THE NEURONAL EFFECTS OF PYRIDOXINE DEFICIENCY ON THE MATURE RAT

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Wayne David Singer

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BY

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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for my parents, Brian and Marilyn

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ABSTRACT

Vitamin B_6 or pyridoxine is essential for the normal development of the central nervous system (CNS). It is the coenzyme of the various amino-acid decarboxylases involved in the formation of various neurotransmitter amines. It is required for proper myelination. Also, the effects of pyridoxine deficiency on the developing rat pup have been investigated. In these animals the deficiency was induced during the critical development of the CNS.

The objective of this thesis was to investigate the effects of pyridoxine deficiency induced after maturation on the CNS. It was found that pyridoxine deficiency was associated with morphological changes such as lesions around the paws, eyes and nose as well as a significant loss of body weight. There was no difference in brain weights. Pyridoxal phosphate levels and glutamic acid decarboxylase (GAD) activity were significantly lowered in all regions of the brain examined. This was in contrast to GAD apoenzyme activity which was greater than that of controls. Histological examination revealed that there was no change in the amount of myelin or myelinated tracts resulting from the deficiency. Examination of the electroencephalogram revealed no significant differences between the control and deficient rats. Investigation of auditory evoked potentials demonstrated a longer but non-significant latency period in deficient animals. The amplitudes of the evoked potentials were also greater in these rats. As previously seen in rat pups, non-parallel changes in monoamine levels were associated with pyridoxine deficiency. A significant reduction of serotonin levels was seen in brain regions of deficient rats while the levels of norepinephrine and dopamine remained at control values.

Since the structural maturation of the brain is considered essentialy complete at weaning in rats, our investigation suggests that some of the effects of pyridoxine deficiency on rats need not be related to the critical preweanling period of development. The effects seen in rat pups were therefore due to pyridoxine deficiency rather than developmental factors but other effects may be related to the developmental stage.

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Abbreviations

Ach	Acetylcholine
AOHH	Amino-oxyacetic Acid
B.S.	Brainstem
Cb	Cerebellum
C.C.	Cerebral Cortex
CNS	Central Nervous System
C.S.	Corpus Striatum
CSF	Cerebrospinal Fluid
DA	Dopamine
DOPA	Dihydroxyphenylalanine
DOPAC	3-4 Dihydroxyphenylacetic acid
E	Epinephrine
EDTA	Ethylene Diamine Tetra Acetic Acid
EEG	Electroencephalogram
E.P.	Evoked Potential
GABA	Gamma-aminobutyric Acid
GABA-T	GABA-transaminase
GAD	Glutamic Acid Decarboxylase
5-HT 1AA	5-hydroxyindoleacetic Acid
5-HT	5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
Hypo	Hypothalamus
L-AMP	Levo-adenosine monophosphate
LC	Locus Coeruleus
LH	Luteinizing Hormone

Mb.	Midbrain
MAO	Monoamine Oxidase
NCS	NCS Tissue Solubilizer
NE	Norepinephrine
OPT	O-pthaldialdehyde
PT	Inorganic Phosphate
PLP	Pyridoxal Phosphate
PNS	Peripheral Nervous System
SSADH	Succinic Semialdehyde
TOH	Tyrosine Hydroxylase
TOPH	Tryptophan Hydroxylase

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 divided into five major sections. The first section deals with the normal development of the central nervous system highlighting the three main stages of development. The second section discusses various aspects of pyridoxine metabolism such as its biochemistry, functions and the effect of pyridoxine deficiency on the developing rat. The last three sections focus on neurotransmitters. They deal with γ-aminobutyric acid, serotonin, and the catecholamines with respect to their function, localization, regulation and the effect of pyridoxine deficiency on them. Finally a brief conclusion summarizes the Review of the Literature, and states the objectives of this investigation.

The section entitled "Experimental" outlines both the methods and 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 methods for measuring pyridoxal phosphate, glutamic acid decarboxylase and myelination. This is followed by several experiments designed to determine the effects of deficiency on electrophysiological parameters such as EEG readings and evoked potentials. Finally, the method for measuring brain monoamines 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 that the effects of pyridoxine deficiency in rats need not be related to the critical preweanling period of development.

II REVIEW OF THE LITERATURE

Development of the Central Nervous System

The development of the central nervous system (CNS) begins essentially as soon as the neural tube appears. The period of development can roughly be divided into three stages namely the period of cell division, the period of cell growth with the formation of axons, dendrites and their interconnections, and finally, the last stage, that of myelination.

The first stage, that of predominantly cell division, can be measured biochemically by the determination of deoxyribonucleic acid (DNA) based on the premise that DNA has predominantly a nuclear localization and is constant in amount in each diploid cell. Thus, the content of brain DNA increases during this period which lasts until the 13th day of life in the rat (Winick and Noble, 1965).

The second stage is characterized by an increase in brain weight, greater than can be accounted for by the increase in cell number. This is also accompanied by increases in ribonucleic acid and protein content (Winick, 1968) and subsequently by an increase in lipids (Yakovlev and Lecours, 1967).

Once the architecture of the brain has been established during the earlier stages of development, myelination quantitatively constitutes the major component of brain growth (Morré et al, 1970). The formation and deposition of myelin is a consequence of the previous development of glial cells (Peters and Vaughn 1970). A glial cell process encircles an axon forming a mesaxon which subsequently elongates progressively around the axon to form the spiral known as the mature myelin sheath. This process of myelination is accompanied by several alterations in the lipid profile of the CNS.

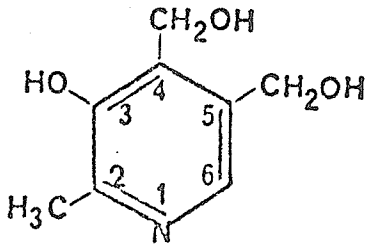
Pyridoxine

The term vitamin B_6 is the generic description for all 3-hydroxy-2-methyl-pyridine derivates exhibiting qualitatively the biological activity of pyridoxine in rats. There are three naturally occuring pyridines namely pyridoxine, pyridoxal, and pyridoxamine which are metabolically and functionally inter-related (Figs. 1 & 2), (Bonjour, 1980). The active form of these vitamins in tissue are the 5'-phosphorylated derivatives. The phosphorylation is accomplished by a phosphokinase whose substrate affinity decreases in the order of pyridoxal, pyridoxine and pyridoxamine. Although the various members of the B_6 family may occur naturally and may to varying degrees be active nutritionally for animals and microorganisms, they are not metabolically active as a coenzyme unless in the form of pyridoxal phosphate or pyridoxamine phosphate. Pyridoxal phosphate is the major co-enzymatic form of pyridoxine (Dakshinamurti, 1981).

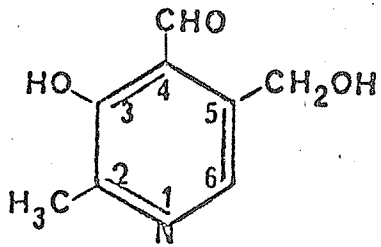
Biochemical Reactions

Since pyridoxal is a carbonyl compound, it reacts as other carbonyl compounds, with primary amines to form a Schiff base, as outlined below:

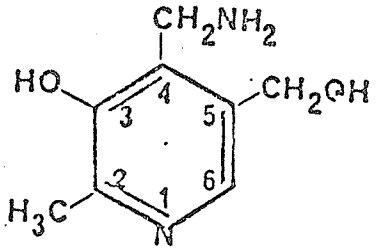
$$R_1 - NH_2 + R_2 - \underset{\underset{O}{\|}}{C} - R_3 \longrightarrow R_2 - \underset{\underset{N - R_1}{\|}}{C} - R_3 + H_2O$$



Pyridoxine



Pyridoxal

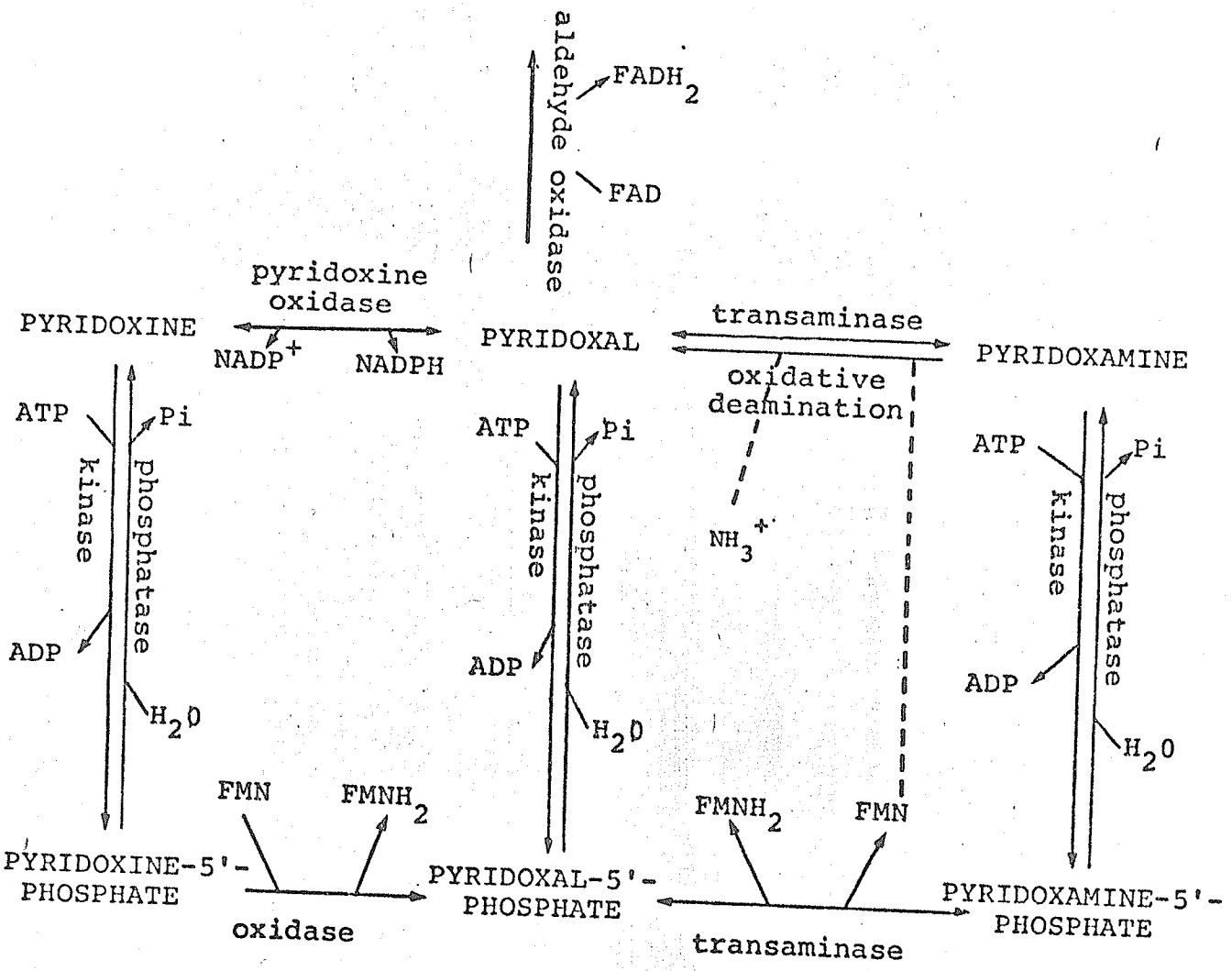


Pyridoxamine

Figure I: Chemical Structure of B_6 Vitamers

Figure 2: Relationship between Vitamin B_6 vitamers (Harris 1968)

4-PYRIDOXIC ACID



As a result, the carbon atom of the substrate which is adjacent to the aldimino group is destabilized. The further reactions can be classified into three groups depending upon the site of elimination and replacement of the substituents. Reactions occurring at the α -carbon atom include those catalyzed by transaminases, racemases of α -amino acid, α -amino acid decarboxylases, the condensation reactions of glycine, the α - β cleavage of β -hydroxy amino acids by enzymes such as δ -amino levulinic acid synthetase, serine hydroxymethylase and sphingosine synthetase. Reactions occurring at the β -carbon atom include those catalyzed by serine and threonine dehydrases, cystathionine synthetase, tryptophanase and kynureninase. Reactions catalyzed by homoserine dehydrase and γ -cystathionase occur at the γ -carbon atom (Dakshinamurti, 1981).

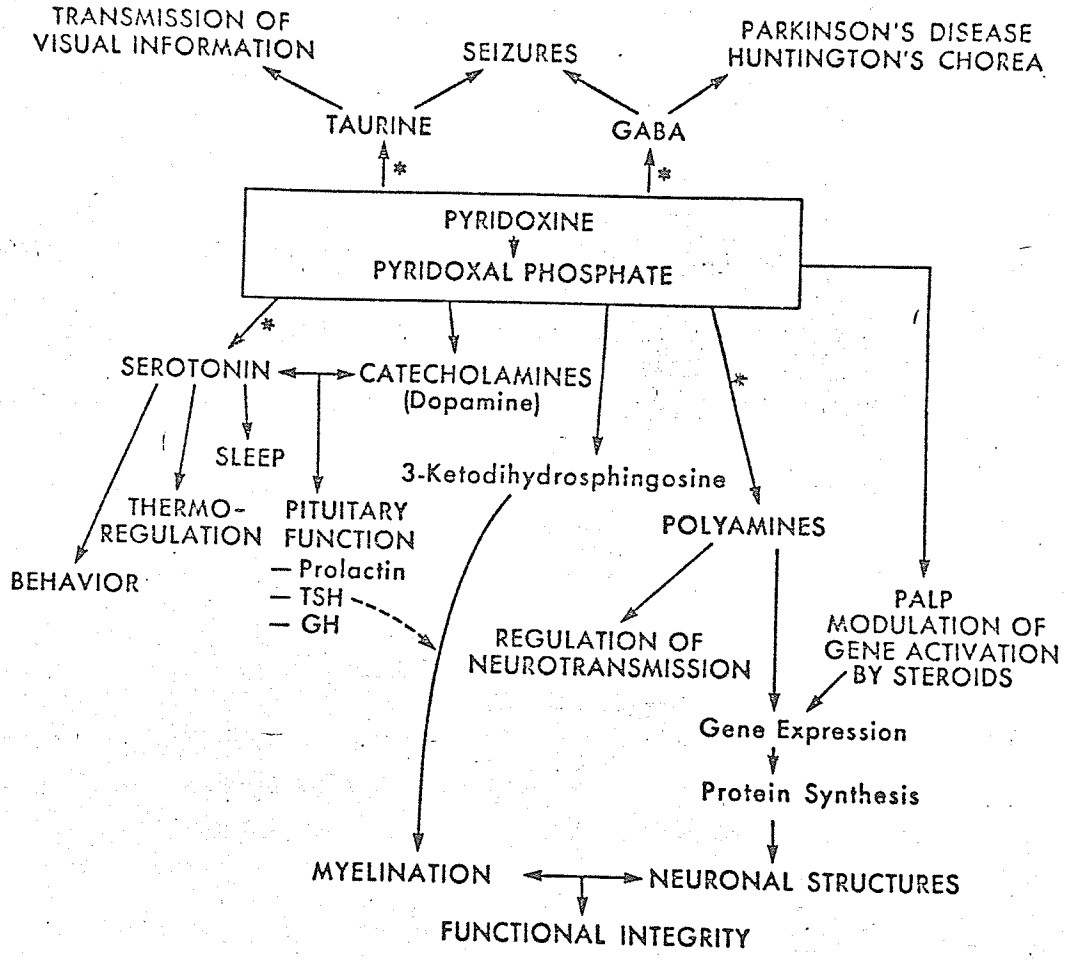
Pyridoxine-dependent enzymes investigated in the nervous system are involved in catabolic reactions of various amino acids. They include the following: glutamic-oxaloacetic aminotransferase, glutamic-pyruvic aminotransferase, amino acid (valine, leucine, isoleucine, cysteine, methionine, ornithine) - α ketoglutarate aminotransferase, γ -aminobutyrate transaminase, cystathionine synthetase, cystathionase, kynureninase, glutamic acid decarboxylase, aromatic amino acid (tyrosine, dihydroxyphenylalanine, 5-hydroxytryptophan, phenylalanine, histidine) decarboxylases, ornithine decarboxylase and cysteine sulfinic acid (cysteic acid) decarboxylase. An enzyme reaction not related to amino acid metabolism but of significance to nervous tissue is dihydrosphingosine synthetase (Dakshinamurti, 1981).

The crucial role played by pyridoxine in the nervous system is evident from the fact that the various putative neurotransmitters - dopamine, norepinephrine, tyramine, tryptamine, serotonin, histamine, γ-amino-butyric acid and taurine - are synthesized and/or metabolized by pyridoxine-dependent enzymes. The role of pyridoxine in the synthesis of sphingolipids and polyamines highlights its importance in the development as well as in the maintenance of the integrity of the nervous system (Dakshinamurti,1981),(Fig. 3). The interaction of biogenic amines and pyridoxal kinase may alter the formation of pyridoxal phosphate (PLP) which in turn may influence the activity of numerous PLP dependent enzymatic reactions in brain (Ebadi and Govitrapong,1979).

Effects of Pyridoxine Deficiency

Pyridoxine deficiency can be produced either by dietary restrictions or through the use of antimetabolites. Vitamin B_6 inhibitors have been used extensively as an aid in the development of pyridoxine deficiency symptoms, in studies on the mechanisms of action of pyridoxal phosphate and in the elucidation of enzymes which require this cofactor. There are several groups of Vitamin B_6 inhibitors. One group represents pyridoxine analogs in which either the ring has been modified or the substituents of the ring have been altered. In another group are various binding agents that inactivate pyridoxine by combination. Of the antagonists of pyridoxine available, 4-deoxypyridoxine has been widely employed. Deoxypyridoxine appears to be converted by pyridoxal kinase to a phosphorylated form, which in turn competes with pyridoxal phosphate for various coenzymes (Sauberlich, 1968).

Figure 3. Involvement of Pyridoxine in CNS (Dakshinamurti 1981)


TRANSMISSION OF VISUAL INFORMATION
SEIZURES
PARKINSON'S DISEASE HUNTINGTON'S CHOREA
TAURINE
GABA
PYRIDOXINE
PYRIDOXAL PHOSPHATE
SEROTONIN
CATECHOLAMINES (Dopamine)
SLEEP
3-Ketodihydrosphingosine
THERMO-REGULATION
PITUITARY FUNCTION
— Prolactin
— TSH
— GH
POLYAMINES
BEHAVIOR
REGULATION OF NEUROTRANSMISSION
PALP MODULATION OF GENE ACTIVATION BY STEROIDS
Gene Expression
Protein Synthesis
MYELINATION
NEURONAL STRUCTURES
FUNCTIONAL INTEGRITY

Meisler and Thanassi (1980) measured the levels of pyridoxal phosphate in plasma, liver, and brain, and the activities of pyridoxine kinase, pyridoxine phosphate phosphatase and pyridoxine phosphate oxidase in liver and brain over a six week period in rats fed pyridoxine sufficient and pyridoxine deficient diets. Consistently significant differences in enzyme activities between the two groups of animals were found only in pyridoxine kinase indicating that this enzyme plays a key role during the development of pyridoxine deficiency. Physical development in rat pups on a pyridoxine deficient diet was impaired (Dakshinamurti and Stephens, 1969). Retardation of growth, delay in the onset of reflexes such as palmer-grasp, vibrassæ placing, visual placing, and auricular startle, and a delay in the onset of advanced neuromotor coordination such as standing and grooming were observed in these pups (Alton-Mackey and Walker 1978). The morphological effects of the deficiency will be discussed later (Methods) as will the effects on neurotransmitter levels. The biochemical effects are well documented (Weber and Wiss 1968, Dakshinamurti and Stephens 1969, Dakshinamurti et al 1971).

Gamma(γ)-Aminobutyric Acid

Phylogenetically, enzymatic degradation of L-glutamate to form γ-aminobutyric acid (GABA) is found throughout living organisms, from bacteria to fungi to animals (Starr 1978). GABA is an inhibitory synaptic transmitter in the central nervous system (CNS). The evidence for GABA being a transmitter can be summarized as follows:
when GABA is applied to neurons it produces electrical and ionic changes similar to those observed with the natural transmitter.
Glutamate decarboxylase (L-glutamate-1-carboxylase, EL.4.1.1.15) (GAD)

is present in nerve endings (associatedwith synaptosomes) and concentrated in regions where GABA seems to act as a transmitter. GABA is released from brain tissue upon stimulation by a calcium dependent mechanism (Sandoval, 1980, Pearce et al, 1980) and it is eliminated from extracellular spaces by a sodium dependent uptake into nerve cells, glial cells or nerve endings (Sze and Lovell, 1970). GABA metabolism may be viewed as follows:

$$\text{glutamic acid} \xrightarrow{\text{GAD}} \text{GABA} + CO_2$$

$$\text{GABA} + \alpha\text{-ketoglutamate} \xrightarrow{\text{GABA-T}} \text{succinic semialdehyde} + \text{glutamate}$$

$$\text{Succinic semialdehyde} + NAD^+(P) \xrightarrow{\text{SSADH}} \text{succinic acid} + NADH(P) + H^+$$

GABA-T = GABA-transaminase

SSADH = succinic semialdehyde dehydrogenase

GAD is the rate limiting enzyme for this pathway

Different organisms possess different forms of GAD. For example, comparison of human and rat brain GAD reveals several differences in the primary structure of the two enzymes as well as differences in the optimum pH of the reaction and the Km values for glutamate and PLP (Maitre and Mandel, 1978). Even within the same organism isozymes exist i.e. mouse GAD in non-neural tissues differ from that found in the CNS (Wu and Roberts, 1974). Alterations in GABA metabolism, such as interference with receptor binding, result in powerful convulsions in experimental animals (Meldrum and Horton, 1971). These were observed immediately when GAD activity, and thus the rate of GABA synthesis, was inhibited to a certain extent. This was independent of total brain GABA levels suggesting that under normal conditions, GABA was not

being released from a storage pool specifically related to its synaptic function but instead that the newly synthesized GABA was being liberated into the synaptic cleft (Sze and Lovell, 1970).

The importance of GABA becomes apparent when one considers that dysfunctions of GABA mediated synaptic transmissions have been implicated in human pathological conditions including Huntington's Chorea, Parkinson's Disease, Alzheimer's Disease, some forms of epilepsy and possibly in schizophrenia (Neal and Iverson, 1969; Enna, 1980). In this discussion I have focused upon the distribution of GAD and GABA in the CNS, peripheral nervous system (PNS) and non-neuronal tissue as well as the regulation of GAD activity.

DISTRIBUTION OF GABA

A) PNS and Non-Neural Tissue

GABA in the pineal gland is not involved in neurotransmission. This is supported by the absence of GABA releasing nerve terminals (Roberts and Anderson, 1979). GABA is taken up exclusively by the gliocyte cells of the gland as shown following both in vitro incubation with ^{3}H-GABA and after in vivo administration of the amino acid by intra-arterial injection. The concentration of GABA in the pineal gland was found to be about 30% of that found in the CNS as determined by the microdansylation technique (Sherman and Gal, 1978). GAD activity is not located in nerve processes entering the pineal gland. Rather, it is associated with intrinsic pineal structures rather than nerve processes whose cell bodies are extra pineal. Significant amounts of GAD were present at 25% of the levels found in the spinal cord suggesting endogenous GABA synthesis within the gland (Roberts and Anderson, 1979). The regulation of GABA and GAD levels, as well as the role of GABA, are being

investigated.

The posterior pituitory gland and the superior-cervical and dorsal root ganglia all show the capacity to actively accumulate exogenous (^{3}H)-GABA (Beart and Schon, 1974). As in the pineal gland, this accumulation occurred exclusively in the glial cells and their processes as opposed to the neural components which were completely devoid of label. The tissues contained significant amounts of endogenous GABA, GAD and GABA-T.

A non-transmitter role for GABA in these regions was suggested where it may act as an intermediate in the metabolic pathway from glutamate to succinate (Beart and Schon, 1974). Evidence has been published that GABA is an neuro-transmitter in the peripheral autonomic nervous system (Jessen et al, 1979). They have shown that a small population of neurons in the myenteric plexus of guinea pigs posses a high affinity uptake mechanism for GABA. Furthermore, the myenteric plexus contains GAD, endogenous GABA and can synthesize and accumulate both ^{3}H - GABA and ^{3}H - homocarnosine from ^{3}H - glutamic acid.

GABA is also found in non-neuronal tissue such as kidney, blood vessels and glial cells (Beart and Schon, 1974). Rat kidney cortex converts glutamate to GABA by a decarboxylation which differs from the corresponding reaction in brain (Lancaster and Whelan, 1973). GABA stimulates respiration in the kidney cortex slices in vitro and the compound crosses the cell membrane by a respiration linked process. In rats, chronic acidosis lowers renal GABA but has no effect on the brain form.

B) Central Nervous System

a) Cerebrospinal fluid (CSF)

Discrepancies were found in the literature with respect to [GABA] in the CSF. In the introduction to GAD and GABA, I mentioned that GABA was related to the etiology of some diseases. For example, the GABA in the CSF and brain of choreic patients was significantly lower than control subjects (Emma and Yamahura, 1977; Palkovits and Saavedra, 1974). This, and the existence of a concentration gradient for GABA within the ventriculospinal fluid system, suggests that CSF GABA concentrations may reflect brain GABA concentrations (Loscher, 1979). It is minimally affected by changes in peripheral GABA (Bohler and Palfeyman, 1979). GABA exists in the CSF in two forms, conjugated and free. The chemical nature of the conjugated form is unknown but its concentration, and that of GABA, are linearly correlated thus allowing indirect monitoring of changes in brain GABA content during drug therapy (Bohler and Palfeyman, 1979). No references were made to GAD concentration or activity.

b) Brain and Spinal Cord

GAD activety has been found in virtually all brain areas examined as well as in the spinal cord. The highest specific activities are localized in substantia nigra, nucleus accumbens, globus pallidus, head of the caudate and dentate nucleus. The majority of GABA-ergic terminals in the globus pallidus belong to striatopallidal fibers (Starr, 1978; Dichiara et al, 1980). Also, a large number of striatoentopendunclular and striatonigial fibers are GABA-ergic, the latter arising preferentially from the posterior part of the caudate-putamen (Fannum and Grofova, 1978; Fibiger, 1980).

Tappaz et al, measured GAD levels in rat brain nuclei and found an uneven distribution i.e. the areas richest in GAD contained ten times as much enzyme as the poorest region (Tapia and Conteras, 1975). High GAD activity (500 pmoles/h/ug protein) was found in the substantia nigia, the colliculi, the olfactory tubercles and olfactory bulb, some rostral limbic system nuclei (nucleus tractus diagnoalis, nucleus accumbens), some hypothalamic nuclei (the dorsal medial nucleus) and in the medial forebrain bundle. Low GAD activity (150 pmoles/h/ug protein) was found in the olfactory tract, hypothalamic median eminance, nuclei of the pons, and reticular formation. Moderate activity was found in most areas. In areas such as the colliculi and olfactory bulb which contains layered structures, an uneven distribution of GAD and GABA have been shown (Tapia and Contreas, 1975; Austin and Mandel, 1979). On the whole, GAD activity is fairly high (100-1000 pmole/h/ug protein) especially when compared with rate limiting enzymes for the synthesis of other neurotransmitters such as tryptophan hydroxylase (.2 - .5 pmoles/h/ug protein) (Tapia and Conteras, 1975). GAD is evenly distributed throughout the cerebral cortex and amygdaloid nuclei. This is in agreement with the idea that short GABA-ergic interneurons synapsing upon bodies of pyramidal cells are scattered throughout the cerebral cortex (Holtfelt and Ljungdahl, 1972; Nahmod and Pirola, 1978). When GABA content was measured in 70 nuclei, it was found to parallel GAD activity. GABA content of the sacral region of the spinal cord is greater than that of the cervical, lumbar and thoracic regions (Heyden and Versteeg, 1979).

REGULATION OF GAD

Alterations in the binding of PLP to the apoenzyme may regulate the activity of GAD *in vivo* and therefore play an important role in controling the rate of GABA synthesis. Glutamate can inactivate GAD by promoting the disociation of PLP from the enzyme (Miller and Walters, 1978). The basis for this is that when a 20,000g supernatant of rat brain is incubated in the presence of glutamate and without exogenous PLP, there was a progressive glutamate concentration-dependent inactivation of the enzyme. This inactivation was not due to enzyme denaturation, substrate contamination, pH effects, depletion of substrate, protein concentration, sulfhydryl reagents or product inhibition. Another indirect method for GAD regulation through PLP is by ATP activation (Tappasz and Palhovits, 1976). In the presence of .1 mM ATP an 80% increase in GAD activity is seen. However it has been reported that when a more purified form of the enzyme is used, this stimulation was removed (Weiner, 1970). This observation was supported when the dialyzed enzyme showed a minimal amount of ATP promoted stimulation of GAD activity. If in addition to ATP 1mM pyridoxal or pyridoxamine was present, four-fold stimulation was observed suggesting that one of these is needed for the ATP effect. Data shows that under these conditions PLP is generated during the assay and that the amount of cofactor produced (16 uM) is sufficient to account for the observed stimulation (Tappasz and Palhovits, 1976). It has been reported that ATP is able to inhibit GAD activity, the extent of which was dependent upon PLP and the amount of inorganic phosphate present (an inverse relationship) (Tursky, 1970; Tunnicliff and Ngu, 1977; Schon and Kelly, 1975). This inhibition may come about

by a block of the association of PLP with the apoenzyme thereby producing a progressive inactivation of the enzyme (Schon and Kelly, 1975).

Martin and Martin (1979) have suggested that Pi relieves the inhibition by ATP, at least in part, by promoting the activation of glutamate apodecarboxylase and that Pi may be an important factor in GAD regulation. Tunnicliff et al were only able to detect stimulation and explain discrepancies by stating that "the machinery for generating PLP is present only in certain subcellular fractions" (Tappasz and Palkovits, 1976). ADP, UTP and GTP are strong inhibitors of GAD activity at high concentrations. AMP and adenosine have no effect.

GABA has no direct effect on GAD activity in the mammalian brain although it has been shown to repress the enzymes synthesis indirectly (Weiner, 1970). Sze and Lovell (1970) presented evidence demonstrating a reduction in the steady state level of GAD in mouse brain when endogenous GABA was increased. They suggested that this may be product repression of the enzyme's synthesis. GABA has also been shown to inhibit brain pyridoxal kinase and this may represent a mechanism for regulating GAD through PLP (McGeer and McGeer, 1975). As GABA is not an inhibitor of vertebrate brain GAD, its effect on the activity of the enzyme in vivo or in tisue culture may be attributable to feedback repression of the enzyme through cellular mechanisms regulating synthesis or degradation of the enzyme. During early CNS development before GAD concentration is significant, GABA may be formed from putrescine by a path involving ornithine decarboxylase in the initial step (DeMillo and Nirenberg, 1976). It has been speculated that gene expression for GAD in GABA-ergic neurons during early development may

be mediated by regulatory events related to ornithine decarboxylase. This model has never been tested (Starr, 1978).

One means by which GABA synthesis may be regulated is through alterations in GAD saturation with cofactor (Miller and Walters, 1979). Recent studies show that the majority of GAD molecules in vivo are in the apoenzyme form even though the brain PLP concentration is high enough to saturate the enzyme (Miller and Walters, 1977). This idea emerged after it was found that there is increased GAD saturation by PLP after death and during homogenation. When these changes were minimized, whole brain GAD appeared no more than 35% saturated by PLP in vivo. This supports the possibility that increased release and utilization of GABA may be associated with increases in the amount of PLP endogenously bound to GAD in nerve terminals (Miller and Walters, 1979).

DOPA has an inhibitory effect on both GAD and GABA-T but only at high concentrations (McGeer and McGeer, 1975). Both brain PLP levels and GAD activity were decreased in rats 24 hours after an oral dose of 125 mg/kg of DOPA. This could indicate some inhibition of pyridoxal kinase by dopa or its metabolites.

Brain GAD may be inducable. Acetylcholine (Ach) can cause GAD induction in vivo in the cerebellar cortex of the developing mouse (Starr, 1978). The effect of chronic Ach treatment on GAD responsivity was also examined. There was a lack of response after two weeks, possibly due to the full maturation of the blood-brain barrier. The mechanism of this increase in GAD activity, presumably enzyme induction initiated by pharmacologically enhanced cholinergic activity, is presently not known.

GAD may be regulated transynaptically. In the striatum, where the nigro-striatal pathways give dopaminergic innervation to GABA-ergic neurons, GAD activity was found to be reduced when pathway degeneration occurs, such as in Parkinson's disease (Floyd and Hornykiewicz, 1973).

Dopaminergic processes within the substantia nigra can also effect endogenous GABA release (van der Heyden et al, 1980-a) in a concentration dependent manner. The presence of 10 uM-DA in the perfusion medium increased GABA release (140%). At 25 uM-DA both stimulation and inhibition of the nigral GABA release was observed. Higher concentrations of dopamine produced a decreased GABA release (50%). The in vivo release of endogenous GABA from rat striatum has also been shown to be inhibited by dopamine (van der Heyden et al, 1980-b).

Recently it has been suggested that brain GAD may be regulated by a protein posphorylation process (Starr, 1978). There are two lines of evidence supporting this theory. Firstly, purified alkaline phosphatase causes a loss of GAD activity. The inactivation is characterized by a reduction in V_{max} with no changes in Km for PLP. A second line of evidence is the demonstration that GAD can be activated by a system that favors phosphorylation (Starr, 1978). Pre-incubation of a mouse cerebellar supernatant preparation in the presence of ATP and Mg^{+2} nearly doubled GAD activity. This activity was retained after dialysis which removed the added ATP and Mg^{+2}. This indicates that the increase may be due to covalent bond formation.

It has also been suggested that phosphorylation and dephosphorylation of presynaptic membrane proteins may form a mechanism for the presynaptic control of GABA release (Brennan and Cantrill, 1980 a & b). As

mentioned previously, GAD activity has been shown to occur in kidney and other non-neural tissue. In contrast to brain GAD,which is inhibited by anions and carboxyl trapping agents and is PLP stimulated, non-neural GAD requires high concentrations of anions (e.g. Cl^-, pyruvate) for maximal activity (Haber and Roberts, 1970). In further contrast to the brain, the kidney enzyme was activated by aminooxyacetic acid (AOAA),a carbonyl trapping agent. Kidney GAD is unaffected by PLP. This form of GAD has been referred to as GAD II by some authors (Beart and Schon, 1974).

Effect of Pyridoxine Deficiency on GABA and GAD

Work done with rat pups has demonstrated a decrease in GABA concentration due to pyridoxine deficiency (Stephens et al, 1971). It was also found that the activity of the GAD holoenzyme was decreased whereas the activity of the apoenzyme was increased in whole brain.

CATECHOLAMINES

Naturally occurring catecholamines have been found in a wide range of animal and vegetable tissues, but are particularly associated with neuronal tissues in animals. In the CNS, norepinephrine (NE) and dopamine (DA) are the two main catecholamines. There is only a small concentration of epinephrine (E) whose function is yet to be fully established.

In animals the most concentrated source of NE and E is the adrenal medulla (Laverty, 1978). Here concentrations of up to 5 mg/g wet weight have been measured. The proportion of E to NE varies between species and also with age. Catecholamines occur in peripheral tissues in the extra medullary chromaffin tissues found predominantly in young children, but mainly in sympathetic ganglion and in all tissues

innervated by the orthosympathetic nervous system. The predominant amine in tissues arising from sympathetic nerves is NE, with usually only 5% of the content being E (Laverty, 1979). DA is found only in low concentrations in most tissues except in ruminants where it appears to be stored in mast cells. Thus, in some tissues, for example sheep, goat or cattle hearts, lungs or gut, high concentrations of dopamine which may be equal to or greater than the concentration of NE, have been reported but are of non-neuronal origin. Functions of catacholamines are summarized in Table I.

DISTRIBUTION IN CNS

A) Norepinephrine

NE has a remarkably extensive projection system that distributes connections widely throughout the brain (Fig. 4).

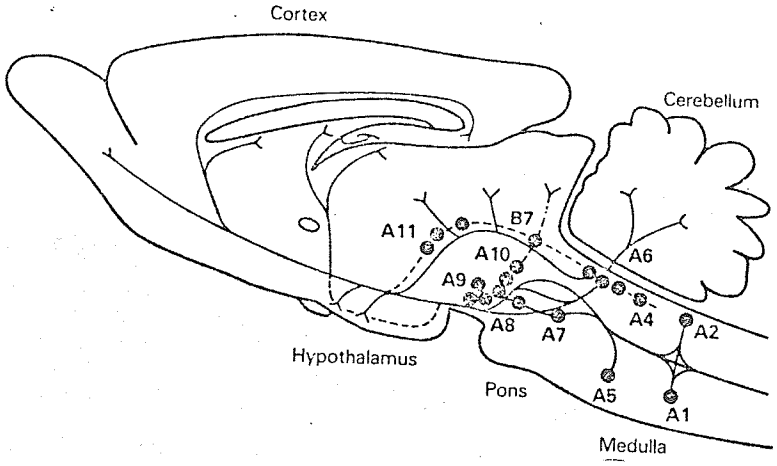
NE cell bodies occur in the medulla and pons and send long processes decending into the spinal cord and ascending to the hypothalamus, thalamus and cortex. Most regions of the brain contain NE with the highest concentrations occurring in the hypothalamus (Van DeKar and Lorens, 1979). Lowest concentrations occur in the cerebellum. General distinct uncrossed fiber pathways connect the cell bodies with the innervated areas.

NE pathways can be divided into two systems, the locus coeruleus (LC) system and the lateral tegmental system. The LC is a prominent nucleus located in the brainstem reticular formation (A6). It is composed entirely of NE producing neurons and is the largest NE nucleus within the mammalian brain (Langer, 1977). Three major pathways migrate from the LC (Fig. 5). The largest ascending pathway projects into the mesencephalic tegmentum, which was referred to by Ungerstedt (1971) as

TABLE I. FUNCTIONS OF CATECHOLAMINES

Transmitter Amine	Functions	
	CNS	General
Norepinephrine	central control of blood pressure	control of normal blood pressure and flow
	control of temperature and food intake	may contribute to hypertension
	possible control of mood or drives	increased release in many stressful conditions
Epinephrine	possible role in central cardiovascular control	metabolic effects through glucose and lipid mobilization
		blood flow through skeletal muscles
		may be implicated in hypertension
		increased release during stress or arousal
Dopamine	involved in motor activity pathways especially dyskinesias and sterotyped activity	no effect except perhaps in the kidney
	possible role in schizophrenia	

Fig. 4. Norepinephrine pathways and amine cell bodies



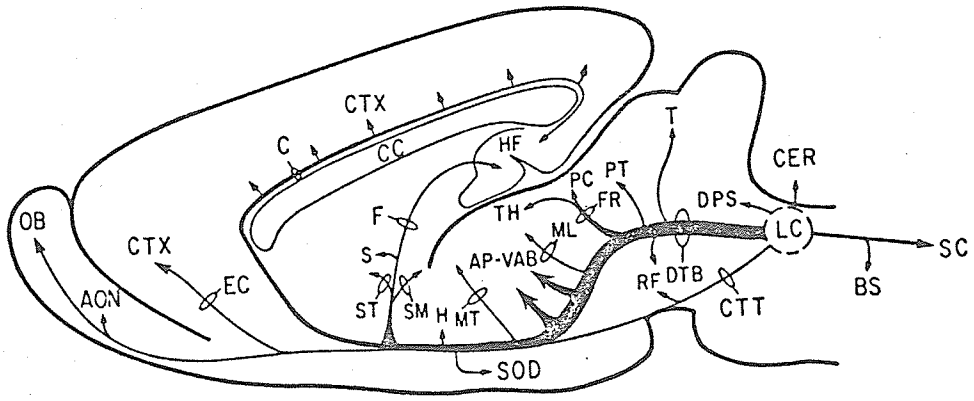


Fig. 5. Diagrams of the Projections of the L.C.

AON-anterior olfactory nucleus; AP-VAB, ansa peduncularis-ventral amygdaloid bundle system; BS brainstem nuclei; C, cingulum; CC, corpus callosum; CER cerebellum; CTT, central tegmental tract; CTX, cerebral neocortex; DPS, dorsal periventricular system; DTB, dorsal catecholamine bundle; EC, external capsul; F, fornix; FR, fasiculus retroflexus; H, hypothalamus; HF, hippocampal formation; LC, locus coeruleus; ML medial lemniscus; MT, mammillothalamic tract; OB, olfactory bulb; PC, posterior commissure; PT, pretectal area; RF, reticular formation; S, septal area; SC, spinal cord, SM stria terminalis; T, tectum; TH, thalamus (Moore and Bloom, 1978).

the dorsal catecholamine bundle. A second projection enters the central grey and ascends as a component of the dorsal longitudinal fasiculus. A third ascending component turns ventrally from the LC to traverse the mesencephalic tegmentum in the central tegmental tract and then ascends through the ventral tegmental area into the medial forebrain bundle. General pathways arise from the dorsal pathway, which appears as a compact group of fibers lying ventral and lateral to the cerebral aqueduct just outside the periaqueductal gray (Moore and Bloom, 1978). Two major groups of fibers leave the LC. the first is a group that ascends in the superior cerebellar peduncle to innervate the cerebellum. The second group descends in the central tegmental bundle through the brainstem to enter the ventral portion of the lateral column of the spinal cord.

Cell bodies of the lateral tegmental NE system run from groups of cell bodies in the medulla and pons (A 1,2,5,7) (Fig.4) into the medial forebrain bundle and innervate the hypothalamus as well as the forebrain in general (Tursky and Lassanova,1978).

The origins and terminations of the two systems are summarized in Table 2. One observation is that NE fibers don't appear to make any close synaptic contacts with other neurons in the brain (Descarriers and Lapierre, 1977). This suggests that NE acts as a more general neuromodulation conveying specific information to individual cells on a 1:1 basis.

B) DOPAMINE

The DA neuron systems are more complex in their anatomy, more diverse in localization, and more numerous, both in terms of definable systems

TABLE 2. Norepinephrine Neuron Systems in the Mammalian Brain

System	Nucleus of Origin	Site(s) of Termination
Locus Coerleus	Locus Coeruleus	Spinal Cord (1,2), brainstem (3), cerebellum (4), hypothalamus (5), thalamus (6), basal telencephalon (7), and the entire isocortex (8)
Lateral Tegmental	Dorsal motorvagus, nucleus tractus solitarius, and adjacent tegmentum, interal tegmentum	spinal cord (2), brainstem (3), hypothalamus (8), basal telencephalon (9).

1 - Kuypers and Maisky, 1975, Ness and Olson 1979.

2 - Moore and Bloom, 1978.

3 - Moore and Bloom, 1979.

4 - Lindvall and Bjorkland, 1974.

5 - Lindvall and Steventi, 1974.

6 - Levitt and Moore, 1978.

7 - Laverty, 1978.

8 - Nygren and Olson, 1977.

9 - Lindvall, 1975.

and in number of neurons, than the NE system. DA neurons are principally located in the upper mesencephalon and diencephalon (Fig. 6). They appear to vary anatomically from systems of neurons without axons (retina, olfactory bulb) and with very restricted projections, to systems with extensive axonal arborizations. The principal morphologic differences, however, between the DA and NE systems is that DA systems appear to be "local" systems with highly specified, topographically organized projections (Moore and Bloom, 1978).

The majority of DA cell bodies occurs in the mesencephalon, particularly in the pons compacter of the substantia nigra (A9), more caudal and dorsal near the red nucleus (A8), and near the midline in the ventral tegmentum adjacent to the interpeduncular nucleus (A10) (Laverty, 1978). From these, fibers extend into the median forebrain bundle which innervate the striatum (caudate nucleus putamen) from A8 and A9. The mesolimbic system from A10 innervates the nucleus accumbens and olfactory tubercles. In addition, dopamine containing terminal fibers have been found in certain cortical areas, particularly the frontal cortex (from A10), the anterior cingulate cortex (from A9) and the entorhinal cortex (Nygren and Olson, 1977).

It has been suggested that all dopamine cell groups are not pharmacologically identical and that different dopamine nuclei may respond differently to psychoactive drugs (Browder et al, 1981). The results not only indicate a medial (A10)-lateral (A9) difference in the sensitivity of DA cells to AMP isomers but also a dorsal-ventral difference. The finding that cells in the dorsal half of the A9 were sensitive to L-AMP indicates that these cells are different from the ventral A9 cells which were insensitive to high doses of L-AMP.

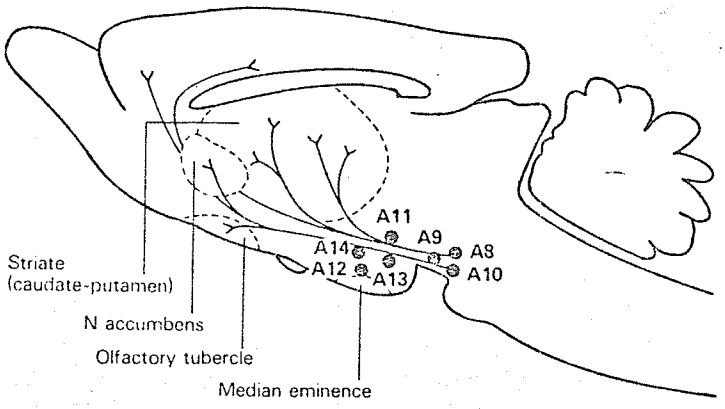


Fig. 6. Principal DA cell groups and fibre pathways

Additional cell bodies occur in the anterior hypothalamus. A group in the arcuate nucleus (A12) supplies short fibers to the infundibulum and anterior pituitary. Further dopaminergic innervation of the median eminence comes from the nigral region (Kizer and Brownstein, 1976). Other cell bodies have been reported in the periventricular nuclei (A14), dorsal hypothalamus (A13) as well as the caudal hypothalamus and thalamus (A11) though these latter cell bodies may include some containing NE (Laverty, 1975). The dopamine neuron system is summarized in Table 3, (Moore and Bloom, 1978).

One aspect of the catecholamine pathways that should be stressed is the anatomical and functional inter-relationships that exist between different pathways of the catecholamine system and between the catecholamine pathways and other neurotransmitter systems. For example, the L.C. supplies innervation to other NE cell bodies such as A2, dopaminergic cell bodies such as A9, and to serotonin cell bodies such as in the medical raphe nucleus (B7). Fibers from the lateral tegmental system innervate the L.C.

Regulation of Catecholamine Synthesis

Pathways of catecholamine synthesis are given in Figure 7.

EH_2 + tyrosine $\longrightarrow$ EH_2 tyrosine (1)

EH_2 + tyrosine + O_2 $\longrightarrow$ E + DOPA + H_2O (2)

Pteridine H_2 + NADPH + H + $\longrightarrow$ pteridine = H_4+NADPH (3)

Pteridine H_4 + E $\rightarrow$ EH_2 + pteridine H_2 (4) (McGeer & McGeer, 1975)

It is thought that during hydroxylation of tyrosine to DOPA, the enzyme protein itself becomes oxidized to an inactive form (step 2). It is reactivated by a reduced pteridine molecule which donates its hydrogen

TABLE 3. DA Neuron Systems in the Mammalian Brain

System	Nucleus of Origin	Site(s) of termination
Meso-telencephalic	Substantia nigra, para	Neostriatum (caudate-putamen)
Nigrostriatal	Compacta; ventral tegmental area	Globus pallidus
Mesocortical	Ventral tegmental area; substantia nigra, para compacta	Isocortex (mesial frontal, anterior cingulate, entorhinal perirhinal) Allocortex (olfactory bulb, anterior olfactory nucleus, olfactory tubercle, piriform cortex, septal area, nucleus accumbens, amygdaloid complex)
Tubero-hypophysical	Arcuate + periventricular hypothalamic nuclei	Neuro-intermediate lobe of pituitary, median eminence
Retinal	Interplexiform cells of retina	Inner and outer plexiform layers of retina
Inserto-hypothalamic	Zona incerta, posterior hypothalamus	Dorsal hypothalamic area, septrum
Periventricular	Medulla in area of dorsal motor vagus, nucleus tractus solitarius, periaqueductal and perigray	Periventricular and periaqueductal gray, tegmentum thalamus, hypothalamus
Olfactory bulb	Periglomerular cells	Glomeruli (mitral cells)

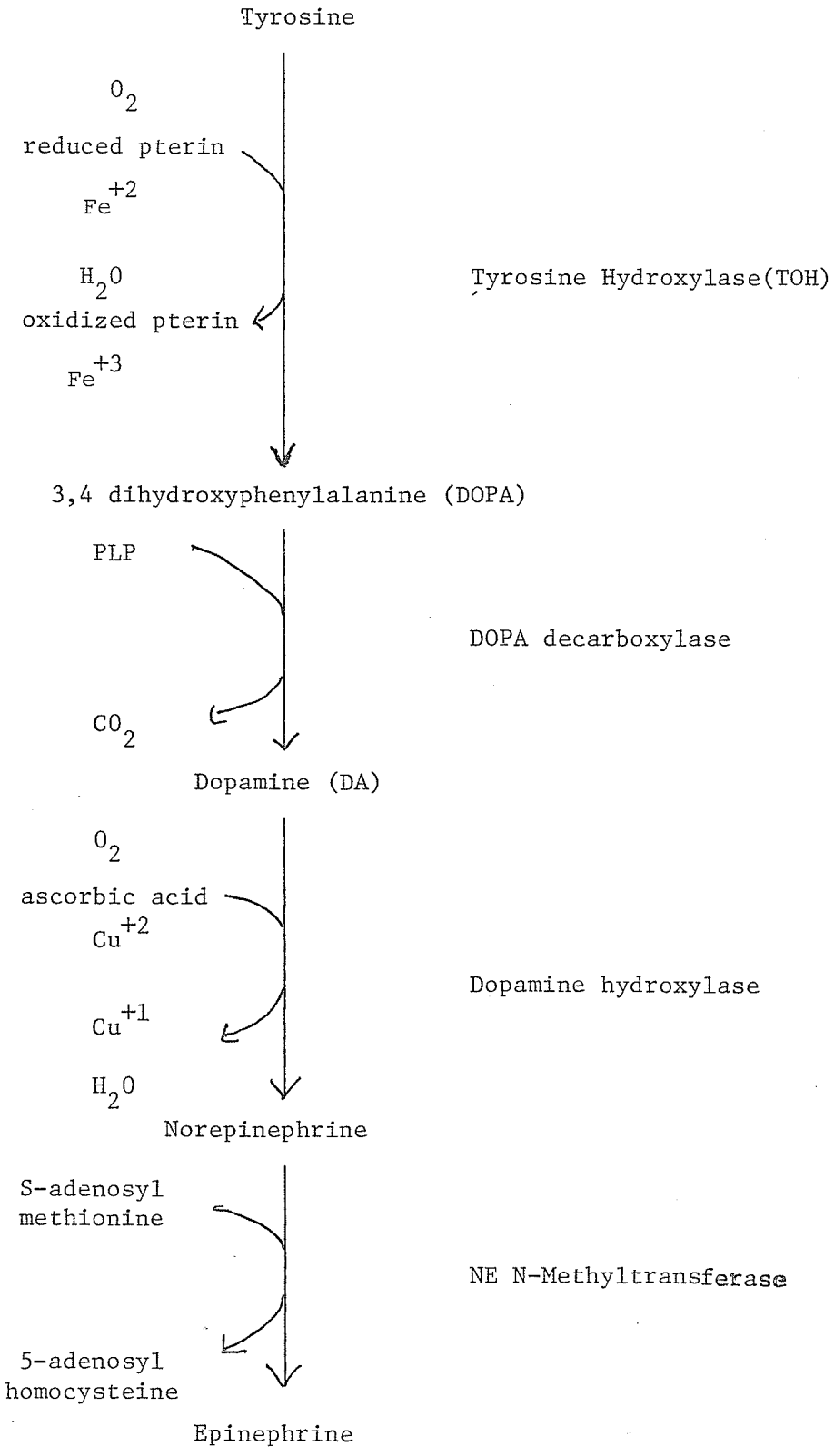


Fig. 7. The biosynthesis of catecholamines in the brain. (Fuller and Steinburg, 1976).

activity.

TOH requires 0_2 and it has been shown that changes in $p0_2$ cannot be ruled out as a factor in the regulation of TOH activity (Davis and Carlsson, 1973).

Tyrosine loads do not have an appreciable effect on central catecholamine synthesis. In vivo levels of tyrosine are of the order of 10^{-4}M. The km for TOH has been variously reported as 2 - 10 x 10^{-5}M for the soluble or unsolubilized enzyme from the brain (Kuczenski and Mandell, 1972).

There is a complex system of control mechanisms affecting transmitter release by an effect on the presynaptic nerve terminal membrane (Laverty, 1978). Autacoids such as angiotensin increase the release of NE whereas prostaglandins and Ach inhibit its release. More significantly, agonists such as NE and DA suppress the release of these catecholamines from the nerve terminal, constituting a precise and elegant means of feedback control of neurotransmitter release through the concentration of neurotransmitter in the synaptic cleft (Langer 1977).

Specific receptors are present in the outer surface of NE nerve endings. These presynaptic receptors are involved in the regulation of transmitter release during nerve stimulation (Langer, 1977). The presynaptic α adreno receptors mediate a negative feedback mechanism which leads to inhibition of transmitter release probably by restricting the Ca^{+2} available for excitation secretion coupling (Dismukes and Mulder, 1976; Dixon and Weiner, 1979). The presynaptic β-adrenoreceptors mediate a positive feedback mechanism which is activated at low frequencies of nerve stimulation leading to an increase in

atoms (step 4). The pteridine molecule must itself be regenerated by another enzyme, pteridine reductase, which donates hydrogens to convert the dihydropteridine back to the tetrahydropteridine (step 3) (McGeer and McGeer, 1975). Pteridine reductase has been demonstrated in adrenal glands and brain (Muguaini and Dahl, 1975).

Short term control of catecholamine synthesis is achieved without any alteration in enzyme protein assayed by in vitro methods (Versteeg and Palkovits, 1976). The enzyme protein is substantially inhibited in vivo probably by being in an oxidized form. There is considerable evidence supporting the concept that feedback inhibition is by newly synthesized unbound NE + DA within the nerve endings interacting with the pteridine cofactor. The cofactor, acting as a hydrogen donor to the oxidized protein, determines the exact rate at which tyrosine hydroxylation takes place (McGeer and McGeer, 1975). Thus the rate of hydroxylation decreases as the DA and /or NE concentration increases. It's thought that the catecholamines compete with the oxidized pteridine for a binding site on pteridine reductase. Since the brain concentration of pterdine is so low, the reductase may play an important modulatory role in the catecholamine feedback mechanism of TOH activity. Nevertheless, it has been suggested that DA + NE levels aren't dependent on the levels of the pteridine cofactor (Seligmann and Martin, 1978). They showed that a 50% reduction in the cofactor pool has no effect on amine synthesis. Catecholamine biosynthesis is not inhibited by the concentration of cofactor. Levine and Lovenberg, (1979) have shown that the presence of high concentrations of hydroxylase cofactor in brain areas known to contain large amounts of tyrosine (striatum) and tryptophan (mesencephalic tegmentum) hydroxylase

activity. This mechanism appears to be mediated through an increase in the levels of cyclic AMP in NE nerve endings. It has been shown that this effect carries over to DA and E (Dietl and Philippu, 1981). That is to say a dual regulation of catecholamines seems to exist in the brain. -adenoreceptors mediate a negative, while B_1- and B_2- receptors mediate a positive feedback mechanism by which the impulse dependent release of catecholamines is controlled. Experiments by Cerrito and Raiteri (1981) suggest that DA reuptake at the presynaptic terminal plays a key role in the control of DA synthesis. The kind of synthesis inhibition here and the classical 'end product' inhibition comes directly from the synaptic cleft, in amounts proportional to the actual synaptic concentration. Thus the role of DA reuptake would be twofold: inactivation of the synaptic transmitter and direct modulation of its synthesis.

Depolarization may increase DA synthesis by reducing the inhibition of TOH by DA (Kapatos and Zigmoid, 1979). Incubation of striatal synaptosomes under deplarizing conditions (veratridine or KCl) increased the release of DA and produced a small increase in DA synthesis. This was not accompanied by an activation of either soluble or membrane bound TOH, but was associated with a large depletion of synaptosomal DA. Thus, depolarization may increase DA synthesis by increasing feedback inhibition of TOH. Catecholamine synthesis and release can also be effected by other transmitters, metabolites and amino acids.

Serotonergic modulation of DA neurons is a generally occurring phenomenon in the brain (Waldemeier, 1980). Exposure of DA neurons to serotonin or 5 hydroxytryptophan leads to an inhibition of dopamine

synthesis, mediated in part by an interneuronal displacement of dopamine from vesicle storage sites, leading to an increase in dopamine induced feedback inhibition of TOH, and in part by a direct inhibition of DOPA decarboxylation (Andrews and Barchar, 1978). Serotonin can also modulate the synthesis of DA in striatal nerve terminals through a presynaptic receptor mechanism (deBelleroche and Bradford, 1980). Glycine can stimulate the efflux of ^{3}H-DA from rat striatum (Kerwin and Pycock, 1979; Reubi and Jessel, 1977). This is a calcium dependent process. GABA utilizing neurons are thought to interact with dopaminergic systems and regulate their activity (Fuxe et al 1975; Moore and Wuerthele, 1979). Giorguieff and Besson (1978) have shown that GABA can specifically stimulate the release of DA endogenously synthesized in dopaminergic terminals. Gundlach and Beart (1981) examined GABA - DA interactions by studying the influence of several gabaergic drugs on nigrostriatal and mesolimbic dopaminergic systems and found that drugs which block the action of synaptically released GABA or reduce control GABA concentrations can result in an elevation of 3-4-dihydroxyphenylacetic acid (DOPAC) concentrations in the nerve terminal and/or somatodentritic regions of both dopaminergic pathways. Such an alteration in the levels of DOPAC has been shown to reflect increased activity in nigrostriatal and a mesolimbic dopaminergic neurons (Roth et al, 1976). There is still controversy as to whether or not the GABA receptors mediating the action of DA are located presynaptically on dopaminergic terminals or on neurons or neuronal afferences within the striatum (Giorguieff and Besson, 1978; Snodgrass and Iverson, 1974).

5-Hydroxytryptamine (Serotonin)

5-hydroxytryptamine (5-HT) in the CNS has been implicated with mental functions, sexual behavior and neuroendocrine functions. (Table 4) (Full and Ungerstedt, 1970; Chase et al, 1976).

Distribution

Although the 5-HT neural tracts are less well traced, it seems as if they have the same principal construction as the NE neurons; that is, large collateral systems with widespread innervation areas (Fig. 8).

The 5-HT neurons form pathways mainly arising from cell bodies of the raphe nucleus in the mesencephalon, ascending in the medial forebrain bundle and terminating in the limbic forebrain, the hypothalamus, the corpus striatum, and the neocortex (Hedner, 1978; Parent et al, 1981). The descending bulbospinal 5-HT neurons arie mainly from 5-HT cell bodies in the raphe nuclei of the medulla oblongata (B1,2) and the 5-HT cell bodies surrounding the pyramidal tract at the cranial level of the medulla oblongata (Fuller and Steinburg, 1976). A particularly large projection of 5-HT axons exists, travelling to the lumbar and sacral part of the spinal cord. It should be mentioned that many of the 5-HT cell bodies of the medulla oblongatad are innervated by NE nerve terminals. These aminergic links should be remembered when considering brain circuitry and interactions between NE and 5-HT neurons. There probably also exist short 5-HT neuronal tracts localized mainly in the pons (B3,5).

High concentrations of 5-HT were measured in all parts of the cat brainstem (Gaudin-Chagal and Ternaux, 1979). The highest amount was detected in the superior colliculi and the part of the mesencephalon

Table 4 - Functions of Serotonin

a) Mental Functions

mood - depressive states may be partly due to a decrease in 5-HT neurotransmission.

wakefulness - destruction of the 5-HT neurons results in insomnia

thought processes - high 5-HT receptor activity may cause hallucinations inasmuch as the psychotometric drugs LSD, dimethyltryptamine, and p-methoxyamphetamine are potent stimulators of central 5-HT receptors

b) Sexual Behaviour

the 5-HT neurons probably inhibit sexual behaviour and other types of hyperactive motor performance such as those seen after stimulation of DA and NE receptor activity.

c) Neuroendocrine Function

the 5-HT neurons may play an important role in inter alia regulation of LH secretion from the anterior pituitary

d) Function in Reflex Activity

descending bulbospinal 5-HT neurons participate in control of autonomic and somatic reflex activity especially in the lumbosacral region

Serotonin also has non-neural roles such as in the pineal gland, where it serves as an intermediate in the synthesis of melatonin (Kaufman, 1974).

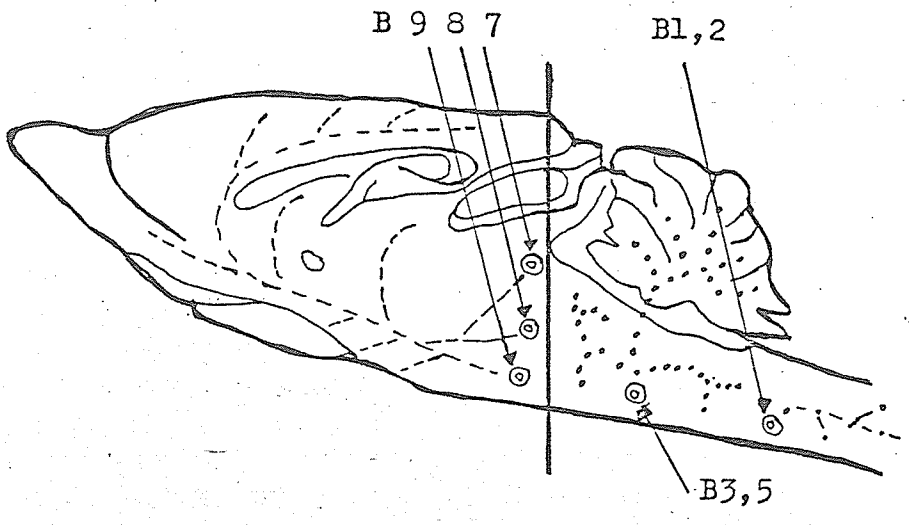


Fig. 8. Illustration of the central 5-HT tracts and cell bodies (Hokfelt and Ljungdahl, 1972)

which includes the dorsalis and centralis superior raphe nuclei. In the cat forebrain, very high quantities of 5-HT were also found in the hypothalamus, caudate nucleus, olfactory gyrus, and piriform gyrus. It should be noted that there is differential serotonergic innervation of individual hypothalamic nuclei and other forebrain regions by the dorsal and median raphe nuclei. Whereas the median raphe nucleus seems to be the primary source of 5-HT fibers to the suprachiasmatic nucleus, the anterior hypothalamic area and medial preoptic area, the 5-HT input to the anterolateral area and arcuate nucleus appear to derive from both the dorsal and median raphe nuclei (Ungerstedt, 1971). The lowest concentration of 5-HT was determined in the cat parieto-occipital cortex and especially in the posterior suprasylvian gyrus and posterior lateral gyri (Gaudin-Chagal and Ternaux, 1979).

Regulation of Synthesis

The pathway of serotonin biosynthesis and catabolism is illustrated in Fig. 9. The rate limiting enzyme of this pathway is tryptophan hydroxylase (TPOH). TPOH, like TOH, is a two-enzyme system, the hydroxylase itself being a mixed-function oxidase, with tetrahydrobiopterin as a cofactor. In the hydroxylation, the pterin is oxidized to dihydrobiopterin, and the second enzyme of the system is dihydrobiopterin reductase, an NADPH-dependent enzyme (Bender, 1978). Although the level of cofactor is not limiting for 5-HT synthesis (Levine et al, 1979) there is still the possibility that its reduction by the reductase could be a rate-determining factor (Craine and Kaufman, 1972). There appear to be two separate forms of TPOH in brain, a soluble form with a k_m of about 10^{-4}M and an absolute requirement for an exogenous pteridine cofactor system, and a particulate enzyme with a k_m of approximately

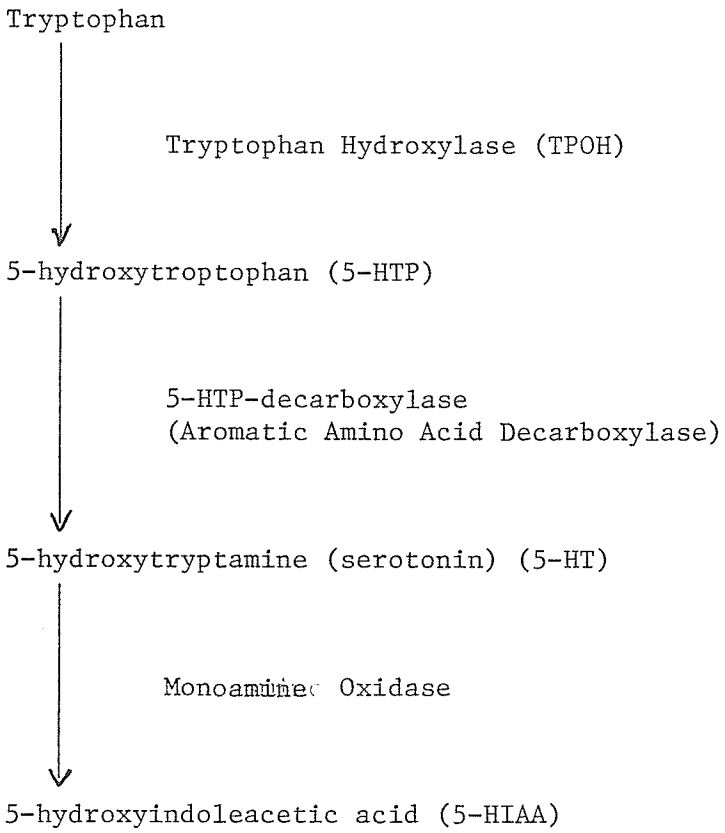


Fig. 9. The pathway of 5-HT biosynthesis and catabolism (Fuller and Steinburg, 1976).

2×10^{-5}M which shows no stimulation by the exogenous cofactor added to the assay medium (McGeer and McGeer, 1975). The soluble and particulate forms of enzyme are localized in different brain regions. The particulate form is in high concentrations in areas containing serotonergic nerve endings and presumably reflects its association with synaptosomes. The soluble form is found in areas containing serotonergic cell bodies.

Substrate concentration may play a major role in the control of tryptophan hydroxylation although it seems unimportant for tyrosine hydroxylation (Tapia and Conteras, 1975; McGeer and McGeer, 1975). The tryptophan level of the brain is normally close to or below the km of TPOH for its substrate. It has been shown that changes in blood tryptophan levels are reflected in changes in brain 5-HT synthesis. Since the blood tryptophan level is affected by the diet, it seems likely that food intake can affect 5-HT synthesis in the brain (Bender, 1978). However, it is also possible to have changes in brain serotonin levels without changes in brain tryptophan as seen in pyridoxine deficient rats (Dakshinamurti et al, 1976). The partial pressure of oxygen in the tissue may also play a role in the control of the hydroxylation of tryptophan since the km for oxygen of TPOH is greater than 10^{-4}M (McGeer and McGeer, 1978). Available information on this point is unclear however because of the complicating effects of oxygen on other enzymes such as MAO and tryptophan pyrrolase of the liver.

Hamon et al (1979) demonstrated that the incubation of brainstem slices from adult rats in a potassium enriched medium containing a 5-HT uptake inhibitor (fluoxetine) significantly increased their capacity to synthesize 5-HT from tryptophan. The measurement of TPOH from incubated

tissues revealed that the increased 5-HT synthesis was associated with an activation of the enzyme. Kinetic analysis indicated that this activation resulted from an increase in the V_{max} of TPOH.

CDP choline is recognized as a brain activator. It was found to decrease the level of serotonin and tryptophan and the rate of synthesis of serotonin in the midbrain, hypothalamus and brainstem. In contrast, it increased the level and the rate of synthesis of DA, and the level of tyrosine in the corpus striatum (Martinet and Pacher, 1979).

Angiotensin II can activate TPOH thus stimulating serotonin release from neuron terminals and accelerating 5-HT synthesis (Musacchio and Wurzburger, 1972). A biphasic effect was observed. At high doses the stimulatory effect depended on conversion of angiotensin II to angiotensin III. At low doses an inhibitory effect was found, possibly dependent on angiotensin II metabolites.

It is well established that neurotransmitter release in the brain is subject to modulation by the action of other locally released transmitters. For example, DA can stimulate the release of (^{3}H)-GABA from rat substantia nigra slices (Perry and Hansen, 1979). In addition to 5-HT containing cell bodies and dendrites, the midbrain raphe receives a NE input from the LC and both GABA containing and possibly substance P containing terminals have been reported in this region (Kerwin and Pycock, 1979). It was found in this area that substance P (100 - 500 uM) stimulated the efflux of ^{3}H-5-HT. It has been demonstrated that GABA neurons inneravating both the substantia nigra and the median raphe nucleus may regulate 5-HT neuronal activity (Forchetti and Meek, 1981; Soubrie et al, 1981).

It is well documented that TPOH is inhibited *in vitro* by DOPA and the catecholamines and it has been known for many years that high doses of DOPA *in vivo* will cause a decrease in brain 5-HT levels, and that high doses of 5-HT will cause a decrease in brain catecholamine levels (McGeer and McGeer, 1975). Intravenous L-dopa also causes a dose dependent depletion of 5-HT in the lumbar region of the rat spinal cord (Commission and Sedgwick, 1979). DOPA may be competing with tryptophan for uptake mechanisms, or some transsynaptic action of catecholamine neurons on the serotonergic neurons may be involved (Heller, 1972). NE projections to the median raphe nucleus from the A1 and A2 cell body groups may also modulate serotonergic neuronal functions (Massari and Jacobowitz, 1979).

5-HT release is modulated by presynaptic autoreceptors (Cerrito and Raiteri, 1979, 1980). The term presynaptic autoreceptor refers to a specific presynaptic site where the transmitter, present in excess in the synaptic cleft, can interact in order to depress its further release. It was found that when 5-HT was added to superfused hypothalamic symptosomes, 5-HT release was inhibited.

A circadian rhythm in the synthesis of 5-HT in rat brain has been reported with the higher rate of synthesis occurring during the light period and lower rates during the dark period. The mechanisms for control of this rhythm are unknown (McGeer and McGeer, 1975).

Lovenberg and Kuhn, (1978) have attempted to determine the potential role of protein phosphorylation in the modulation of TPOH. They found that the activity of TPOH can be altered by an apparent phosphorylation process requiring ATP and Mg^{+2}. Kinetic studies indicate that ATP - Mg^{+2} increases the affinity of the enzyme for its cofactor. This

process is independent of cyclic nucleotides thus if protein phosphorylation underlies the ATP-Mg^{+2} activation, it is probably mediated by a cyclic nucleotide-independent protein kinase. Calcium in high concentrations will also activate TPOH (Hanon and Hery, 1977). The Ca^{+2} effect is manifested kinetically as an increase in affinity of the enzyme for both substrate and cofactor. This is mediated by a Ca^{++} dependent protease. Limited tryptic digestion of TPOH has effects on the activity and kinetics of the hydroxylase which are identical to those of Ca^{+2}, supporting the contention that proteolytic cleavage stimulates TPOH (Hanon and Hery, 1977). Ca^{+2} can alter the activity of the hydroxylase by two different mechanisms. First, Ca^{+2} stimulates a protease which acts directly on TPOH (as above). Second, Ca^{+2} stimulates a protease which produces an active protein kinase from its proenzyme. This cyclic nucleotide-independent protein kinase, in the presence of ATP + Mg^{+2}, phosphorylates TPOH or some other protein leading subsequently to the activation of the hydroxylate (Lovenberg and Kuhn, 1978). Additional experiments are necessary to determine the precise roles that Ca^{+2}, ATP and Mg^{+2} play in activating TPOH.

A marked increase in (^{3}H) -5 HT was seen during the local depolarization of 5-HT terminals with HCl (60 mM) or during stimulation of 5-HT cell bodies in the nucleus raphe dorsalis with L-glutamic acid (5×10^{-5}M) (Hery and Glowinski, 1970).

Effect of Pyridoxine Deficiency on DA, NE and 5-HT Levels

Previous work in our laboratory has focused upon the levels of these transmitters in whole brain samples from rat pups. A significant decrease in brain serotonin of the pyridoxine deficient growing rats was seen. However, the brain levels of DA and NE were not altered (Dakshinamurti

et al, 1976). In this further investigation we have studied the effect of pyridoxine deficiency instituted in rats after myelination was completed. The general idea is to examine the effect of neurotransmitter deficiency brought about by pyridoxine deficiency in the adult rat on the function of the CNS.

ELECTROPHYSIOLOGY

The electroencephalogram (EEG), a record of the variations in potential recorded from the brain, can be used in the analysis of the background electrical activity of the brain. It is possible to obtain unipolar recordings which measure the potential difference between a cortical electrode and a theoretically indifferent electrode on the same part of the body or skull where there is no interfering activity. Bipolar recordings which are a record of potential fluctuations between two cortical electrodes, can also be obtained (Willis and Grossman, 1978).

The activity recorded in the EEG is mostly that of the superficial layers of the cortical grey substance (Ganong, 1979). These layers contain relatively few cell bodies. Potential changes in the cortical EEG are due to current flow in the fluctuating dipoles formed on the dendrites of the cortical cells and cell bodies. The dendrites of the cortical cells are a forest of similarly orientated densely packed units in the superficial layers of the cerebral cortex. Dendrites are the site of non propagated hyperpolarizing and hypopolarizing local potential changes. As excitatory and inhibitory endings on the dendrites of each cell becomes active, current flows into and out of these current sinks and sources from the rest of the dendritic processes and

the cell body. The cell - dendrite relationship is therefore that of a constantly shifting dipole. Current flow in this dipole would be expected to produce wavelike potential fluctuations in a volume conductor.

The electrical events that occur in the cortex after stimulation of a sense organ can be monitored with an exploring electrode connected to a reference electrode at an indifferent point some distance away. If the exploring electrode is over the primary receiving area for the particular sense, a surface positive wave appears. This is followed by a small negative wave and then a larger more prolonged positive deflection. The first positive - negative sequence is the primary evoked response, the second is the diffuse secondary response.

The primary evoked response is highly specific in its location and can be observed only where the pathway from a particular sense organ ends (Ganong, 1979). The positive-negative sequence recorded from the surface of the cortex is due to the fact that the superficial cortical layers were positive relative to the initial negativity, then negative to the deep hyperpolarization.

In unanesthetized animals the primary E.P. is largely obscured by the spontaneous activity of the brain, but can be demonstrated by special techniques.

The diffuse secondary response is sometimes followed by a negative wave or series of waves. Unlike the primary response, it is not highly localized. It appears at the same time over most of the cortex and in many other parts of the brain. The secondary response is due to activity ascending below the cortex. The pathway involved appears to

be the non-specific thalamic projection system from the midline and related nuclei.

Previous electrophysiological work in our laboratory focused upon rat pups in which the EEG and auditory evoked potentials in B_6 deficient and normal rat pups were analysed (Stephens et al, 1971). The EEG of B_6 deficient animals showed spike activity, presumably indicative of the existence of seizures in many of these rats. Evoked potentials presented abnormalities in their latency, wave form and response to repetitive stimuli, but the extent to which they were affected depended upon the intensity of the deficiency.

In this study we have used neuronally mature pyridoxine deficient and normal rats to compare the EEG activity as well as changes in the evoked potential (wave form, latency and amplitude). EEG voltage differences are measured by computer while changes in E.P.'s are detected by measurement of the post activation depression of the E.P. These data are obtained by increasing the frequency of auditory stimulation from 0.2 to 6.0 Hz.

Myelination

In normal CNS development myelination quantitatively constitutes the major component of brain growth. Sphingosine, the basic unit of all sphingolipids is formed from palmitaldehyde by condensation with serine in a PLP dependent reaction. Cerebrosides and sulfatides are the characteristic sphingolipids of the myelin sheath (Brady et al, 1958). Since sphingosine synthesis is a PLP dependent, a deficiency of vitamin B_6 prior to and during the period of myelination has serious effects on brain development (Morré et al, 1978). Pyridoxine deficiency also leads to an impairment of elongation of long chain

fatty acids. These very long chain fatty acids (lignoceric and cerebronic acids etc) are major components of myelin specific lipids (Chauhan and Dakshinamurti, 1979). Histological methods and light microscopy will be used to determine if placing neuronally mature animals on a pyridoxine free diet results in a breakdown and loss of myelin in the CNS and PNS.

CONCLUSIONS

This review of the literature demonstrates the essential role of pyridoxine for the normal development of the CNS in the growing rat. Pyridoxine deficiency interferes with transmitter synthesis, myelination, lipid synthesis, protein metabolism and EEG patterns. Few experiments have been reported on rats in which B_6 deficiency is produced after neuronal maturation, including myelination is completed. I have focused my investigation upon the effects of pryidoxine deficiency in these rats.

III. EXPERIMENTAL

i) Methods

A. Production of Pyridoxine Deficiency

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.

At 4-5 weeks of age, male Sprague Dawley rats were fed ad libitum a pyridoxine deficient diet (Tables 5 + 6). In order to enhance the deficiency symptoms the antimetabolite 4-deoxypyridoxine (0.25 g/kg) was added to the diet. Deoxypyridoxine appears to be converted by pyridoxal kinase to a phosphorylated form which in turn competes with the coenzyme pyridoxal phosphate for non-productive binding sites on various enzymes. (Harris 1968) Deficiency symptoms usually appeared after 3-4 months on the diet used in this study when clear symptoms of pyridoxine deficiency were seen. Rats fed Purina Laboratory Chow served as controls. They were age matched with the deficient rats.

B. Criteria for Pyridoxine Deficiency

Owing to the numerous enzymes requiring pyridoxal phosphate, a large variety of biochemical lesions occur in vitamin B_6 deficiency. These include red blood cell and plasma glutamic-pyruvic and glutamate-oxaloacetic transaminase activities, as well as levels of PLP, γ-aminobutyric acid, and glutamic acid decarboxylase activity in brain of similar deficient rats (Daskhinamurti and Stephens, 1969; Stephens et al, 1971). Morphologically, lesions in rats such as acrodynia, are observed mainly on the dorsa of the paws. (Harris 1968). They are characterized by hyperkeratosis and loss of subcutaneous fat. The lesions on the extremities are accompanied by lesions around the ears, nose, chin, submental region, and occasionally the upper thorax. At a later stage of the

TABLE 5

Percentage Composition of Pyridoxine Deficient Diet

Vitamin free Casein	30.00
Dextrose	39.85
Corn Oil (Mazola)	5.00
Salt mix, number 446 (ICN Nutritional Biochemicals)	4.00
Vitamin mix (No B_6) (Table 6)	1.00
Choline Chloride	0.15

TABLE 6

Composition of Vitamin Mixture (without Pyridoxine)	
P-Aminobenzoic Acid	11.00 g
Ascorbic Acid	99.20 g
Biotin	0.05 g
Calcium Pantothenate	6.60 g
Folic Acid	0.20 g
Inosital	11.00 g
Menadione	5.00 g
Niacin	9.90 g
Riboflavin	2.60 g
Thiamine H Cl	10.00 g
∝ -Tocopherol (250 I. units/g)	10.00 g
Vitamins A and D, concentrate (500,000 units A/g concentrate0 (5000 units 0/2 concentrate)	4.00 g
Dextrose	827.85 g
Vitamin B12 (Triturate in 0.170 manitol)	3.00 g
Total weight	1000 g

deficiency, atrophy of the hair follicles and sebacious glands occurs. Because biochemical lesions occur before morphological ones, the criteria of pyridoxine deficiency in this study included the appearance of these lesions, examination of body and brain weights and the measurement of pyridoxal phosphate levels.

C. Enzymatic Determination of Pyridoxal Phosphate (PLP)

The procedure is based on the pyridoxal phosphate (PLP) dependent enzymatic decarboxylation of L-tyrosine by tyrosine decarboxylase (L-tyrosine carboxy-lyase, E.C. 4.1.1.25). This is a modification of the procedure developed by Daskhinamurti and Stephens, (1969). The reaction is as follows:

$$\text{L- (1-}^{14}\text{C) tyrosine} \xrightarrow[\text{tyrosine decarboxylase apoenzyme}]{\text{PLP}} \text{tyramine} + {}^{14}CO_2$$

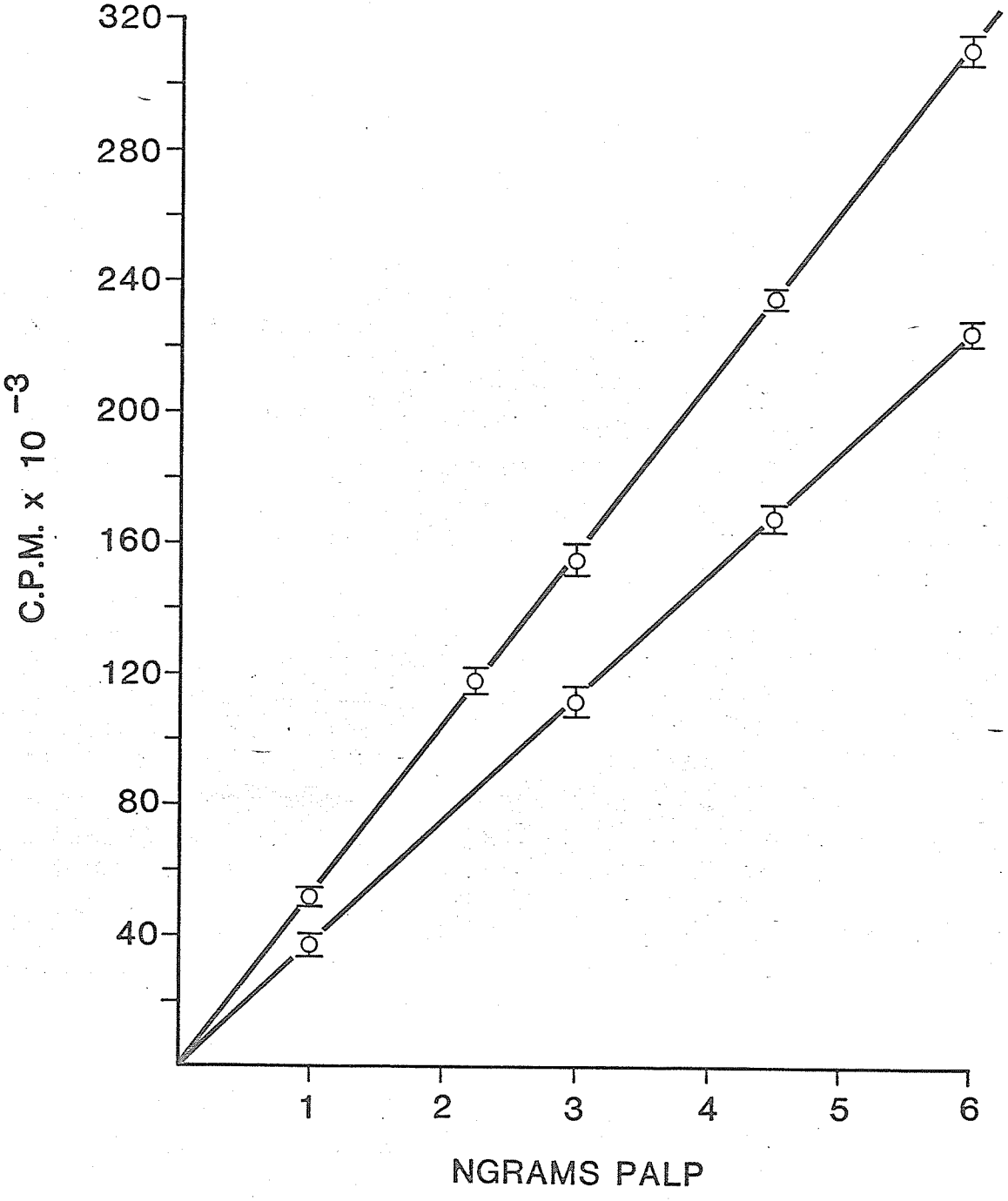
Radioactive $^{14}CO_2$ evolved was trapped by NCS in a center well suspended over the reaction mixture. PLP present in the reaction mixture was proportional to the $^{14}CO_2$ produced. Calibration plots were included with each set of assay. Since the commercial enzyme (tyrosine apodecarboxylase) was not totally free of the coenzyme (PLP), different batches of enzyme gave different curves although the response to PLP was always linear (Figure 10).

Reagents:

- L- tyrosine - 1- ^{14}C (New England Nuclear) solution: 0.008M, in potassium acetate buffer, 0.8M pH 5.5, specific activity 10.1 mCi/millimole.

- PLP stock solution: 1.12×10^{-4}M, 3.031 mg PLP (Sigma Chemicals Co.) dissolved in 100 ml of double distilled water. This solution was refrigerated in the dark and is stable for 2 days. In the assay, the stock

Figure 10: Calibration curve for the enzymatic determination of PLP. The two curves represent two different enzyme batches. Blank values subtracted from data.


320
280
240
200
160
120
80
40
C.P.M. x 10 -3
1
2
3
4
5
6
NGRAMS PALP

solution was diluted 1000 fold with potassium acetate buffer, 0.01 M, containing 0.005M ethylenediaminetetra acetate (EDTA), pH 5.5 to give a concentration of 30 mg/ml.

- NCS solubilizer (Amersham)

- Tyrosine decarboxylase apoenzyme (Sigma Chemical Company):
Aliquots of the apoenzyme were dissolved in potassium acetate buffer, 0.01M, containing 0.005 M EDTA, pH 5.5 just before use.

- OCS Organic counting scintillant (Amersham).

Procedure:

To the test tubes, in triplicate, were added 0.1 ml of tyrosine apodecarboxylase (0.75 units) in potassium acetate buffer, 0.01M, containing 0.005M EDTA pH 5.5, 0.0-0.2 ml (0-6 ng) of the standard PLP solution or brain extract (as prepared in the next section), 0.6 ml of L-tyrosine-1-^{14}C solution and water in a final volume of 1 ml. The tyrosine 1-^{14}C solution was added after the apoenzyme had been equilibrated with PLP or brain extract for 15 minutes at 37^{o}C in a shaking water bath (equilibration increases the reaction rate by 15%). The flasks were then stoppered with rubber septum (Kontes Glass Company) containing polyethylene center wells and incubated at 37^{o}C for 10 minutes. The reaction was terminated by the injection of 1.0 ml of 10% trichloroacetic acid through the septums. Then 0.3 mls of NCS was injected into the center well of each flask, and the flasks were shaken at 37^{o}C for one hour. The center well from each flask was carefully removed and transferred to a scintillation vial containing 10 mls of OCS. The radioactivity was counted in a Beckman LS-250 Liquid Scintillation System. Two reaction blanks were run, one without PLP to correct for the endogenous PLP in the apoenzyme preparation and one without the enzyme.

The concentration of PLP in brain was calculated using the equation:

$$\frac{\text{unknown PLP (ng) (from calibration curve) x volume of brain extract}}{\text{volume of brain extract in incubation medium x brain weight in grams x 1000}}$$

this gives ugrams of PLP/gram wet weight of the tissue.

Preparation of Brain Extract for Determination of PLP

Rats were sacrificed by microwave radiation, decapitated and the brain removed. The brain was dissected into three parts, the cerebellum, brainstem (pons and medulla oblongata) and the remaining section. The regions were weighed and homogenized. Homogenization medium was prepared by mixing 20 ml of double distilled water with 1.7 ml of 10% m-phosphoric acid. Brain tissue was homogenized in 16 ml of medium per gram of brain wet weight (Minard, 1967), using a motor driven homogenizer with a Teflon pestle. The homogenate was spun on a desk centrifuge and the pellet discarded. The volume of the supernatent was recorded for PLP calculations and the pH adjusted to 5.5. Aliquots were taken for determination of PLP as specified above.

D. Determination of L-Glutamate Decarboxylase (GAD)(L-Glutamate 1-Carboxylase, EC4.1.1.15) Activity in Brain

The method employed for GAD determination was similar to that for PLP. The enzyme catalyses the reaction: L(1-^{14}C) glutamic acid $\xrightarrow[\text{PLP}]{\text{GAD}}$ γ-aminobutyric acid + $^{14}CO_2$. The $^{14}CO_2$ liberated was collected and used for the determination of GAD activity. The method used is a modification of that described by Nistico et al (1979).

Activity of L-glutamate decarboxylase holoenzyme in B_6 deficient and normal rats was determined in the absence of PLP, whereas that of the apoenzyme was determined by adding PLP to the incubation flask as specified below.

Reagents:

- L (1-^{14}C) glutamic acid substrate (New England Nuclear Corp.) 0.5 mM of L-glutamic acid, (.57 uCi per ml) dissolved in 0.1 M potassium phosphate buffer pH 6.5

- PLP solution, (Sigma Chemical Company) 1 x 10^{-5}M in phosphate buffer 0.1M, pH 6.5

- Potassium phosphate buffer, 0.1M, pH 6.5

-Sucrose solution 0.25M, containing 0.25% of freshly added Triton X-100 (detergent)

- 2N perchloric acid solution (Fisher Chemical Company)

- NCS solubilizing solution (Amersham)

- Organic counting scintillant (OCS) (Amersham)

Procedure:

Rats were decapitated and the brain dissected into three regions, the cerebellum, brainstem (pons and medulla) and the rest of the brain. These regions were weighed and homogenized in a motor driven glass homogenizer with the Teflon pestle, in 10 volumes of ice cold sucrose solution (0.25M), as specified above. Triton X-100 was added to promote maximal liberation of enzyme from the tissue. Homogenates were centrifuged at 1000 xg for 10 minutes in a Sorvall Centrifuge and the supernatants poured through cheesecloth and used. The final pH of the supernatant was 6.5 and aliquots were used for determination of the decarboxylase activity.

The activities of GAD were determined immediately after dissection in the presence and in the absence of exogenous PLP. The activity determined in the absence of externally added PLP reflects the amount of endogenous PLP present in this preparation. This is referred to as the holoenzyme. The

activity determined in the presence of an excess of externally added PLP is referred to as the apoenzyme activity. The enzyme activity measured under these conditions reflects the amount of the glutamic acid decarboxylase apoprotein present.

Determination of holoenzyme activity was performed by adding to the incubation mixture either 0.06 ml of phosphate buffer (0.1M pH 6.5) and 0.45 ml of homogenate, or 0.30 ml of phosphate buffer and 0.075 ml of homogenate. For apodecarboxylase activity, either 0.075ml of homogenate was used or .045 ml of homogenate was mixed with 0.03ml of phosphate buffer. To each flask 0.03ml of PLP solution was added. Therefore, for each homogenate 4 flasks were used: 2 for holoenzyme activity (0.045 and 0.075ml homogenate) and two for apoenzyme activity (0.045 and 0.075ml homogenate). Blanks contained only 0.105ml of phosphate buffer (0.1M, pH 6.5).

The flasks were pre-incubated in a shaking water bath at 37°C for 15 minutes, with gentle shaking. The injection of 0.045 ml of substrate marked the initiation of the reaction. The test tubes were then quickly stoppered with a rubber septum through which a plastic center well was inserted. The reaction proceeded for 30 minutes at 37°C and was terminated by injection of 0.06 ml of 2N perchloric acid. The center well was filled with 0.3 ml NCS and the flasks were shaken for 4 hours after which the NCS was transferred to scintillation vials containing OCS and radioactivity counted as described in the case of PLP determination. The glutamic acid decarboxylase activity was calculated according to the following equation:

$$\frac{\text{(dpm of unknown - dpm blank) x final volume of homogenate}}{\text{dpm per u mole of L-GLU substrate} \times \text{time of reation} \times \text{volume homogenate in incubation flask} \times \text{brain wet weight in grams}}$$

The results were expressed as munits/gram brain/minute.

E. Effect of Pyridoxine Deficiency on Myelin and Myelinated Tracts

Histological methods and light microscopy were used to determine if placing neuronally mature anumals on a pyridoxine free diet would result in a breakdown and loss of myelin in the CNS and PNS. Four different methods were employed (with or without preservation of lipids) to assure that the results were not artifacts of a particular procedure.

Reagents:

Sodium Cacodylate	Fisher Chemicals
Osmium tetroxide (Os04)	Polysciences
812 Resin	Pelco
Spielmyers stain (Iron Hematoxylon)	Fisher Chemicals
Sorenson's buffer (Armed Forces Manual)	
JB4 Resin	Ingram and Bell

10% Buffered Formalin

Sodium Phosphate (Dibasic)	6.5 gm
Sodium Phosphate (Monobasic)	4.0 gm
Formalin	250 cc
Distilled water	750 cc

Procedure:

Rats were anesthetized with Sodium Pentobarbital (35 mg/kg) (Valenstien 1961). The anesthetized rats were then perfused through the left ventricle with 0.9% NaCl for two minutes followed by 10% neutral buffered formalin for twelve minutes at room temperature. The disected brains and pituitaries were immersed (fixed) in 10% neutral buffered formalin.

i) Epon Resin (Osmium post-fixation)

After five hours in fixative, small pieces of the optic nerve, trigeminal nerve and ganglion, and corpus callosum at the optic chiasm level were washed in 0.15M Sodium Cacodylate buffer pH 7.4. The pieces were osmicated (1% OsO4) in Sodium Cacodylate buffer for one and three quarter hours at room temperature. This is followed by a buffer wash, alcohol and propylene oxide dehydration and embedding in 812 Epon Resin. Samples were embedded in flat molds so that corpus callosum axons would be both cross and longitudinally sectioned and stained with Toluidine blue. The optic and trigeminal nerves were cross sectioned. One-half micron sections were cut and studied.

ii) Frozen Sections

Frozen sections were cut after blocks of the fixed brain were frozen in liquid nitrogen without isopentane (Armed Forces Manual). Four micron sections of the right cerebral hemisphere behind the optic chiasm were cut in a cryostat at $-20^{o}C$. In an attempt to preserve lipids, dried sections were not fixed in ethanol. Several different stains were used (Luxol Fast Blue, OsO4, Spielmyer's Stain of Myelin (iron hematoxylon).

iii) Paraffin Sections

Sections were immersed in fixative for two days followed by buffer for two days. The Dioxane procedure with lengthened times in paraffin was

tried and sections were cut at four microns (Armed Forces Manual). The left side of the cerebral hemisphere behind the optic chiasm was stained with Luxol Fast Blue. Other regions such as the trigeminal nerve, pituitory, medulla and spinal cord were also saved in paraffin.

iv) JB4 Sections (with or without long osmium exposures)

After two days in fixative followed by a buffer wash (0.15M Sorenson's Buffer) the tissue was cut in two mm wide segments through the cerebrum at the level of the optic chiasm. The right side of each brain was osmicated and the left side was left overnight in buffer and then osmicated. The tissue was then washed in buffer, dehydrated in alcohol and embedded in JB4 resin. Sections were cut at two microns.

F. Electrophysiological Experiments

Rats used in these experiments were obtained in the same way as for the biochemical experiments and were used at 4-5 months of age. Rats were anesthetized by a subcutaneous injection of urethane at a dose of 1350 mg per kg body weight. After the rats were anesthetized, their heads were immobilized in the prone position and the skull surface was exposed by disecting the overlying muscle, subcutaneous tissue and skin that covered it. A dental drill was then used to perforate the skull at the points where the electrodes would be implanted. Electrodes were implanted on the dura-mater at the points shown in Figure 11 and electrode wires were soldered to pin connectors made from dual in-line integrated circuit sockets. There was one reference electrode for unipolar recordings (1), two electrodes in the right auditory cortex (2,3) and two electrodes in the left auditory cortex (4,5). The locations of the electrodes were the following measured from the bregma as shown in Figure 11; (1) Y = 4mm X = 2mm (2) Y = -3 X = 9 (3) Y = -4.5 X = 8

FIGURE 11

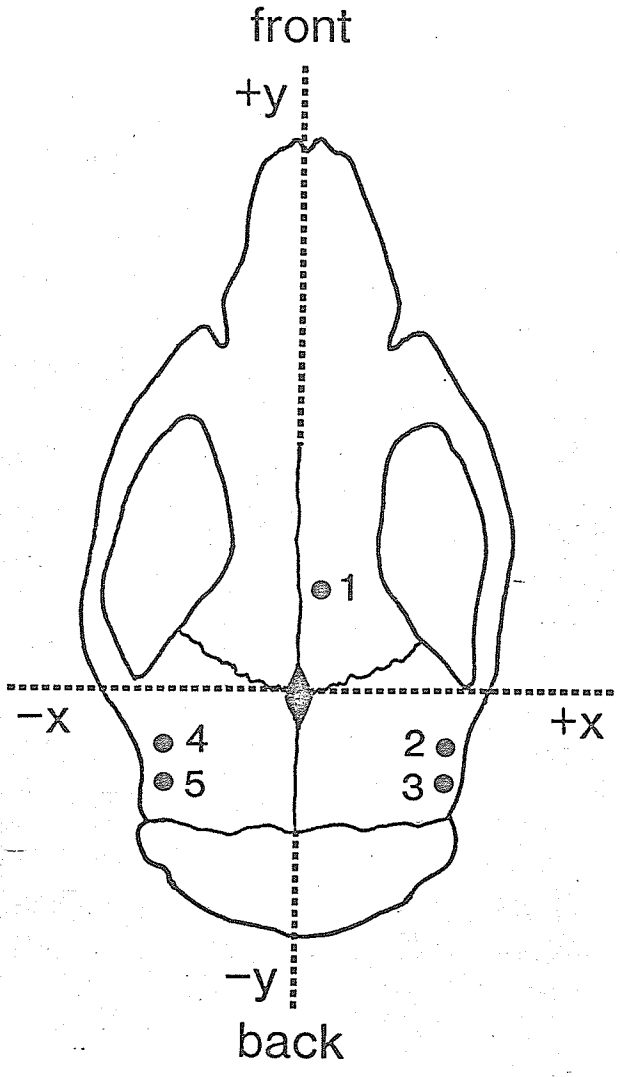


Diagram of rat skull viewed from above.

Electrodes: 1 −reference

2, 3, 4, 5 −auditory cortex on right and left sides of brain

(4) y = -3 X = -8 (5) y = -4 x = -9

Recordings were taken immediately after the operation was completed, with animals under the influence of the anesthetic. Unipolar and bipolar recordings of the EEG were taken from auditory areas with a Grass 6 channel EEG machine. The EEG analog signal and the trigger pulse were recorded on Hewlett-Packard 3960 Instrumental FM tape recorder (15/16 in/sec tape speed) for further computer processing. The fast Fourier transform (FFT) was performed off line on a PDP8/E computer with 16K words of memory using the Rothman's version of the FFT algorithm (Rothman,1968). Samples of 10.24 sec duration for each channel of EEG signal were digitized at a frequency of 100 Hz. The FFT transform for each sample generated a 256 point spectrum per channel between 0.098 Hz per point. The large amount of frequency spectra data generated was reduced to nine integrated values each representing one of eight frequency bands and a total sum, as described in Fig. 20. Therefore, the 512 point spectrum produced from two channels of 10.24 second epochs was reduced to 18 data values. Presently, all power spectra information is automatically transferred and stored on DEC magnetic disk for further statistical evaluation. Both intra- and inter-individual statistical evaluation were performed on an PDP8/E computer using one-way analyses of variance and Duncan's multiple range test. Evoked responses were obatined from unipolar recordings from the auditory areas and they were triggered by clicks generated from an Audio Generator Model 377 at the frequencies indicated in the respective figures (Results); the clicks had a duration of 4.0 milliseconds. The evoked responses were averaged with a DIDAC 800

Intertechnique computer. Each tracing on the DIDAC oscilloscope represented 100 stimuli.

G. Determination of Brain Monoamine Levels

Brain monoamines were determined using the method of Jacobowitz and Richardson, (1975). The levels of dopamine (DA), norepinephrine (NE) and serotonin (5-HT) were determined flurometrically in small tissue samples disected from the brain. The fluorescence of the monoamines was developed with iodine(catecholamines) and ortho-phthaldialdehyde (OPT) and read at the characteristic excitation and emission wavelengths for each compound. Internal as well as external standards were used for maximal accuracy.

Reagents:

n-butanol

heptane

0.1 M phosphate buffer pH 6.5: (prepared fresh every 2 or 3 days by adding 32 ml of stock A (7.1 gm sodium phosphate dibasic up to 500 ml with distilled water) to 68 ml of stock B (6.9 gm sodium phosphate monobasic up to 500 ml with distilled water)

Versene:(4g disodium ethylene-diaminetetra-acetic acid (EDTA) were dissolved in:

(95 ml of distilled water and the pH adjusted to between 6.0 and 6.5 with ion sodium hydroxide before bringing the volume up to 100 ml)

Iodine solution: (prepared from 4.8 g potassium iodine plus 0.25 g iodine dissolved to 100 ml with distilled water)

Sodium sulfite: (made fresh daily by dissolving 2.5 g sodium sulfite (Fisher Scientific Co.) in 100ml 4N sodium hydroxide.)

OPT solution: (made fresh daily by dissolving 50 mg OPT (Sigma Chemical Co.) in 100 ml. of absolute methanol (Fisher Scientific Co.)

a) Brain Preparation

i) Disection

Using microwave irradiation, rats were killed between 1100 - 1300 hours. They were decapitated, the brains removed and the hypothalamus (Hypo), cerebellum (Cb), brainstem (B.S.), midbrain,(Mb), corpus striatum (C.S.) and cerebral cortex (C.C.) were frozen in dry ice (Glowinski and Iverson, 1966). Brain parts were stored in polyethylene vials at -70°C until time of preparation.

ii) Preparation for Assay

NE, DA, and 5-HT were prepared as follows. Using a 5ml repipet, 5 ml of butanol was pipetted into a homogenizing tube and placed in ice. Each tissue sample was rapidly weighed and added to the butanol with an appropriate amount of 0.01N HCl (Table 7), so that the tissue water and 0.01N HCl was equal to 0.75 ml (assuming tissue weight is 70% water) in 5 ml of butanol. The tissue samples were homogenized in glass homogenizing tubes with a motor driven teflon pestle. Samples were homogenized until the tissue was uniformly distributed throughout the butanol (1 -2 minutes depending on the weight of the tissue). The homogenate was poured into a test tube and centrifuged (Servall refrigerated-automatic, 1500 rpm) for 10 minutes to sediment the tissue debris thus leaving a clear supernatant. A 2ml aliquot of butanol supernatant was added to a test tube containing 1.5 ml (use 5ml repipet) of 0.1M phosphate buffer in water. For analysis of 5-HT, an additional 2ml of butanol supernatant was added to a test tube containing 5ml (5ml repipet) of

TABLE 7

VOLUME OF 0.01 N HCl FOR TISSUES IN A GIVEN WEIGHT RANGE

Weight in g between	HCl in ml	Weight in g between	HCl in ml
0.0000 & 0.0071	0.75	0.2500 & 0.2643	0.57
0.0071 & 0.0214	0.74	0.2643 & 0.2786	0.56
0.0214 & 0.0357	0.73	0.2786 & 0.2929	0.55
0.0357 & 0.0500	0.72	0.2929 & 0.3071	0.54
0.0500 & 0.0643	0.71	0.3071 & 0.3214	0.53
0.0643 & 0.0786	0.70	0.3214 & 0.3357	0.52
0.0786 & 0.0929	0.69	0.3357 & 0.3500	0.51
0.0929 & 0.1071	0.68	0.3500 & 0.3643	0.50
0.1071 & 0.1214	0.67	0.3643 & 0.3786	0.49
0.1214 & 0.1357	0.66	0.3786 & 0.3929	0.48
0.1357 & 0.1500	0.65	0.3929 & 0.4071	0.47
0.1500 & 0.1643	0.64	0.4071 & 0.4214	0.46
0.1643 & 0.1786	0.63	0.4214 & 0.4357	0.45
0.1786 & 0.1929	0.62	0.4357 & 0.4500	0.44
0.1929 & 0.2071	0.61	0.4500 & 0.4643	0.43
0.2071 & 0.2214	0.60	0.4643 & 0.4786	0.42
0.2214 & 0.2357	0.59	0.4786 & 0.4929	0.41
0.2357 & 0.2500	0.58	0.4929 & 0.5000*	0.40

*Weights between 0.5 and 1g divide weight by 2, find HCl for ½ original weight, put twice this amount of HCl in 10ml butanol (e.g. tissue weights 0.7100 - 2 = 0.3550, HCl for 0.3550 = 0.50 ml x 2 = 1 ml HCl in 10 ml butanol). For weights between 1 & 1.5 g, find HCl for 1/3 original weight, put 3 times this amount of HCl in 15 ml butanol.

0.1N HCl in ice water. Both mixtures were shaken (Vortex) for 20 seconds. NE an DA were extracted into the phosphate buffer and 5-HT was extracted into the 0.1N HCl. Centrifugation (3000 rpm) separated the organic and aqueous layers and, a vacuum line with a liquid trap was used to aspirate the top organic layer including any tissue debri, leaving as much of the aqueous layer as was possible. A 1.0 ml (1ml repipet) sample of the phosphate extract was placed in a 12 x 75 mm test tube. A 0.3 ml (1 ml repipet) sample of the 0.1N HCl extract was added to a 12 x 75mm test tube. It was found that these aliquots could be frozen at this point and assayed the following day without any adverse effect.

b) Preparation of Standards

Standards were prepared as follows. Separate standard stock solutions of the catecholamines and serotonin (400 ug of base per ml of 0.01N HCl) were mixed. These stock solutions were good for one week in the freezer. All subsequent solutions were kept in ice water. A 0.5 ml (1ml repipet) sample of the NE-DA stock (400 ug NE and 400 ug DA) was placed in a 5ml Volumetric flask with added 0.01N HCl to volume. A 0.5ml aliquot sample of this solution was transferred into a 25ml volumetric flask with 0.01N HCl added to volume. This solution was the stock standard (800 mg/ml). For standard curve readings 0.5 ml (400 mg), 0.25 ml (200 ng), 0.125ml (100 ng), 0.0625ml (50ng) and 0.03125 ml (25ng) were transferred to test tubes with 0.01N HCL added for a final volume of 0.75 ml. 5-HT standards were prepared as above. A reagent blank (Ong 5-HT) was made by adding 0.75ml of 0.01N HCl to 5ml of butanol. The 5-HT and catecholemine standards were processed for assay as in the brain preparation.

c) Oxidation of catecholamines

The NE and DA in the phosphate buffer extract were oxidized into fluorophores by adding 0.25ml (1ml repipet) Versene and briefly vortexed. Then, at exactly 2 minute intervals: (1) 0.2 ml iodine, vortex briefly (2) 0.25ml alkaline sodium sulfite, vortex briefly and (3) 0.3ml 5N acetic acid were added and vortexed briefly. Test tubes were placed in boiling water for 5 minutes then in ice water for 1 minute. NE fluorescence was immediately read in an Amnico-Bowman spectrophotofluorometer excitation 385/emission 485 nm. DA fluorescence was read at least 20 minutes later at 320/385 nm. Blank readings were obtained by adding, at exactly 2 minute intervals, first the alkaline sulfite, then the iodine and finally the acetic acid. By reversing the order in which the iodine and alkaline sulfite are added, the oxidation of NE and DA into fluorophores was prevented.

d) Oxidation of Serotonin

The highly fluorescent condensation product of OPT and the 5-HT in the 0.1N HCl extract was prepared by adding 0.2ml of OPT solution followed immediately by 1.5ml of concentrated (10N) HCl. Test tubes were vortexed briefly and placed in boiling water for 10 minutes, then cooled to room temperature in tap water. 5-HT fluorescence was read at 360/470 nm.

e) Calculations

The amount of NE, DA, or 5-HT in ug/gm brain region was determined as follows:

$$[\text{monoamine}] = \frac{\text{ng monoamines (graph)}}{\text{weight of sample(gm)}} \times \text{correction factor}$$

$$\text{correction factor} = \frac{\text{(brain and internal standard)-(brain alone)}}{\text{external standard}}$$

where $[\text{internal standard}] = [\text{external standard}]$

ii RESULTS

EFFECTS OF PYRIDOXINE DEFICIENCY ON THE MATURE RAT

A. Morphological Effects

The clinical symptoms of a pyridoxine deficiency are seen in Figure 12, As described in the methods section, lesions of the paws, eye areas, and nose are evident. This was accompanied by hair loss around the nose.

B. Effects on Body and Brain Weight

Table 8 shows the body and brain weight values for a group of rats analyzed between 4-5 months of age. The control rats, fed a commercial ration had a mean weight of 283.8 ± 20.6 grams. Similar value for rats on a vitamin B_6 deficient diet was 168.2 ± 25.6 grams. The difference between the two means was statistically significant ($p < .01$). With respect to brain weight the difference between the means of the two groups were not statistically significant for any region.

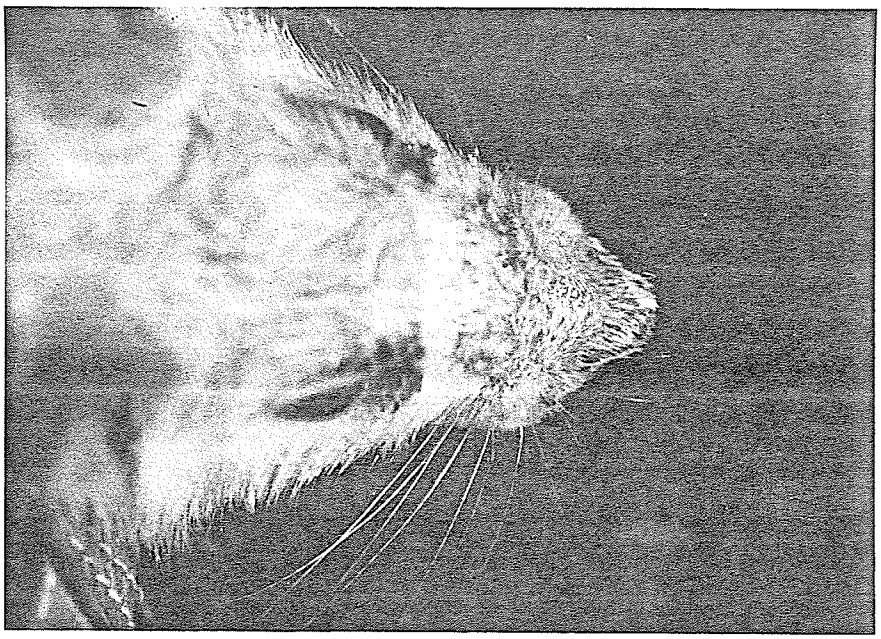
C. Effect on the Concentrations of PLP Levels

Table 9 shows that the brain PLP concentration in the pyridoxine supplemented groups had mean values of 1.47 ± 0.40, 0.90 ± .33, and 1.12 ± .4 ng/gram tissue for the brainstem, cerebellum and brain (minus these regions) respectively. Similar value for rats on a Vitamin B_6 deficient diet were 0.4 ± .1, 0.43 ± 0.2 and 0.32 ± 0.1 ng/gram brain region. The difference between the two means in each group were statistically significant ($p < .001$, $p < .005$, $p < .002$).

D. Effect on the Activity of L-Glutaminic Acid Decarboxylase of Brains

The results are shown in Tables 10, 11 and 12. Values under the column subtitled "no added PLP" represent holoenzyme activity, while values in the column subtitled "added PLP" represent apoenzyme activity. When comparing the pyridoxine supplemented and deficient animals in the six

Figure 12. MORPHOLOGICAL EFFECTS OF PYRIDOXINE DEFICIENCY



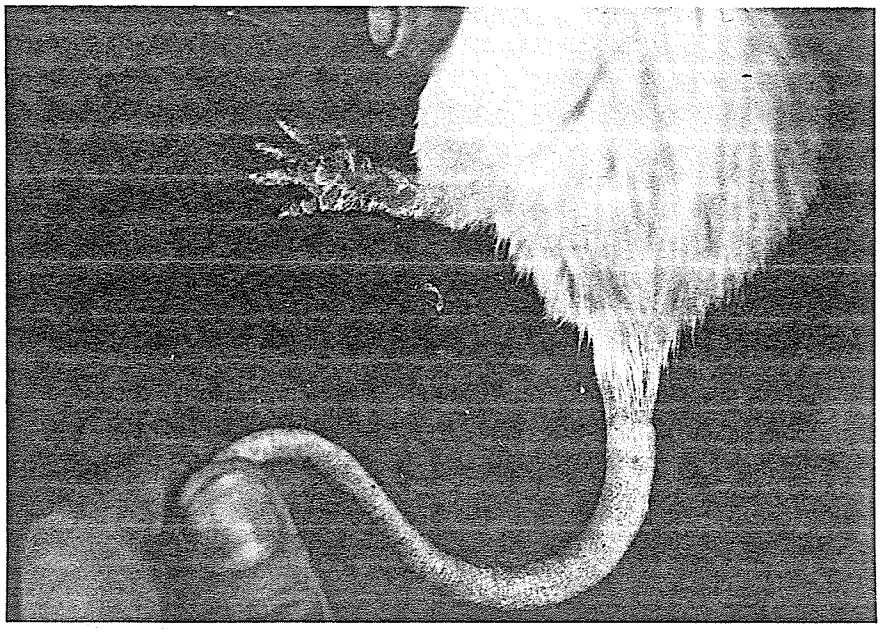


TABLE 8

EFFECT OF PYRIDOXINE DEFICIENCY ON BODY AND BRAIN WEIGHT

Status of Animals	Age of Rats (months)	Body Weight (g)	Brain Weight (g)
Pyridoxine (6) supplemented	4 - 5	283.8 ± 20.9	1.65 ± .01
Pyridoxine (7) deficient	4 - 5	168.2 ± 25.6	1.7 ± .04
Statistical significance		$p < .01$	NS

Values represent means ± standard deviation for the number of animals within brackets.

TABLE 9

EFFECT OF PYRIDOXINE DEFICIENCY ON THE LEVELS OF PLP IN RAT BRAIN

Status of Rats	Age of Rats (months)	PLP Levels (ng/g of wet tissue)		
		Cerebellum	Brainstem	Brain(-2 regions)
Pyridoxine supplemented	4 - 5	0.9 ± .3(7)	1.46 ± .40 (6)	1.12 ± .4 (7)
Pyridoxine deficient	4 - 5	0.43 ± .2(7)	0.44 ± .1 (7)	0.32 ± .1 (7)
Statistical significance		$p < .005$	$p < .001$	$p < .002$

Values represent means ± standard deviation for the number of animals within brackets.

TABLE 10

EFFECT OF PYRIDOXINE DEFICIENCY ON THE ACTIVITY OF GAD IN BRAIN (minus cerebellum and brainstem)

Status of Rats	Age of Rats (months)	Wt.of Brain (g)	GAD Activity (m units g wet tissue)	
			No added PLP	Added PLP
Pyridoxine supplemented	4	1.33 ± 0.1	92.1 ± 6.3 (5)	232.4 ± 59.5 (6)
Pyridoxine deficient	4	1.24 ± .1	28.1 ± 7.8 (5)	291.2 ± 53 (5)
Statistical significance			$p < .001$	NS

Values represent means ± standard deviation for the number of animals within brackets.

One enzyme unit decarboxylases 1 umole of L-glutamic acid/minute

TABLE 11

EFFECT OF PYRIDOXINE DEFICIENTY ON THE ACTIVITY OF GAD IN BRAINSTEM

Status of Rats	Age of Rats (months)	Wt. of Brainstem (g)	GAD Activity (m units/g wet tissue)	
			No added PLP	Added PLP
Pyridoxine supplemented	4	.26 ± .03	67.3 ± 13.8 (5)	266.7 ± 47 (5)
Pyridoxine deficient	4	.25 ± .01	23.8 ± 1.5 (5)	295.8 ± 39 (5)
Statistical significance			$p < .005$	$p < .025$

Values represent means ± standard deviation for the number of animals within brackets.

One enzyme unit decarboxylase 1 umole of L-glutamic acid/minute.

TABLE 12

EFFECT OF PYRIDOXINE DEFICIENCY ON THE ACTIVITY OF GAD IN CEREBELLUM

Status of Rats	Age of Rats (months)	Wt. of cerebellum (g)	GAD Activity (m units/g wet tissue)	
			No added PLP	Added PLP
Pyridoxine	4	.29 ± .021	78.6 ± 18.5 (5)	166.9 ± 33.9 (6)
Pyridoxine deficient	4	.26 ± .01	30.8 ± 5.4 (5)	296.8 ± 71.6 (5)
Statistical significance			$p < .005$	$p < .005$

Values represent means ± standard deviation for the number of animals within brackets.

One enzyme unit decarboxylase 1 umole of L-glutamic acid/minute.

groups, the difference in means was seen to be statistically significant with the only exception being the brain (minus brainstem and cerebellum). This may have been significant if a larger sample size had been used. In the case of holoenzymes the pyridoxine deficient brain regions all had significantly lower GAD activity than the control ($p < .005$). When apoenzyme acitivty was determined, the pyridoxine deficient groups had the higher activity ($p < .025$) except for the above exception.

E. Effect of Pyridoxine Deficiency on Brain Monoamines

The effects of dietary pyridoxine deficiency on the various brain monamines are presented as histograms in Figures 13, 14 and 15. The results are summarized in Table 13. In all regions examined, no significant differences between the catecholamine levels of the control and deficient rats were found. The cerebellar concentration of NE for example was 1.17 ± 0.3 ug/g for control animals compared to 1.27 ± 0.4 ug/g for deficient rats. The cerebellar DA content was 2.5 ± 0.5 ug/g for controls compared to 2.5 ± 0.6 ug/g for deficients. This is in contrast to the changes seen in 5-HT levels. The differences here were statistically significant. The cerebellar 5-HT content for example was 1.46 ± 0.2 ug/g for controls compared to 0.32 ± 0.1 ug/g for the pyridoxine deficient rats ($p < .001$). The numbers obtained in these experiments were found to be generally higher than those previously reported in the literature (Tables 14,15 and 16). The differences in the values could be due to different methods used for assay as well as the method of sacrifice. We have used microwave irradiation followed by a fluoro metric determination of the monoamines. Blank et al (1979) demonstrated a significantly lower value for decapitated animals as compared to the microwave heated group upon examination of striatal DA levels as quantitated by gas chromatography with mass fragmentometry.

Figure 13. Effect of pyridoxine deficiency in the mature rat on brain norepinephrine. (ng/g Fresh Weight)
Standard deviation is indicated by bars on the histograms.
There were 5 - 6 animals in each group.

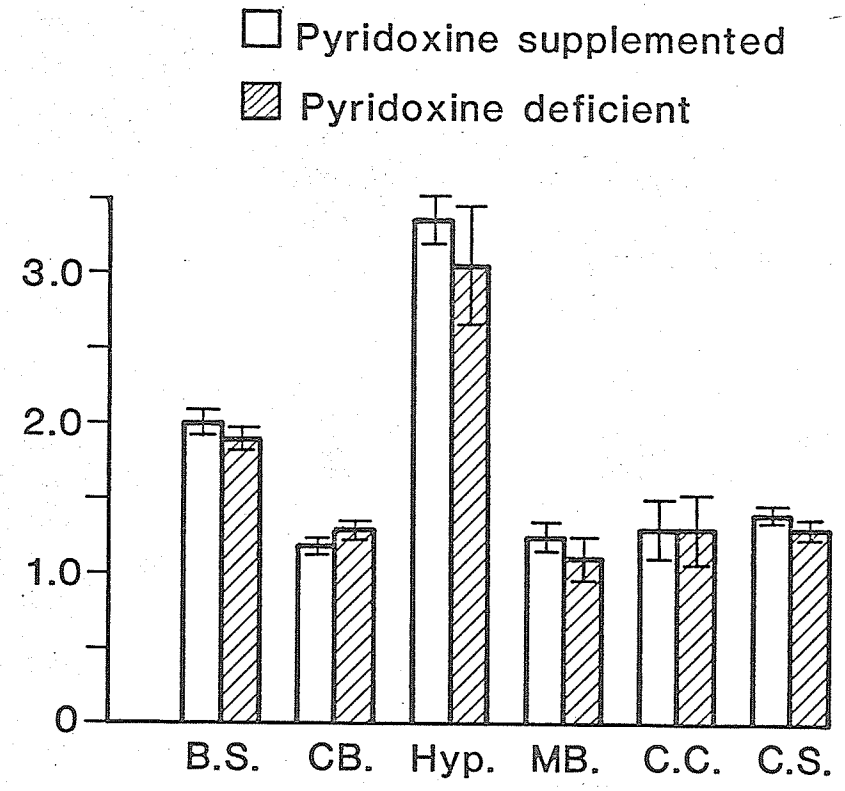

Pyridoxine supplemented
Pyridoxine deficient
3.0
2.0
1.0
0
B.S.
CB.
Hyp.
MB.
C.C.
C.S.

Figure 14. Effect of pyridoxine deficiency in the mature rat on brain dopamine. (ng/g Fresh Weight)
Standard deviation is indicated by bars on the histograms
There were 5 - 6 animals in each group.

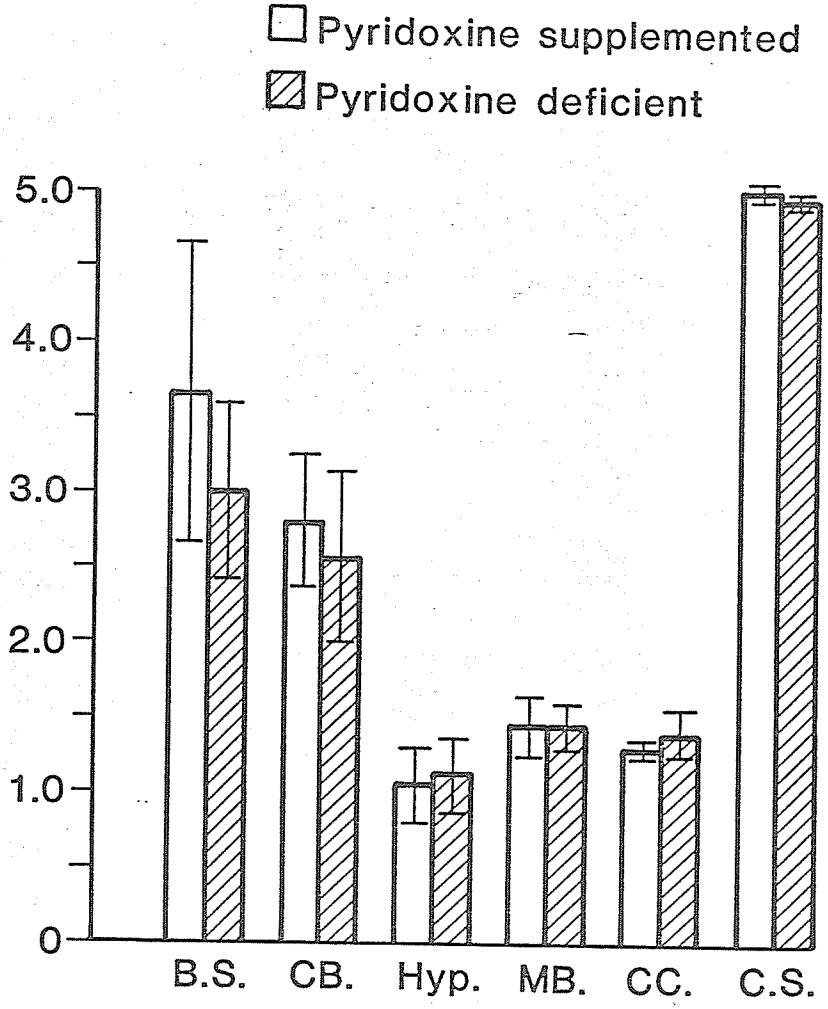

Pyridoxine supplemented
Pyridoxine deficient
5.0
4.0
3.0
2.0
1.0
0
B.S.
CB.
Hyp.
MB.
CC.
C.S.

Figure 15. Effect of pyridoxine deficiency in the mature rat on brain serotonin. (ng/g . Fresh Weight)
Standard deviation is indicated by bars on the histograms
There were 5 - 6 animals in each group

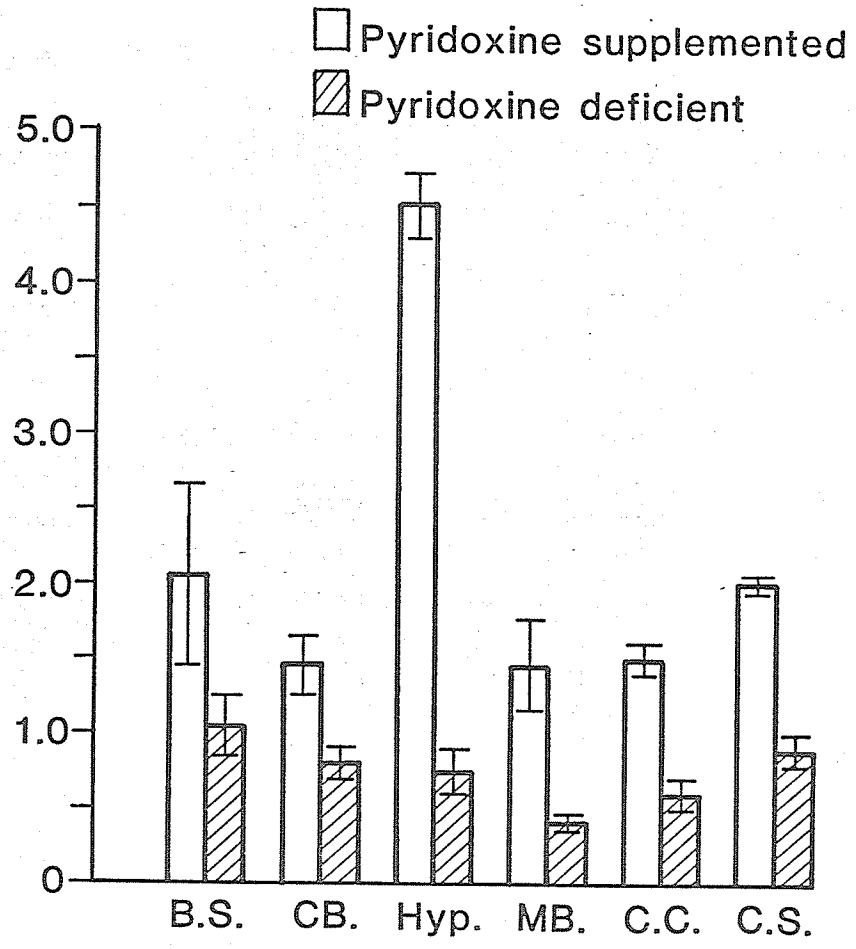

Pyridoxine supplemented
Pyridoxine deficient
5.0
4.0
3.0
2.0
1.0
0
B.S.
CB.
Hyp.
MB.
C.C.
C.S.

TABLE 13

SUMMARY OF THE EFFECTS OF B_6 DEFICIENCY ON BRAIN MONOAMINE LEVELS

Region	Serotin			Norepinephrine			Dopamine		
	Normal	Deficient	Stat. sig.	Normal	Deficient	Stat. sig.	Normal	Deficient	Stat. Sig.
Brainstem	2.02±.7	1.04±.2	$p < .015$	2.01±.6	1.89±.6	NS	3.66±1.0	2.98±.6	NS
Cerebellum	1.46±.2	0.32±.1	$p < 0.001$	1.17±.3	1.27±.4	NS	2.8±.5	2.53±.6	NS
Hypothal-anus	4.53±.2	0.77±.1	$p < 0.001$	3.34±.2	3.03±.4	NS	1.06@.2	1.13±.2	NS
Midbrain	1.45±.3	0.42±.04	$p < .001$	1.23±.1	1.11±.1	NS	1.46±.2	1.46±.2	NS
Cortex	1.48±.1	0.6±.1	$p < 0.001$	1.3 ±.2	1.29±.2	NS	1.3 ±.1	1.43±.2	NS
Corpus Striatum	2.0±.04	0.4±.1	$p < 0.001$	1.4±.1	1.3±.1	NS	4.49±.4	4.49±.5	NS

Values represent means ± standard deviation 5-6 rats were used for each group.

TABLE 14 CONTENT OF NE FROM VARIOUS AREAS OF RAT BRAIN

NE (ug/g)

Brain Area

Reference	C.C.	C.S.	Mb.	B.S.	Cb.	Hypo	Method of Sacrifice	Method of Assay
Own data	1.3 ± .2	1.4±.1	1.2 ± 1	2.0 ± .6	1.2 ± .3	3.3 ± .2	M Wave	Fluro.
Blank et al (1979)	0.26 ± .03	0.12±.01	0.49±.02	0.46±.02	0.41±.03		Decap.	HPLC
Blank et al (1979)	0.29 ± .01	0.12±.01	0.46±.02	0.44±.03	.46±.02		M Wave	HPLC
Jacobowitz & Richardson (1979)	0.14 ± .01	0.33±.02	0.43±.01	0.43±.03	0.2 ±.01	2.0 ± .1	Decap.	Fluro.
Cheng & Wooten (1980)		0.11±.01			0.23±.08	1.3 ± .2	Decap.	Radio-enz.

TABLE 15. CONTENT OF DA FROM VARIOUS AREAS OF RAT BRAIN

DA (ug/g)

Brain Area

Reference	C.C.	C.S.	Mb.	B.S.	Cb.	Hypo	Method of Sacrifice	Method of Assay
Own data	1.3±.1	4.98±.14	1.46±.2	3.66±1.0	2.4±.46	1.06±.2	M Wave	Fluro.
Cheng & Wooten (1980)		4.5±.5			0.3±.01	0.05±.1	Decap	Radio-enz.
Jacobwitz & Richardson (1979)	0.21±.01	4.9±.2	0.13±.01			0.03±.02	Decap	Fluro.
Blank et al (1979)	.70±.06	4.6±.1	0.095±.01	0.08±.012	0.02±.002		Decap	HPLC
Blank et al (1979)	0.68±.06	4.7±.6	0.112±.01	0.05±.01	.03±.003		Decap	HPLC
Blank et al (1979)	0.62±.04	7.8±.2	0.12±.01	0.07±.01	.02 ±.002		M Wave	HPLC
Blank et al (1979)	0.62±.06	8.4±.6	0.13±.01	0.05±.01	.03 ±.002		M Wave	HPLC

TABLE 16. CONTENTS OF 5-HT FROM VARIOUS AREAS OF RAT BRAIN

Reference	5-HT (ug/g) Brain Area C.C.	C.S.	Mb.	B.S.	Cb.	Hypo.	Method of Sacrifice	Method of Assay
Own data	1.48±.1	2.0±.04	1.45±.2	2.0±.7	1.46±.2	4.5±.23	M Wave	Fluro.
Jacobowitz & Richardson (1979)	0.49±.03	1.05±.1	1.1±.03	1.1±.1	0.33±.02	2.03±.13	Decap	Fluro
Tadano et al (1980)	0.33±.02	0.50±.03	0.51±.03	0.45±.01	0.1±.01	0.54±.03	Decap	Fluro
Curzon and Green (1970)	0.53	1.84	1.04	1.25	0.387	2.64	Decap	Fluro
Miller et al (1970)	0.651		1.06		0.193	0.86	Decap	Fluro
Saavedra et al (1973)	0.56	0.56	0.97	0.83	.07	0.99	Decap	Radio-enz

F. Effect on Myelin and Myelinated Tracts

Pictures of representative sections comparing normal and pyridoxine deficient brain areas are shown in Figures 16, 17 and 18.

One-half micron epon cut sections of the trigeminal nerve and ganglion showed possible differences in peripheral nerve myelin thickness but this wasn't obvious at low magnification. Examination of optic nerve and corpus callosum cross sections (at 1000x) showed no detectable differences in CNS myelin. Luxol Fast blue, Osmium tetroxide and Spielmyers stain for myelin were tried on the frozen sections. None of these stains revealed any differences in the myelinated tracts of the control and deficient animals. No differences were detectable when paraffin sections were studied. Examination of the JB4 sections showed that the osmicated tissue didn't infiltrate properly and was hard to cut but did have blackening of the myelin due to the extended time in osmium. When non-osmicated tissue was stained with toluene blue, the myelin didn't pick up the blue color. No detectable differences between normal and deficient rats were seen.

Within the limits of light microscopy by all known methods (with or without preservation of lipids), no detectable difference in myelin or myelinated tracts was seen. Cellular differences were not investigated and may indeed be present.

G. Electrophysiological Experiments

a) Effect on the Electroencephalogram

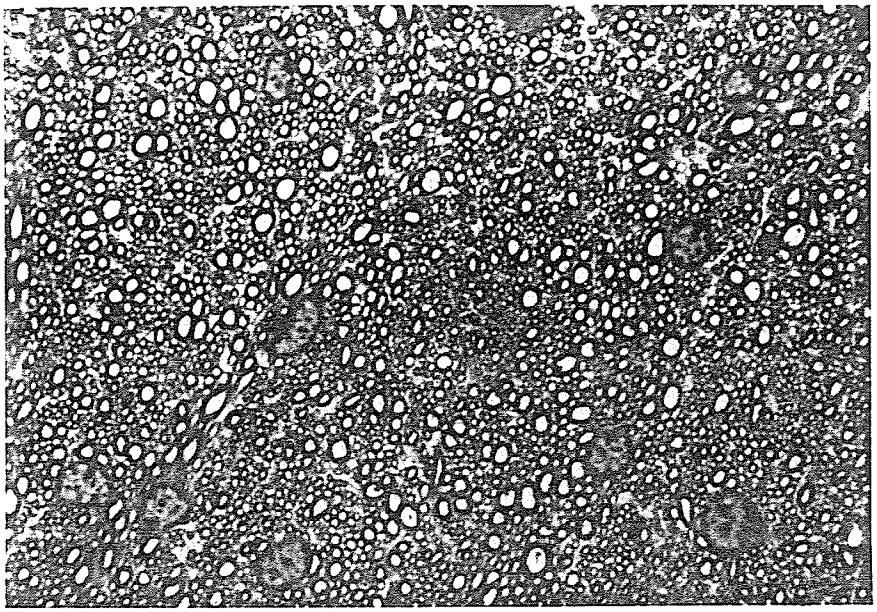
Figure 19 compares tracings of the EEG patterns of control and B_6 deficient rats. Examination of the tracing revealed no differences between the two groups of rats.

FIGURE 16

½ micron EPON cut cross sections of the corpus callosum stained with toluidine blue.

magnification = 1400X

CONTROL



DEFICIENT

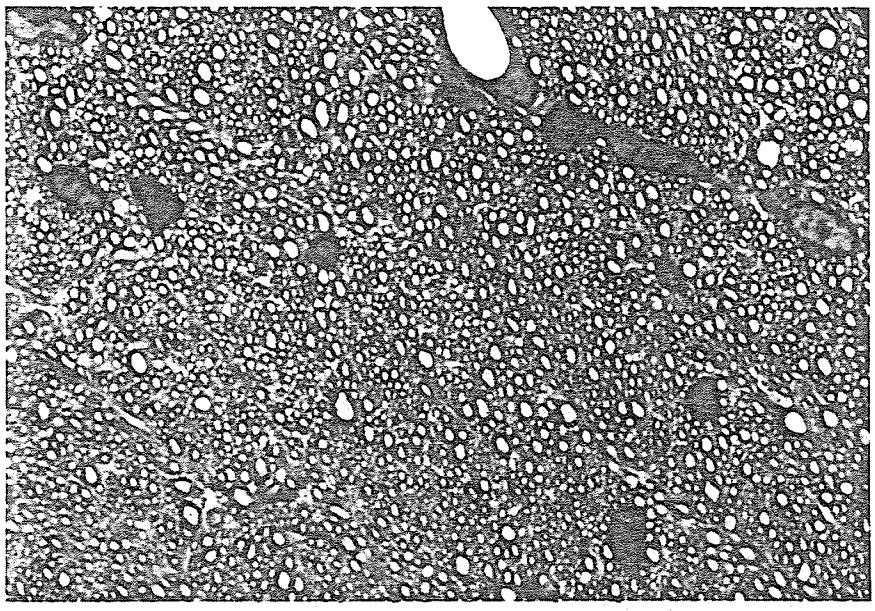
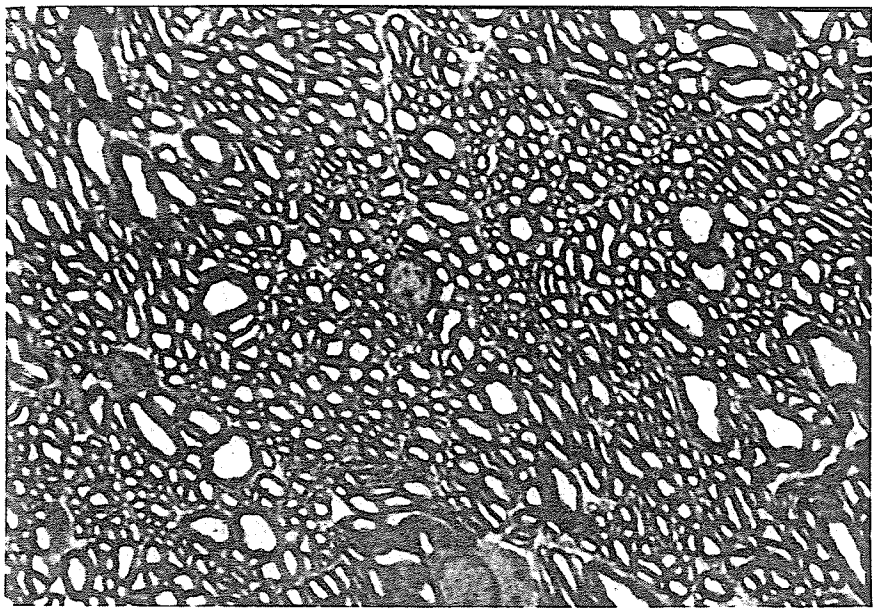


FIGURE 17

½ micron EPON cut cross section of the optic nerve stained with toluidine blue. magnification = 1400X

CONTROL



DEFICIENT

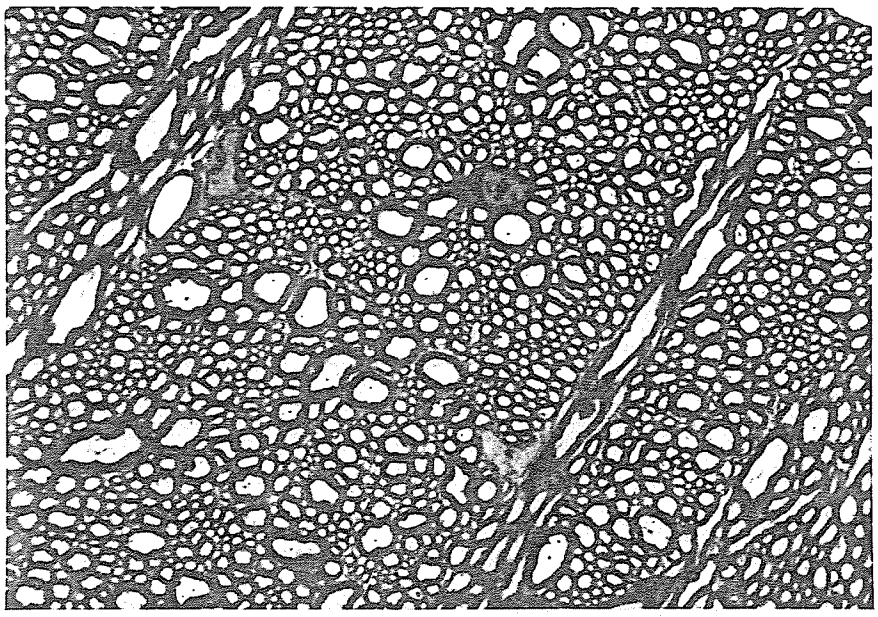
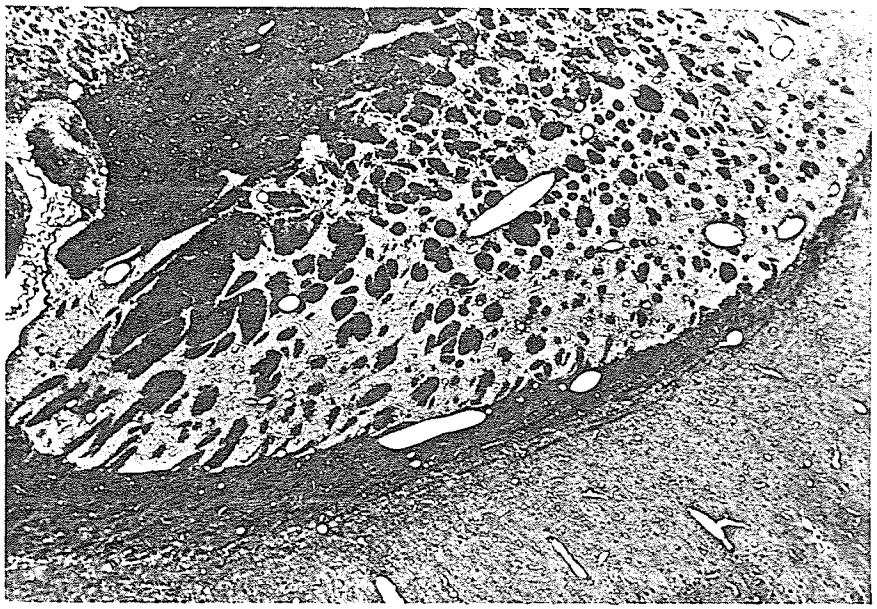


FIGURE 18

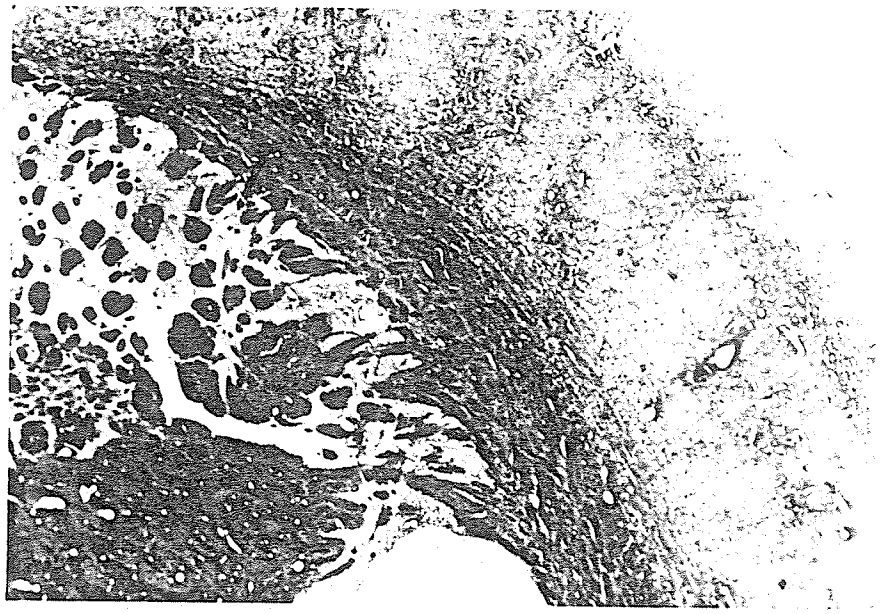
4 micron paraffin sections of the corpus callosum and internal capsule fibres of the cerebrum. Stained with Luxol fast blue.

magnification = 35X

CONTROL



DEFICIENT



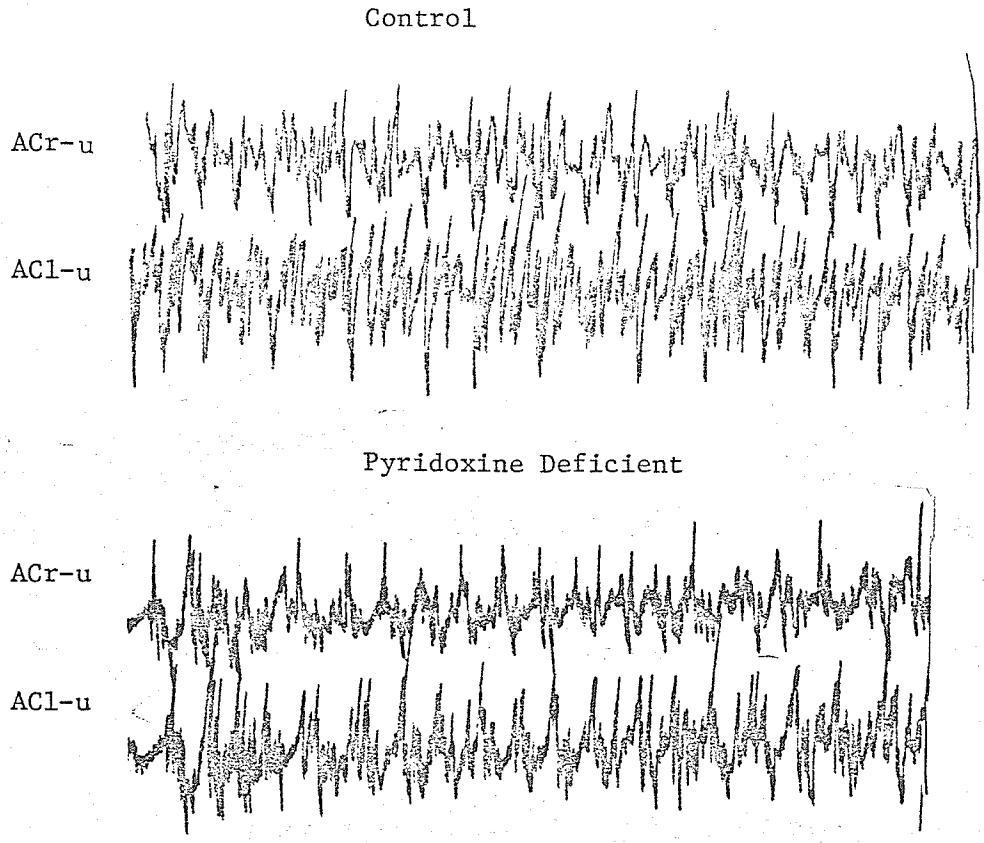


FIGURE 19: Electroencephalograms of a pyridoxine supplemented (upper figure) and deficient rat (lower figure).

AC = auditory cortex; r = right; l = left; u = unipolar

The frequency spectrum of the EEG in control and test animals were analyzed (Fig. 20 and 21). Of the nine bands examined there was no significant difference between the two groups. Differences in the power spectrum (Fig. 22) showed that in view of the standard error, there were no significant differences at any band although lower values were exhibited in the test group especially for D1, D2, T1, and T2.

b) Effect on Auditory Evoked Potentials

Immediately following the EEG recording the same rats were tested for auditory E.P. Figure 23 is an example of recorded E.P. for a pyridoxine supplemented (control) and a pyridoxine deficient rat. After the application of stimulus there was a latency period in which no electrical activity was recorded followed by an initial downward deflection (initial negative wave). This is followed by the secondary phase of the E.P. consisting of late positive - negative oscillations. It was found that the period of latency in control rats was consistantly shorter than those for the test animals. The difference between the two groups was not statistically significant. In both groups of rats, the latency period increased with increasing frequencies (Table 17, Figues 24 and 25).

In contrast to Table 17, the pyridoxine deficient rats differed from their controls in the effect of increasing the frequency of auditory stimuli on evoked potentials (Table 18, Figures 26 and 27). An increase in the frequency of clicks from 0.2 to 6.0 Hz decreased the amplitude of the evoked potentials in both groups. However, there was a more pronounced decrease in amplitude in the B_6 deficient rats. Except at 2.0 Hz, the mean amplitudes of the evoked potentials were greater for the deficients than for the controls. The difference in amplitudes was statistically significant at frequencies between 0.2 and 0.6 Hz.

Figure 20. Frequency specturm of the EEG in the group of control rats (N=9). Frequency bands are indicated on horizontal scale: D1 = 0.10 - 148 Hz, D2 = 156 - 351 Hz, T1 - 361 - 5.57 Hz, T2 = 5.66 - 7.52 Hz, A1 - 7.62 - 9.47 Hz, A2 = 9.57 - 12.50 Hz, B1 = 12.60 - 17.48 Hz, B2 = 12.58 - 25.0 Hz, SUM = 1.56 - 25.0 Hz.

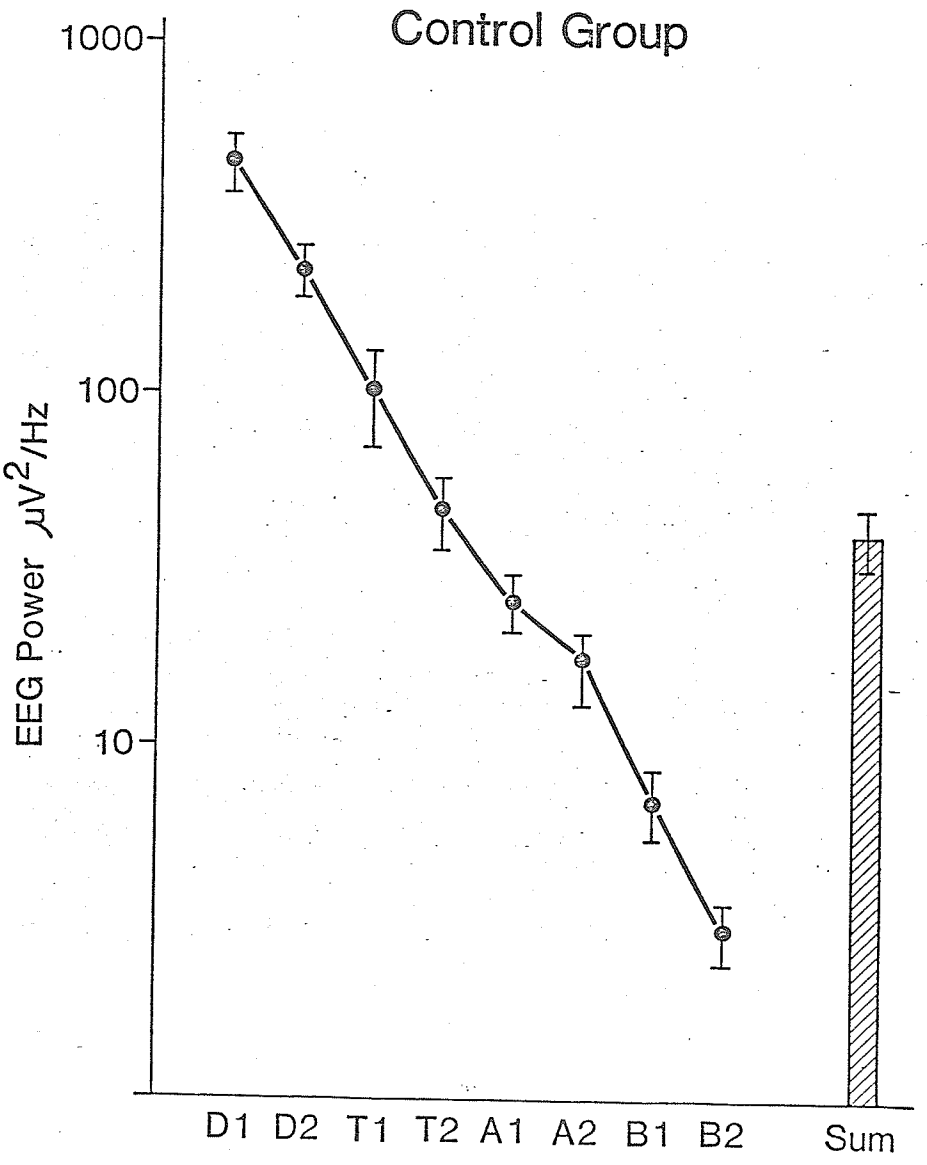

Control Group
1000
100
10
EEG Power μV²/Hz
D1 D2 T1 T2 A1 A2 B1 B2
Sum

Figure 21. Frequency spectrum of the EEG in the group of pyridoxine deficient rats (N=9 Horizontal scale = frequency bonds). (See Fig. 20). Vertical lines indicate S.E.

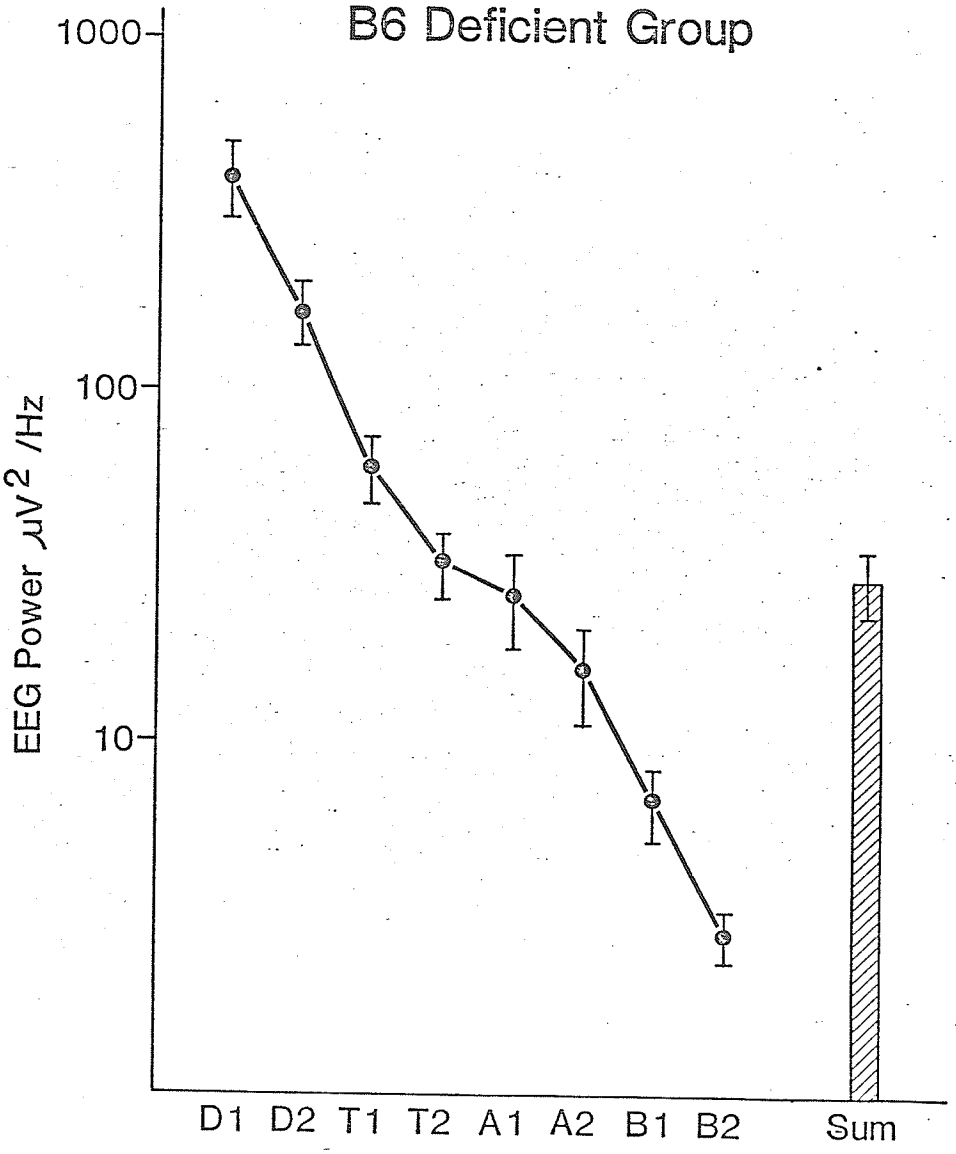
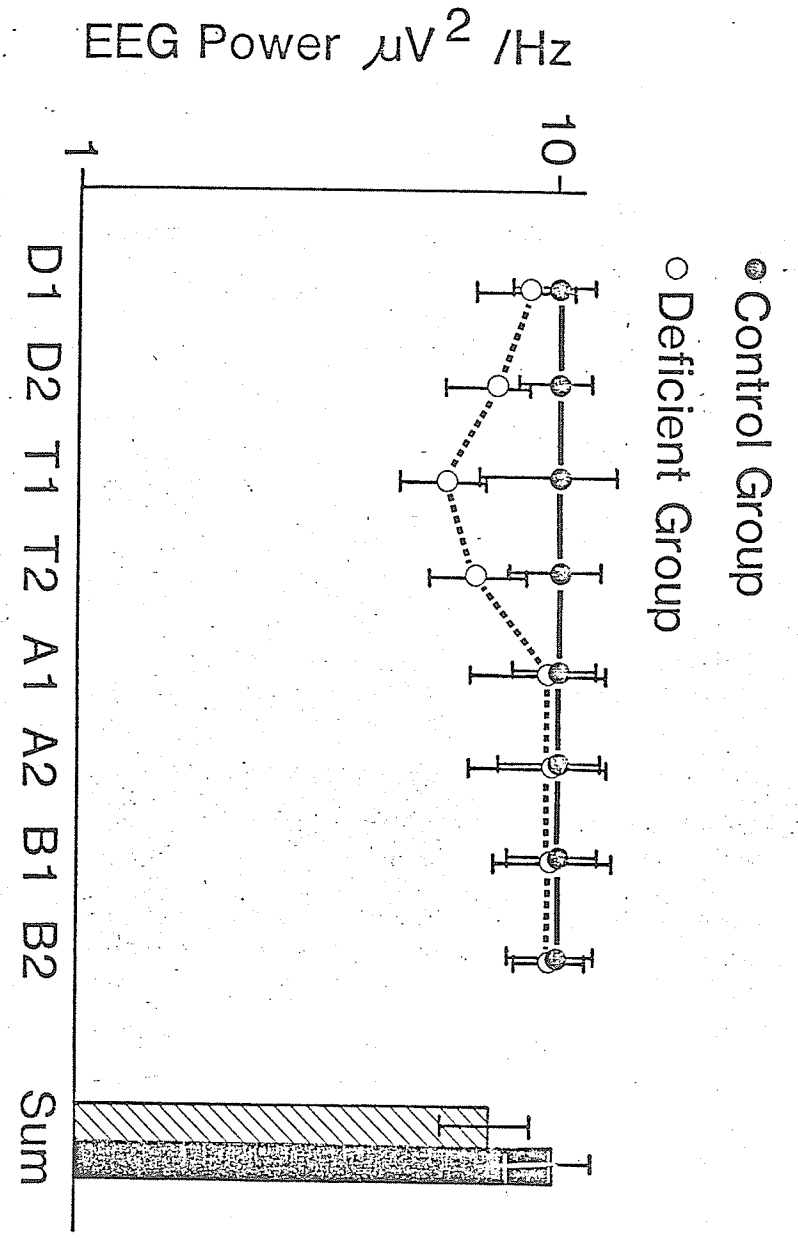
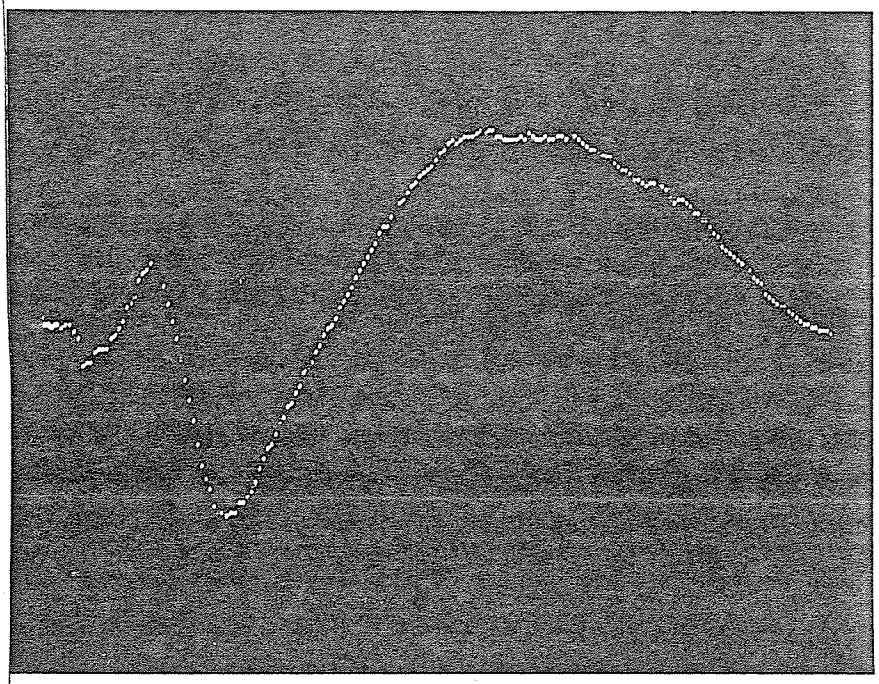

B6 Deficient Group
1000
100
10
EEG Power μV2 /Hz
D1 D2 T1 T2 A1 A2 B1 B2
Sum

Figure 22. Differences in power spectrum (u V^2/Hz) between groups of control rats (indicated by solid line) and pyridoxine deficient rats (indicated by broken line). Values of EEG in control rats were taken as 100%. Horizontal scale = frequency bands (See Fig. 20). Vertical lines indicate S.E.


EEG Power μV² /Hz
10
1
D1 D2 T1 T2 A1 A2 B1 B2 Sum
● Control Group
○ Deficient Group

Control

mag=1x



Deficient

mag=½x

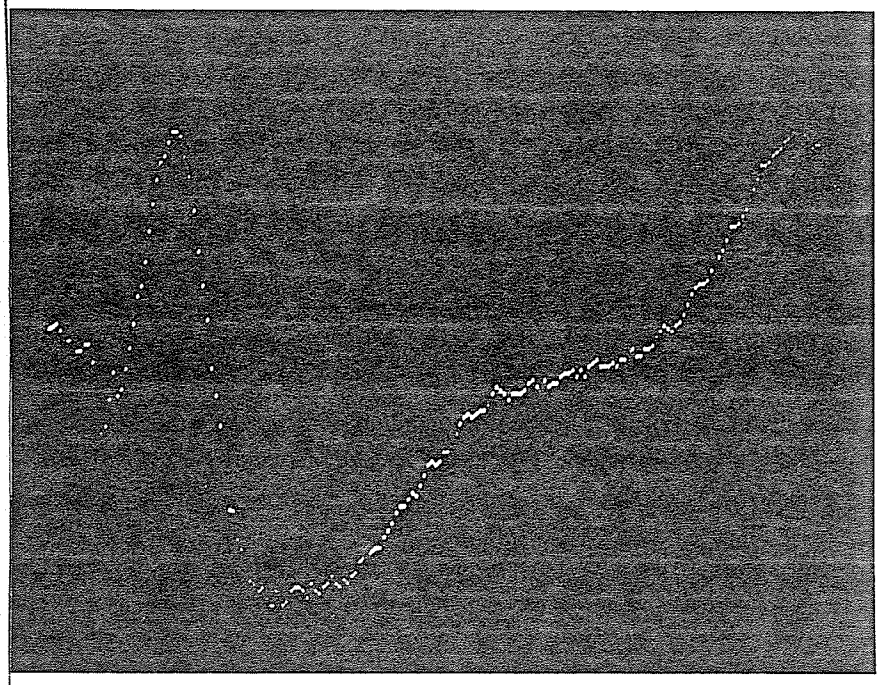


Figure 23. Auditory evoked potentials in control (upper photograph) and pyroxidine deficient (lower photograph) rats under urethane anaesthesia. Stimuli were clicks at a frequency of 0.4 Hz.

TABLE 17

COMPARISON OF LATENCY AT VARIOUS FREQUENCIES

Frequency (H2)

Latency (mSec)	0.2	0.4	0.6	0.8	2.0	4.0	6.0
Rat Control (6)	17.8 ± 2.3	18.5±3.0	19.5±1.4	19.7±1.51	20.3±1.2	21 ±.9	21.5± 1.2
Deficient Rats (7)	19.1 ± 1.7	19.7±2.4	20.00±1.9	20.7±1.4	21.4±1.6	22.1±1.6	21.9± 1.8
Statistical significance	NS	NS	NS	NS	NS	NS	NS

Values represent means ± standard deviation for the number of animals within brackets.

Figure 24. Effect of increasing frequency of clicks (abscissa) on the latency (ordinate) of auditory evoked potentials in control rats under urethane anaesthesia. Plotted points denote means ± standard deviation.

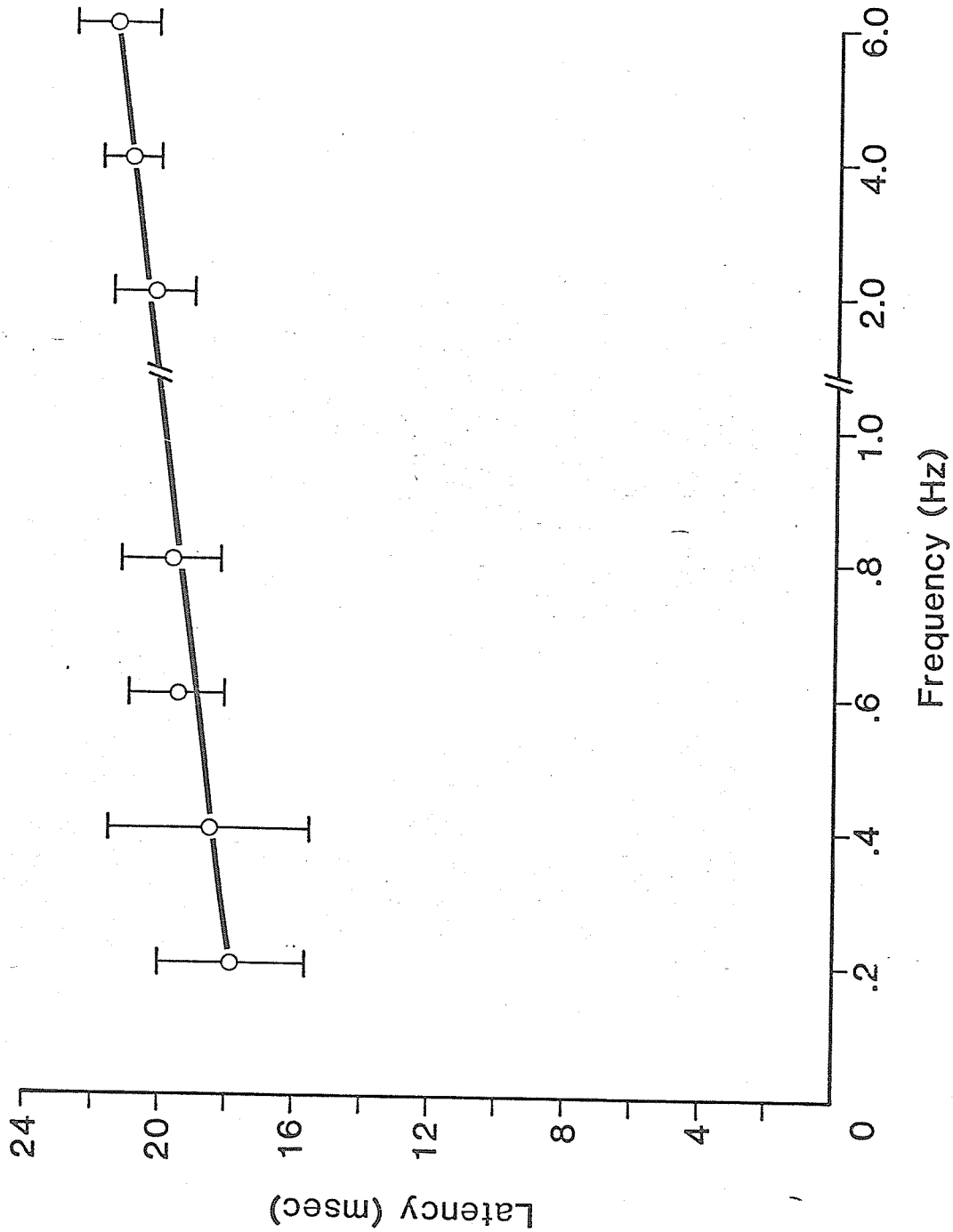

Latency (msec)
24
20
16
12
8
4
0
.2
.4
.6
.8
1.0
2.0
4.0
6.0
Frequency (Hz)

Figure 25. Effect of increasing frequency of clicks (abscissa) on the latency (ordinate) of auditory potentials deficient rats under urethane anesthesia. Plotted points denote means ± standard deviation.

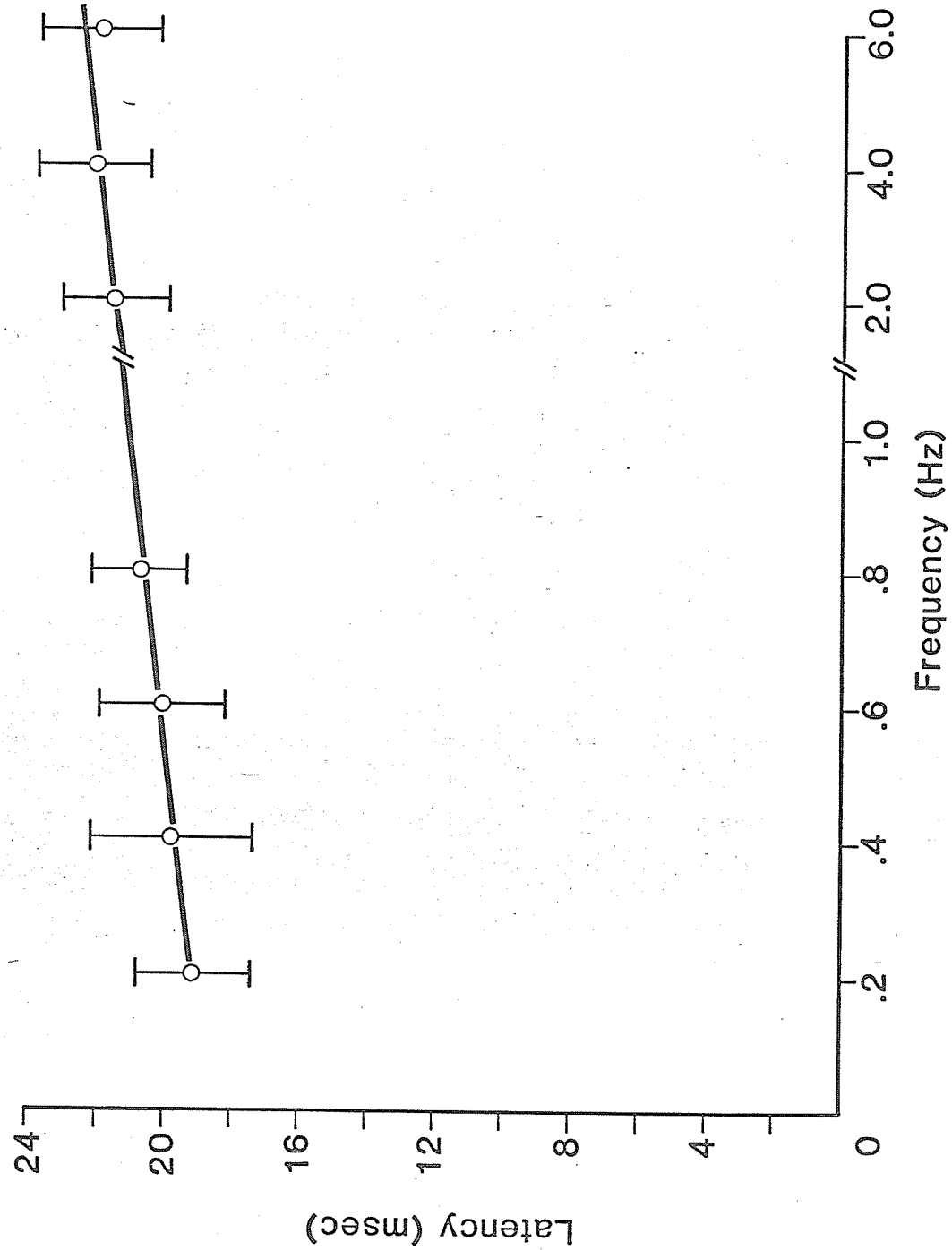

Latency (msec)
24
20
16
12
8
4
0
.2
.4
.6
.8
1.0
2.0
4.0
6.0
Frequency (Hz)

TABLE 18

COMPARISON OF E.P. AMPLITUDE FOR VARIOUS FREQUENCIES

Frequency (Hz)							
Amplitude (mm)	0.2	0.4	0.6	0.8	2.0	4.0	6.0
Deficient Rats (7)	22.6±6.4	21.4±5.4	22.9±9.8	10.6±5.8	7.1±4.0	6.3±3.2	5.9±2.1
Control Rats (6)	6.8±4.8	8.5±5.4	7±6.3	8.3±7.0	7.4±5.0	4.8±2.2	4.6±2.3
Statistical sign.	$p < .01$	$p < .01$	$p < .01$	NS	NS	NS	NS

Values represent means ± standard deviation for the number of animals within brackets

Figure 26. Effect of increasing frequency of clicks (abscissa) on the amplitude (ordinate) of auditory evoked potentials in control rats under urethane anesthesia. Plotted points denote means ± standard deviation.

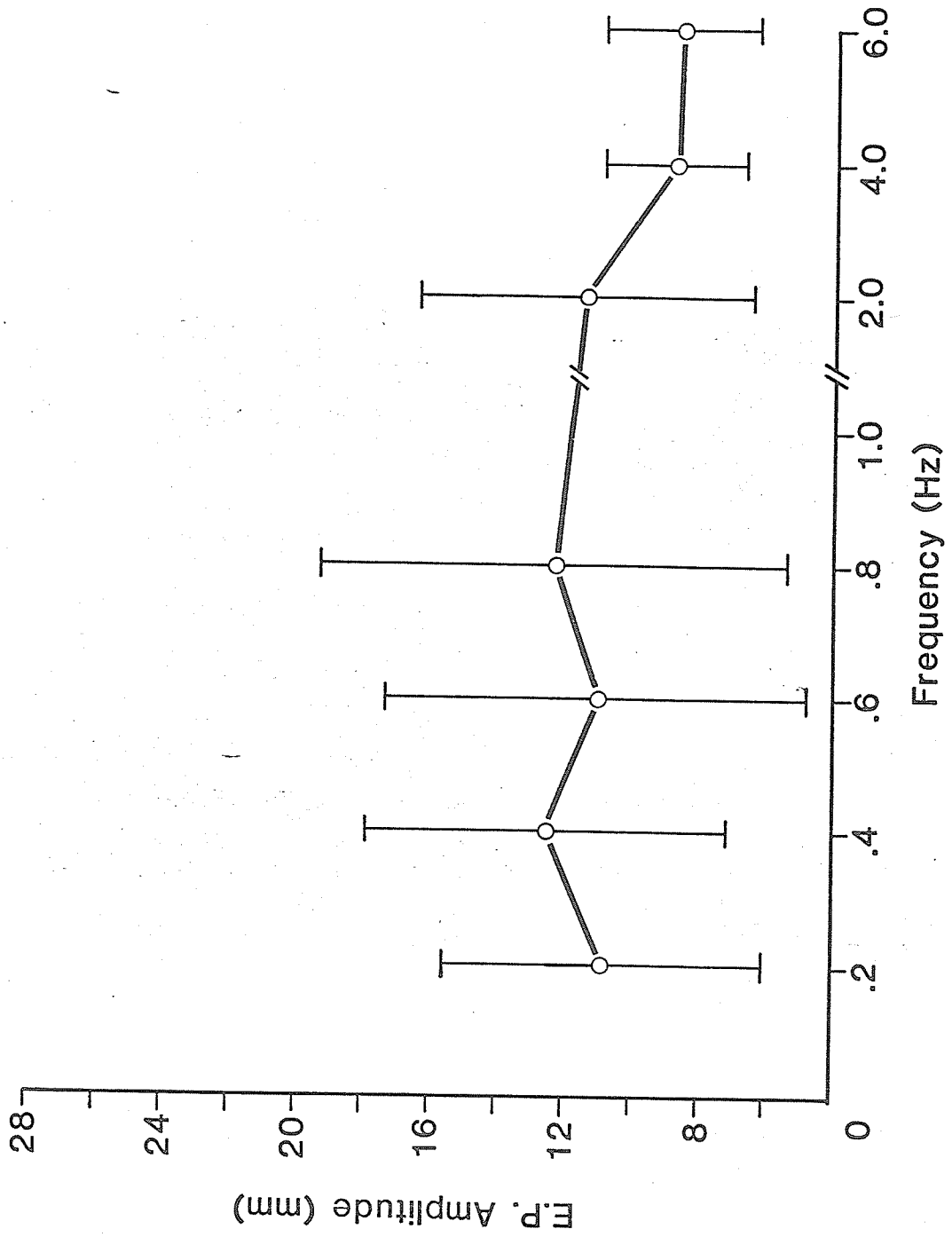
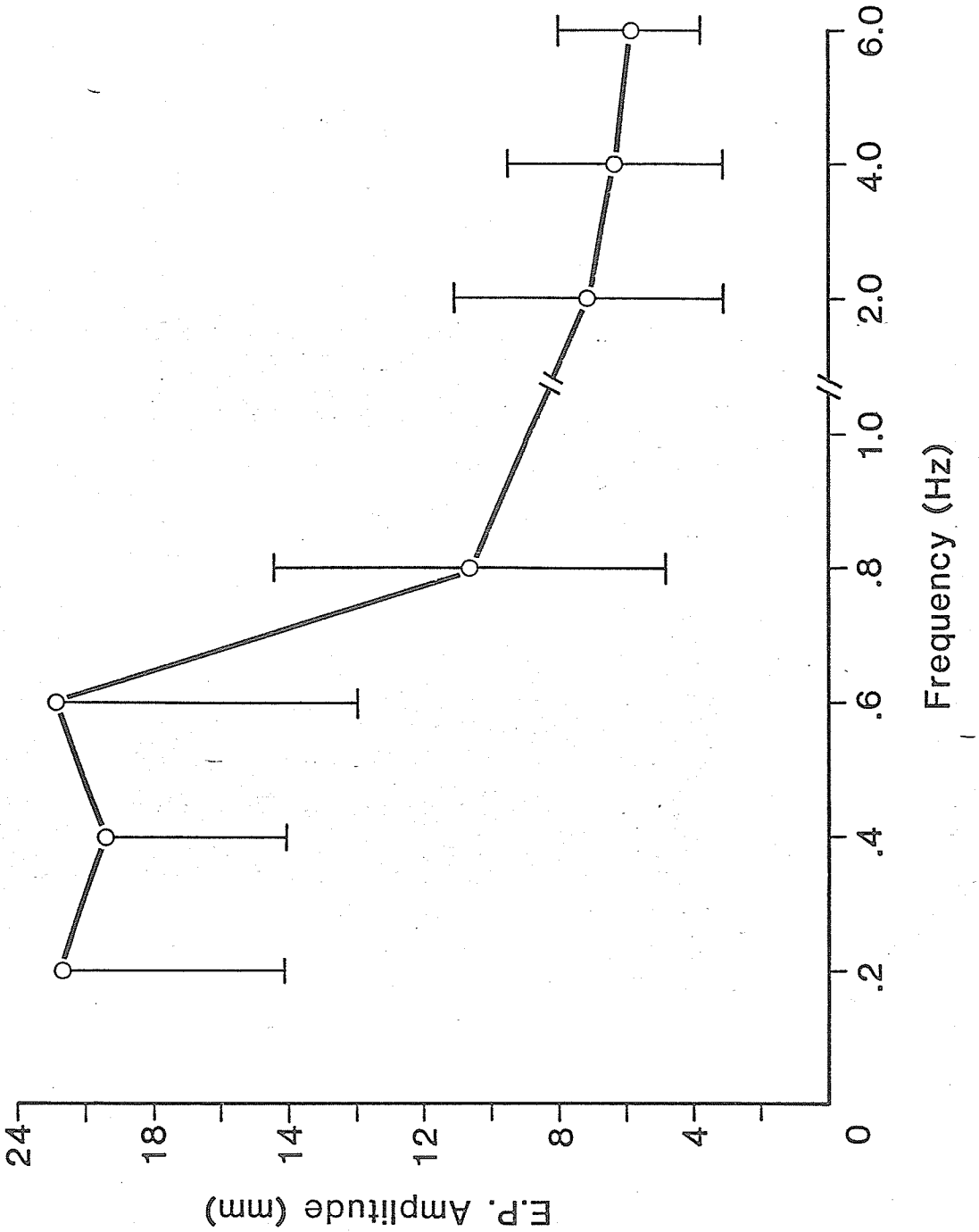

E.P. Amplitude (mm)
28
24
20
16
12
8
0
.2
.4
.6
.8
1.0
2.0
4.0
6.0
Frequency (Hz)

Figure 27. Effect of increasing frequency of clicks (abscissa) on the amplitude (ordinate) or auditory evoked potentials in pyroxidine deficient rats under urethane anesthesia. Plotted points denote means ± standard deviation.


E.P. Amplitude (mm)
24
18
14
12
8
4
0
.2
.4
.6
.8
1.0
2.0
4.0
6.0
Frequency (Hz)

IV DISCUSSION

The rats on the pyridoxine deficient diet had obvious physical lesions. There were no visible behavioural differences between the deficient and control groups of animals during handling or when placed in a new cage. In contrast to the results with rat pups, there were no significant differences in brain weights between the adult pyridoxine deficient and control groups. This was expected in that neuronal proliferation and myelination were completed when the deficient group was started on the pyridoxine deficient diet. We also found no histological evidence indicating a breakdown of myelin or myelinated tracts. Although cellular differences in the cells involved in myelination were not investigated by electron microscopy in this study, they may indeed be present. This is in contrast to the observation in pyridoxine deficient rat pups from which electron micrographs have shown markedly less myelination than control pups (Moore et al, 1978). Some myelinated axons of the deficient pups exhibited an unusual appearance of whorls of lamina separated by cytoplasmic space occupied by widely separated lamina. Pyridoxine deficiency was confirmed upon measurement of pyridoxal phosphate levels. In all regions examined, PLP levels in deficient rats were significantly lower than those of controls. This was also seen in rat pups (Stephens et al, 1971). Our control values were comparable to those found in the literature (Ebadi and Bifano, 1978).

We have found a decrease in the concentration of GAD holoenzyme presumably due to a lack of cofactor. In contrast, the concentration of GAD apoenzyme tended to be significantly greater than that of controls. This increase in apoenzyme appears to be an exception to usual findings with other PLP enzymes where, under B_6 deficient conditions, the addition of PLP to the assay system only restored the maximal activity to normal

levels (Chatagner, 1970). For example, in developing brains of pyridoxine deficient rats, no increase in apoenzyme levels occurs with aromatic L-aminoacid decarboxylase (Eberle and Eiduson, 1968), 5-HT decarboxylase, GABA transaminase (Bayoumi and Smith, 1972) asparate amino-transferase, alanine aminotransferase (Bayoumi, 1973) tyrosine aminotransferase (Eidusen et al, 1972). The increase in GAD apoenzyme seemed to be fairly specific suggesting enzyme induction (Stephens et al, 1971; Bayoumi and Smith, 1973).

Having established the pyridoxine-deficient status, we investigated the effects of such deficiency on various electrophysiological parameters. It has been reported that the EEG of deficient rat pups displayed higher EEG voltages and a higher dominant frequency than controls (Stephens et al, 1971; Hoff and Castor, 1975). We found no significant differences in voltage potentials of normal and deficient mature rats due to the large standard error between groups. In some cases (such as T_1) there was only a slight overlapping of the upper and lower limits of the standard errors.

Three types of parameters can be analyzed; the latency and shape of the evoked potential and the response to repetitive stimulation. In contrast to what has been observed in rat pups (Stephens et al, 1971), the latency and shape of the evoked potentials were similar in the mature animals whether they were pyridoxine deficient or control. The delay in latency seen in rat pups may be due to the lack of myelination associated with pyridoxine deficiency prior to and including the period of rapid myelination (Moore et al, 1978). A decrease in latency of the evoked potentials was invariably noticed with maturation of the CNS (Moore et al, 1967, Ellingson 1967, Marty, 1967) and was associated with increased

axon diameter and rapid myelination of afferent fibers as well as biochemical maturation of synaptic transmission (Mollines,1967). In our experiments rats were made deficient after myelination was completed. Stewart et al, (1973) have studied the effects of post weanling pyridoxine deficiency on CNS function in rats. The latency of visually evoked cortical responses was employed as a measure of CNS reaction to stimulation in their study. The deficient animals in their study showed statistically significant increases in the latency. The latencies demonstrated by deficient rats in our studies were not statistically significant. In our studies however the evoked responses were produced by auditory stimulation and were analyzed for the effects of frequency stimulation upon response amplitude, a variable not studied in Stewart et al's investigations. With increasing frequencies, the duration of latency for onset of the auditory evoked response in our study was found to increase. The latency of the visual response seen in the study of Stewart et al decreased as the intensity of the photostimulator increased. Their data show that imposition of pyridoxine deficiency in rats after completion of CNS development can produce alterations in CNS functions as reflected in the latencies of the cortical evoked responses. Both Stewart's study and ours showed a depletion of PLP of about 50% or more in the brains from the deficients as compared with control rats. They also found the increase in latency to be reversible by pyridoxine treatment. This was not attempted in our study. Because of the anorexia associated with pyridoxine deficiency, the possibility that under-nutrition might be partially responsible for alterations in evoked responses must be considered. Stewart et al (1968) have shown that pair-fed control rats failed to show any significant increases

in the latency of cortical evoked responses. Stephens et al (1971) have also found that this dietary restriction during the pre- and post-weanling period failed to produce any alteration in the evoked response of the animals. These findings, along with the previously mentioned reversibility of alterations in latency following injection of pyridoxine in deficient animals, suggests that the altered brain function as reflected in the cortical evoked response is related to the lack of pyridoxine per se and not some indirect effects of the deficiency.

We have found that the amplitude of the E.P.'s in deficient rats were almost consistently greater than in control animals. There was also a greater drop in the E.P. amplitude in these rats following the increasing frequency of repetitive stimuli. The former result is in contrast to what was observed in rat pups, that is, a higher amplitude for control pups. One marked difference between the experiments described here and those done previously in the rat pups is that urethane was used instead of nembutal. The use of urethane may account for the difference in that it may anesthetize the rats by acting in a manner different from nembutal thus somehow inhibiting the evoked potential amplitude in controls.

The results of this investigation have confirmed a nonparallel change in brain level of the monoamines in the pyridoxine deficient mature 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 those found by Dakshinamurti et al (1976) in pyridoxine deficient rat pups. That pyridoxine is the factor responsible for the decrease in brain serotonin is clearly indicated, as the general undernutrition of

pyridoxine supplemented rats on restricted dietary intake did not result in a similar decrease of brain serotonin (Sereni et al, 1966). Further, the normal levels of both the catecholamines reported in pyridoxine deficient rat pups were in striking contrast to the decrease in these amines in rats subjected to undernutrition perinatally, as reported by Shoemaker and Wurtman (1971). Dakshinamurti et al (1976) have demonstrated that the decrease in 5-HT did not result from a decrease either in brain level of tryptophan or the activity of tryptophan hydroxylase. Increased degradation of serotonin measured by the level of its metabolite, 5-hydroxyindoleacetic acid was also excluded, thus suggesting the possibility that the decarboxylation of 5-hydroxytryptophan is decreased in pyridoxine deficiency.

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