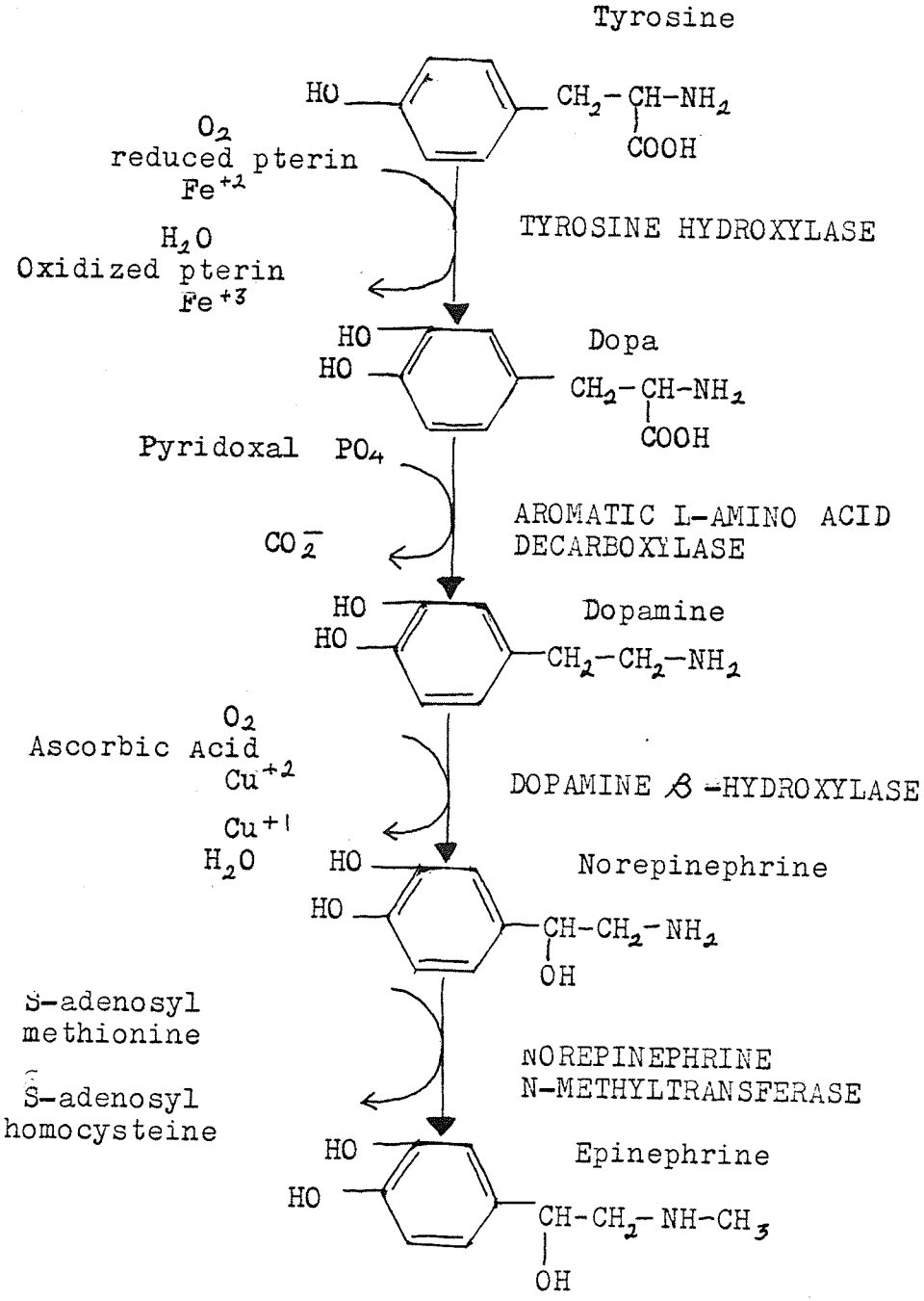



Fig. 9. The biosynthesis of catecholamines in the brain. (Fuller and Steinberg, 1976).

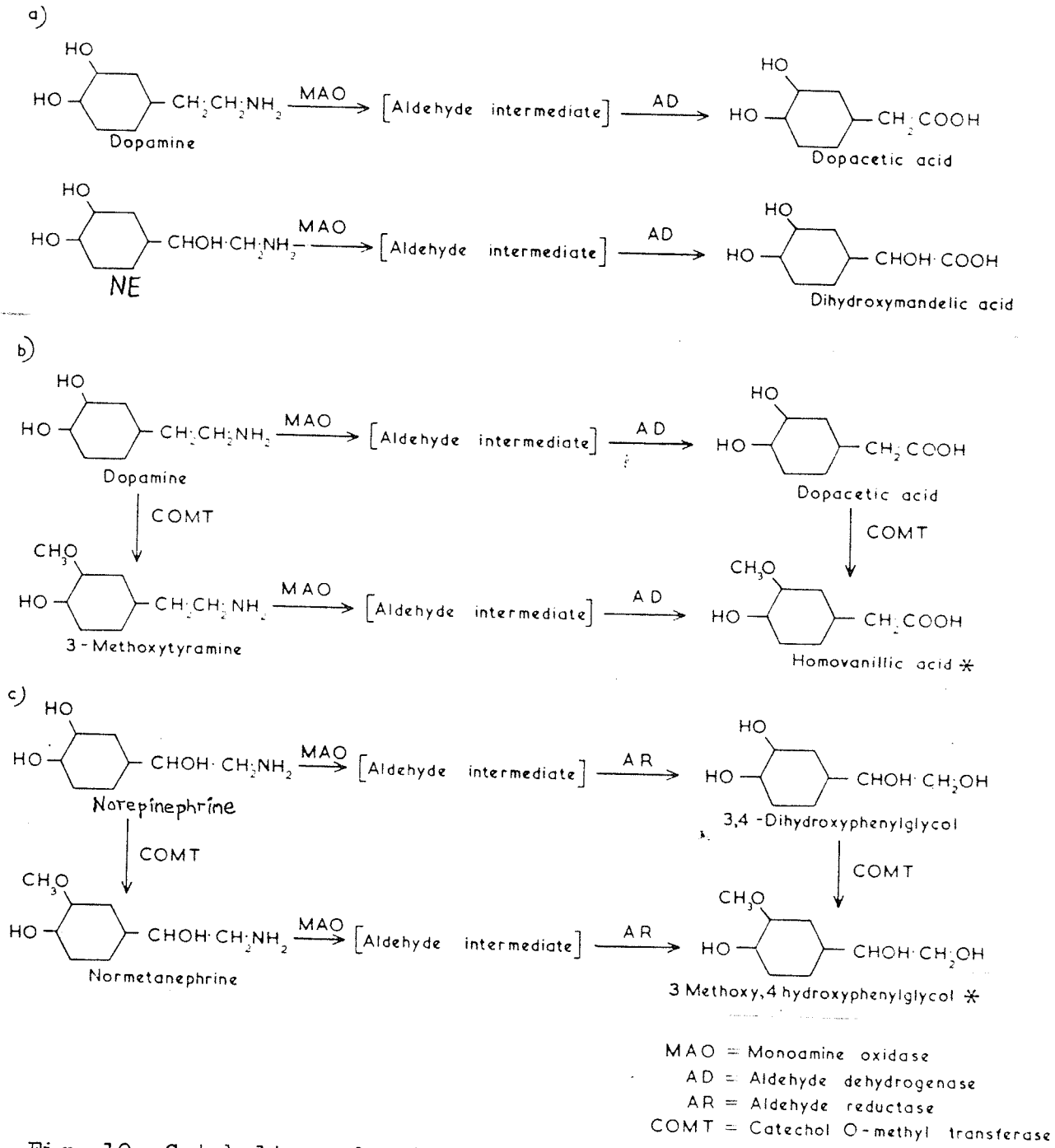



Fig. 10. Catabolism of catecholamines in the brain (Curzon, 1972)
(a) Monoamine oxidase pathway with oxidation of aldehyde intermediate.
(b) Combined monoamine oxidase and catechol-O-methyl transferase pathway with oxidation of aldehyde intermediate-major pathway of dopamine catabolism.
(c) As (b) but with reduction of aldehyde intermediate-major pathway of noradrenaline catabolism

* = Principal terminal amine metabolite in brain.

## B. Tyrosine Hydroxylase

Tyrosine hydroxylase (TOH) (EC1.10.31) is thought to be the rate-limiting step in catecholamine biosynthesis (Nagatsu et al, 1964). Not only are its concentration, activity, and rate of reaction very low compared to DOPADC and DBH (Levitt et al, 1965; Udenfriend, 1966), but pharmacological or physiological manipulations of the rate of brain catecholamine synthesis are invariably associated with parallel changes in TOH activity (Cegrell et al, 1970; Carlsson et al, 1972). Furthermore, significant correlations between TOH activity and catecholamine concentration or turnover have been demonstrated in various brain regions (Bacopoulos and Bhatnagar, 1977).

One method of short-term regulation of TOH activity is feedback inhibition by DA or NE. Thus, the rate of TYR hydroxylation decreases as the DA and/or NE concentrations increase (Nagatsu et al, 1964; Spector et al, 1967; Neff and Costa, 1968). For instance, Spector et al (1967) found that a rise in NE concentrations due to MAO inhibition causes a reduced synthesis of NE from TYR-$^{14}$C, whereas the production of NE from DOPA occurs at a normal rate. Feedback inhibition of the adrenal TOH appears to be noncompetitive with TYR, but competitive with the hydroxylase's reduced pteridine cofactor (Kuczenski and Mandell, 1972; Nagatsu et al, 1972; Simon et al, 1978). In rat brain, Musacchio et al (1971) found that DA was twice as effective an inhibitor of TOH as NE. It is thought that the catecholamines compete with the oxidized pteridine for a binding site on dihydropteridine reductase (loc. cit.). Since the brain concentration of pteridines is so low, the reductase may play an important modulatory role in the catecholamine feedback inhibition of TOH activity. Nevertheless,

a very recent study by Sherman and Gál (1978) suggests that the synthesis of brain NE, DA and 5-HT is not dependent on the levels of pteridine cofactor. They showed that a 50% reduction in the cofactor pool has no effect on amine synthesis.

Besides TYR and the reduced pteridine, TOH also requires molecular $O_2$. Its $K_m$ for $O_2$ is well below atmospheric $O_2$ concentrations, but the concentration of $O_2$ at the site of the enzyme is unknown. However, it is known that $pO_2$ varies in different brain regions (Costa and Meek, 1974), and that the hydroxylation of TYR *in vivo* is decreased when rats breathe a concentration of $O_2$ lower than normal (Davis and Carlsson, 1973). Thus, changes in $pO_2$ cannot be ruled out as a factor in the regulation of TOH activity.

It has also been shown recently that an increase in neuronal depolarization can actually bring about a change in the physical properties of TOH, causing it to have an increased affinity for pterin cofactor (Murrin et al, 1976). In addition, allosteric regulation via interaction of the hydroxylase with membrane components has been suggested (Lovenberg and Victor, 1974). Just as multiple forms of TPOH have been suggested, Nagatsu et al (1964) observed that TOH in adrenal extracts also exists in both soluble and particulate forms. Studies involving histofluorescence in noradrenergic neurons in the nucleus locus coeruleus have provided evidence that two forms of the enzyme may exist *in vivo* (Mandell, 1978).

Finally, studies involving developmental changes in TOH activity have yielded results which are similar to those studying developmental changes in TPOH. At birth in the rat, TOH activity is less than half its adult activity (Lanier et al, 1976), and thus, like TPOH, is definitely rate-limiting.

C. Availability of Tyrosine

Wurtman et al have shown that elevations in brain tyrosine (TYR) (after inhibition of DOPA decarboxylation) are associated with parallel changes in DOPA synthesis (Wurtman et al, 1974; Gibson and Wurtman, 1977). Such elevations were produced either by consumption of a single protein-containing meal or by intraperitoneal injection of TYR. They thus concluded that TOH is unsaturated under normal conditions.

More recent work which studies the rate of accumulation of a NE metabolite after probenecid administration provides evidence that brain TYR levels affect not only the synthesis, but also the turnover of brain NE (Gibson and Wurtman, 1978).

However, brain TYR levels in the neonate rat are much higher than those found in the adult. Thus, any possibility of TYR availability as a rate-limiting step in brain NE synthesis in the neonate is eliminated.

D. 3,4-Dihydroxyphenylalanine Decarboxylase

3,4-dihydroxyphenylalanine decarboxylase (DOPADC) (EC 4.1.1.26) activity, like 5-HTPDC activity, is not considered to be rate-limiting in monoamine synthesis. For example, Udenfriend (1966) quotes a $V_{MAX}$ for guinea-pig kidney DOPADC that is over 200-fold greater than the $V_{MAX}$ for beef adrenal TOH. Also, DOPADC inhibitors fail to have any appreciable effect on NE or DA levels (Spector, 1966). These studies provide a strong argument that the hydroxylation rather than the decarboxylation is the rate-limiting step. Further evidence is found in the developmental pattern of DOPADC. Lamprecht and Coyle (1972) reported that even though its specific activity at birth was only 40%

of the adult level, it exhibits a sharp increase which far exceeds TOH activities.

Nevertheless, as previously discussed for 5-HTPDC, the same problems in purification and dependency on PALP as a coenzyme apply to DOPADC. Failure to obtain a pure preparation has led to difficulties in interpretation of studies regarding regulation of its activity.

## E. Catechol-O-Methyl Transferase

Catechol-O-methyl transferase (COMT) (EC 2.1.1.6) is one of the major biological inactivation processes for catecholamines. Its exact role in the regulation of NE and DA concentrations is still unclear. There is now evidence for intraneuronal COMT in brain. This is based on the fact that one third of the enzyme activity is found in the synaptosomal fraction (Guldberg and Marsden, 1975).
Also, Km measurements after partial purification with ammonium sulphate suggest that there may be two forms of COMT in rat brain. Furthermore, the Km value of the enzyme in the synaptosomal fraction differed from that in the supernatant (loc. cit.). Thus, researchers speculate that some of the supernatant enzyme may be the extraneuronal COMT which functions in the inactivation of NE and DA released from nerve endings. The synaptosomal COMT, on the other hand, is thought to exist within the nerve endings. It probably aids the mitochondrial MAO in the breakdown of NE and DA within the neuron.

Even though there is little information about the ontogenic aspects of COMT, Mirkin (1970) did find that in rat brain its activity at birth was only 20% that of the adult. COMT activity in the chick heart increases to peak levels at hatching and then gradually falls to very low levels by 6 weeks after birth. In the fetal and newborn chick, its

level of activity is inversely proportional to heart rate (Coyle, 1973). Thus, COMT is thought to play an important role in the inactivation of catecholamines during certain stages of development.

F. Feedback Control Via a Neuronal Loop

It is postulated that dopaminergic neurons, like serotnergic neurons, have a neuronal loop that participates in feedback control of monoamine synthesis (Costa and Meek, 1974). The dopaminergic neurons are thought to have presynaptic inhibitory DA receptors on their terminals (Figure 7). While activation of these receptors inhibits the release and synthesis of DA, axotomy results in an increase of DA synthesis due to a decrease in stimulation of the inhibitory receptor.

An older hypothesis which attempts to explain the mechanism of dopaminergic feedback states that this feedback is mediated by postsynaptic DA-receptors via a strionigral neuronal loop (Carlsson and Lindqvist, 1963). Recently, Tissari et al (1978) have used an *in vivo* experimental model which is very useful in testing the two hypotheses. This model involves the local injection of kainic acid which selectively destroys striatal neuronal perikaria and postsynaptic DA-receptors, leaving axons and synaptic terminals of afferent neurons intact. This study suggests a dual mechanism of DA synthesis, providing evidence for both hypotheses.

Kellogg and Wennerstrom (1974) have presented evidence to suggest that even though functioning DA-containing and NE-containing neurons exist in rat brain from the first day of life, the feedback control of DA and NE by receptor stimulation is not established until 4 weeks of age. Thus, such neuronal loops are probably not significant in the regulation

of monoamine metabolism during the development of the CNS.

G. Competing Metabolic Pathways

David et al (1974) have found that the extraneuronal AADC will decarboxylate TYR to form tyramine, especially when the plasma level of TYR is high. TYR can also undergo transamination in both brain and liver (Minatogawa et al, 1973). Hepatic tyrosine aminotransferase varies in amount in response to the time of day and to hydrocortisone and glucagon levels. Nevertheless, Benkert and Matussek (1970) found that although induction of the enzyme by administration of these hormones decreased TYR levels in plasma and brain, the brain levels of NE were not significantly lower.

H. Long-Term Regulatory Mechanisms

Long-term neuronal stimulation or deprivation has been found to alter NE and DA levels via a change in the actual amounts of either TOH or DBH. Cold exposure, immobilization stress, chronic reserpine administration, and electroshock will all produce an increase in the in vitro levels of TOH (McGeer and McGeer, 1975). Since these increases can be prevented by using protein synthesis inhibitors, it may be concluded that actual enzyme induction is the mechanism by which they occur.

However, it has been shown in the caudate that increases in TOH after cold exposure or chronic reserpine injection are not affected by protein synthesis inhibition (Zivkovic et al, 1973). Furthermore, Dunn et al (1978) have found that adrenocorticotrophic hormone effects on catecholamine turnover in mouse brain are not even mediated by changes in TOH activity at all. Thus, some mechanism different from the long-term increase of enzyme activity must be involved.

# Aromatic L-Amino Acid Decarboxylase

## A. Biochemical Reactions

A single enzyme, aromatic L-amino acid decarboxylase (AADC), is presently thought to be responsible for the decarboxylation of several amino acids. These include DOPA, 5-HTP, m-TYR, and 5-hydroxy-N-acetyltryptophan (Lovenberg et al, 1962; Christenson et al, 1972; McGeer and McGeer, 1975). Distinctly separate enzymes catalyze the decarboxylation of other aromatic amino acids such as histidine, TRY., phenylalanine, and p-TYR and of aliphatic amino acids such as glutamic acid (ibid. ). However, all of these decarboxylases are dependent on PALP as a coenzyme. For example, PALP was shown to restore the *in vitro* glutamic acid decarboxylase activity in cerebral tissues from rats exposed to a $B_6$-deficient diet for 8 weeks (Roberts et al, 1951). Also, many of these reactions are involved in the synthesis of putative neurotransmitters such as GABA, DA, NE, and 5-HT. Thus, studies of the effects of pyridoxine deficiency are valuable tools in attempts to understand the regulation of brain monoamine metabolism.

## B. Effect of Pyridoxine Deficiency

Because pyridoxine is found in a wide variety of foods, a primary deficiency of pyridoxine has never been reported. Nevertheless, there are numerous reports of convulsions in infants who were fed milk that had a low pyridoxine level (Coursin, 1954). These convulsive seizures were corrected by the administration of pyridoxine. Side effects such as depression and changes in sleep pattern and mood have been associated with oral contraceptive drugs. These effects have also been corrected by the administration of pyridoxine (Baumblatt and Winston, 1970). Green et al (1970) suggest that the induction of tryptophan

-2, 3-dioxygenase by estrogens decreases the availability of both TRY and PALP for brain 5-HT synthesis. Unfortunately, there is still no information on the levels of brain monoamines or the activity of AADC in patients using oral contraceptives.

Experimental pyridoxine deficiency may be produced in two ways. The most common method involves feeding the animals a pyridoxine-deficient diet. The second method involves the administration of drugs which act as pyridoxine antagonists or anti-metabolites. Many of these drugs are actually being used therapeutically. Examples include isonicotinic acid hydrazide (isoniazid) and cycloserine which are used in the treatment of pulmonary tuberculosis, and penicillamine which is used in the treatment of Wilson's disease. Administration of pyridoxine has been found to alleviate the symptoms of neurological toxicity associated with all three of these anti-metabolites (Dakshinamurti, 1977).

In addition to these drugs, others such as 4-deoxypyridoxine, amino-oxyacetate, 4-methoxymethylpyridoxine, iproniazid, and thiosemicarbazide have also been used to produce experimental pyridoxine deficiency (Bilodeau, 1965; Minard, 1967). Although little information is available as yet on the effect of these drugs on brain AADC activity, a more recent study by Gey and Georgi (1974) does report a decrease in its DOPA-decarboxylating activity in rat brain after a single intraperitoneal injection of either amino-oxyacetate or thiosemicarbazide. This decreased activity just preceded the convulsions produced by such agents, and was coexistent with a decrease in PALP levels.

Several studies involving dietary pyridoxine deficiency have investigated its effect on brain monoamine metabolism. Based on a series of investigations, Sourkes (1972) concluded that pyridoxine deficiency has no effect on the steady state concentration of catecholamines of various tissues.

However, Wiss and Weber (1964) reported a decrease in AADC activity in the liver of pyridoxine deficient rats, although the brain decarboxylase was little affected. Then Eberle and Eiduson (1968) found a decrease in both liver and brain AADC during pyridoxine deficiency, the brain still less affected than the liver.

Discrepancies are common in the studies investigating the brain AADC. Attempts at its purification have as yet been unsuccessful. Sims et al (1973) have found that DOPADC activity and 5-HTPDC activity are influenced very differently by experimental conditions such as changes of pH, temperature, and, most importantly, concentration of PALP. They conclude that two distinct decarboxylases may have evolved in neuroepithelial tissue. In view of the role of PALP as the coenzyme of the decarboxylase (s), we shall briefly review the evidence for and against the existence of a single brain AADC enzyme.

## C. Evidence Supporting the Existence of One Enzyme

The enzyme DOPADC was first demonstrated in the kidneys of guinea pigs and rabbits (Holtz et al, 1938). Both DOPADC and 5-HTPDC activities have since been found in a variety of mammalian tissues, namely, liver, brain, kidney, heart, stomach, and adrenal (David et al, 1974). Early work with only partially purified extracts suggested that these were distinct enzymes (Clark et al, 1954). However, as analytical methods improved, a number of reports appeared which indicated that one enzyme was responsible for the decarboxylation of both L-DOPA and 5-HTP (Dairman et al, 1975). For example, Huntzman et al (1961) reported that the ratio of DOPADC activity to that of 5-HTPDC in feline brain was relatively constant on a gross regional basis. Lovenberg et al (1962) first proposed the name ''aromatic L-amino acid

decarboxylase'', since they found that a partially purified enzyme from guinea pig kidney decarboxylated a large number of aromatic L-amino acids, including DOPA and 5-HTP.

Christenson et al (1970) succeeded in purifying to homogeneity a decarboxylase from hog kidney. Because it was capable of decarboxylating all of the naturally occurring aromatic amino acids, including L-DOPA and 5-HTP, this finding strongly supported the one enzyme hypothesis. They then used this purified enzyme as an antigen to produce a monospecific antiserum to the decarboxylase (Christenson et al, 1972). Immunotitrations of this antiserum with extracts from a number of tissues of various species resulted in a proportionate removal of 5-HTP and L-DOPA decarboxylating activities. They thus concluded that there is indeed just one enzyme. Nevertheless, they did report that 10 times more antibody per unit of enzyme activity was required for the complete inhibition of the rat brain enzyme than for the total inhibition of the enzyme in pig kidney. More recently, Hökfelt et al (1973a,b) using an immunohistochemical technique, concluded that in the rat CNS the decarboxylase in 5-HT and catecholamine containing neurons was immunologically undistinguishable.

<u>In vivo</u> evidence supporting the existence of one enzyme stems from a study by Dairman et al (1975) where they report a proportionate loss of L-DOPA and 5-HTP decarboxylating activity in rat brain and spinal cord following intracisternal administration of 5,6-dihydroxytryptamine or 6-hydroxydopamine. These drugs are thought to specifically destroy serotonergic and catecholaminergic nerve endings respectively. On the basis of these findings, Dairman (ibid.) states that if two distinct decarboxylases do exist, they would have to be present in both 5-HT and catecholamine neurons. He concludes that such a situation would offer no obvious functional advantage.

## D. Evidence Supporting the Existence of Distinct Enzymes

The first evidence supporting the existence of more than one decarboxylase for aromatic amino acids in different organ systems was provided by Awapara et al in 1962. They found that a partially purified DOPADC preparation from liver exhibited significant differences from the kidney decarboxylase. These differences included pH optima, substrate specificity, and cofactor requirements.

More recently, Sims et al (1973) have studied the DOPADC and 5-HTPDC activities in rat brain and have found that they are influenced differently by changes in pH, temperature, substrate concentration, and levels of PALP. They also noted that the two brain enzyme activities respond differently to reducing substances, detergent, and a wide variety of storage conditions. Furthermore, the regional distributions of the two brain decarboxylases did not parallel one another. Their study of the subcellular localization revealed that DOPADC is found predominantly in the high speed supernatant, whereas most of the 5-HTPDC activity is associated with the synaptosomal fraction (ibid.).

Sims and Bloom (1973) have presented *in vivo* evidence which suggests that the two decarboxylase activities in brain tissue are catalyzed by distinct enzymes. Their study involved the use of 6-hydroxydopamine, similar to the work by Dairman et al (1975) which was discussed earlier. While Dairman's group found a proportionate loss of the two decarboxylating activities after administration of the drug, Sims and Bloom found a "disparate" loss in DOPADC activity with a significant increase in 5-HTPDC activity (loc cit.). Because they determined both activities in the same tissue preparation using their previously defined optimal assay condition, Sims and Bloom conclude that two different proteins catalyze the decarboxylation of 5-HTP and DOPA in the brain.

# Conclusions

This review of the literature shows that the mechanisms involved in the regulation of monoamine metabolism during the course of brain development are poorly understood. These mechanisms are of considerable importance, since NE, DA, and 5-HT are thought to function as neurotransmitters in the CNS. The preceding discussion also points out that there are conflicting reports regarding the exact nature and regulation of the ''aromatic amino acid decarboxylase'' which is thought to be involved in the biosynthesis of both the catecholamines and 5-HT. Since this enzyme requires PALP as a coenzyme, I have investigated the effects of both dietary and drug-induced pyridoxine deficiency on brain monoamine metabolism in the growing rat.

# III. Experimental

# Methods

## A. Production of Pyridoxine Deficiency

Sperm-positive female Holtzman rats were housed in individual cages and fed the pyridoxine-supplemented diet containing 5 mg pyridoxine per kilo of diet for the period of gestation (Dakshinamurti and Stephens, 1969). The composition of the diets is given in Tables 1, 2, and 3. After delivery, the control dams continued to get the pyridoxine-supplemented diet during the weaning period, following which the weanlings continued on the same diet until they were sacrificed. The same number of pups (8) was left with the dam in each group (loc. cit.). The experimental group of rats was fed the pyridoxine-deficient diet for the same period as rats on the control diet. Cycles of 12 hour artificial lighting of the animal room followed 12 hours of darkness (18:00 h to 6:00 h).

Pyridoxine deficiency was also produced by administering the pyridoxine antagonist, 4-deoxypyridoxine. Male Holtzman rats between 60 and 65 grams were divided into 2 groups. One group received daily intraperitoneal injections (5 mg/100 g) of 4-deoxypyridoxine, while the other group received daily injections of saline and weekly injections (5 mg/kg) of pyridoxine. All the animals were fed the pyridoxine-deficient diet for the period of injections (10 weeks) after which they were sacrificed.

## B. Criteria of Pyridoxine Deficiency

Several biochemical criteria of pyridoxine deficiency have been reported (Dakshinamurti and Stephens, 1969; Stephens et al, 1971). These include red blood cell and plasma glutamic-pyruvic and glutamic-oxaloacetic transaminase activities, and levels of PALP, $\gamma$-aminobutyric acid, and glutamic acid decarboxylase activity in brain of similar deficient rats.

Table 1

Percentage Composition of Pyridoxine Deficient Diet

| | | |
|---|---|---|
| Vitamin free Casein | | 30.0 |
| Dextrose | | 59.85 |
| Corn Oil | (Mazola) | 5.0 |
| Salt mix. number 446 | (General Biochemicals) | 4.0 |
| Vitamin mix. (no $B_6$) | (Table 2) | 1.0 |
| Choline chloride | | 0.15 |

Table 2

Composition of Vitamin Mixture (Without Pyridoxine)

| | |
|---|---|
| Vitamins A and D, concentrate (500000 units A/g conc.) (5000 units D/g conc.) | 4.0g |
| Alpha Tocopherol (250 I units/g) | 10.0g |
| Ascorbic acid | 99.2g |
| Inositol | 11.0g |
| Menadione | 5.0g |
| Para amino benzoic acid | 11.0g |
| Niacin | 9.9g |
| Riboflavin | 2.2g |
| Thiamine HCl | 10.0g |
| Calcium Pantothenate | 6.6g |
| Folic acid | 0.2g |
| Vitamin $B_{12}$ (triturate in 0.1% mannitol) | 3.0g |
| Biotin | 0.05g |
| Dextrose | 827.85g |
| Total weight | 1000.0g |

Table 3

Percentage Composition of Pyridoxine Supplemented Diet

| | | |
|---|---|---|
| Vitamin free Casein | | 30.0 |
| Dextrose | | 59.85 |
| Corn Oil | (Mazola) | 5.0 |
| Salt mix. number 446 | (General Biochemicals) | 4.0 |
| Vitamin mix. (no $B_6$) | (Table 2 ) | 1.0 |
| Choline chloride | | 0.15 |
| Vitamin $B_6$ | (in mg) | 0.5 |

Therefore, the criteria of pyridoxine deficiency in this study simply included body and brain weights, motility, and deep body temperature. Open field activity (motility) was measured to test for relative degrees of hyper-or hypokinesia in experimental and control animals. It was measured in sound shielded plastic boxes (20 x 18 x 22 cm) which were mounted on radiofrequency activity monitors. Animals were tested individually over a 10 min. period after a 5 min acclimation. Rectal temperature was measured using a thermistor probe and an electronic thermometer.

C. Determination of Brain Monoamine Levels

Brain monoamines were determined by the method of Shellenberger and Gordon (1971). This method involves the adsorption of the catecholamines onto alumina in order to separate them from the 5-HT. The fluorescence of the monoamines is then developed with iodine (catecholamines) and ninhydrin (5-HT), and read at the characteristic excitation and emission wavelengths for each compound. Internal as well as external standards are used for maximal accuracy with recoveries in the order of 80 - 90% for each.

a) Extraction from brain

Reagent:

-Perchloric acid (0.4N with 1.0 g sodium metabisulfite and 0.5 g ethylenediamine tetraacetic acid (EDTA) per liter).

Procedure:

Animals were killed by decapitation and the whole brains were removed rapidly. The brains were washed quickly in a hypertonic buffer, frozen in liquid nitrogen, weighed (in a Sartorius balance), and then homogenized in 5 volumes of the perchloric acid solution using Duall tissue

grinders. Internal standards were added and mixed by a passage of the pestle through the homogenate. The homogenates were left to stand in ice for 20 min and then centrifuged at 25,000 g for 20 min.

The supernatants were saved and the pellets were rehomogenized in 3 volumes of the perchloric acid. After a second centrifugation, the supernatants from each sample were pooled and adjusted to final volume.

b) Isolation of catecholamines

Reagents:

-Aluminum oxide (Alumina-Woelm Neutral Activity, Grade 1). This was prepared in quantities of about 250 g as described by Anton and Sayre (1964) and Shellenberger and Gordon (1971).

-Tricine solution (Tricine, Calbiochem, A grade). This was prepared by dissolving 17.9 g of Tricine and 25 g of disodium EDTA in each liter of 0.525 N sodium hydroxide.

-Perchloric acid solution (0.05 N).

Procedure:

The tissue extracts were adjusted to pH 7.5 with the Tricine solution. They were then placed in glass tubes which contained 300 mg of the activated alumina, shaken for 30 min in the cold room (0-4° C), and then centrifuged at 500 g for 10 min. The supernatants were transferred to 50 ml centrifuge tubes for the extraction and assay of 5-HT.

The alumina was washed 5 times with 10 ml distilled water and centrifuged after the last wash at 500 g for 10 min. These last washes were aspirated off and 4 ml of 0.05 N perchloric acid added. The tubes were then shaken for 30 min and centrifuged at 500 g for 10 min. Then three 1.0 ml samples were taken for oxidation and development of fluorescence.

c) Isolation of 5-HT

Reagents:

-Borate buffer (0.5 M, pH 10, saturated with sodium chloride and n-heptanol).

-n-Heptanol was prepared by redistilling reagent - grade heptyl alcohol at 174 - 176°C to remove impurities which caused high blanks and lowered recoveries.

-Phosphate buffer (0.05 M, pH 7) was prepared with 2.70 g $NaH_2PO_4$ and 4.34 g $Na_2HPO_4$ (anhydrous) per liter.

Procedure:

To the supernatants mentioned above, 5.0 g of sodium chloride were added. The tubes were then shaken for 10 min. To these salt-saturated solutions, 20 ml of n-heptanol and 0.5 g of potassium carbonate were added. Each of the tubes was immediately capped and shaken for 10 min. The aqueous phases were aspirated off, and the heptanol was washed with 10 ml of borate buffer (pH 10) by shaking the tubes for 5 min. The tubes were then centrifuged at 500 g for 5 min. 15 ml of the heptanol were transferred into similar 50 ml polypropylene centrifuge tubes which contained 3.0 ml of 0.05 M phosphate buffer. These tubes were shaken for 5 min and centrifuged at 500 g for 5 min. After the heptanol was removed completely, two 1.0 ml samples of the phosphate buffer were taken from each tube for duplicate determinations.

d) Estimation of NE and DA

Reagents:

-Phosphate buffer - EDTA solution was prepared by adding 9.0 g of disodium EDTA to 1.0 liter of 0.1 M phosphate buffer and adjusting the pH to 7.0 with 5.0 N sodium hydroxide.

-Iodine reagent (o.1 N) was prepared by dissolving 2.0 g of potassium iodide and 0.5 g iodine in a final volume of

40 ml.

-Alkaline sodium sulfite solution (2.5%) was made by diluting 1 ml of a solution containing 250 mg sodium sulfite/ml, to 10 ml with 5.0 N sodium hydroxide.

-Glacial acetic acid.

Procedure:

1.5 ml of 0.1 M phosphate buffer - EDTA solution were added to the 1.0 ml samples of NE and DA that were isolated as described above. External standards (200 ng NE and 400 ng DA) were also included in the assay. After 0.2 ml of the iodine reagent was added, the tubes were shaken immediately and allowed to stand for exactly 2 min. Then 0.5 ml of the alkaline sodium sulfite solution was added, and the tubes were shaken and allowed to stand for another 2 min. The samples were acidified to pH 4.4 - 4.8 with 0.4 ml of glacial acetic acid and heated in an oven maintained at 100°C for 5 min. The tubes were cooled in an ice bath for 5 min. The NE fluorescence was then read at room temperature in a spectrophotofluorometer with the activation peak at 380 nm and the emission peak at 485 nm.

The samples were returned to the oven and heated at 100°C for an additional 45 min to develop the DA fluorescence. They were then placed in an ice bath. Their fluorescence was read immediately with the excitation peak at 300 nm and the emission peak at 381 nm.

e) Estimation of 5-HT

Reagent:

-Ninhydrin (0.1 M).

Procedure:

To the 1.0 ml samples of 5-HT (isolated as described above) was added 0.1 ml of the 0.1 M ninhydrin reagent.

External standard tubes (300 ng 5-HT) were also assayed. The tubes were heated in an oven at 100° C for 30 min. and left at room temperature for 1 hour, after which their fluorescence was read with the excitation peak at 375 nm and the emission peak at 490 nm. Finally, 0.1 ml of the alkaline sulfite reagent was added to each tube. After allowing the tubes to stand for 20 min, their fluorescence was read again.

Calculation:

The monoamine concentration was calculated as shown below:

$$\frac{\left[\begin{array}{l}\text{conc. of}\\ \text{standard (ng)}\end{array}\right] \times \left[\begin{array}{l}\text{relative fluorescence}\\ \text{of (sample - blank)}\end{array}\right] \times \left[\begin{array}{l}\text{dilution}\\ \text{factor}\end{array}\right]}{\left[\begin{array}{l}\text{relative fluorescence}\\ \text{of ( standard-blank)}\end{array}\right] \times \left[\begin{array}{l}\text{brain wet weight}\\ \text{(g)}\end{array}\right]}$$

This gives the concentration of the monoamine in nanograms per gram of brain wet weight.

The statistical analysis of all results was carried out using the Student t-test (Dixon and Massey, 1957).

## D. Specificity of Pyridoxine Deficiency

The specificity of pyridoxine deficiency as the cause of any changes in brain monoamine levels was established in recovery experiments. Three week old pyridoxine-deficient rats were randomized to remove interlitter differences. They were then divided into two groups. One group was given a single intraperitoneal injection of pyridoxine (5 mg/kg), while the other group was given saline. They were continued on the deficient diet and sacrificed 7 days later.

In another experiment, pyridoxine-supplemented rats

were ''pair-weighed'' with pyridoxine-deficient rats (Stephens et al, 1971). The pyridoxine-supplemented dams and later the weanlings were offered amounts of diet that maintained them in the same weight range as the pyridoxine-deficient rats until they were sacrificed at 4 weeks of age.

## E. Determination of Brain 5-Hydroxyindoleacetic Acid

Brain levels of 5-hydroxyindoleacetic acid (5-HIAA) were determined by the method of Curzon and Green (1970). It involves a butanol-heptane extraction and development of fluorescence with o-phthalaldehyde (OPT). Recoveries of internal and external standards are between 75 and 80%.

Reagents:

-Acidified n-butanol (0.85 ml concentrated hydrochloric acid in 1 liter of n-butanol).
-OPT (0.004% in 10 N hydrochloric acid).
-Cysteine (0.1% in 0.1 N '' '' ).
-Cysteine (1% in deionized water).
-OPT (0.1% in methanol).
-n-Heptane
-Phosphate buffer (0.5 M, pH 7.0).
-Sodium periodate (0.02%).

Procedure:

Whole brain was homogenized in 10 volumes of cold acidified n-butanol and centrifuged for 10 min at 500 g. 2.5 ml of the supernatant was pipetted into a 25 ml glass stoppered tube and shaken for 10 min with 5 ml n-heptane and 0.4 ml 0.1% cysteine. The phases were separated by centrifugation as before. 5 ml of the organic phase was retained for determination of 5-HIAA.

To determine 5-HT, 0.6 ml of 0.004% OPT was added to two 0.1 ml samples of the aqueous phase. After mixing and then heating in a boiling water bath for 15 min, the tubes

were placed in an ice bath. Their fluorescence was measured with the excitation peak at 360 nm and the emission peak at 470 nm. Blanks were prepared by reacting 0.6 ml of the OPT solution with 0.1 ml of 0.1% cysteine solution only.

To determine 5-HIAA, the 5 ml of the organic phase was pipetted into a 25 ml glass stoppered tube which contained 0.6 ml 0.5 M phosphate buffer. The tube was shaken for 10 min and centrifuged for 5 min at 500 g, and then two 0.2 ml portions of the aqueous phase were pipetted into two test tubes, A and B. 0.02 ml of 1% cysteine solution and 0.02 ml of 0.02% sodium periodate solution was added to tubes A and B respectively. 0.4 ml of concentrated hydrochloric acid was added to both A and B. Then 0.02 ml of 0.1% OPT solution and 0.02 ml of the periodate solution was added to tube A. After 30 min, 0.02 ml of the cysteine and OPT solutions was added to tube B. The tubes were then placed into a boiling water bath for 15 min and then into an ice bath. Their fluorescence was read with excitation and emission peaks of 350 nm and 475 nm respectively.

The concentrations of 5-HT and 5-HIAA were calculated by using the same formula as that for the monoamines discussed in the previous section.

## F. Pargyline and Probenecid Administration

Pargyline, a monoamine oxidase inhibitor, was injected intraperitoneally at a dose of 250 mg/kg body weight. The animals were sacrificed three hours later, and brain monoamines were determined by the method of Shellenberger and Gordon (1971) described previously.

In another experiment, probenecid was injected at a dose of 250 mg/kg in order to block the transport of 5-HIAA from the brain tissue (Lycke and Roos, 1972). The animals were sacrificed three hours later and brain 5-HIAA was measured by the method of Curzon and Green (1970) described above.

This method was also used to determine brain 5-HIAA levels after pargyline administration.

G. Determination of Tryptophan in Plasma

Total plasma TRY was determined by the method of Denckla and Dewey (1967). Their fluorometric analysis consists basically of a condensation of TRY with formaldehyde and trichloroacetic acid (TCA) to yield a highly fluorescent compound, norharman. Although the actual yield of norharman from TRY is only 65-70%, recovery of external standards is at least 85%.

Reagents:

-TCA (10%) containing 3 x $10^{-4}$M ferric chloride.
-Ferric chloride (3 x $10^{-4}$M) in $10^{-4}$M hydrochloric acid.
-TCA (75%).
-TCA (10%).
-Formaldehyde (1.8%).

Procedure:

Blood was collected in heparinized tubes from the neck wound after decapitation of the animal. It was then centrifuged in an International Centrifuge (Model-HR-I; head SS-34) for 10 min at 1200 rpm. Duplicate 0.02 ml samples of plasma were pipetted into 3 ml polyethylene centrifuge tubes. 1.8 ml of the TCA and ferric chloride solution were added, and the tubes were mixed and spun at 20,000 g for 10 min. The supernatants were then decanted completely into 5 ml glass stoppered centrifuge tubes, and 0.2 ml of formaldehyde was added. The tubes were firmly stoppered, placed in a 99-101° C water bath for 1 hour, and then cooled to room temperature. After the sample volume was adjusted to 2 ml with 10% TCA, the fluorescence was read at excitation and emission wavelengths of 373 nm and 452 nm, respectively.

Calculation:

The total plasma TRY concentration was calculated in μg/ml as shown below:

$$\frac{\left[\text{conc. of TRY standard } (\mu g)\right] \times \left[\text{relative fluorescence of (sample-blank)}\right] \times \left[\text{dilution factor}\right]}{\left[\text{relative fluorescence of (standard-blank)}\right]}$$

Free TRY in plasma was determined by the method of Knott and Curzon (1972) in which 0.4 - 0.5 ml of plasma ultrafiltrate was prepared by centrifuging 1.0 ml of plasma in a 'CF 50 Diaflo' membrane cone (Amicon) at 800 g for 30 min at room temperature. Then fluorescence was developed by converting the TRY to norharman as described previously.

## H. Determination of Tryptophan in Brain

Brain levels of TRY were determined by the method of Curzon et al (1972). After decapitation, each brain was homogenized in 10 volumes of cold acidified n-butanol and centrifuged for 10 min at 1000 g. 5 ml of the supernatant was pipetted into a 25 ml glass stoppered tube and shaken for 10 min with 10 ml n-heptane and 1.0 ml of a freshly prepared 0.1% solution of L-cysteine hydrochloride in 0.1 M hydrochloric acid. The phases were separated by centrifugation for 10 min at 1000 g. Then brain TRY was determined by the method of Denkla and Dewey (1967) as modified above.

## I. Assay of Tryptophan-5-Hydroxylase

The nonisotopic method of Gál and Patterson (1973) was used for the assay of brainstem tryptophan-5-hydroxylase, using tetrahydrobiopterin (6-MP$H_4$) as the cofactor. The aromatic amino acid decarboxylase inhibitor, NSD-1034 was inclu-

ded in the incubation at a concentration of 0.2 mM.

Reagents:

-Tris-acetate buffer (0.05 M, pH 7.6) containing $10^{-3}$M 2-mercaptoethanol.

-Tris-acetate buffer (500 mM, pH 7.6).

-2-Mercaptoethanol (10 mM).

-Pargyline hydrochloride (2 mM).

-L-Tryptophan (0.88 mM).

-6-$MPH_4$(1.6 mM).

-Catalase (100 $\mu$g/ml).

-Cysteine (1%).

-OPT (0.004% in 10 N hydrochloric acid).

-NSD-1034 (0.2 mM).

Procedure:

The brainstem was removed from the rest of the brain after decapitation of the animal. It was homogenized in 3 volumes of 0.05 M Trisacetate (pH 7.6) containing $10^{-3}$M 2-mercaptoethanol and centrifuged at 30,000 g for 12 min.

The incubation mixture had a final volume of 1 ml. It contained 50 mM Tris-acetate (pH 7.6), 1 mM 2-mercaptoethanol, 0.2 mM pargyline, 0.5-3 mg protein, 0.088 mM L-tryptophan, 0.16 mM 6-$MPH_4$, and 10 $\mu$g catalase. Protein content was determined spectrophotometrically (Warburg and Christian, 1941). The mixture was incubated at 37°C in a shaking Dubnoff water bath for 30 min, and was stopped by immersion in boiling water for 5 min. It was then transferred into tubes, and the incubation flask was washed with 1 ml of water. This wash was combined with the incubation mixture. The denatured protein was removed by centrifugation at 2000 g for 5 min.

Then 0.5 ml of the supernatant was transferred into test tubes containing 0.1 ml 1% cysteine solution. The sample was mixed with 1.15 ml of 10 N hydrochloric acid and

0.5 ml of OPT reagent, and then heated in boiling water for 15 min. After cooling in an ice bath, fluorescence was read with excitation and emission peaks of 360 nm and 470 nm respectively. Each assay included internal and external 5-HTP and 5-HT standards, and one sample was incubated without substrate in order to correct for endogenous 5-hydroxyindoles.

Calculation:

The activity of tryptophan-5-hydroxylase was calculated as nanomoles of 5-HTP produced per hour per mg protein, according to the following formula:

$$\frac{\left[\begin{array}{l}\text{conc. of 5-HTP}\\ \text{standard}\end{array}\right] \times \left[\begin{array}{l}\text{relative fluorescence}\\ \text{of (sample-blank)}\end{array}\right] \times \left[\begin{array}{l}\text{dilution}\\ \text{factor}\end{array}\right] \times 2}{\left[\begin{array}{l}\text{relative fluorescence}\\ \text{of (standard-blank)}\end{array}\right] \times \left[\begin{array}{l}\text{M.W. of}\\ \text{standard}\\ (220.2)\end{array}\right] \times \left[\begin{array}{l}\text{conc. of}\\ \text{protein (mg)}\end{array}\right]}$$

# Results

A. Effect of Dietary Pyridoxine Deficiency on the Body Weight and Brain Weight of the Growing Rat

The body weights and brain weights of pyridoxine deficient and control (+ pyridoxine) animals of various ages are given in Table 4. The statistical analyses gave P values of less than 0.05 or less than 0.005. Thus, the difference between the control and deficient group was statistically significant for each of the ages shown in the table.

B. Effect of Dietary Pyridoxine Deficiency on the Deep Body Temperature and Motility of the Growing Rat

The deep body temperature and motility of deficient and control animals of various ages are given in Table 5. The difference between the control and deficient group was statistically significant for each of the ages studied ($P < 0.005$).

C. Effect of Pyridoxine Deficiency on Brain Monoamines in the Growing Rat

The effects of dietary pyridoxine deficiency on the various brain monoamines are presented as histograms in Figures 11-13. There is a gradual rise in the brain levels of NE with age in both the deficient and the control (pyridoxine-supplemented) groups (Fig. 11). There was no difference between these groups in the brain levels of NE.

Changes in brain DA levels with age (Fig. 12) were not as great as seen for NE. The brain DA levels of the deficient rats were not different from those of the control animals.

The brain 5-HT levels of pyridoxine-deficient and pyridoxine-supplemented rats are shown in Fig. 13. There was

Table 4. Effect of Dietary Pyridoxine Deficiency on the Body Weight and Brain Weight of the Growing Rat

| Weeks on Diet | Body Weight (G) | | Brain Weight (G) | |
|---|---|---|---|---|
| | $-B_6$ | $+B_6$ | $-B_6$ | $+B_6$ |
| 3 | 39.5[a]±1.1 | 70.4±3.9 | 1.30[a]±0.01 | 1.44±0.02 |
| 5 | 59.0[a]±2.9 | 92.8±4.8 | 1.32[a]±0.02 | 1.46±0.01 |
| 6 | 58.8[a]±1.5 | 109.7±7.2 | 1.34[a]±0.03 | 1.47±0.02 |
| 7 | 61.4[a]±8.2 | 143.8±11.8 | 1.44[+]±0.04 | 1.49±0.07 |
| 8 | 42.8[a]±4.6 | 168.4±8.6 | 1.40[a]±0.06 | 1.67±0.03 |

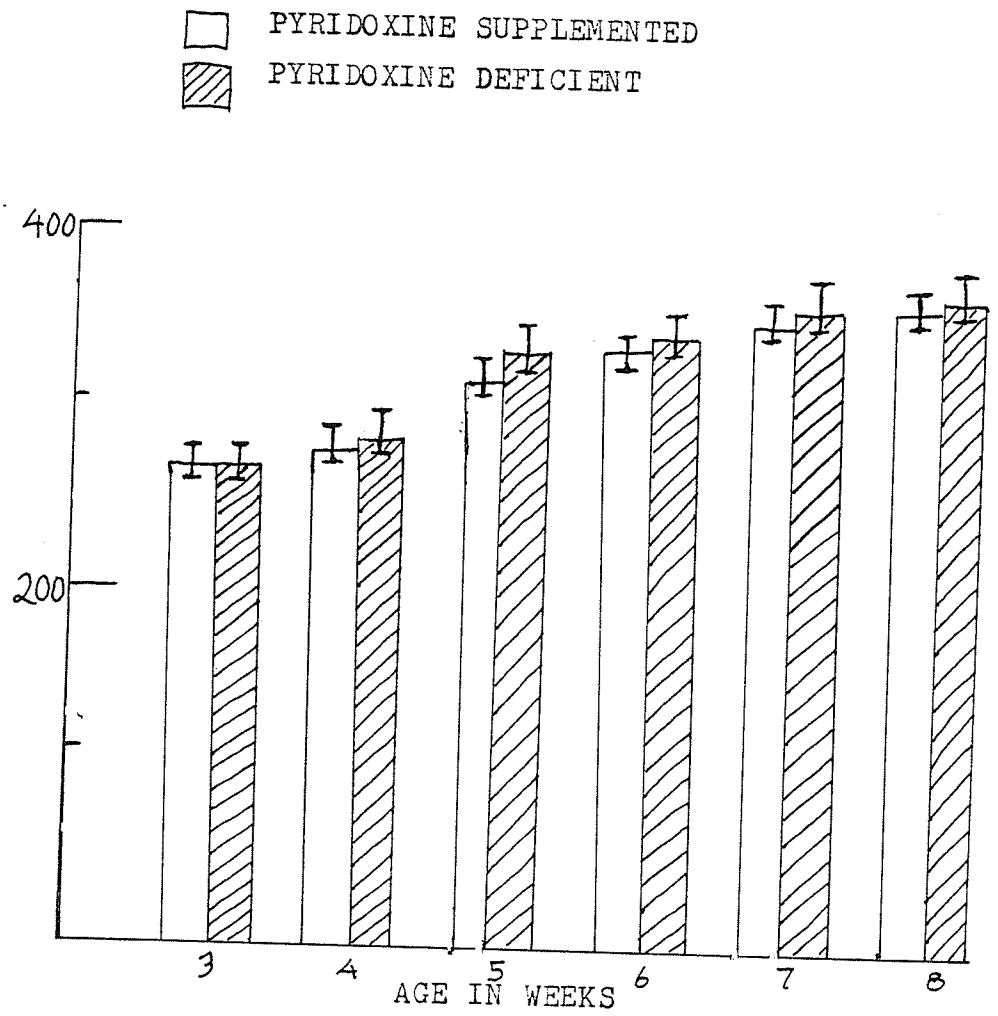
Values are means ±SD of 8 rats in each group.
[a]$p<0.005$ with respect to $+B_6$ controls, [+]$p<0.05$ with respect to $+B_6$ controls

Table 5. Effect of Dietary Pyridoxine Deficiency on the Deep Body Temperature and Motility of the Growing Rat

| Weeks on Diet | Body Temp. (°C) | | Motility (counts/10 min) | |
|---|---|---|---|---|
| | $-B_6$ | $+B_6$ | $-B_6$ | $+B_6$ |
| 3 | 35.8[a]±0.2 | 38.1±0.3 | | |
| 5 | 36.4[a]±0.3 | 38.2±0.2 | | |
| 6 | 35.9[a]±0.4 | 37.6±0.3 | 141[a]±34 | 301±15 |
| 7 | 35.8[a]±0.5 | 37.3±0.3 | 123[a]±25 | 471±48 |
| 8 | 35.2[a]±0.2 | 37.8±0.2 | 114[a]±18 | 358±34 |

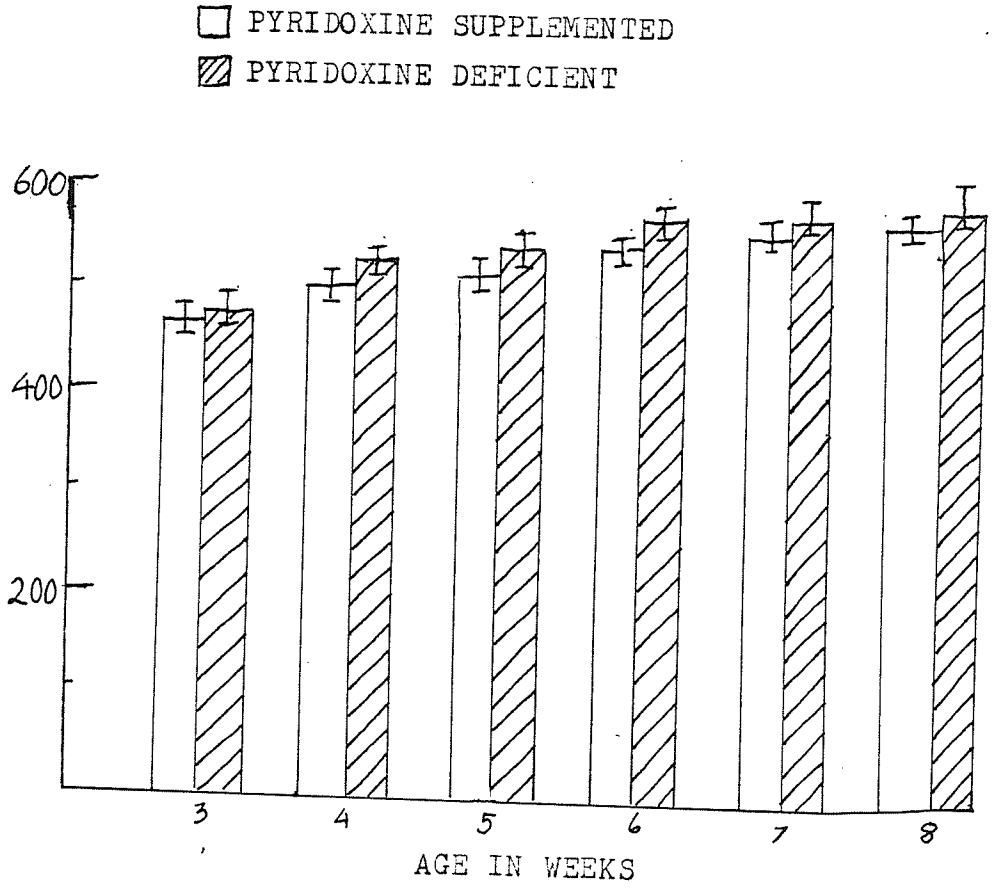
Values are means ± SD of 8 rats in each group.
[a]$P < 0.005$ with respect to $+B_6$ controls

Fig. 11. Effect of Dietary Pyridoxine Deficiency in the Growing Rat on Brain Norepinephrine (ng/g Fresh Weight)



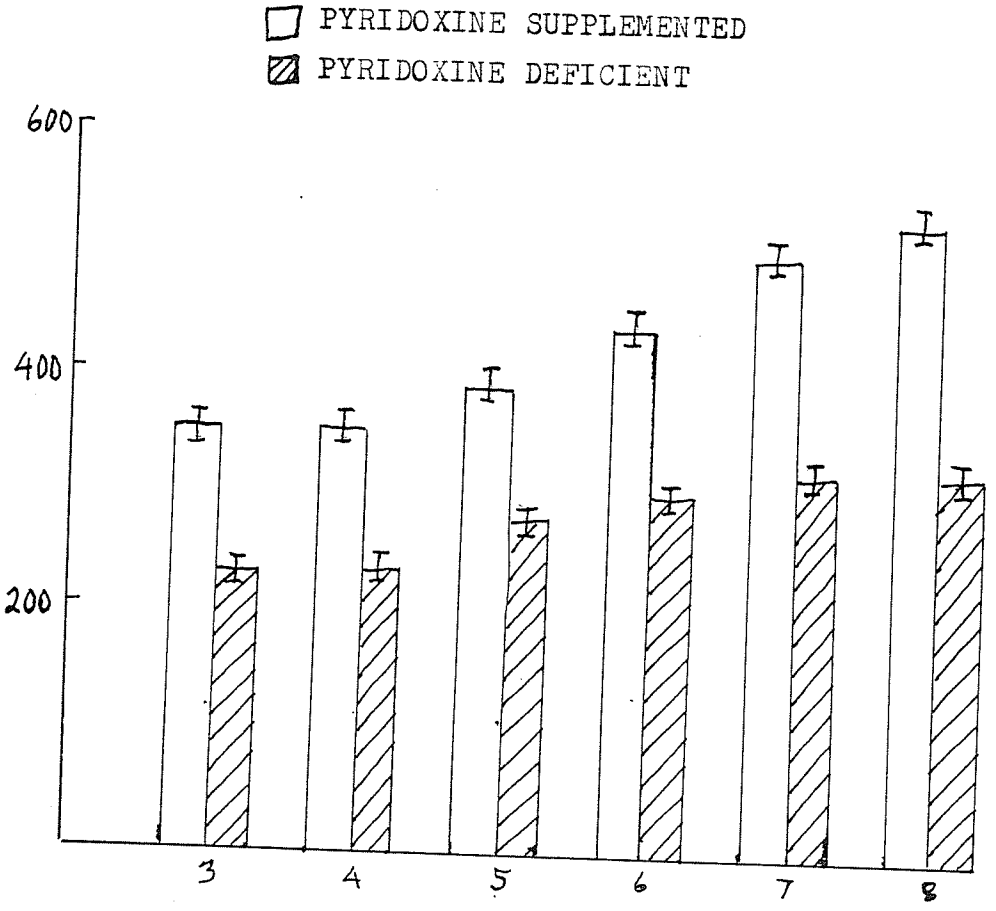

Standard deviation is indicated by bars on the histograms. There were 10 animals in each group.

Fig. 12. Effect of Dietary Pyridoxine Deficiency in the Growing Rat on Brain Dopamine (ng/g Fresh Weight)




Standard deviation is indicated by bars on the histograms. There were 10 animals in each group.

Fig. 13. Effect of Pyridoxine Deficiency in the Growing Rat on Brain Serotonin (μg/g Fresh Weight)




Standard deviation is indicated by bars on the histograms. There were 10 animals in each group.

a consistent increase with age of brain 5-HT in the control group, but this was not as evident in the deficient group. However, there was a very significant decrease in brain 5-HT levels of the pyridoxine-deficient rats throughout the experimental period from 3 to 8 weeks ($P < 0.005$). The difference between deficient and control groups was of the order of 40%.

Brain monoamine levels in deoxypyridoxine-treated rats are shown in Table 6. These values are the means $\pm$ SD of 12 rats in each group. The brain NE and DA levels of the deoxypyridoxine-treated rats were not different from those of the control (pyridoxine-treated) animals. However, there was a significant decrease in the brain 5-HT levels of the deoxypyridoxine-treated rats ($p < 0.05$).

## D. Effect of Dietary Restriction or Pyridoxine Treatment on Body and Brain Weight and Brain Serotonin

The possible effect of inanition and malnutrition on brain levels of 5-HT was investigated in a ''pair-weighing'' experiment, the results of which are seen in Table 7. The reduced brain weights and brain 5-HT levels in the deficient animals were both statistically significant with P values of 0.01 and 0.001 respectively.

The results of a recovery experiment are seen in Table 8. Pyridoxine administration to deficient rats increased both the body weight and the brain 5-HT levels significantly. Even though this experiment established the specificity of pyridoxine deficiency as the cause of the decrease in brain 5-HT, the recovery was not complete. Rats that were on the pyridoxine-supplemented diet still had higher levels of brain 5-HT ($403 \pm 16$ ng/g) than the deficient rats that were injected with pyridoxine ($347 \pm 10$ ng/g).

Table 6. Brain Monoamines in Deoxypyridoxine-Treated Rats

| Treatment | Norepinephrine (ng/g) | Dopamine (ng/g) | Serotonin (ng/g) |
|---|---|---|---|
| Deoxypyridoxine (12) | 389 ± 36 | 723 ± 40 | 369 ± 35* |
| Pyridoxine (12) | 330 ± 24 | 717 ± 46 | 458 ± 45 |

*$p < 0.05$

Table 7. Effect of Pyridoxine Deficiency or Dietary Restriction on Body and Brain Weight and Brain Serotonin

| Animal Status | Age (weeks) | Body Wt. (g) | Brain Wt. (g) | Brain 5-HT (ng/g) |
|---|---|---|---|---|
| On $B_6$ Def. Diet | 4 | 59.6±1.7 | 1.46±0.01[a] | 273±14[+] |
| On Restricted, $+B_6$ Diet | 4 | 59.6±1.3 | 1.50±0.01 | 399±16 |

Values are means ± SD of 8 rats in each group. [a]$P < 0.01$ and [+]$P < 0.001$ with respect to $+B_6$ controls

Table 8. Effect of Pyridoxine Treatment on Body and Brain Weight and Brain Serotonin

| Animal Status | Age (weeks) | Body Wt. (g) | Brain Wt. (g) | Brain 5-HT (ng/g) |
|---|---|---|---|---|
| $B_6$-deficient | 4 | 53.8±3.0 | 1.43±0.02 | 280±12 |
| Pyridoxine injected | 4 | 75.8±2.0 | 1.48±0.02 | 347±10[a] |
| $B_6$-supplemented | 4 | 87.1±5.4 | 1.55±0.04 | 403±16 |

Values are means ± SD of 8 rats in each group. [a] $p < 0.01$ with respect to $B_6$ group

## E. Effect of Pargyline Treatment on Brain Monoamines

The results presented in Table 9 show that the actual levels of all monoamines are higher after pargyline administration due to inhibition of their oxidation. Nevertheless, pyridoxine-deficient rats showed only a 2.1 fold increase in 5-HT levels in 3 hours, while the pyridoxine-supplemented rats had a 3.4 fold increase. Also, only the brain 5-HT levels are significantly decreased in the pyridoxine-deficient animals ($P < 0.005$). The brain NE and DA levels showed no statistically significant differences between the deficients and the controls.

## F. Effect of Probenecid and Pargyline Treatments on Brain 5-Hydroxyindoleacetic Acid

The effect of probenecid treatment on brain 5-HIAA levels is seen in Table 10. The concentration of 5-HIAA in brain was increased in both the deficient and control rats after probenecid treatment, but the brain levels of 5-HIAA of the deficient rats were still significantly lower than those of the controls ($P < 0.025$).

The results in Table 10 show that inhibition of MAO by pargyline resulted in a considerable reduction of brain 5-HIAA concentration in both the pyridoxine-deficient and pyridoxine-supplemented animals. However, brain 5-HIAA levels of the deficient animals were once again significantly lower than those of the controls following pargyline treatment.

## G. Effect of Pyridoxine Deficiency on Plasma and Brain Tryptophan Levels

Total and free plasma levels as well as brain levels

Table 9. Effect of Pargyline Treatment on Brain Monoamines

| Dietary Status of Animal | Age (weeks) | 5-HT (ng/g) | | NE (ng/g) | | DA (ng/g) | |
|---|---|---|---|---|---|---|---|
| | | Untreated | Treated | Untreated | Treated | Untreated | Treated |
| $B_6$-deficient | 4 | 280±12[a] | 588±19[+] | 363±23 | 662±10 | 447±36 | 627± 9 |
| $B_6$-supplemented | 4 | 378±14 | 1288±49 | 416±14 | 602±23 | 482± 8 | 681±34 |

Values are means ± SD of 8 rats in each group. [a]$P<0.01$ and [+]$P<0.005$ with respect to $+B_6$ controls

Table 10. Effect of Probenecid and Pargyline Treatments on Brain 5-Hydroxyindoleacetic Acid

| Dietary Status of Animal | Age (weeks) | Brain 5-HIAA (ng/g) Untreated | Probenecid Treated | Pargyline Treated |
|---|---|---|---|---|
| $B_6$-deficient | 4 | 208±29[a] | 575±43[+] | 88±15[a] |
| $B_6$-supplemented | 4 | 397±15 | 732±32 | 157± 6 |

Values are means ± SD of 8 rats in group. [a] $p < 0.005$ and [+] $p < 0.025$ with respect to $+B_6$ controls

of TRY in pyridoxine-deficient and supplemented groups are shown in Table 11. The fraction of plasma TRY not bound by albumin was about 20% of the total plasma TRY in both groups. Similarly, both groups exhibited no statistically significant differences in either plasma levels of total and free TRY or brain levels of TRY (Table 11).

Similar results were obtained when TRY levels were determined after deoxypyridoxine treatment. These results are seen in Table 12. Each group contained 10 animals. Plasma levels of total and free TRY as well as the brain level of TRY were not significantly altered after deoxypyridoxine administration.

## H. Effect of Dietary Pyridoxine Deficiency on Brainstem Tryptophan-5-Hydroxylase Activity

The results of an experiment investigating the effect of dietary pyridoxine deficiency on brainstem TPOH activity are seen in Table 13. These results show no significant difference in the activity of this enzyme between pyridoxine-deficient and pyridoxine-supplemented rats ($P > 0.10$).

Table 11. Plasma and Brain Tryptophan Levels

| Weeks on Diet | Plasma Tryptophan ($\mu$g/ml) | | | | Brain Tryptophan ($\mu$g/g wet weight) | |
|---|---|---|---|---|---|---|
| | Total | | Free | | | |
| | $-B_6$ | $+B_6$ | $-B_6$ | $+B_6$ | $-B_6$ | $+B_6$ |
| 3 | 13.60±1.15 | 12.21±1.01 | 2.38±0.13 | 2.64±0.21 | 3.24±0.16 | 2.93±0.22 |
| 4 | 9.35±0.79 | 11.07±0.50 | 1.79±0.32 | 2.17±0.14 | 3.21±0.23 | 3.69±0.15 |
| 5 | 13.58±1.33 | 12.26±0.89 | 2.59±0.14 | 2.69±0.27 | 3.54±0.31 | 3.28±0.18 |
| 6 | 11.86±0.75 | 11.55±0.43 | 2.76±0.08 | 2.49±0.19 | 3.33±0.23 | 3.48±0.29 |

Values are means ± SD of 8 rats in each group. No statistical significance between values for $+B_6$ and $-B_6$ groups

Table 12. Plasma and Brain Tryptophan Levels in Deoxypyridoxine-Treated Rats

| Treatment | Plasma Tryptophan (μg/ml) | | Brain Tryptophan (μg/g) |
|---|---|---|---|
| | Total | Free | |
| Deoxypyridoxine (10) | 17.52 ± 0.61 | 1.83 ± 0.18 | 3.53 ± 0.31 |
| Pyridoxine (10) | 18.32 ± 0.91 | 1.83 ± 0.19 | 3.28 ± 0.19 |

Table 13. Brainstem Tryptophan-5-Hydroxylase Activities

| Dietary Status of Animal | Tryptophan-5-hydroxylase activity * (n mole/hr/mg protein) |
|---|---|
| $B_6$-deficient | $2.98 \pm 0.15^{+}$ |
| $B_6$-supplemented | $2.54 \pm 0.15$ |

*Values are means ± SD of 7 experiments in each group; $^{+}p > 0.10$ with respect to $+B_6$ controls.

# IV. Discussion

The results of this investigation have shown a nonparallel change in the brain level of the monoamines in the pyridoxine-deficient growing rat. There was a very significant decrease in brain 5-HT with no alteration in the NE and DA levels. These results are in agreement with a report by Sourkes (1972) in which there was no change in the steady state concentration of catecholamines of various tissues in pyridoxine deficiency.

The young rats also exhibited a marked impairment of growth as previously reported by Eberle and Eiduson (1968) and Dakshinamurti and Stephens (1969). Changes in motility were also significant. The deficient animals were less irritable and less aggressive than the controls. This reduced motility is specific to pyridoxine deficiency, since rats deficient in other vitamins such as biotin are quite aggressive.

Modigh (1974) and Myers (1975) have presented evidence that a serotonergic mechanism is involved in thermoregulation in the rat. Results of the present study show a decrease in deep body temperature in the pyridoxine-deficient rats. Therefore, this hypothermia might be related to the decrease in the brain 5-HT levels in these animals.

The ''pair-weighing'' experiment in this investigation clearly indicates that pyridoxine deficiency rather than general malnutrition is the factor responsible for the decrease in brain 5-HT. Although the rats on restricted dietary intake were undernourished, their brain 5-HT levels were not decreased. Normal brain 5-HT levels have also been reported by Sereni et al (1966) in 35-day old rats that had been undernourished since birth. However, Ahmed and Rahman (1975) found no effect of malnutrition in rats on any of the brain monoamines. In their study the animals were malnourished during intrauterine or post-natal life up to 42 days of age. In addition, Shoemaker and Wurtman (1971) have found a decrease in the catecholamines of undernourished rats. The normal levels of both the catecholamines and the decrease in brain 5-HT levels in the

present investigation are in sharp contrast to these studies. These differences may be due to the different methods used to produce undernutrition in the experimental animals.

Recovery experiments were also used in order to establish the specificity of pyridoxine deficiency as the cause of the decrease in brain 5-HT levels. Although the recovery was not complete, the increase in brain 5-HT levels in the deficients one week after a single injection of pyridoxine was statistically significant ($P<0.01$).

Further proof of the specificity of the deficiency was obtained by injecting rats with deoxypyridoxine, a pyridoxine antagonist. These animals exhibited the same nonparallel changes in brain monoamines, i.e. a very significant reduction in 5-HT with no change in NE or DA.

In order to investigate the possibility that the reduction in brain levels of 5-HT in the pyridoxine-deficient animals might have resulted from increased catabolism, MAO was inhibited in vivo using pargyline. This possibility was eliminated, since analysis of brain monoamines revealed the same reduction in brain 5-HT with no significant changes in the catecholamines of the deficient animals. Further investigation of both 5-HT and 5-HIAA by means of a different extraction method developed by Curzon and Green (1970) provided additional evidence that the catabolism of 5-HT is not altered in pyridoxine-deficient rats. Finally, the effects of probenecid administration showed that there was no increase in elimination of 5-HIAA from the brain to the CSF in pyridoxine deficiency.

An analysis of free and total TRY in plasma as well as brain TRY levels revealed no significant differences between the pyridoxine-supplemented and pyridoxine-deficient animals. Similar results were obtained when the deficiency was produced with deoxypyridoxine. Thus, substrate availability did not appear to be the cause of the decreased brain levels of

5-HT in the deficient rats.

A study of TPOH which is thought to be the rate-limiting enzyme in 5-HT synthesis showed no significant difference in its activity between pyridoxine-deficient and pyridoxine-supplemented rats. This suggests the possibility that the decreased synthesis of brain 5-HT in the pyridoxine-deficient growing rat is due to a decrease in the decarboxylation of 5-HTP.

As mentioned in the Review of the Literature, attempts at purification of the ''aromatic L-amino acid decarboxylase'' have so far been unsuccessful. Difficulties and differences in the conditions used for its assay were also discussed, as well as evidence for and against the existence of one enzyme. The differences in regional and subcellular distribution of the DOPA and 5-HTP decarboxylating activities reported by Sims (1974) indicate the possibility that the two decarboxylases are distinct enzymes. The specific effect of pyridoxine deficiency in the growing rat on just the decarboxylation of 5-HTP with no apparent effect on that of DOPA seems to support the existence of two separate enzymes.

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